# Disruption of Androgen Metabolism, Regulation and Effects: Involvement of Steroidogenic Enzymes

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# I. Abbreviations

| | |
|---|---|
| 11β-HSD1: | 11β-Hydroxysteroid Dehydrogenase Type 1 |
| 11β-HSD2: | 11β-Hydroxysteroid Dehydrogenase Type 2 |
| 17β-HSD3: | 17β-Hydroxysteroid Dehydrogenase Type 3 |
| 17β-HSD5: | 17β-Hydroxysteroid Dehydrogenase Type 5 |
| AAS: | Anabolic Androgenic Steroids |
| AKR: | Aldo-Keto Reductase |
| Androstenedione: | Δ4-androstene-3, 17-dione |
| AP1: | Activator protein 1 |
| AR: | Androgen Receptor |
| ARE: | Androgen Responsive Elements |
| ATF-2: | Activation Transcription Factor 2 (ATF2) |
| BMI: | Body Mass Index |
| bp: | base pairs |
| CI: | Confidence Interval |
| CPY: | Cytochrome P450 |
| Ct: | Cycle Threshold |
| CVD: | Cardio Vascular Disease |
| DEX: | Dexamethasone |
| DHEA: | Dehydroepiandrosterone |
| DHEAS: | Dehydroepiandrosterone Sulfate |
| DHT: | Dihydrotestosterone |
| DMT2: | Diabetes Mellitus Type 2 |
| DSD: | Disorder of Sex Development |
| EIA: | Enzyme Immunoassay |
| ELISA: | Enzyme Linked Immunosorbant Assay |
| ER: | Estrogen Receptor |
| ERK: | Extracellular Signal-Regulated Kinase |
| ESI: | Electro Spray Ionisation |
| GC: | Gas Chromatography |
| GR: | Glucocorticoid Receptor |
| H6PDH: | Hexose-6 Phosphate Dehydrogenase |

| | |
|---|---|
| HDL: | High Density Lipoprotein |
| HSD: | Hydroxysteroid Dehydrogenase |
| IKK: | I kappa B-Kinase |
| IUPAP: | International Union of Pure and Applied Chemistry |
| JNK: | c-Jun N-Terminal Kinase |
| LC: | Liquid Chromoatography |
| LH: | Luteinizing Hormon |
| MAP2K: | MAPK Kinase |
| MAP3K: | MAPK Kinase Kinase |
| MAPK: | Mitogen Activated Protein Kinase |
| MR: | Mineralocorticoid Receptor |
| mRNA: | Messenger RNA |
| MS/MS: | Tandem Mass Spectometry |
| MS: | Mass Spectrometry |
| $NAD^+$: | Nicotinamide Adenine Dinucleotide |
| NADP: | Nicotinamide Adenine Dinucleotide Phosphate |
| NADPH: | Nicotinamide Adenine Dinucleotide Phosphate Hydroxyl |
| NR: | Nuclear Receptor |
| PBS: | Phosphate Buffered Saline |
| PC: | Prostate Cancer |
| PR: | Progesterone Receptor |
| PSA: | Prostate Specific Antigen |
| RA: | Retinoic acid |
| RE: | Response Elements |
| RIA: | Radioimmunoassay |
| RIP: | Receptor Interacting Protein |
| rtPCR: | Real Time Polymerase Chain Reaction |
| RXR: | Retinoid X Receptor |
| S.D.: | Standard Deviation |
| S: | Steroid |
| SAME: | Syndrome of Apparent Mineralocorticoid Excess |
| SDR: | Short Chain Dehydrogenase Reductases |
| StAR: | Steroidogenic Acute Regulatory Protein |

| | |
|---|---|
| TBT: | Tributyltin |
| TF: | Transcription Factor |
| TLC: | Thin Layer Chromatography |
| TNFR1: | Tumor Necrosis Factor Receptor 1 |
| TNF-α: | Tumor necrosis factor-α |
| TPT: | Triphenyltin |
| TRADD: | TNFR-Associated Death Domain |
| UPLC: | Ultra High Pressure Liquid Chromatography |
| WHO: | World Health Organisation |

# 1. Summary

Communication between organs and tissues is predominately controlled by hormones. Hormones regulate a vast variety of physiological and behavioural activities, including metabolism, growth and development, reproduction, sleep and mood. Steroid hormones are characterized by their sterane backbone and are regulated by distinct enzymes which control the balance between their active and their inactive forms. It is a common feature of steroid hormones to bind to nuclear receptors in order to exert their action. Steroid hormones are classified into five groups, depending on the receptor they bind: glucocorticoids, mineralocorticoids, androgens, estrogens and progestogens. Most research within the field of steroidogenesis focuses on the receptors, since they play a pivotal role in converting the chemical signal from the active steroid hormone into a biological response. The present studies of this thesis focus on the enzymes which selectively control and regulate the availability of active ligand for receptor binding. The two major enzyme superfamilies responsible for the interconversion of the active to the inactive hormones are the aldo-keto-reductases (AKRs) and the short chain dehydrogenase/reductases (SDRs), which both belong to the hydroxysteroid dehydrogenases (HSDs). Additionally, a smaller family, consisting of the 5α-steroid reductases play distinct roles in androgen formation. The balance between the inactive and active steroid hormones within the same class is important (e.g. cortisone and cortisol). However, the balance of active steroids from different classes (e.g. mineralocorticoids and glucocorticoids) also plays an important role in (patho)physiology. For example, glucocorticoids play a role in psychological stress and depression which are risk factors in androgen sensitive diseases, such as cancer and male and female infertility.

The first project described in my thesis investigated the impact of anabolic androgenic steroids (ASS) on the enzyme activity of 11β-hydroyxsteroid deydrogenase 2 (11β-HSD2). ASS are known to induce cardiovascular complications. The underlying mechanisms remain largely unknown. Using enzyme activity assays we observed that fluoxymesterone, a widely used ASS, potently inhibited 11β-HSD2-dependent inactivation of cortisol to cortisone. Furthermore, using LC-MS/MS we could show that fluoxymesterone is metabolized to 11-oxofluoxymesterone by human 11β-HSD2. Structural modelling revealed that the binding modes for fluoxymesterone and cortisol are similar, suggesting that fluoxymesterone may act as a competitive inhibitor of 11β-HSD2. No direct modulation of the mineralocorticoid receptor (MR) could be observed in transactivation assays. Since cortisol is able to potently activate the MR, we suggested that fluoxymesterone-induced inhibition of 11β-HSD2 could contribute to cortisol-induced MR activation, leading to electrolyte dysbalance and elevated blood pressure and subsequent cardiovascular disease development. The inhibitory potential of ASS in rat kidney microsomes and in cells expressing recombinant mouse 11β-HSD2 revealed a much weaker inhibition, revealing important species differences. This study unveiled potential pathways involved in adverse cardiac outcomes as a result of ASS misuse. It furthermore highlights the importance of species differences, especially within the field of steroidogenesis.

The second study presented in my thesis investigated the pathways involved in the generation and metabolism of androgens in Leydig cells. Currently, Leydig cell are needed to investigate the androgen axis, thus, their characterisation with respect to this axis is vital. Our investigation in two

important Leydig cell lines, the well-established MA-10 cells and the more recently established BLTK-1 cells, showed that there are marked differences regarding androgen metabolism between these two cell lines. Enzyme activity assays showed that 17β-hydroxysteroid dehydrogenase type 3 (17β-HSD3) -dependent formation of testosterone from androstenedione is not the predominant pathway in BLTK-1 cells. This observation was supported be the low expression of *HSD17B3* mRNA in BLTK-1 cells. We further investigated the specific pathway by which the BLTK-1 cells degrade androstenedione. Liquid chromatography/ mass spectrometry LC-MS/MS measurements confirmed that BLTK-1 cells predominately reduce androstenedione to androsterone via the intermediate metabolite, 5α-androstanedione. This alternative pathway is part of the "back-door" pathway, which ultimately leads to the formation of 5α-Dihydrotestosterone (DHT) and which has not been shown before in an established cell model. Under stress conditions, cells are able to switch pathways from the well-known 17β-HSD3-mediated androstenedione reduction to testosterone to the back-door pathway. In addition to characterizing the pathways in two different Leydig cell lines, we compared and tested different methodologies to specifically quantify androgen metabolites. Our results emphasize that for complex steroid matrices, LC-MS/MS measurement is the method of choice. Enzyme immunoassay need to be evaluated carefully (since they may show a high cross-reactivity, especially when metabolites of the back-door pathways are involved). Tin layer chromatography (TLC) should only be carried in validated two-dimensional or even in three-dimensional systems. Our study was able to demonstrate that the MA-10 and the BLTLK-1 cells both are valuable models. However, they should be used only for investigation a specific pathway.

In the third study presented in my thesis, we investigated the transcriptional regulation of the *HSD17B3* promoter. 17β-HSD3 is the key enzyme for testosterone formation of the front-door pathway. With the ultimate goal to identify compounds interfering with testosterone formation we constructed a MA-10 Leydig cell line stably expressing a 2.8 kilo base sequence of the putative human *HSD17B3* promoter under the control of a luciferase reporter gene. Using this tool, I carried out two projects:

A) We could show using transactivation assays, that TNF-α strongly activates the *HSD17B3* promoter via the p38 MAPK pathway. Importantly, this activation could not be reversed by the synthetic glucocorticoid dexamethasone. The results from our novel reporter assay were supported both on the mRNA-level and by enzyme activity measurements. The key conclusion from this study was the identification of a pathway which may link cancer-related inflammation with elevated testosterone levels, subsequently contributing to the growth and progression of androgen dependant tumors.

B) The mechanisms of imposex induction in aquatic organisms are still disputed. Using the screening tool described above, we showed that the retinoid X receptor (RXR) ligand 9-cis retinoic acid and specific organotins are able to activate the human *HSD17B3* promoter. This finding suggests that organotins exert pro-androgenic effects. We propose in a future study to address a possible link between two established yet controversial theories of imposex onset in aquatic organisms: the involvement of RXR and the elevation of testosterone levels.

In conclusion, the results presented in this thesis significantly extend our knowledge on the roles of SDRs and emphasize the importance of studying pre-rector regulation. Within the complex field of steroid measurement, this thesis provides important data and novel information. Further, the importance of considering species-specific differences when trying to extrapolate steroidigenic effects observed in animal models to humans is underlined.

# 2. Introduction

## 2.1 Steroid hormones

Steroids form a class of organic compounds derived from the hydrocarbon sterane (Cyclopentanoperhydrophenanthren). Steroids share a common characteristic structure, consisting of a chain of three cyclohexane rings (A-C) and one cyclopentane ring (D) (Figure 1). Steroids are widespread among animals, plants and fungi. Their biochemical roles are very diverse. Steroids can act as hormones, vitamins, bile acids, plant toxins and others. Synthetic steroids show a strong affinity to bind to nuclear receptors and are therefore popular scaffolds for drug research (Gupta *et al.*, 2013). The pharmacological applications of steroids cover antibiotic drugs, anticancer drugs, anti-inflammatory drugs and others. Furthermore, a large number of doping agents were synthesized from the steroid scaffold.

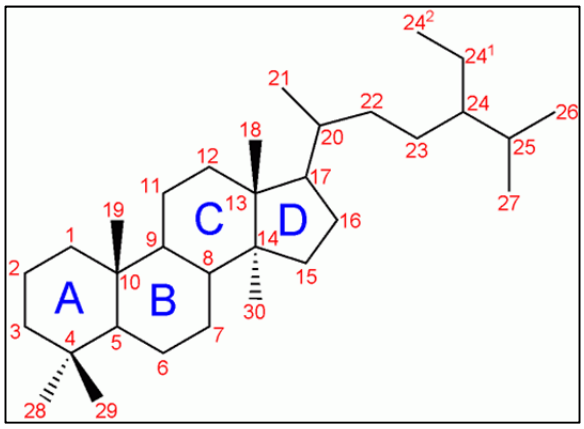


*Figure 1: Steroid structure including numbering according to IUPAC*
*http://www.chem.qmul.ac.uk/iupac/steroid/3S01.html*

Steroids acting as hormones are known as steroid hormones. Steroid hormones usually exist in a balance between their active and inactive forms. The active form can be produced either by endocrine or intracrine actions. Steroid hormones are produced from cholesterol in specific tissues referred to as the endocrine glands. In the human body, the major endocrine glands secreting steroid hormones are the adrenals and the gonads (ovaries and testes). After secretion, the hormones are delivered via the circulation to their specific site of action (Luu-The, 2013). This system of targeting receptors on cells distant from the site of hormone production is referred to as the endocrine system. In contrast, intracrine tissues are not able to transform cholesterol into active steroid hormones. However, intracrine tissues form active steroids from inactive precursors depending on the enzymes they express. Inactive precursors are found in the circulation and originate from endocrine glands and form a part of a homeostasic feedback system which balances the levels of inactive and active hormones in the body (Labrie *et al.*, 1997a; Labrie *et al.*, 1997b; Labrie *et al.*, 1997c).

Steroid hormones exert their action by binding to their cognate nuclear receptor (NR). Once the steroid (S) is bound to a NR, the receptor dimerizes, translocates into the nucleus where it directly binds to specific response elements (RE) on the DNA, which initiates the transcription of the corresponding target gene (Carson-Jurica *et al.*, 1990). This process is illustrated in Figure 2.

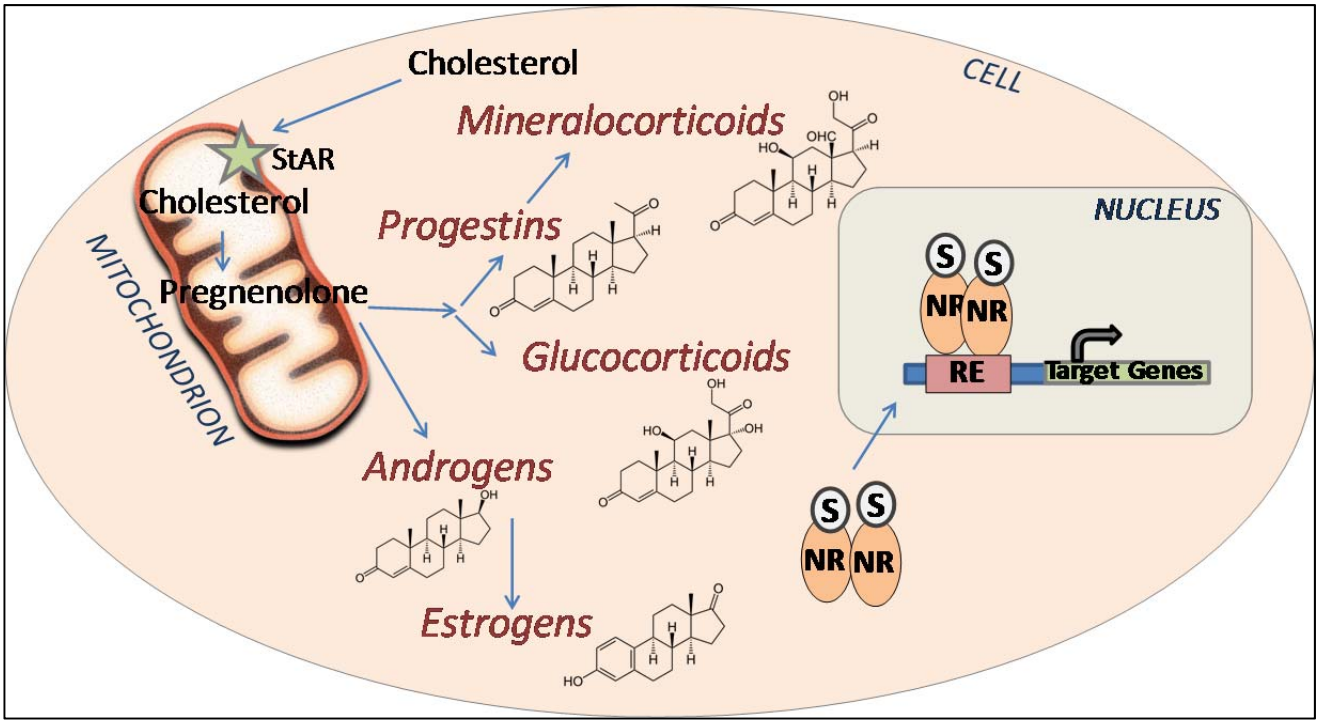


*Figure 2: Schematic overview of steroidogenic targeting.*

Steroids target specific nuclear receptors, therefore each individual steroids has a unique spectrum of activity. Steroid hormones are divided into at least five classes (Table 1), which include the major active and inactive forms (Miller, 1988).

| | **Scaffold (number of carbons)** | **Major inactive steroid** | **Major active steroid(s)** |
|---|---|---|---|
| **Glucocorticoids** | 21 | Cortisone (human)<br>11-Dehydrocorticosterone (rodent) | Cortisol (human)<br>Corticosterone (rodent) |
| **Mineralocorticoids** | 21 | *no inactive precursor steroid* | 11-Deoxycorticosterone<br>*and*<br>Aldosterone |
| **Androgens** | 19 | Androstenedione | Testosterone/<br>Dihydrotestosterone |
| **Estrogens** | 18 | Estrone | Estradiol |
| **Progestogens *or* Progestins** | 21 | Pregnenolone | Progesterone |

*Table 1: Overview of steroid classification*

## 2.2 Steroidogenesis

Steroidogenesis, the understanding and unveiling of the mechanism of biosynthesis of steroid hormones, is a highly competitive field within biochemical research. Major research efforts have focused on the cloning of steroidogenic enzymes, identifying their multiple isoforms, characterizing known and novel pathways responsible for the formation of active steroids and investigating tissue specific expression of steroidogenic enzymes in health and disease (Payne and Hales, 2004).

Figure 3 shows a schematic overview of the most abundant steroid hormones in humans and the enzymes responsible for their formation (Luu-The, 2013; Payne and Hales, 2004).

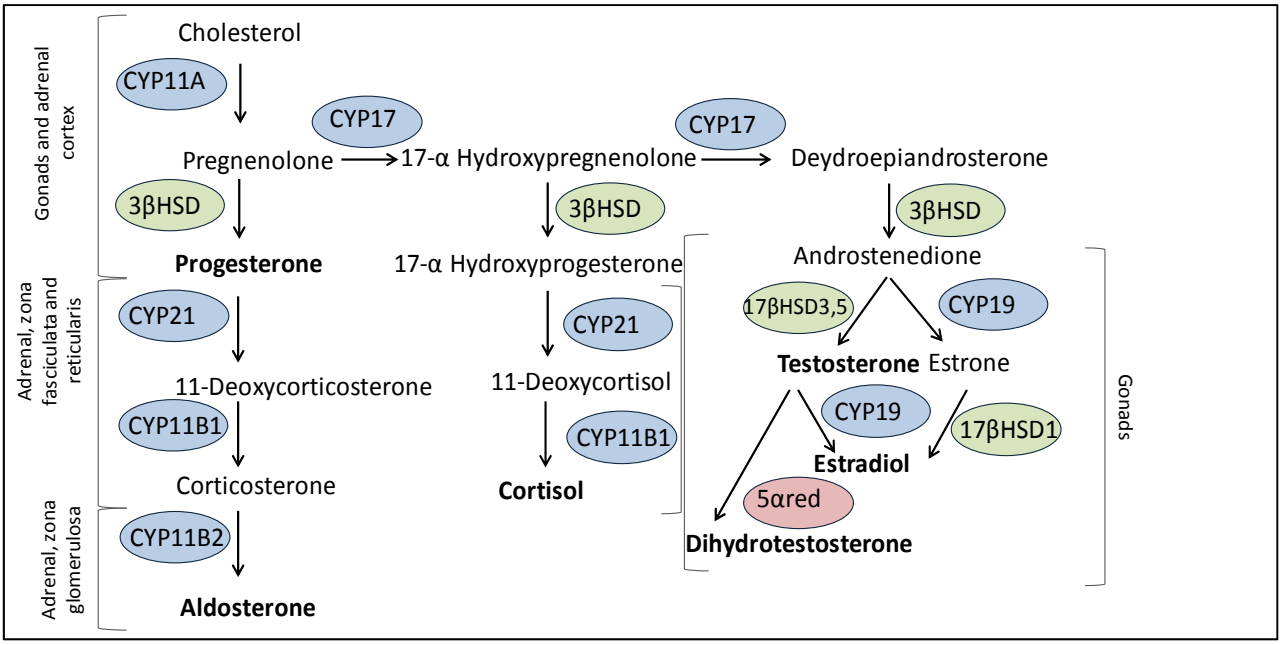


*Figure 3: Schematic overview of the steroidogenesis. The main active steroids are in bold, cytochromes are in blue, HSDs in green, and the 5α-steroid reductase is in red.*

The cytochrome P450 (CYP) enzymes involved in steroidogenesis are membrane bound proteins associated with either the mitochondrial membrane (CYP11A, CYP11B1, CYP11B2) or the microsomal endoplasmic reticulum (CYP17, CYP19, CYP21) (Nelson *et al.*, 1996). Hydroxysteroid dehydrogenases consist of a multitude of enzymatic superfamilies, three of which; aldo-ketoreductases (AKR) (17βHSD5), short chain dehydrogenases/ reductases (3β-HSDs and 17β-HSDs) and steroid 5α-reductases (5α-reductase 1 and 2) play an important role in steroidogenesis (Jornvall *et al.*, 1995; Krozowski, 1994; Stiles and Russell, 2010).

## 2.3 Steroid hormones in health and disease

The binding of steroids to their cognate nuclear receptor impacts on many physiological pathways including development, metabolism, immune system, psychology and behavior both for example, in health and disease (Knoedler and Denver, 2014). Isoforms of the glucocorticoid receptor (GR) are expressed ubiquitously in all types of tissues. Since glucocorticoids play important anti-inflammatory, anti-proliferative, pro-apoptotic and anti-angiogenic roles, synthetic derivatives of them are very effective in treating various diseases and have been a focus of pharmaceutical research for decades. Androgens play a key role in male sexual and reproductive development and health (MacLusky *et al.*, 1997; Marcell *et al.*, 2011). Additionally, androgens are key determinants in the prevention and development of cardiovascular disease (Mercuro *et al.*, 2010; Vitale *et al.*, 2009). The role of androgens and progestins in the development and progression of a variety of cancers are widely acknowledged, and are the focus of many research groups worldwide. The major role of aldosterone is the regulation of sodium and potassium homeostasis. Therefore, aldosterone is a key regulator of blood pressure and is implicated in many diseases involving the kidney such as aldosterone-dependent kidney damage (Zennaro *et al.*, 2013) (Wu *et al.*, 2009). A dysregulation of aldosterone levels results in blood pressure-independent pathologies such as myocardial fibrosis (Freel *et al.*, 2012) and diabetes mellitus (Reincke *et al.*, 2010). The pathological effects of steroid hormones arise due to a misbalance in the levels of active/ inactive hormones and /or an impaired receptor sensitivity and disturbed signaling. Furthermore, the mineralocorticoid receptor (MR) plays a role in the regulation of immune functions and an excessive activity has been associated with oxidative stress and inflammation (Odermatt and Kratschmar, 2012). To conclude, the enzymes activating and deactivating the steroid hormones play key roles in every aspect of development, health and disease.

## 2.4 Hydroxysteroid dehydrogenases are key pre-receptor control elements regulating the balance between active and inactive hormones

Hydroxysteroid dehydrogenases (HSDs) are enzymes which catalyze positional and stereospecific reactions on ketone or hydroxyl groups on the steroid hormone (Penning, 2011). In steroid hormone target tissues, HSDs co-exist in pairs which interconvert potent active steroids to the cognate inactive metabolite and vice versa. Therefore, HSDs act as an intracellular switch which regulates the amount of ligand available for the nuclear receptor (Labrie *et al.*, 2000; Penning, 2003). This selective control is maintained by the HSDs which function preferentially as either nicotinamid adenine dinucleotide phosphate hydrogen (NADPH)-dependent ketosteroid reductases or as $NAD^+$-dependent hydroxysteroid oxidases (Penning, 2011). The HSDs responsible for the interconversions belong to two large gene superfamilies; the aldo-keto reductases (AKRs) and the short chain dehydrogenase reductases (SDRs) (Bauman *et al.*, 2004) (Jornvall *et al.*, 1995). Figures 4a and 4b show the two superfamilies and how they function to control the cellular levels of nuclear receptor ligand availability.

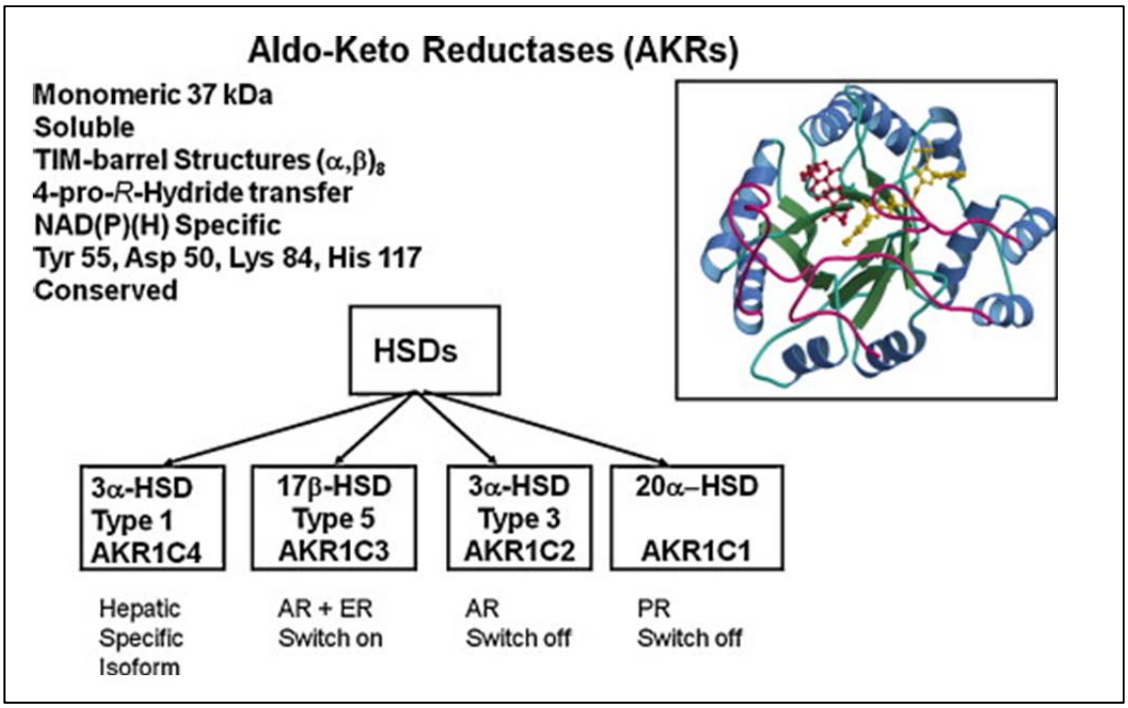


*Figure 4a: Overview of the AKR superfamily (Penning, 2011)*

*(used with permission from Elsevier)*

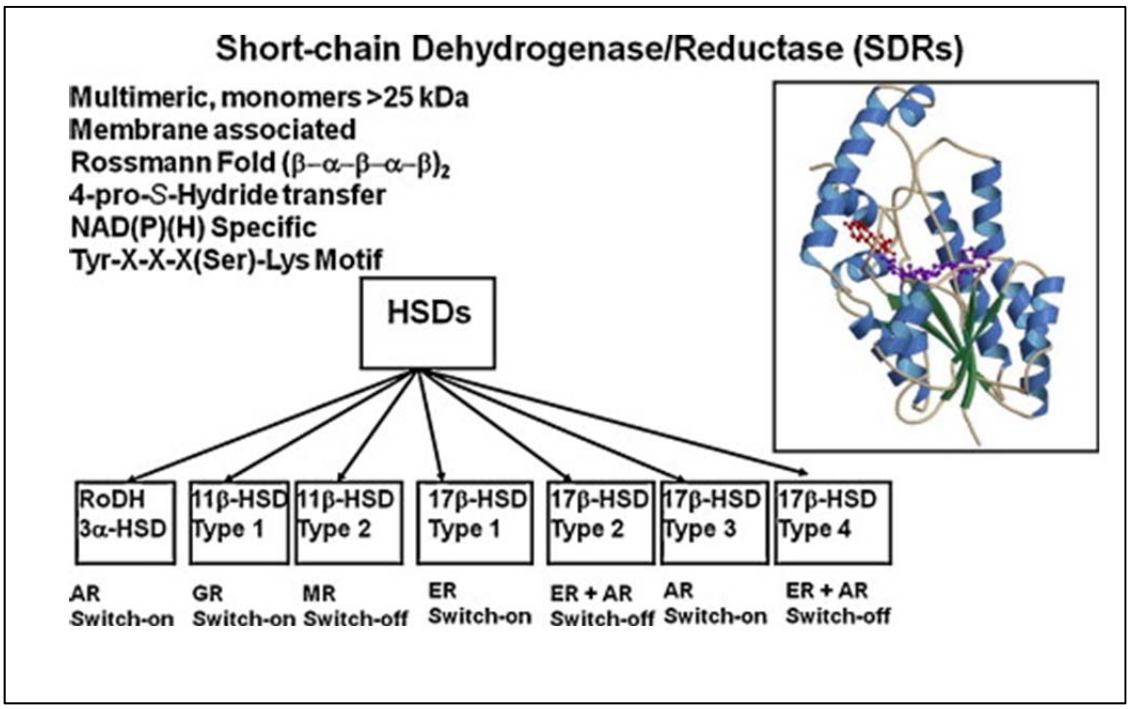


*Figure 4b: Overview of the SDR superfamily (Penning, 2011)*

*(used with permission from Elsevier)*

## 2.5 11β-Hydroxysteroid dehydrogenase type 1 and type 2

The interconversion of cortisone and cortisol in humans and of 11-dehydrocorticosterone and corticosterone and in rodents is catalyzed by the enzymes 11β-hydroxysteroid dehydrogenase (11β-HSD) type 1 and type 2, respectively (Funder *et al.*, 1988). The catalysis of the inactive steroid cortisone to the potent GR and MR agonist cortisol by 11β-HSD1 was first reported in 1953 (Amelung *et al.*, 1953). Initially, it was considered that the oxidation of cortisol and the reduction of cortisone were catalyzed by a single microsomal enzyme. More than 30 years later, in 1985, evidence confirmed the enzymatic oxidase and the reductase activity, were by two independent processes and therefore two enzymes were described (Lakshmi and Monder, 1985). 11β-HSD1 catalyzed the reduction of cortisone and 11β-HSD2 catalyzed the oxidation of cortisol. *In vivo*, 11β-HSD2 is most probably a strictly unidirectional enzyme. However, Rebuffat et al. could show that 11β-HSD2 is a reversible enzyme and is able to reduce 11-ketodexamethasone, a synthetic glucocorticoid to dexamethasone in HEK-293 cells transiently expressing 11β-HSD2 (Rebuffat *et al.*, 2004). *In vivo*, 11β-HSD1 acts predominantly as a reductase, however, in states of NADPH deficiency, it can exhibit dehydrogenase activity (Lavery *et al.*, 2006). Figure 5 shows the interconversion of cortisone and cortisol catalyzed by 11β-HSD1 and 11β-HSD2.

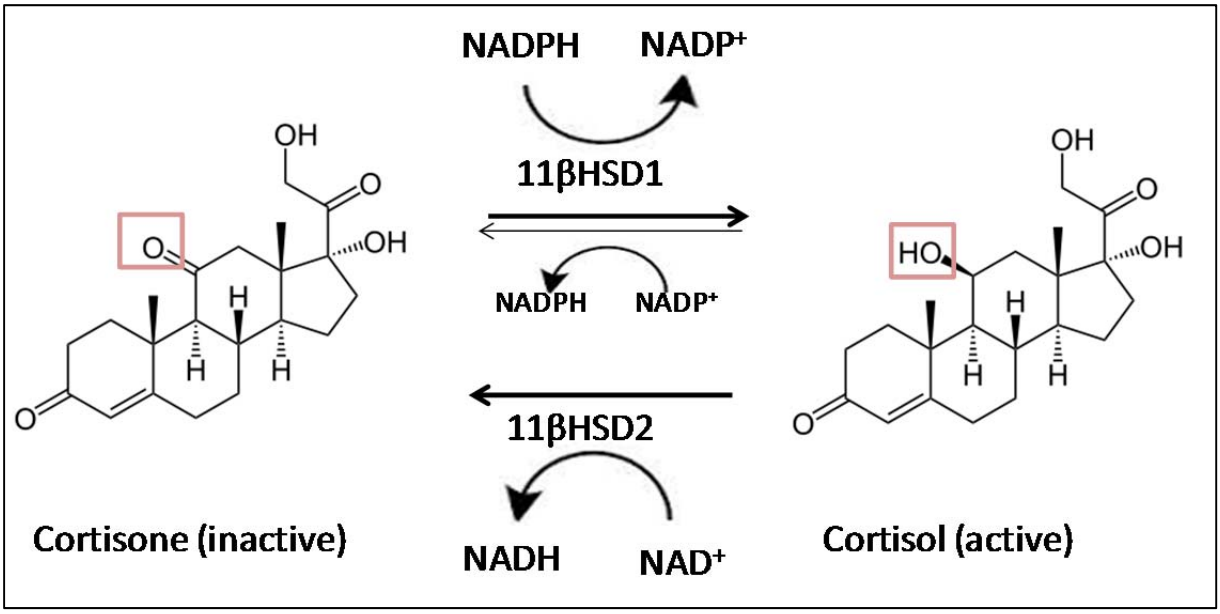


*Figure 5: Interconversion of cortisone and cortisol catalyzed by 11β-HSD1 and 11β-HSD2,*

### 2.5.1 Properties and expression of 11β-hydroxysteroid dehydrogenases

The two kinetically distinct forms of 11β-HSD (11β-HSD1 and 11β-HSD2) are differentiated by their metabolic directionality, cofactor specificity and tissue distribution. Cortisone shows no biological activity, explained by negligible affinity for GR and MR (Agarwal *et al.*, 1989; Tannin *et al.*, 1991; Walker *et al.*, 1992). 11β-HSD1 expression and activity is present in most tissues, but is most abundant in the liver. The $K_m$ of 11β-HSD1 for cortisone is within the micromolar range, which is much higher than that of 11β-HSD2 for cortisol, which is in the nanomolar range. 11β-HSD1 oxidizes the reduced form of NADPH. 11β-HSD1 knockout mice show elevated levels of corticosterone (corticosterone in rodents is the equivalent to cortisone in human). These mice show reduced glucocorticoid responses, including a strong down regulation of gluconeogenesis following fasting. The

lipid profiles in the 11β-HSD1 knockout mice were improved, and there was no effect on blood pressure reported (Holmes *et al.*, 2001; Kotelevtsev *et al.*, 1997; Morton *et al.*, 2001). In contrast, the expression of 11β-HSD2 is limited. Immunohistochemical studies show that 11β-HSD2 is localized in a distinct set of organs and tissues which are primarily involved in the transepithelial transport of sodium such as the distal renal tubules (mainly cortical collecting duct), distal colon, sweat and salivary glands. In addition, 11β-HSD2 is also expressed in the vascular wall and in the placenta (Albiston *et al.*, 1994; Benediktsson *et al.*, 1993; Krozowski *et al.*, 1995; Naray-Fejes-Toth *et al.*, 1991).

### 2.5.2 11β-Hydroxysteroid dehydrogenase type 2 is co-expressed with the mineralocorticoid receptor in epithelial cells

In 1983, Krozowski and Funder showed that the *in vitro* affinities of the MR towards aldosterone and cortisol were identical (Krozowski and Funder, 1983). However, in contrast, the MR in specific tissues is selective for aldosterone. Edwards et al. later showed that the organs with aldosterone selectivity such as the kidney have much higher levels of 11β-HSD2 than MR expressing tissue with no aldosterone selectivity (Edwards *et al.*, 1988). It soon became clear that there are two distinct types of MR expressing tissues: Firstly, tissues co expressing 11β-HSD2 and MR; the role of 11β-HSD2 in these tissues is to serve as a “gate-keeper” to maintain aldosterone selectivity and to protect the MR from high glucocorticoid concentrations (Edwards *et al.*, 1988; Funder *et al.*, 1988); secondly, tissues in certain brain regions (hippocampus), immune cells (microglia, macrophages) and others, where the MR function is mainly regulated by glucocorticoids (Roland *et al.*, 1995) (Odermatt and Kratschmar, 2012).

### 2.5.3 11β-Hydroxysteroid dehydrogenases 1 interacts with hexose-6 phosphate dehydrogenase

A further level of tissue-specific glucocorticoid regulation was revealed following the discovery that hexose-6 phosphate dehydrogenase (H6PDH) plays an important role in the determination of the reaction direction of 11β-HSD1 (Atanasov *et al.*, 2004; Banhegyi *et al.*, 2004). It was shown that in tissues where 11β-HSD1 is co-expressed with H6PDH, such as the liver, adipose tissue or skeletal muscles (Gomez-Sanchez *et al.*, 2008), 11β-HSD1 functions as a reductase. H6PDH is a key enzyme in the pentose phosphate pathway in the endoplasmic reticulum and catalyzes the reaction of glucose-6-phosphate to 6-phosphogluconolactone to generate NADPH (Mason *et al.*, 1999; Ozols, 1993). Dzyakanshik et al showed in microsomal preparations of HEK-293 cells expressing 11β-HSD1 that a $NADPH/NADP^+$ ratio of ten or higher was required for efficient 11β-HSD1 reductase activity (Dzyakanchuk *et al.*, 2009). Such observations suggest that an intraluminal change in the $NADPH/NADP^+$ redox coupling causes a shift in the activity of 11β-HSD1 from reductase to dehydrogenase.

### 2.5.4 11β-Hydroxysteroid dehydrogenases in health and disease

Glucocorticoids are involved in almost every aspect of development, physiologically and psychologically, and in health as well an in disease. Cortisol is involved in lipid synthesis and it plays an important role in carbohydrate metabolism and protein turnover. Glucocorticoids are key regulators of stress responses, neuronal functions, cell growth and differentiation, blood pressure and immune functions (Sapolsky *et al.*, 2000). The importance of a functioning glucocorticoid regulatory system is

demonstrated in the GR knock-out mice, which die within hours after birth (Cole *et al.*, 1995). Beyond the primary function of 11β-HSD1 as a key switch to provide active glucocorticoid hormones and modulate local glucocorticoid dependent gene expression, 11β-HSD1 acts as key enzyme in the regulation of metabolic functions. Elevated 11β-HSD1 activity has been associated with metabolic disorders. Obesity studies in genetically obese rodents (Zucker rats and Leptin-deficient *(ob/ob)* mice) have shown that 11β-HSD1 mRNA and enzyme activity are significantly increased in adipose tissue. (Livingstone *et al.*, 2000; Masuzaki *et al.*, 2001). These findings could be confirmed in obese humans (Rask *et al.*, 2002). It has also been shown, in contrast, the levels of hepatic 11β-HSD1 mRNA and activity are reduced (Rask *et al.*, 2002; Stewart *et al.*, 1999). Transgenic mice with adipose specific 11β-HSD1 overexpression were generated to study the adverse effects of 11β-HSD1 up regulation (Masuzaki *et al.*, 2001). These mice showed a marked insulin resistance, glucose intolerance, elevated levels of free fatty acids and triglycerides, apparent leptin resistance and angiotensin overexpression driven hypertension (Masuzaki *et al.*, 2001; Masuzaki *et al.*, 2003). All these findings support the research carried out in 11β-HSD1 knock-out mice. 11β-HSD1-deficient mice are resistant against hypoglycemia which normally appears upon stress or high fat diet (Kotelevtsev *et al.*, 1997). Generally, 11β-HSD1-deficient mice show a cardioprotective phenotype, with reduced serum triglycerides and raised high density lipoprotein cholesterol (Morton *et al.*, 2001). Rare human cortisone reductase deficiency is presented by excessive adrenal androgen production as a consequence of enhanced metabolic clearance of cortisol and compensatory HPA axis activation (Phillipov *et al.*, 1996). Drugs selectively targeting 11β-HSD1 for cardioprotective effects are currently being investigated (Barf *et al.*, 2002). However, the role of 11β-HSD1 seems to be diverse and is yet not fully understood. Accordingly, 11β-HSD1 inhibition might bear risks and lead to unwanted effects.

Deficiency of renal 11β-HSD2 allows glucocorticoids to constantly activate the MR in the distal tubule. This deficiency, which is named syndrome of apparent mineralocorticoid excess (SAME) (Ulick *et al.*, 1979) results in sodium retention, severe hypertension and hypokalemia (Edwards *et al.*, 1988; Stewart *et al.*, 1988; Stewart *et al.*, 1987). To have a model for studying this severe hypertensive condition, Kotselev et al. produced 11β-HSD2 knockout mice. (Kotelevtsev *et al.*, 1999) Fifty percent of the homozygous mice died within two days following birth, often due to sudden cardiac arrest. Surviving adult animals suffered multiple conditions caused by severe hyperkalemia (Kotelevtsev *et al.*, 1999), the most distinct phenotype being hypotonic polyuria (Berl *et al.*, 1977). Blood pressure was elevated markedly in both, male and female 11β-HSD2-deficient mice.

Besides the roles discussed above, within the regulation of the metabolism, homeostatic processes and energy supply, 11β-HSD1 is an important enzymes in the central nervous system. Glucocorticoids affect neurotransmitters, receptors, channels and metabolism in the brain (De Kloet *et al.*, 1991). Some regions of the brain such as the hippocampus, are especially sensitive to glucocorticoids (McEwen, 1999). 11β-HSD1 is highly expressed in these areas, whereas 11β-HSD2 is almost absent (Robson *et al.*, 1998). Aging 11β-HSD1-deficient mice have better learning abilities compared with aged-matched wild type mice (Penning, 2011). It is hypothesized, that the reduced interhippocampal corticosterone levels in the knock-out mice may be the reason for the improved learning abilities (Penning, 2011).

## 2.6 Focus on the short chain dehydrogenase/reductase family member 17β-hydroxysteroid dehydrogenase type 3

### 2.6.1 Properties and expression of 17β-hydroxysteroid dehydrogenase type 3

In 1994, Geissler et al reported the isolation of the cDNA and the gene encoding for the short-chain dehydrogenase/reductase 17β-Hydroxysteroid dehydrogenase type 3 (17β-HSD3) (Ulick *et al.*, 1979). They were the first to show that 17β-HSD3 is a testicular enzyme which catalyzes the reduction of the weak androgen Δ4-androstene-3, 17-dione to the potent androgen testosterone utilizing NADPH as cofactor. Testosterone can be further converted to dihydrotestosterone (DHT) by members of the 5α-reductase family (Stewart *et al.*, 1988) (Figure 6).

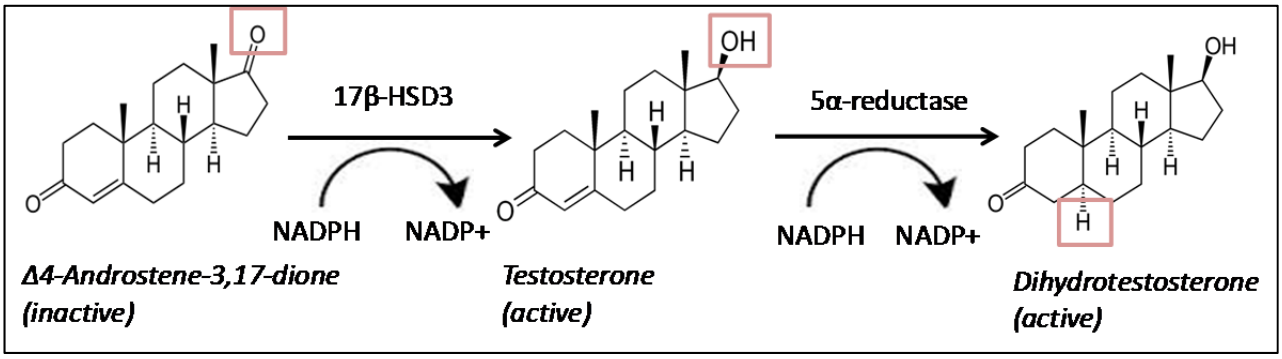


*Figure 6: Conversion of androstenedione into testosterone and dihydrotestosterone.*

The target receptor of testosterone, the androgen receptor (AR), is unique among the hormone receptors because it has two biologically high affinity binding hormones that are different in their physiological potency (Stewart *et al.*, 1987). Testosterone, the product of 17β-HSD3 and 17β-HSD5 (organ specific expression) is the major androgen in muscle and shows anabolic properties starting in puberty. DHT also has its own distinct tasks during male development. Human male fetal genital development is impaired in individuals with normal testosterone levels but no DHT expression, the result of a genetic defect in 5α-reductase (Bormann *et al.*, 2011).

Often conversion from androstenedione to testosterone is determined as a direct measure for 17β-HSD3 expression. This method is flawed, since is does not distinguish between the conversion of androstenedione to testosterone by 17β-HSD3 and 17β-HSD5. A decade ago, it was believed that 17β-HSD3 is exclusively expressed in the Leydig cells of the testis (Geissler *et al.*, 1994; Payne and Hales, 2004). More recent reviews provide evidence that 17β-HSD3 is predominately, but not exclusively expressed in the testis (George *et al.*, 2010). An interesting study carried out by Corbould et al. investigated whether 17β-HSD3 is co expressed with CYP19 (aromatase) in preadipocytes in women and whether the expression pattern of these two enzymes is different in subcutaneous versus intra-abdominal adipose tissue (Corbould *et al.*, 2002). They observed the presence of 17β-HSD3 mRNA in preadipocytes but not in mature adipocytes.

Interestingly, generalized obesity (Body mass index (BMI) as measure) or central obesity (judged by waist circumference) was correlated with the ratio of 17β-HSD3 mRNA: aromatase mRNA. This effect differed in adipose tissue derived from abdominal and intra-abdominal sites. A study carried out in

women showed an expression pattern suggesting that obesity is associated with a decline in 17β-HSD3 mediated testosterone production in subcutaneous abdominal adipose tissue and an increase in 17β-HSD3 mediated testosterone production in the intra-abdominal adipose tissue (Corbould *et al.*, 2002). 17β-HSD3 is also expressed in the brain, in sebaceous glands and in bones (George *et al.*, 2010).

### 2.6.2 17β-Hydroxysteroid dehydrogenase type 3 deficiency

Testosterone is produced in the Leydig cells of the male embryo as early as eight weeks of gestation (Jost, 1970). It is primarily used to stabilize the Wolffian duct (Tong *et al.*, 1996), an embryonic structure forming the epididymis, vas deferens and the seminal vesicles. Later, testosterone and DHT induce the formation of the male genitalia (penis, prostate, urethra and scrotum) (Wilson, 1978). 17β-HSD3, 5α-reductase and AR are key elements in the production and the signaling of testosterone and DHT and dysregulation in any of these factors, including 17β-HSD3, can result in the 46 XY disorder of sex development (DSD) (George *et al.*, 2010). The definition of DSD is quite broad and includes any congenital condition in which the development of chromosomal, gonadal or anatomical sex is atypical (Houk *et al.*, 2006; Hughes *et al.*, 2006). DSDs are characterized into three distinct categories; sex chromosome DSD, 46 XX DSD and 46 XY DSD. The designation 46 XY DSD was proposed to replace the former term of *male pseudohermaproditsm,* according to the consensus statement (Houk *et al.*, 2006; Hughes *et al.*, 2006). Commonly, patients with the 46 XY DSD suffer from androgen insensitivity syndrome. However, a small number of 46 XY DSD patients have deficiencies in 17β-HSD3 or 5α-reductase (George *et al.*, 2010). A deficiency of 17β-HSD3 leads to an autosomal recessive form of 46 XY DSD which was first described in 1971 by Saez et al.(Saez *et al.*, 1971; Saez *et al.*, 1972). Mutations in the *HSD17B3* gene can lead to a broad spectrum of 46 XY DSD, ranging from completely undervirilized external female genitalia, ambiguous to predominantly male genitalia with a micropenis and hypospadias (birth defect of urethra) (Boehmer *et al.*, 1999). Twenty seven different mutations in the *HSD17B3* gene have been identified and characterized (Mains *et al.*, 2008). 17β-HSD3 mutations range from missense mutations, exonic deletions and mutations within the intrinsic splice site (George *et al.*, 2010). 1 in 5000 people worldwide carry DSD mutations, a figure surprisingly high (Sax, 2002; Thyen *et al.*, 2006). 17β-HSD3 deficiency is present in about 1 in 147'000 newborns in Europe (Boehmer *et al.*, 1999). Among the Gaza Strip Arab population, the prevalence is reported to be as high as 1 in 100-300 newborns because of the high consanguinity (Rosler, 2006; Rosler *et al.*, 1996).

Only about 50% of the children suffering from 46 XY DSD get a definitive diagnosis, therefore, it is difficult to determine the true prevalence (Hughes *et al.*, 2006). The clinical presence at birth is often atypical, since female external genitalia are present (Rosler, 2006). Therefore, the phenotype of the mutation often remains unnoticed at birth. The newborns are usually registered as females and it is not until adolescence that they are correctly diagnosed (Rosler, 2006; Twesten *et al.*, 2000). At the time of puberty, DSD patients show varying degrees of virilisation, including development of a male body habitus, increased body hair and a deepening of their voice (Lee *et al.*, 2007). The impaired late onset development of the penis may be due to peripheral conversion of testosterone (Balducci *et al.*, 1985). The female to male gender change is relatively frequent in 46 XY DSD sufferers, at approximately 39-

64 % (Hiort *et al.*, 2003). If the 17β-HSD3 deficiency is discovered early, patients commonly undergo gonadoectomy in childhood (prepubertally) in order to and maintain the female phenotype and gender role (Bertelloni *et al.*, 2009).

## 2.7 Focus on the aldo-keto reductase family member 17β-hydroxysteroid dehydrogenase type 5 (AKR1C3)

*In vitro*, 17β-HSD5 catalyzes the interconversion of the alcohol group to the carbonyl group and vice versa at position 17 on the steroid backbone. Due to the high ratio of the cofactor NADPH to $NAD^+$ 17β-HSD5 mainly acts as a reductase *in vivo* (Penning *et al.*, 2000). Lin et al. cloned 17β-HSD5 from a human prostate cDNA library and proposed that the recombinant enzyme reduced the potent androgen 5α-DHT to the weaker androgen 3α-androstanediol (Lin *et al.*, 1997). The reverse reaction could not be shown. Later, Dufort, Luu-The and others showed that 17β-HSD5 catalyzes the same reaction as 17β-HSD3; the reduction from the weak androgen androstenedione to the potent androgen testosterone (Dufort *et al.*, 1999; Labrie *et al.*, 1997c). Furthermore, they showed that the activity of 17β-HSD5 is destroyed upon homogenization of cells or tissue samples and therefore suggested that the 17β-HSD5 enzymatic reaction proposed by Lin et al. was incorrect. Dufort et al. hypothesized that the reaction observed by Lin et al was instead catalyzed by 3α-HSD. This hypothesis was later confirmed (Luu-The *et al.*, 2001; Qin *et al.*, 2006) and it is now generally accepted that 17β-HSD5, in addition and equally to 17β-HSD3, catalyzes the reaction from androstenedione to testosterone. However, 17β-HSD5 and 17β-HSD3 differ in their tissue distribution. 17β-HSD3 is mainly expressed in the testis, whilst 17β-HSD5 is mainly present in the prostate and the mammary glands (Penning *et al.*, 2000). Other tissues where 17β-HSD5 is expressed include lung, liver, uterus, small intestine, brain and to a very low extent in testis (Penning *et al.*, 2000).

### 2.7.1 The role of 17β-hydroxysteroid dehydrogenase type 5 (AKR1C3) in prostate cancer

Prostate cancer is amongst the most frequently diagnosed cancers worldwide and one of the leading causes of cancer-related deaths in men worldwide (Jemal *et al.*, 2011). Although there are still many questions to be answered in respect to the initiation and progression of prostate cancer, two major mechanisms, which both result in the activation of the androgen axis have been studied extensively (Knudsen and Penning, 2010). The first mechanism involves the over-activation of AR by amplification, overexpression or mutations of the AR (Lapouge *et al.*, 2008; Reddy *et al.*, 2006). The second mechanism is focuses on AR ligands and involves either their *de novo* synthesis or the increased enzymatic conversions to active androgens (Hofland *et al.*, 2010; Pfeiffer *et al.*, 2011). Since 17β-HSD5 is highly expressed in the prostate, it plays an important role in the synthesis of testosterone which is a strong stimulus for growth, activation, proliferation and metastasis of prostate cancer cells. Furthermore, upon inhibition of the 5α-reductase by finasteride, a cornerstone drug to treat prostate cancer, it was shown *in vitro* in the prostate cancer cell line LnCap that 17β-HSD5 was up regulated (Pfeiffer *et al.*, 2011). This cellular adaption process in response to testosterone and DHT deprivation can result in a resistance to the 5α-reductase inhibitor finasteride (Byrns *et al.*, 2012). Increased angiogenesis markers and proliferation in the prostate cancer cell line PC-3 was shown by Dozmorov et al.(Dozmorov *et al.*, 2010). Several studies have reported a strong up regulation of 17β-

HSD5 in different types of prostate cancers (Fung *et al.*, 2006; Nakamura *et al.*, 2005). In conclusion, the evidence shows that there is a correlation between the expression levels of 17β-HSD5 and the progression of prostate cancer, however, the distinct mechanism of action linking these observed correlations remain unclear and will be in the focus of many researchers in the future.

## 2.8 The pleiotropic effects of testosterone

Testosterone, the product of the reduction of androstenedione catalyzed by the enzymes 17β-HSD3 and 17β-HSD5, exerts a vast amount of biological actions and plays key roles in many physiological and pathological processes throughout life, starting in the early embryonic phase. Testosterone levels in plasma or serum vary over three orders of magnitude depending on age, gender and health (Rosner *et al.*, 2007). Testosterone levels also follow a circadian rhythm, therefore, fixed standard levels do no not exist (Rosner *et al.*, 2007). Only 1-3 % of the circulating testosterone is free, the rest is plasma protein bound and there is no convention whether the free or the bound testosterone is the more useful measure (Rosner *et al.*, 2007). Mean total testosterone levels in healthy, middle aged men are between 300 and 600 ng/dl (10.4-20.8 nM/ L). Corresponding female levels are between 20 and 40 ng/dl (0.7-1.4 nM/L).

### 2.8.1 Male embryonic development is testosterone dependent

Normal gonad and genitalia development takes place in three sequential stages; the undifferentiated stage where XX and XY embryos show the same structures, the gonadal determination phase and finally the phase where internal and external genitalia are differentiated (McLachlan *et al.*, 2002). In the presence of testicular hormones (mainly testosterone and DHT) the differential phase is driven towards the male development pathway. Jost's (Jost, 1970) conclusions from very early experiments have been confirmed by recent observations: The adequate production and action of testosterone is the key factor for the development and differentiation of the internal and external genitalia in males (Walker, 2009).

### 2.8.2 Male fertility and sexual health is driven by testosterone

Male fertility is primarily dependent on spermatogenesis. Testosterone is essential for the production of spermatozoa (McLachlan *et al.*, 2002). Most testosterone is produced locally by Leydig cells. Testosterone levels in adult testis is remarkably stable (Walker, 2009). Besides its key role in fertility testosterone is also responsible for many different aspects of sexual function, such as sexual desire and erectile functions (Finkelstein *et al.*, 2013).

### 2.8.3 The role of testosterone in metabolism

Testosterone, besides its most obvious and known roles in fertility and sexuality, is an important regulator of carbohydrate, fat and protein metabolism (Kelly and Jones, 2013). The metabolic syndrome, which is defined as a cluster of risk factors for cardiovascular diseases and diabetes mellitus type 2 (DMT2), is an emerging problem worldwide (Alberti *et al.*, 2009). Following a joint statement of international diabetes and heart associations, to be diagnosed with the metabolic syndrome, patients must exhibit a minimum of three of the following criteria (Alberti *et al.*, 2009):

- Elevated waist circumference (ethnic-specific)
- Elevated triglycerides (≥ 150 mg/dL (1.7 mmol/L) or drug treatment for elevated triglycerides
- Reduced high density lipoprotein (HDL) (in males ≤ 50 mg/dL (1.3 mmol/L), in females ≤ 40 mg/dL (1.0 mmol/L)) or drug treatment for reduced HDL
- Elevated blood pressure (systolic ≥ 130 and/or diastolic ≥ 85 mm Hg) or antihypertensive drug treatment in a patient with a history of hypertension
- Elevated fasting glucose (≥ 100 mg/dL) or treatment for elevated glucose

The metabolic syndrome is a condition recognized by the World Health Organization (WHO) to be associated with an increased risk of myocardial infarction, stroke and cardiovascular death. The central biochemical defect associated with the metabolic syndrome and DMT2 is a reduced sensitivity to insulin, known as insulin resistance (Alberti *et al.*, 2009).

Epidemiological studies show, that up to 40% of men with DMT2 have testosterone deficiency and that endogenous, both total and free testosterone was lower in men with metabolic syndrome compared to healthy men (Corona *et al.*, 2011). Despite the focused research on this topic, the relationship between testosterone and metabolic syndrome is not yet fully understood. It seems that there is a bidirectional effect of this disease, where obesity induces androgen deficiency and hypogonadism (associated with low androgen levels) contributes to obesity (Cohen, 1999; Laaksonen *et al.*, 2005).
Cardiovascular disease (CVD) linked with the metabolic syndrome is a major cause of morbidity and mortality in older men (Shores *et al.*, 2014). CVD in elder man may be related to decreased testosterone levels, which are associated with the metabolic syndrome (Haring *et al.*, 2011; Muller *et al.*, 2005; Svartberg *et al.*, 2006). Although the evidence suggests, that low levels of testosterone are associated with risk factors, it was not known whether low testosterone is independently associated with a higher risk for CVDs. To investigate this question, Shores et al. recently published a longitudinal cohort study to evaluate whether total testosterone, unbound (free) testosterone, DHT and unbound (free) DHT were associated with incident CVD and mortality in men who were free of CVD at the time of blood collection (Shores *et al.*, 2014). The study showed that total and free testosterone were not associated with CVD events while the levels total and free DHT showed curvilinear associations (high risk at low levels, lower risk at medium levels, high risk at high levels).

### 2.8.4 Testosterone and prostate cancer - a paradigm shift

It has been more than 40 years since the relationship between serum testosterone levels and prostate cancer progression was first described by Hodges and Huggins (Huggins and Hodges, 1972). Androgen deprivation therapy is today the cornerstone of treatment for men with advanced prostate cancer. Testosterone replacement therapy in elder man is therefore contraindicated if prostate cancer is diagnosed (Apostolova *et al.*, 2005; Legeza *et al.*, 2013).

However, in multiple longitudinal studies, an association between prostate cancer and endogenous testosterone levels has never been found (Dohle *et al.*, 2003). Multiple studies in animal systems show a dose response curve for prostate growth with respect to androgen concentrations (Dohle *et al.*,

2003). However, at higher concentrations, a plateau in prostate tissue growth is reached and any further increase in androgen concentration produces little or no further growth (Dohle *et al.*, 2003). Overall, there is no evidence to support the long standing assumption that high circulating testosterone levels directly contribute to the development of prostate carcinoma (Giovanni Luca *et al.*, 2013). However, prostate cancer tumors are extremely sensitive to variations in testosterone concentrations in the serum. There is evidence, that prostate cancer behaves in an androgen dependent manner at low testosterone concentrations and becomes androgen indifferent at higher concentrations. Saturation models describing the relationships of prostate tumor growth and testosterone concentrations proposed by Morgentaler and Traish (Helsen *et al.*, 2014) are primarily based on the finite capacity of the AR to bind androgens (Wilson, 1975; Wilson and Walker, 1969).

### 2.8.5 The role of testosterone in male breast cancer is not yet understood

The role of testosterone in male breast cancer development in not yet fully understood (Samson *et al.*, 2010). Several studies and case reports indicate a relationship between breast cancer and testosterone levels (M. Samson, 2010; Samson *et al.*, 2010). It is hereby important to mention, that especially the amount of testosterone produced locally (intracrine) within the breast cancer tissue is critical for the growth of the tumor (Jobling *et al.*, 2002; Labrie *et al.*, 2000). Furthermore, it was suggested that high testosterone levels may lead to an increase in aromatization and therefore to higher estradiol levels. Estradiol may stimulate the estrogen receptor (ER) in the breast tissue and increase the risk of male breast cancer (Andersson and Russell, 1990). These aspects are also relevant regarding triple-negative, AR positive breast cancer in women.

### 2.8.6 Testosterone and cognition

In respect to the increasing life expectancy worldwide, age-related dementia is a growing problem. Age related cognitive decline and dementia is not fully understood. Serum testosterone decreases with age in men (Auchus, 2004). From the age of 30 years, on average per year, total testosterone decreases by 0.2- 1% and free testosterone by 2-3% (Kamrath *et al.*, 2012). There is evidence that sex hormones such as testosterone or estradiol can exhibit protective functions in the brain, especially in regions which are known to have a high density of AR (Attard *et al.*, 2012; Mahendroo *et al.*, 2004). Through its binding to AR, testosterone or its downstream metabolite estrogen act as neuroprotectors by modulating neuronal damage caused by oxidative stress (Rahman and Huhtaniemi, 2004) as well as reducing neuronal apoptosis (Saez, 1994). Oxidative stress and apoptosis both play important roles in dementia. Clinical trials performed by Cherrier et al., examining the effects of testosterone supplementation on memory function in an eugonadal man showed a significantly improved memory and visuo-spatial function compared to the placebo groups (Baek *et al.*, 2006; Nagpal *et al.*, 1994). In other studies, curvilinear associations between free testosterone levels and working memory (McEwan, 2013), verbal memory (Mangelsdorf *et al.*, 1995) and attention (Khorasanizadeh and Rastinejad, 2001) have been found, suggesting the beneficial effects of testosterone at the appropriate doses. Overall, there is growing evidence to support the hypothesis that testosterone exerts neuroprotective effects and exhibits positive effects on cognition and memory. However, the results are not conclusive since it appears that serum levels of testosterone in relation to its beneficial

effects are curvilinear, with the beneficial effects of testosterone shown at medium serum concentrations, whereas there are no beneficial effects at low and high levels of testosterone.

### 2.8.7 The Janus face of testosterone when it comes to muscles, bones and strengths - clinic and doping

Testosterone is a member of the anabolic androgenic steroids (AAS) which are known to promote muscle building. In myocytes, testosterone or its synthetic derivatives bind to the AR initiating a cascade of anabolic processes. At a supraphysiological dose of 300 mg testosterone enanthate per week, muscle strength and endurance are markedly increased in healthy young men after only three weeks compared with placebo (Yamana, 2010). Testosterone therapy is used in the clinic to aid muscle building and development in primary and secondary hypogonadism. It has been shown that in men with pulmonary diseases (Shores *et al.*, 2014) or in elderly men in rehabilitation (Muller *et al.*, 2012) that muscle strength or physical function improved after testosterone replacement therapy. In concordance with this finding, two placebo controlled trials in frail, elderly men and in men with limited mobility showed beneficial effects both on leg- and chest-press as an indication of muscle strength, upon testosterone administration compared to the placebo group (Davis *et al.*, 2011; Rosenfield and Otto, 1972). In contrast, no improvement in muscle strength could be observed in two placebo controlled studies in *healthy* elderly men (Davis *et al.*, 2011; Zouboulis *et al.*, 2007). Focusing on strength and fitness, testosterone also plays an important role in the maintenance of bone density in man. Low androgen levels caused by hypogonadism or androgen deprivation therapy is clearly related to a higher incidence of osteoporosis and rapid bone loss (Stepan *et al.*, 1989). Studies have shown bone structural parameters such as the microarchitecture and bone mineral density are impaired in men with hypogonadism (Benito *et al.*, 2003). In men with low testosterone levels at the start of the study, significant increased lumbar spine bone density was observed (Zouboulis *et al.*, 2007). However, another clinical trial in men with testosterone levels in the low-normal range showed that testosterone replacement therapy did not change bone density (Davis *et al.*, 2011). It appears that the muscle strength, endurance and bone density can only be positively influenced by testosterone replacement therapy if the endogenous testosterone level is lower than the normal, age dependent value.

Despite the known and lethal side effects, testosterone and its derivatives are very popular doping agents. They are historically the “first” doping agents introduced in the 1950s to enhance performance among weightlifters (Duntas and Popovic, 2013). In later chapters of this thesis, I will introduce ASS in more detail.

# 3. Project 1:
# Evaluation of anabolic androgenic steroids regarding their effect on 11β-hydroxysteroid dehydrogenase 2-dependent glucocorticoid inactivation

## 3.1 Introduction anabolic androgenic steroids

Anabolic androgenic steroids (AAS) are synthetic steroids, derivatives of the male sex hormone testosterone (androgen), which promote the development of male sexual characteristics (androgenic effect) and growth of skeletal muscle (anabolic effect). Since the fifties when AAS were first introduced and used predominately by weight-lifting top athletes, there has been a huge drive to modify and improving these substances. Today, more than 1000 testosterone derivatives are known. Since testosterone undergoes a rapid hepatic metabolism when administered orally, a lot of new compounds that show improved bioavailability and metabolic stability were designed (van Amsterdam *et al.*, 2010).

According to their metabolic and chemical properties, AAS are classified into three groups (Hall, 2005):

a) Class A: 17β-hydroxylated testosterone esters. These steroids show improved lipid solubility. Intramuscular dosing is required.
b) Class B: Testosterone derivatives which are alkylated at the 17α-hydroxyl position. These compounds can be administered orally.
c) Class C: Derivatives alkylated at ring A, B or C (Figure 1) of the steroid backbone. These steroids are orally available and resist hepatic metabolism.

### 3.1.1 The different types of ASS users - prevalence data

Whereas ASS were initially solely used by elite athletes and bodybuilders, ASS abuse spread into different population groups within the last three decades (Kanayama *et al.*, 2008). Surprisingly, it is even estimated, that elite athletes form the smallest group of ASS abusers (Bahrke and Yesalis, 2004). The highest ASS abuse prevalence occurs within people participating in recreational sport (Bahrke *et al.*, 2000). Men abuse ASS about three times more than women (Bahrke *et al.*, 2000). Another subgroup of ASS users is formed by individuals who consume ASS either for occupational or for aesthetic purposes (Bahrke and Yesalis, 2004; Monaghan, 2002). A study from 2007 (Cohen *et al.*, 2007) shows another, completely different profile of a typical ASS user: Cohen et al. recruited 1955 males via sport- and muscle-building related webpages. The internet was chosen on purpose, to guarantee the most possible anonymity expecting to obtain honest answers. According to this study, the typical ASS user holds a university degree (minimum Bachelor's degree), earns an above-average income, is full time employed and has an average age of 30 years. Most of these users never consumed ASS in their childhood.

Reviewing the literature, it becomes obvious, that it is impossible to classify ASS users, since they are represented in every social class independent of education-level, income-class or childhood. A recent meta-analysis including 187 studies aims to obtain an overall lifetime prevalence of ASS misuse classified by geographical regions (Sagoe *et al.*, 2014). To get an overview on AAS-use prevalence data, an excerpt from this study's results is shown in Table 2.

| Region | *N* | % p | 95% CI |
|---|---|---|---|
| Middle East | 7 | 21.7 | 13.9- 32.9 |
| South America | 5 | 4.8 | 1.2-16.7 |
| Europe | 81 | 3.8 | 2.4-5.8 |
| North America | 126 | 3.0 | 2.7-3.4 |
| Oceania | 38 | 2.6 | 2.1-3.3 |
| Africa | 11 | 2.4 | 1.2-4.8 |
| Asia | 1 | 0.2 | 0.0-3.5 |

*Table 2: Prevalence of AAS misuse classified by geographical region is shown.*
*N; Number of studies, %p: lifetime prevalence of AAS misuse, 95% CI: 95 % Confidence Interval*

### 3.1.2 Abuse of AAS - what are the consequences?

While testosterone is often administered in the clinic (e.g. testosterone replacement therapy due to hypogonadism), ASS are normally misused. AAS's are usually administered in combinations, by either "stacking" or "pyramiding" (Hall, 2005). Stacking refers to a practice where two or more ASS are taken at the same time (orally, intramuscular or both), while pyramiding is a circular way of ASS intake, where doses are gradually increased and then decreased during one cycle. There is no scientific data to proof that stacking or pyramiding shows synergism regarding desired effects. Abusers take AAS within the gram range, which results in steroid plasma levels that are more than 100 times higher than the physiological range.

Adverse effects can be divided into acute and chronic effects, however, especially for the chronic effects, it is difficult to attribute them to one substance, as AAS are usually abused in combination with other ASS, with alcohol or with other legal and illegal drugs. The Swedish institute of health and national information service installed an anti-doping hotline with its main goal to educate and to prevent doping abuse. Eklof et al. summarized information from incoming calls over a 7-year period of time (Eklof *et al.*, 2003) and gained valuable information about acute side effects of ASS abuse. The ten most commonly reported side effects in males were increased aggressiveness, depression, acne, gynecomastia, anxiousness, potency problems, testicular atrophy, sleep disorders, fluid retention and mood disturbances. Side effects affecting women included menstruation disturbances, facial hair growth, voice deepening and clitoris enlargement (Eklof *et al.*, 2003). All the above listed side effects can be explained by over activation of the androgen axis.

Chronic health consequences associated with AAS abuse include cardiovascular diseases (Kanayama *et al.*, 2008). The underlying mechanisms of AAS-induced cardiovascular problems remains poorly understood. It was suggested that ASS may be directly toxic to cardiac tissue resulting in

cardiomyopathy characterized by impaired systolic and diastolic function. (D'Andrea *et al.*, 2007; Kasikcioglu *et al.*, 2009). Second, AAS intake leads to cholesterol dysbalance, it increases low-density lipoprotein cholesterol and decreases high-density lipoprotein cholesterol (Glazer, 1991; Hartgens *et al.*, 2004; Kouri *et al.*, 1996). Such lipid abnormalities are known to be major risk factors for coronary heart disease (Grundy *et al.*, 2004).

Another well-known adverse effect of AAS is hepatotoxicity including hepatic neoplasm (Sanchez-Osorio *et al.*, 2008; Socas *et al.*, 2005). Suspected chronic side effects of AAS misuse include prostate cancer (Gittes, 1991; Roberts and Essenhigh, 1986) although causality of this is questioned (Morgentaler, 2007; Morgentaler, 2006). Further, evidence for adverse renal (Bryden *et al.*, 1995; Martorana *et al.*, 1999), immunologic and musculoskeletal effects were reported (Maravelias *et al.*, 2005; Modlinski and Fields, 2006).

## 3.2 Are AAS inhibitors of 11β-hydroxysteroid dehydrogenase 2 and may therefore cause cortisol-induced mineralocorticoid receptor activation?

Within the AAS project I tested whether AAS, due to a lack of target specifity, inhibit 11β-HSD2 dependent glucocorticoid inactivation, which could lead later to MR (over)activation. Cortisol-induced MR activation may lead to electrolyte disturbances and therefore contributes to the development of hypertension and cardiovascular disease. I tested the inhibitory potential of several different ASS on 11β-HSD2. For my experiments with radiolabeled cortisone I used HEK-293 cells stably expressing 11β-HSD2. Potential modulation of the MR activity was studied using transactivation assays. The transactivation experiments suggested that fluoxymesterone did not directly module the MR. However, experiments in other cell models need to be performed to address the possible contribution of transport activity. Testing several ASS, I showed that fluoxymesterone was the most potent inhibitor of human 11β-HSD2, inhibiting the enzyme in the low nanomolar range. Furthermore, in LC-MS/MS studies, we showed, that fluoxymesterone is a substrate of 11β-HSD2 and is oxidized to 11-oxofluoxymesterone. 3D-modelling of 11β-HSD2, its endogenous substrate cortisol and fluoxymesterone confirmed that fluoxymesterone potently binds to 11β-HSD2 and inhibits the enzyme in a competitive manner. Since adverse effects are usually studied in rodents as a next step, I tested whether fluoxymesterone would inhibit rat and mouse 11β-HSD2 *in vitro*. Measurements in rat microsomes and in HEK-293 cells stably expressing the murine 11β-HSD2 shows that fluoxymesterone inhibited the rodent enzymes about 80-fold less. The results of our study suggested a new possible mechanism of ASS induced cardiotoxicity and emphasize on the necessity to carefully evaluate animal models with respect to species differences.

The paper of the above mentioned study forms the basis of this chapter.

## 3.3 Published Paper:

***The anabolic androgenic steroid fluoxymesterone inhibits 11β-hydroxysteroid dehydrogenase 2–dependent glucocorticoid inactivation***

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# The Anabolic Androgenic Steroid Fluoxymesterone Inhibits 11β-Hydroxysteroid Dehydrogenase 2–Dependent Glucocorticoid Inactivation

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**Anabolic androgenic steroids (AAS) are testosterone derivatives used either clinically, in elite sports, or for body shaping with the goal to increase muscle size and strength. Clinically developed compounds and nonclinically tested designer steroids often marketed as food supplements are widely used. Despite the considerable evidence for various adverse effects of AAS use, the underlying molecular mechanisms are insufficiently understood. Here, we investigated whether some AAS, as a result of a lack of target selectivity, might inhibit 11β-hydroxysteroid dehydrogenase 2 (11β-HSD2)–dependent inactivation of glucocorticoids. Using recombinant human 11β-HSD2, we observed inhibitory effects for several AAS. Whereas oxymetholone, oxymesterone, danazol, and testosterone showed medium inhibitory potential, fluoxymesterone was a potent inhibitor of human 11β-HSD2 (half-maximal inhibitory concentration [$IC_{50}$] of 60–100nM in cell lysates; $IC_{50}$ of 160nM in intact SW-620, and 530nM in MCF-7 cells). Measurements with rat kidney microsomes and lysates of cells expressing recombinant mouse 11β-HSD2 revealed much weaker inhibition by the AAS tested, indicating that the adverse effects of AAS-dependent 11β-HSD2 inhibition cannot be investigated in rats and mice. Furthermore, we provide evidence that fluoxymesterone is metabolized to 11-oxofluoxymesterone by human 11β-HSD2. Structural modeling revealed similar binding modes for fluoxymesterone and cortisol, supporting a competitive mode of inhibition of 11β-HSD2–dependent cortisol oxidation by this AAS. No direct modulation of mineralocorticoid receptor (MR) function was observed. Thus, 11β-HSD2 inhibition by fluoxymesterone may cause cortisol-induced MR activation, thereby leading to electrolyte disturbances and contributing to the development of hypertension and cardiovascular disease.**

*Key Words:* **anabolic androgenic steroid; fluoxymesterone; testosterone; 11β-hydroxysteroid dehydrogenase; hypertension; glucocorticoid; cardiovascular disease.**

The misuse of anabolic androgenic steroids (AAS) represents a serious issue both in sports and in specific subsets of the population (Basaria, 2010). AAS are not only used as ergogenic drugs by athletes to enhance performance during competition but also for bodybuilding and for improving body image. Estimates suggested that the lifetime prevalence of AAS use in the United States is about 3% and that up to 6% of high school boys and 2% of girls use AAS (Basaria, 2010; Buckley *et al.*, 1988; Hall, 2005; Kanayama *et al.*, 2009; Yesalis *et al.*, 1997). In addition to endogenously occurring steroids and synthetic AAS developed for clinical applications such as muscle wasting in human immunodeficiency virus infection and aging, an increasing number of designer steroids that have never been clinically tested often are present in dietary supplements, mostly without proper labeling (Kuhn, 2002; Parr and Schanzer, 2010).

The use of AAS has been associated with a series of adverse effects, including hepatotoxicity, psychological disturbances, virilization, suppressed spermatogenesis, gynecomastia, as well as premature mortality mainly as a result of cardiovascular complications (Handelsman, 2011; Parssinen *et al.*, 2000). AAS exert direct effects on cardiomyocytes and vascular cells and indirect effects by decreasing high-density lipoprotein cholesterol and leading to early abnormalities in blood clotting factors (Ansell *et al.*, 1993; Calof *et al.*, 2005; Glazer, 1991). An impaired systolic and diastolic function has been directly associated with the duration and dose of AAS use in a recent study in power athletes (D'Andrea *et al.*, 2007). Furthermore, AAS use has been associated with increased fluid retention and elevated blood pressure (Sullivan *et al.*, 1998). Despite the recognition of the serious adverse cardiovascular effects of AAS misuse, the underlying molecular mechanisms remain largely unknown.

Impaired corticosteroid metabolism and activation of mineralocorticoid receptors (MR) have been associated with

cardiovascular disease (Briet and Schiffrin, 2010; Hadoke *et al*., 2009; Lastra *et al*., 2010). Two clinical hallmark studies supplementing the standard therapy (angiotensin-converting enzyme inhibitor, loop diuretic, and digoxin) with the MR antagonists spironolactone (Randomized Aldactone Evaluation Study, Pitt *et al*., 1999) or eplerenone (Eplerenone Post-Acute Myocardial Infarction Heart Failure Efficacy and Survival Study, Pitt *et al*., 2003b) revealed significantly reduced mortality in patients with myocardial infarction. Moreover, in patients with essential hypertension and left ventricular hypertrophy (4E-left ventricular hypertrophy study, Pitt *et al*., 2003a), the administration of the MR antagonist eplerenone lowered blood pressure and reduced left ventricular hypertrophy. Importantly, aldosterone levels were low in these patients, suggesting activation of MR by glucocorticoids, alternative ligands, or ligand-independent receptor activation (Frey *et al*., 2004; Odermatt and Atanasov, 2009).

Glucocorticoid-induced MR activation is especially relevant in situations of impaired activity of 11β-hydroxysteroid dehydrogenase 2 (11β-HSD2), which converts active 11β-hydroxyglucocorticoids (cortisol and corticosterone) into their inactive 11-keto forms (cortisone and 11-dehydrocorticosterone) (Frey *et al*., 2004; Odermatt and Kratschmar, 2012). In addition to distal colon and renal cortical collecting ducts, coexpression of 11β-HSD2 and MR has been reported in the atria and the ventricles of the heart and in endothelial cells and vascular smooth muscle cells of the aorta (Caprio *et al*., 2008; Klusonova *et al*., 2009; Naray-Fejes-Toth and Fejes-Toth, 2007). Inhibition of 11β-HSD2 is expected to cause excessive cortisol-induced MR activation in these tissues. The impact of impaired 11β-HSD2 activity has been investigated in apolipoprotein E (−/−)/11β-HSD2 (−/−) double knockout mice where the loss of 11β-HSD2 stimulates inflammation and strikingly promotes atherosclerosis in an MR-dependent manner (Deuchar *et al*., 2011). In humans, genetic defects in the gene encoding 11β-HSD2 result in cortisol-induced MR activation and severe hypertension, ultimately leading to death by cardiovascular complications (Ferrari, 2010).

In the present study, we tested the hypothesis that some widely used AAS compounds might inhibit 11β-HSD2 and/or directly modulate MR activity.

## MATERIALS AND METHODS

***Materials.*** Methenolone was purchased from Cerilliant Corporation (Round Rock, TX), formebolone from LPB Instituto Farmaceutico S.p.A. (Cinisello, Milano, Italy), and other steroids from the National Analytical Reference Laboratory (Pymble, Australia) and from Steraloids (Newport, RI). The reagents and solvents used were of analytical and high performance liquid chromatography grade. [1,2,6,7-3H]-cortisol was purchased from PerkinElmer (Waltham, MA). Cell culture media were purchased from Invitrogen (Carlsbad, CA); all other chemicals were from Fluka AG (Buchs, Switzerland) of the highest grade available.

***Cell culture.*** HEK-293 cells stably transfected with human 11β-HSD2 (Schweizer *et al*., 2003), human SW-620 colon carcinoma cells, and human MCF-7 breast cancer cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 4.5 g/l glucose, 50 U/ml penicillin/streptomycin, 2mM glutamine, and 1mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4.

***Determination of inhibition of human and mouse 11β-HSD2 using cell lysates.*** The conversion of cortisol to cortisone by 11β-HSD2 was measured as described previously (Gaware *et al*., 2011). Briefly, lysates of stably transfected HEK-293 cells were incubated for 10 min (human 11β-HSD2) or 20 min (mouse 11β-HSD2) at 37°C in a total volume of 22 µl of TS2 buffer (100mM NaCl, 1mM ethylene glycol tetraacetic acid [EGTA], 1mM EDTA, 1mM $MgCl_2$, 250mM sucrose, and 20mM Tris-HCl, pH 7.4) containing a final concentration of 200nM cortisol, of which 10% was radiolabeled, 500µM nicotinamide adenine dinucleotide ($NAD^+$), and vehicle or inhibitor at the desired concentration. Reactions were terminated by adding methanol containing 2mM unlabeled cortisol and cortisone, followed by separation of steroids by thin-layer chromatography (TLC) and analysis by scintillation counting. In addition, the inhibition of the conversion of cortisol to cortisone and fluoxymesterone to 11-oxofluoxymesterone by human 11β-HSD2 in cell lysates was assessed by liquid chromatography-mass spectrometry (for LC-MS method, see below). Lysates of HEK-293 cells stably expressing human 11β-HSD2 were incubated for 10 min at 37°C in a total volume of 500 µl at a final concentration of 200nM cortisol, 500µM $NAD^+$, and various concentrations of fluoxymesterone. To assess inhibition of fluoxymesterone oxidation, lysates were incubated for 1 h at 37°C with 500nM fluoxymesterone and various concentrations of cortisol. Reactions were stopped by shock freezing in a dry ice-ethanol bath. Stock solutions of all inhibitors were prepared in dimethylsulfoxide (DMSO) or methanol. Further dilutions were prepared prior to use in TS2 buffer to yield final concentrations between 0.6nM and 10µM.

***Determination of 11β-HSD2 inhibition using rat kidney microsomes.*** Rat kidney microsomes were prepared basically as described earlier (Senesi *et al*., 2010). Briefly, the kidneys of male Sprague Dawley rats were homogenized, and the microsomal fraction was isolated by fractional centrifugation. The microsomes were resuspended at a protein concentration of 1 mg protein/ml of buffer containing 100mM NaCl, 1mM EGTA, 1mM EDTA, 1mM $MgCl_2$, 250mM sucrose and 20mM Tris-HCl, pH 7.4, and frozen at −70°C. For 11β-HSD2 activity measurements, microsomes at a final protein amount of 10 µg per reaction were incubated for 10 min at 37°C as described above for cell lysates.

***Determination of 11β-HSD2 inhibition in intact SW-620 and MCF-7 cells.*** 11β-HSD2 activity in intact SW-620 cells was measured as described earlier (Gaware *et al*., 2011). Briefly, 100,000 cells per well of a 96-well plate were incubated in 50 µl steroid-free DMEM containing vehicle or inhibitor at the desired concentration, 10 nCi radiolabeled cortisol and 50nM unlabeled cortisol. SW-620 and MCF-7 cells were incubated for 2 and 4 h, respectively, at 37°C, followed by separation of steroids by TLC and scintillation counting.

***Transactivation assay.*** HEK-293 cells (200,000 cells per well) were seeded in poly-L-lysine–coated 24-well plates, incubated for 16 h, and transfected using calcium phosphate precipitation with pMMTV-lacZ β-galactosidase reporter (0.20 µg/well), pCMV-LUC luciferase transfection control (0.04 µg/well), and human recombinant MR (0.35 µg/well). Cells were washed twice with DMEM 6 h posttransfection, followed by incubation for another 18 h to allow sufficient expression. Cells were then washed once with steroid- and serum-free DMEM (DMEMsf) and cultivated at least 3 h at 37°C. The culture medium was replaced with fresh DMEMsf containing DMSO (0.05%) or aldosterone (10nM) in the presence or absence of fluoxymesterone (1µM). After incubation for 24 h, cells were washed once with PBS and lysed with 60 µl lysis buffer of the Tropix kit (Applied Biosystems, Foster City, CA) supplemented with 0.5mM dithiothreitol. Lysed samples were frozen at −80°C for at least 20 min. Lysates (20 µl) were analyzed for β-galactosidase activity using the Tropix kit. Luciferase activity was analyzed in 20 µl samples using a luciferin solution (Kratschmar *et al*., 2011).

***Measurement of fluoxymesterone metabolism by LC-MS.*** Lysates of untransfected HEK-293 cells and HEK-293 cells stably expressing human 11β-HSD2 were incubated for 1 h at 37°C in a total volume of 500 µl at a final concentration of 1µM fluoxymesterone or cortisol and 500µM $NAD^+$. The reactions were stopped by shock freezing using a dry ice-ethanol bath.

After thawing, samples were centrifuged at 4°C for 10 min at 500 × g and supernatants transferred onto a solid phase extraction column (Oasis HBL 1 cc [30 mg] Waters WAT094225) preconditioned with 1 ml of methanol and 1 ml of distilled water. After washing twice with 1 ml of water, steroids were eluted by twice adding 1 ml of methanol. The combined eluate was evaporated to dryness and reconstituted with 100 µl of methanol.

Steroids were resolved on an Atlantis T3 (3 µm, 2.1 × 150 mm) column (Waters, Milford, MA) at 30°C using an Agilent model 1200 Infinity Series chromatograph (Agilent Technologies, Basel, Switzerland). The mobile phase consisted of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile). To separate fluoxymesterone and 11-oxofluoxymesterone, a linear gradient starting from 55% solvent A and 45% solvent B to 5% solvent A and 95% solvent B from 0 to 12 min was used. To separate cortisol and cortisone, a linear gradient starting from 85% solvent A and 15% solvent B to 60% solvent A and 40% solvent B from 0 to 15 min was used. The flow rate was maintained at 0.4 ml/min.

The LC was interfaced to an Agilent 6490 triple Quad mass spectrometer (MS). The MS was operated in atmospheric pressure electrospray positive ionization mode, with nebulizer pressure and nebulizer gas flow rate of 45 psi and 10 l/h, respectively, a source temperature of 350°C and capillary and cone voltage of 4 kW and 190 V, respectively. Data acquisition was performed by Mass Hunter workstation (Version B01.04). Metabolites were identified by comparing their retention time and mass to charge ratio (*m/z*) with those of authentic standards.

Qualitative determination of steroids was performed by selected positive ionization multiple reaction monitoring. Fluoxymesterone (*m/z* 337.1) and 11-oxofluoxymesterone (*m/z* 335.1) eluted at 9.1 and 9.7 min, respectively. For the quantitative determination of fluoxymesterone, prednisolone (*m/z* 361.1, retention time 7.3 min) was used as internal standard. Metabolites were quantified from calibration curves of the ratio of the peak area of the authentic fluoxymesterone standard and the internal prednisolone standard incubated with lysates of untransfected HEK-293 cells at a total protein concentration identical to that of the experimental setting and plotted against the concentration of authentic standards (normalization to the internal standard). A reference standard for 11-oxofluoxymesterone was not available and needs to be synthesized for confirmation of the structure and exact quantification. Cortisone (precursor and product ion at *m/z* 361 and 163) and cortisol (precursor and product ion at *m/z* 363 and 121) eluted at 8.5 and 8.9 min, respectively.

***Determination of the binding mode of steroids in 11β-HSD2 by molecular modeling.*** The 2D structures of fluoxymesterone, testosterone, cortisone, and cortisol were generated using ChemBioDraw Ultra 12.0 (CambridgeSoft, U.K., 1986–2009, www.cambridgesoft.com). Two-dimensional structures were transformed into 3D using CORINA (Molecular Networks GmbH, Erlangen, Germany). These four compounds were docked into the previously generated homology model of 11β-HSD2 (Gaware *et al*., 2011) using GOLD (Jones *et al*., 1997; Verdonk *et al*., 2003). The binding site was defined by a 10 Å sphere with the hydroxyl group of Tyr232 in the center. To optimize the ligand orientation, two protein hydrogen bond constraints were defined: one to the catalytically active Tyr232 and the second one to Tyr226. During the docking, the protein was handled as rigid and ligands as flexible. The ligand flexibility was increased by allowing the ring corner to flip. GoldScore was used as a scoring function, and 10 docking solutions for each ligand were calculated. Atom types for the protein as well as for the ligands were automatically determined by the program. LigandScout (Wolber and Langer, 2005) was used for visualizing the docking results and for the analysis of the protein-ligand interactions.

## RESULTS

### *Inhibition of 11β-HSD2 by Anabolic Androgenic Steroids Measured in Cell Lysates*

In order to test the hypothesis that AAS might cause adverse effects by disrupting corticosteroid hormone action, we tested several frequently used AAS for potential inhibition of the 11β-HSD2–dependent conversion of cortisol to cortisone as well as for their impact on MR transactivation. The activity of human 11β-HSD2 was assessed using lysates of stably transfected HEK-293 cells and by determining the conversion of radio-labeled cortisol to cortisone. At a high concentration (10µM), only norbolethone and methenolone did not have any inhibitory effect (Table 1). Dehydroepiandrosterone, mesterolone, and formebolone showed weak inhibitory effects with estimated Half-maximal inhibitory concentration ($IC_{50}$) values of 10µM or higher. For the compounds that showed less than 40% remaining activity at this concentration, $IC_{50}$ values were determined. This revealed potent inhibition by the 11β-hydroxylated steroid fluoxymesterone with $IC_{50}$ of 60 ± 27nM and medium inhibitory effects for testosterone (Fig. 1A), oxymesterone, danazol, and oxymetholone. The inhibition of 11β-HSD2–mediated cortisol oxidation by fluoxymesterone was confirmed in an assay using unlabeled cortisol and quantification of the product cortisone by LC-MS. A slightly higher $IC_{50}$ value of 100 ± 28nM was obtained (data not shown).

Adverse effects are usually studied in a next step in rodents. In preparing for animal studies, we tested whether fluoxymesterone would inhibit rat and mouse 11β-HSD2 *in vitro*. Measurements with rat kidney microsomes revealed an approximately 80-fold lower inhibitory potential with an $IC_{50}$ of 4.9 ± 0.8µM (Fig. 1B). Similarly, an $IC_{50}$ of 5.4 ± 0.4µM was obtained for fluoxymesterone inhibition of 11β-HSD2 using lysates of HEK-293 cells stably expressing the mouse enzyme (Fig. 1C). Testosterone was about 10-fold less potent toward the rat and the mouse enzymes with $IC_{50}$ of 14.9 ± 4.3 and 15.3 ± 3.5µM, respectively. The weak

**TABLE 1**
**Inhibition of 11β-HSD2 by Anabolic Androgenic Steroids**

| Compound | Remaining cortisol to cortisone conversion at 10µM (%) | $IC_{50}$ (nM) |
|---|---|---|
| Glycyrrhetinic acid | 2.4 ± 1.8 | 256 ± 33[a] |
| Testosterone | 12 ± 12 | 1370 ± 60 |
| Dehydroepiandrosterone | 78 ± 12[b] | nd |
| Danazol | 23 ± 17 | 740 ± 110 |
| Fluoxymesterone | 1.9 ± 1.5 | 60 ± 27 |
| Formebolone | 51 ± 12 | nd |
| Mesterolone | 74 ± 6 | nd |
| Methenolone | 90 ± 3 | nd |
| Norbolethone | 92 ± 13 | nd |
| Oxymesterone | 2.6 ± 3.5 | 960 ± 140 |
| Oxymetholone | 6.4 ± 4.5 | 710 ± 50 |

*Notes*. The 11β-HSD2–dependent conversion of cortisol (50nM) to cortisone was measured in cell lysates in the presence of 500µM of $NAD^+$ and various concentrations of AAS or vehicle (0.1% DMSO). Glycyrrhetinic acid was included as reference compound. Inhibitory activities represent $IC_{50}$ ± SD from at least three independent experiments. nd, not determined.

[a]Value taken from Kratschmar *et al*. (2011).

[b]Determined at 20µM.

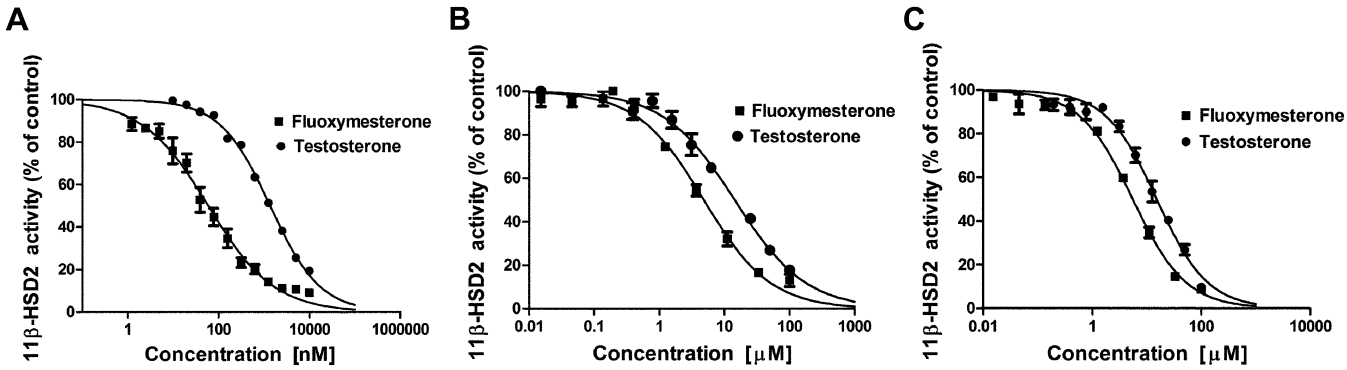


**FIG. 1.** Inhibition of 11β-HSD2 activity by fluoxymesterone measured in cell lysates. (A) Lysates of HEK-293 cells stably expressing recombinant human 11β-HSD2 were incubated for 10 min at 37°C with 50nM of radiolabeled cortisol and increasing concentrations of fluoxymesterone (squares) or testosterone (circles), followed by determination of cortisone formation. Similarly, inhibition by fluoxymesterone and testosterone was measured using rat kidney microsomes (B) and lysates of HEK-293 cells stably expressing mouse 11β-HSD2 (C). Values were normalized to the activity observed in the presence of vehicle (0.1% DMSO). Data represent mean ± SD from three independent experiments.

inhibitory effects of fluoxymesterone and testosterone on the rodent 11β-HSD2 enzymes make it unlikely to study the adverse effects in these species, and animal experiments were not performed.

### *Inhibition of 11β-HSD2 in Intact SW-620 and MCF-7 Cells*

The effect of fluoxymesterone on 11β-HSD2 activity was next determined in intact cells. Two distinct cell models, human colon carcinoma SW-620 cells and human breast cancer MCF-7 cells with relatively high endogenous expression of the enzyme were applied. Cell models with lower expression may not be suitable to determine direct effects on enzyme activity due to interference with altered gene expression. Fluoxymesterone potently inhibited 11β-HSD2 in a concentration-dependent manner with $IC_{50}$ of 160 ± 10nM in SW-620 cells and $IC_{50}$ of 530 ± 70nM in MCF-7 cells (Fig. 2). Although the known reference compound glycyrrhetinic acid also showed potent inhibition, testosterone did not significantly inhibit 11β-HSD2 at concentrations up to 10μM in intact SW-620 or MCF-7 cells.

### *Fluoxymesterone Does Not Directly Modulate MR Activation*

To see whether fluoxymesterone might directly modulate MR activity, transactivation assays in HEK-293 cells transiently expressing MR and a galactosidase reporter under the control of the mouse mammary tumor virus promoter were performed. Cells were incubated with 1μM of fluoxymesterone alone or together with 10nM aldosterone. The reporter gene response was indistinguishable if cells were treated with either aldosterone alone or in combination with fluoxymesterone (Fig. 3). Furthermore, basal receptor activity measured by DMSO treatment (0.05%) was not changed by fluoxymesterone. Similarly, the other tested AAS (see Table 1) did not activate or antagonize MR function, indicating that these steroids have no direct effect on the receptor.

### *Fluoxymesterone Is Metabolized by 11β-HSD2*

Because fluoxymesterone possesses an 11β-hydroxyl group, we hypothesized that this steroid may be a substrate and therefore competitively inhibit the enzyme. Therefore, we established an LC-MS–based method using the multiple

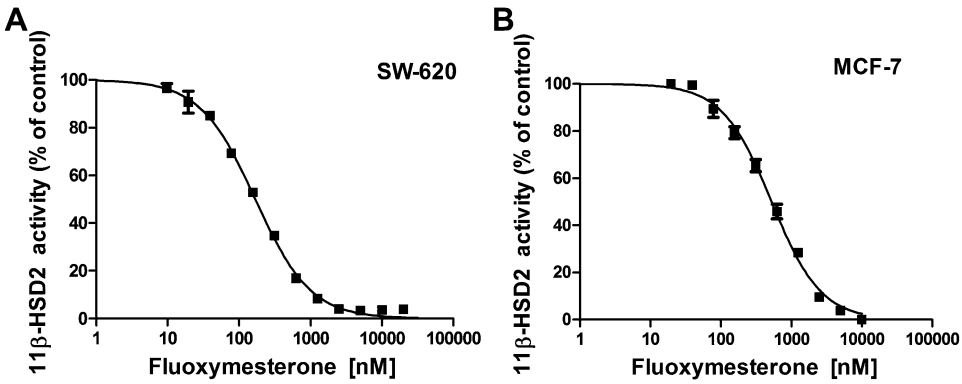


**FIG. 2.** Inhibition of 11β-HSD2 activity by fluoxymesterone in intact SW-620 and MCF-7 cells. Intact SW-620 and MCF-7 cells, with endogenous expression of 11β-HSD2, were incubated for 2 and 4 h, respectively, with 50nM of radiolabeled cortisol and increasing concentrations of fluoxymesterone. Values were normalized to the activity observed in the presence of vehicle (0.1% DMSO). Data represent mean ± SD from three independent experiments.

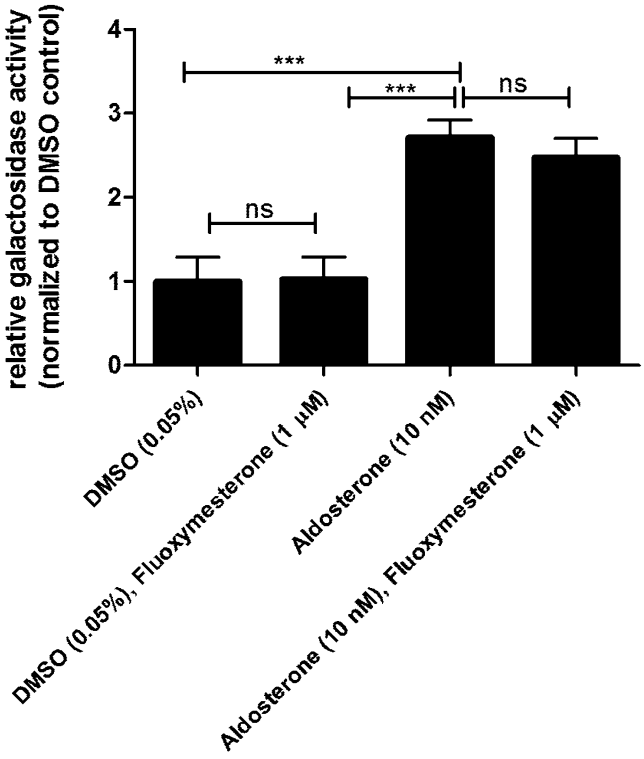


**FIG. 3.** Effect of fluoxymesterone on MR transactivation. HEK-293 cells were transfected with pMMTV-LacZ reporter, pCMV-LUC control plasmid, and human MR expression plasmid. Cells were incubated for 24 h in the presence or absence of 10nM aldosterone with or without 1μM fluoxymesterone, followed by determination of galactosidase and luciferase activities. Galactosidase reporter activity was normalized to the internal luciferase control. Data were normalized to vehicle control (0.05% DMSO) and represent mean ± SD from a representative experiment performed in triplicate.

reaction monitoring mode for the qualitative and quantitative determination of fluoxymesterone (*m/z* 337.1) and its metabolite 11-oxofluoxymesterone (*m/z* 335.1) (see Materials and Methods section). Fluoxymesterone and 11-oxofluoxymesterone were clearly separated with retention times of 9.1 and 9.7 min, respectively (Fig. 4). Similarly, conditions were established to separate and quantify cortisol (*m/z* 363, retention time 8.5 min) and cortisone (*m/z* 361, retention time 8.9 min). Upon incubation for 1 h with lysates of HEK-293 cells stably expressing 11β-HSD2, approximately 50% of the supplied fluoxymesterone (1μM) was converted. In comparison, under the same conditions, approximately 80% of cortisol was converted. No conversion was observed when lysates of untransfected HEK-293 cells were used. Thus, although both steroids are substrates of 11β-HSD2 (Fig. 5), the enzyme more efficiently oxidizes cortisol.

To further support the hypothesis that fluoxymesterone and cortisol bind to and are metabolized by 11β-HSD2, we studied the impact of cortisol on the conversion of fluoxymesterone (at a final concentration of 500nM) to 11-oxofluoxymesterone in cell lysates. A concentration-dependent inhibition with an $IC_{50}$ of 730 ± 190nM was obtained (data not shown).

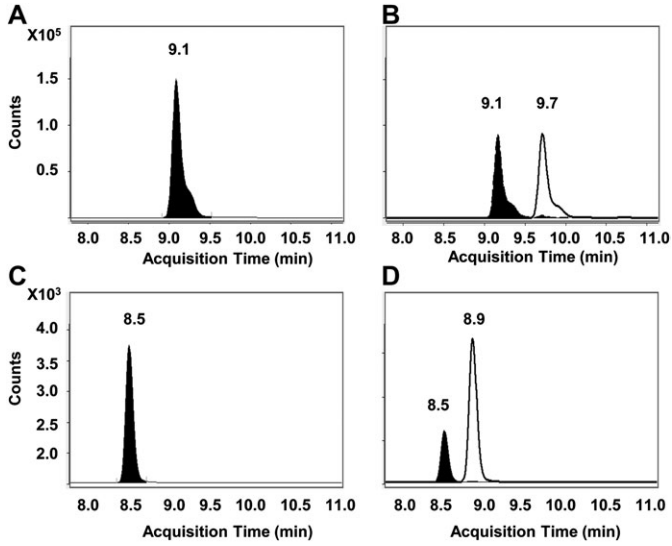


**FIG. 4.** Representative LC-MS chromatogram showing the metabolism of fluoxymesterone and cortisol by 11β-HSD2. Lysates of untransfected HEK-293 cells (A and C) and lysates of 11β-HSD2–expressing HEK-293 cells (B and D) were incubated for 2 h with 1μM of fluoxymesterone (A and B) or cortisol (C and D), followed by analysis of steroid metabolites by LC-MS. The area under the peak of the substrate is indicated in black and that of the product in white color. Fluoxymesterone (*m/z* 337) eluted at 9.1 min and 11-oxofluoxymesterone (*m/z* 335) at 9.7 min. Cortisol (*m/z* 363, 121) eluted at 8.5 min and cortisone (*m/z* 361) at 8.9 min.

### *Comparison of the Binding Mode of Steroids in 11β-HSD2*

All four compounds were docked into the 11β-HSD2 active site. They were all positioned in the vicinity of the catalytic residue Tyr232, close to the catalytic triad Ser219-Tyr232-Lys236 (Fig. 6A). The binding interactions of each compound were investigated in detail to explain the difference in their activities. The automatically created pharmacophoric interaction pattern showed different hydrogen-bonding patterns for each compound (Figs. 6B–E).

The natural ligand cortisol was predicted to have favorable binding interactions. The 11β-hydroxy group interacts with the

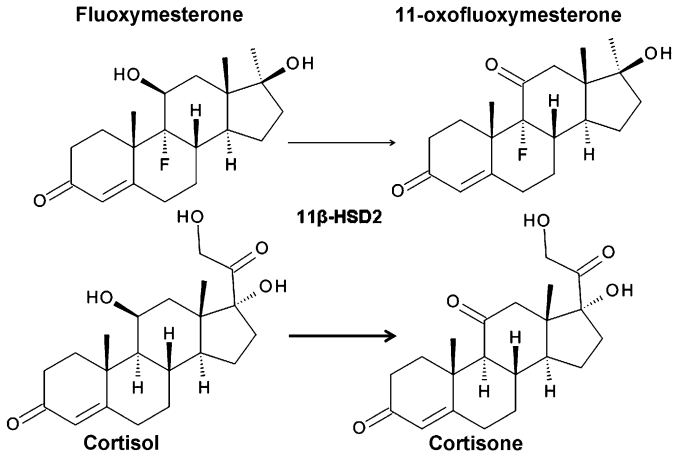


**FIG. 5.** Structures of 11β-HSD2 substrates and products.

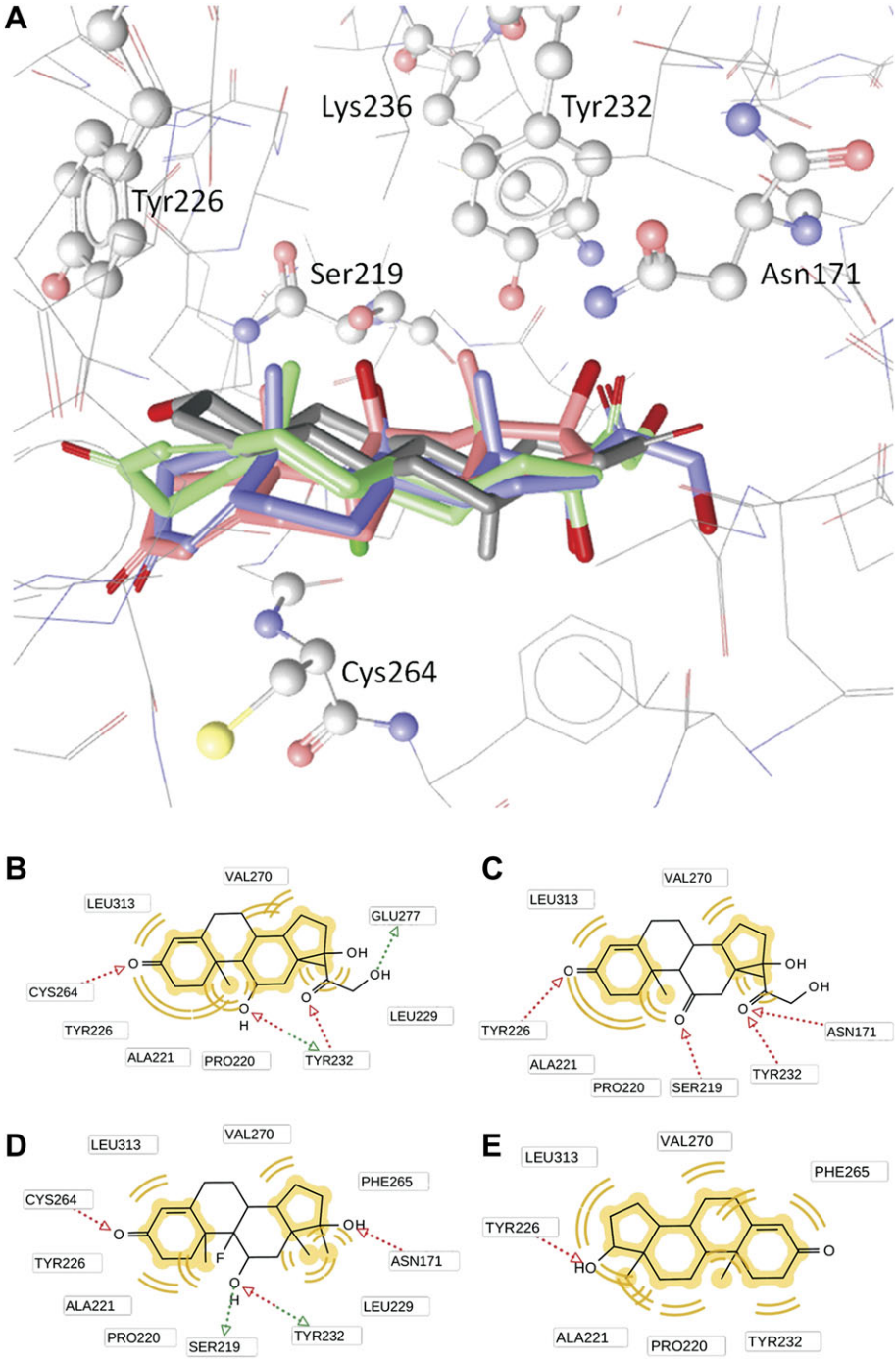


**FIG. 6.** Modeling of the binding of steroids to 11β-HSD2. (A) Cortisol (blue), cortisone (green), fluoxymesterone (red), and testosterone (gray) docked into the ligand-binding pocket of 11β-HSD2. The catalytic triad as well as other amino acid residues with which common interactions were observed are depicted in ball and stick style. (B–D) Two-dimensional representations of the binding interactions of steroids with 11β-HSD2. Binding interactions of cortisol (B), cortisone (C), fluoxymesterone (D), and testosterone (E) with 11β-HSD2 are shown. The hydrophobic interactions are represented in yellow, hydrogen bond acceptors in red, and hydrogen bond donors in green.

catalytic residue Tyr232, and the ligand was anchored to the binding pocket with three other hydrogen bonds. The interaction pattern of cortisone, the end product of the reaction catalyzed by 11β-HSD2, differed to the one of cortisol in terms that the product moved the 11-keto group away from the catalytically active residues. The most active inhibitor, fluoxymesterone, was observed to be anchored to the binding pocket with four hydrogen bonds. The hydrogen bonds between Tyr232, Ser219,

and the 11-hydroxy group of fluoxymesterone indicated that this molecule can be transformed into its 11-keto form by the enzyme. In fact, biological data showed that fluoxymesterone is a substrate of 11β-HSD2, but with a lower conversion rate than cortisol. This lower conversion rate leads to a competitive inhibition of 11β-HSD2 by fluoxymesterone.

Interestingly, testosterone was predicted to adapt a different binding mode compared with the other three compounds. It was located in the same area as the others; however, it had a flipped binding mode. Whereas cortisol, cortisone, and fluoxymesterone had their 3-keto groups pointing toward Tyr226, the 3-keto group of testosterone superimposed onto position 17 of the other steroids. No other hydrogen bond between testosterone and the protein was observed. This kind of binding mode as well as fewer hydrogen bonds and hydrophobic interactions with the protein could be an explanation for the weaker 11β-HSD2 inhibition of testosterone.

## DISCUSSION

In this study, we analyzed several well-known and widely used AAS for their potential to inhibit 11β-HSD2 and identified fluoxymesterone as a potent inhibitor. The $IC_{50}$ values of 60–100nM in cell lysates and 160 and 530nM measured in intact SW-620 and MCF-7 cells, respectively, are in a comparable range with the inhibitory potential of glycyrrhetinic acid, which has been demonstrated in both animals and humans to inhibit 11β-HSD2 and cause glucocorticoid-mediated MR activation, potassium excretion, sodium and water retention, and increased blood pressure (Ferrari, 2010; Ferrari *et al*., 2001; Serra *et al*., 2002). As shown for fluoxymesterone in the present study, glycyrrhetinic acid also does not directly modulate MR function but competitively inhibits 11β-HSD2, thereby leading to glucocorticoid-mediated MR activation.

Oral fluoxymesterone, which is marketed as halotestin in the United States for the treatment of hypogonadism in men and of certain hormone-dependent mammary tumors in women, can exacerbate health states where edema plays important roles, such as congestive heart failure (as described in the package leaflet, http://www.pfizer.com/files/products/uspi_halotestin.pdf). A possible mechanistic explanation for these warnings concerning fluid retention states includes the involvement of MR activation. Following oral administration, fluoxymesterone is rapidly metabolized in the liver. The adverse effects described above can be expected to be much more pronounced upon intravenous application of the drug, which is the preferred route of administration in sports and bodybuilding.

Inhibition of 11β-HSD2 in the renal distal tubules and cortical collecting ducts results in glucocorticoid-induced MR activation with subsequently increased activities of $Na^+/K^+$-ATPases at the basolateral membrane and of $Na^+$ channels (ENaC) on the apical membrane of epithelial cells, resulting in potassium excretion and sodium and water retention (Ferrari, 2010). In the vasculature, decreased 11β-HSD2 activity leads to vasoconstriction and elevated blood pressure, probably by a mechanism involving the activation of glucocorticoid receptor rather than MR (Goodwin *et al*., 2008). Furthermore, inhibition of 11β-HSD2 in vascular smooth muscle cells and endothelial cells of the heart may lead to paracrine activation of MR in cardiomyocytes. In a recent study using transgenic mice, Deuchar *et al*. (2011) showed that the ablation of 11β-HSD2 in apolipoprotein E knockout mice aggravated the progression of atherosclerosis by a mechanism involving MR activation and stimulation of inflammation in the vascular endothelium. The use of AAS has been associated with reduced levels of high-density lipoproteins and an increased ratio of low-density lipoproteins to high-density lipoproteins (Glazer, 1991; Thompson *et al*., 1989). Thus, AAS that inhibit 11β-HSD2, like fluoxymesterone, may be especially prone to cause adverse cardiovascular effects.

Regarding the inhibitory mechanism, both the 3D structural modeling and the fact that 11β-HSD2 can metabolize fluoxymesterone to 11-oxofluoxymesterone support a competitive inhibition of cortisol oxidation. The 11β-hydroxyl on fluoxymesterone has important stabilizing interactions that are absent in testosterone and other AAS. Interestingly, formebolone also has an 11β-hydroxyl but is a very weak 11β-HSD2 inhibitor, probably as a result of the methyl group in ring-A, which leads to steric hindrance and prevents optimal stabilization in the binding pocket. Although 11β-HSD2 can oxidize fluoxymesterone at position 11, this reaction represents a minor contribution to the overall metabolism of this steroid (Pozo *et al*., 2008). Compared with cortisol, fluoxymesterone was approximately three to five times less efficiently converted to its 11-oxo form, resembling the conversion of dexamethasone, which also has a fluor atom at position 9 (Rebuffat *et al*., 2004). A reference compound needs to be synthesized to test whether 11β-HSD2 might be able to reduce 11-oxofluoxymesterone as previously observed for 11-oxodexamethasone. This, together with the extensive hepatic metabolism, would explain why 11-oxo derivatives of fluoxymesterone are minor metabolites of fluoxymesterone in human urine (Pozo *et al*., 2008).

The potent inhibition of human but not rat and mouse 11β-HSD2 by fluoxymesterone emphasizes the importance to assess species-specific differences using recombinant enzymes and enzyme preparations from native tissues before conducting animal studies, both from an ethical point of view and to save resources. Clinical studies will need to investigate the potential of fluoxymesterone to inhibit 11β-HSD2 activity *in vivo*. This can be achieved by measuring its impact on the ratio of 11β-hydroxy- to 11-oxoglucocorticoids in plasma and their corresponding tetrahydro metabolites in 24-h urine samples and by examining the patients for the presence of low-renin and low-aldosterone hypertension.

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## 3.4 Further results and outlook concerning 11β-hydroxysteroid dehydrogenase type 2 inhibition by androgenic steroids

### 3.4.1 Androstenedione potently inhibits 11β-hydroxysteroid dehydrogenase type 2

The AAS project started by a screening of different steroids and determination of their $IC_{50}$ against 11β-HSD2. In addition to synthetic compounds, the inhibitory potential of several endogenous steroids was determined. The results are shown in Table 1 of the fluoxymesterone study (Furstenberger *et al.*, 2012). An important observation not shown and not discussed in this study is the high inhibitory potential of the endogenous steroid androstenedione. Androstenedione was shown to inhibit 11β-HSD2 with an IC50 in the low nanomolar range (Figure 7).

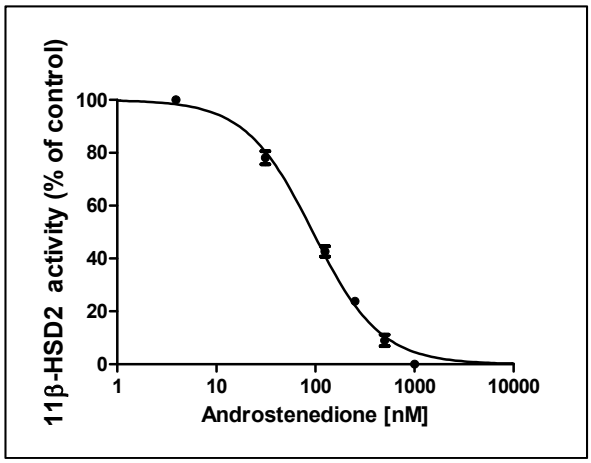


*Figure 7: Conversion of cortisone to cortisone upon androstenedione incubation.*

The potent inhibition of 11β-HSD2 by androstenedione could only be shown in lysates of HEK-293 cells stably expressing 11β-HSD2. However, in intact cells, no relevant inhibitory effect of 11β-HSD2 could be detected. We know from previous studies carried out in our group (Nashev *et al.*, 2010) that androstenedione is able to penetrate the cell membrane of HEK-293 cells. Nashev et al. measured the 17β-HSD3 mediated conversion of androstenedione to testosterone in HEK-293 cells transiently expressing 17β-HSD3. Furthermore, the conversion of androstenedione to testosterone could be inhibited by using the UV-filter benzophenone-1. This raises the question why androstenedione only inhibits 11β-HSD2 in cell lysates where the enzyme is directly accessible. The most obvious difference in our experimental setting compared to the experiments by Nashev et al. was the transfection conditions of the cell line. It has been shown that transfection and medium conditions may alter the expression of transport proteins in HEK-293 cells (Ahlin *et al.*, 2009). Consequently, we repeated the experiment in transiently transfected cells. However, the outcome did not differ from the original experiment. Androstenedione showed no relevant inhibitory potential towards 11β-HSD2. We decided to test another cell line and repeated the experiment using SW-620 cells, a human colon adenocarcinoma cell endogenously expressing 11β-HSD2. Unfortunately, we could also not detect any inhibition of 11β-HSD2 upon incubation with androstenedione. In summary, we observed a very potent inhibition by the endogenous steroid androstenedione of the 11β-HSD2 dependent cortisol inactivation in cell lysates, but we are unable to confirm this inhibitory effect in intact cells. What experiments need to be performed to evaluate whether the inhibition might be important *in vivo*? If

inhibition of 11β-HSD2 occurs *in vivo*, subsequent cortisol-induced MR activation might play a role in the development and progression of hypertension. Possible reasons for the lack of inhibition in intact cells could be due to rapid androstenedione efflux from the cells mediated by transport proteins. A study performed in different commonly used cell lines focuses on the expression profiles of important *xenobiotica*-transporting proteins (Ahlin *et al.*, 2009). Though Ahlin et al. suggests that HEK-293 cells express a relatively low amount of transport proteins compared with other cell lines (such as for example CaCo-2 cells),they could still identify 21 drug transporters expressed in HEK-293 cells. In future studies, it will important, therefore, to inhibit specific transporters to see whether they play a relevant role in androstenedione efflux. Due to distinct species differences in steroid induced 11β-HSD2 inhibition (described before (Furstenberger *et al.*, 2012)), *in vivo* rodent experiments might not be meaningful and translational relevance to humans may be poor. To investigate whether the androstenedione induced 11β-HSD2 inhibition is relevant *in vivo* and might have an influence on subsequent MR activation, a humanized mouse might provide useful data. Additionally, a clinic study with administration of androstenedione and analysis of plasma samples for the ratio of cortisol to cortisone would help to further test for cortisol-induced MR activation.

# 4. Project 2: Evaluation of testosterone production in Leydig cell models and characterization of current methods by measuring androgen levels

## 4.1 Introduction to endogenous androgens

Most androgens mediate their actions through the androgen receptor (AR), which belongs to the superfamily of the steroid/thyroid hormone nuclear receptors. The AR acts as a ligand inducible transcription factor (Mangelsdorf *et al.*, 1995). Testosterone and the more potent DHT are the two native ligands of the AR. Ligand-AR complexes bind as homodimers to androgen responsive elements (ARE) in target gene promoters. Consensus ARE are composed of two core 5′-AGAACA-3′ motifs separated by a space of three base pairs (Khorasanizadeh and Rastinejad, 2001). Testosterone is the most extensively studied androgen, however, serum levels show large variations, both within different organisms but also within the same organism depending on health and age. Furthermore, testosterone levels are also influenced by a multitude of factors such nutrition and the circadian rhythm (Deslypere and Vermeulen, 1984). Research focusing on the different factors that influence testosterone levels and their subsequent impact on disease development is a competitive field. Investigations in relation to testosterone levels include psychological stress (Bhongade *et al.*, 2014), seasonal variations (Smith *et al.*, 2013), autoimmune diseases (Costa *et al.*, 2014), anorexia (Smiarowska *et al.*, 2014) and various types and stages of cancers (Orsted *et al.*, 2014; Thomas *et al.*, 2014). To conclude, the levels of testosterone show broad inter-individual variations and therefore clinical values need to be evaluated within the context of the specific physiological and psychological state of the individual.

DHT is a much more potent AR ligand than testosterone. DHT is formed in endocrine tissues (prostate, skin, hair follicles) from androstenedione via testosterone (front-door pathway) or via 5α-androstanedione (back-door pathway) (Luu-The, 2013). Another source of serum DHT is the liver, which also expresses steroidogenic enzymes at high levels (Shores *et al.*, 2014). DHT levels are roughly 3-5% of the testosterone levels. Although testosterone and DHT both bind to the AR, the downstream response is not always identical and this may be due to changes of the quaternary structure of the AR upon binding testosterone or DHT (Baek *et al.*, 2006). Androstenedione is predominately produced in the testis (in women in the ovaries) by 3β-HSD-mediated conversion from DHEA. 3β-HSD is much more effective in converting androstenedione from DHEA than 17β-HSD3 is converting testosterone from androstenedione, suggesting that the formation of the inactive precursor androstenedione does not represent the rate-limiting step, but rather that it is produced in excess (Luu-The, 2013).

Dehydroepiandrosterone (DHEA) and its sulfate ester DHEAS are the most abundant steroids in humans (Davis *et al.*, 2011), with plasma concentrations in the micromolar range. DHEA is produced from cholesterol mainly in the adrenals but also in the testis, the ovaries, skin, and brain (Davis *et al.*, 2011; Zouboulis *et al.*, 2007). Most of the DHEAS is loosely bound to the plasma protein albumin (Panjari and Davis, 2007). Currently, no specific high affinity plasma binding protein has been

identified (Legrain *et al.*, 2000). DHEA but not DHEAS is bound by the sex hormone binding globulin (Panjari and Davis, 2007). DHEA supplementation combined with gonadotropin is used in reproductive medicine as a way to treat female infertility (Casson *et al.*, 2000). There is evidence that low serum levels of DHEA correlate with coronary heart disease, however, there is not sufficient data to show that DHEA supplementation would have any cardiovascular benefit (Thijs *et al.*, 2003).

A key feature of steroids is their ability to tightly regulate physiological functions. Small changes in systemic/ local steroid concentrations allow steroids to switch on or off physiological processes. Table 3 gives a general overview on the magnitude of concentrations and variations of abundant steroids in human plasma.

| | | |
|---|---|---|
| **Testosterone** | Male prepuberty | **0 - 1 nmol/l** (Albin and Norjavaara, 2013) |
| | Male pubertal | **7 - 13 nmol/l** (Albin and Norjavaara, 2013) |
| | Male < 60 years | **15.9 - 29.8 nmol/l** (Deslypere and Vermeulen, 1984) |
| | Male > 60 years | **11.5 - 22.6 nmol/l** (Deslypere and Vermeulen, 1984) |
| | Female premenopausal | **1.3-2.9 nmol/l** (Davison *et al.*, 2005) |
| | Female postmenopausal | **0.2- 2.0 nmol/l** (Davison *et al.*, 2005) |
| **Androstenedione** | Male adult | **1.39-5.23nmol/l** (Rosenfield and Otto, 1972) |
| | Female adult | **1.05- 6.94 nmol/L** (Burger, 2002) |
| **Dihydrotestosterone (DHT)** | Male adult | **0.6-2.4 nmol/l** (Muller *et al.*, 2012) |
| | Female adult | **0.1-1.0 nmol/l** (Burger, 2002) |
| **Dehydroepiandrosterone (DHEA)** | Male adult | **2.07- 4.87 µmol/l** (Landt *et al.*, 2011) |
| | Female adult | **0.3- 12.5 µmol/l** (Davison *et al.*, 2005) |

*Table 3: Overview of the most abundant steroids and their plasma concentrations.*

## 4.2 Technical methods of steroid measurements

Lack of standardized steroid hormone quantification assays are a major limitation in steroid research (Stanczyk *et al.*, 2007). It is difficult to reliably compare steroid concentrations between different studies that have been measured using different methods. Steroid measurements (in plasma or in other matrices) pose various challenges:

- Steroid concentrations vary depending on age, gender and health status (Rosner *et al.*, 2007).
- Steroids are very difficult to specifically identify in biochemical assays, since their structures and their chemical properties are very similar.
- Steroids in plasma are both free or protein bound and it is questioned whether total or free steroid is the more useful measurement.
- There are generally no age- or gender-corrected reference values for steroids measurements in clinical applications.

There are only a few methods available for the measurement of steroids and/or steroidogenic metabolism. The two most commonly used methods are immunoassay and mass spectrometry.

### 4.2.1 Immunoassays

Immunoassays are the most widely used method to measure steroids. Currently, the popular immunoassay of choice is the enzyme linked-immunosorbant assays (ELISA). However, the majority of our knowledge about the physiological roles of steroids in humans derive from studies in which circulating steroid hormones were measured by Radioimmunoassay (RIA) (Stanczyk *et al.*, 2007). Since RIAs generate radioactive waste, they have become less popular (Rosner *et al.*, 2007). Immunoassays can be carried out either directly without sample preparation, or following extraction and chromatographic separation of the steroids. The latter technique is more advantageous, since it allows the measurement of multiple steroids from a single sample. Generally, immunoassay measurements are more accurate and sensitive if the steroids are separated and purified prior to the measurement. However, such protocols are often labor intensive, time consuming, expensive and due to the complexity of the sample preparation, may increase the probability of human error. For steroid measurements, commercially available kits are used either in conjunction with an automated instrument or manually processed. The limit of quantification for testosterone in commercialized kits varies depending on the supplier and can reach the low picogramm/ml range (for example: *Enzo life science*; 5.67 pg/ml; *Cayman*; 6 pg/ml; *Biovendor Research and Diagnostic Products*: 22 pg/ml; *Abcam*: 70 pg/ml (limit of quantification as indicated by the manufacturer)). Commercially manufactured immunoassay kits are technically simple to carry out and relatively fast. However, both the kit manufacturer and the testing laboratory rarely thoroughly validate the assay method (Stanczyk *et al.*, 2003). Several studies evaluating the reliability of testosterone measurements with commercial kits have been carried out. These studies focused on the impact of different matrices which may result in an over- or underestimation of the testosterone levels (Wheeler *et al.*, 1996). They also compare immunoassays with other methods such as gas chromatography (Fitzgerald and Herold, 1996) or RIA (Boots *et al.*, 1998) and finally, determine the precision of intra- and inter-assay measurements (Boots *et al.*, 1998; Stanczyk *et al.*, 2003). Generally, it can be concluded that there are marked variations between the kits from different manufacturers, and that a remarkable number of kits do not fulfill all the specifications given by the manufacturer (Stanczyk *et al.*, 2007).

### 4.2.2 Mass spectrometry

For the MS measurement of steroids, either gas (GC) or liquid chromatography (LC) methods are used for the initial separation of steroids (Rosner *et al.*, 2007). The main advantage of mass spectrometry is that the steroids can be both identified and quantified. MS is also remarkably accurate, as long as the assay is thoroughly validated. However, MS methods are relatively expensive, time consuming and require considerable expertise. Despite these challenges, MS is a much preferred method in the steroid field. MS methods developed for routine measurements are very popular (Owen *et al.*, 2013) and sophisticated new methods are published frequently (Keevil, 2013; Singh *et al.*, 2014).

## 4.3 Overview of the testis and testicular cell types

The human testis is an ellipsoidal paired organ located in the scrotum. The two testicles are enclosed by connective tissue capsules for protection. Multiple connective tissue septums divide the testicular tissue in 250-350 lobes called the *lobuli testi.* These lobuli testi contain spiral winded tubules, the *seminiferous tubules.* The seminiferous tubules are protected by connective tissue and open into the *rete testis,* which is a network of tubules located in the hilum of the testicle (*mediastinum testis*) in the basal side of the testicle. About 10 *efferent ductules* are formed from the rete testis and open into the semen ducts (Thews, 1999). The inner walls of the seminiferous tubules are predominately constructed from Sertoli cells which nourish the germ cells and guide them through their development from spermatogonim to spermatozoon. Sertoli cells control this process by producing androgen binding protein that can bind testosterone, DHT and the estrogen estradiol, all of which are needed for spermatogenesis (Gershagen *et al.*, 1989; Kaur *et al.*, 2014). The outer wall of the seminiferous tubules is formed by peritubular myoid cells. In the interstitial space in between the tubules, the androgen producing Leydig cells are located (Thews, 1999). Besides the Leydig cells, there are blood capillaries and cells of the immune system present in the interstitium. Figure 8 gives an overview of the anatomy of the testis and of the different cell types present in testicular tissue.

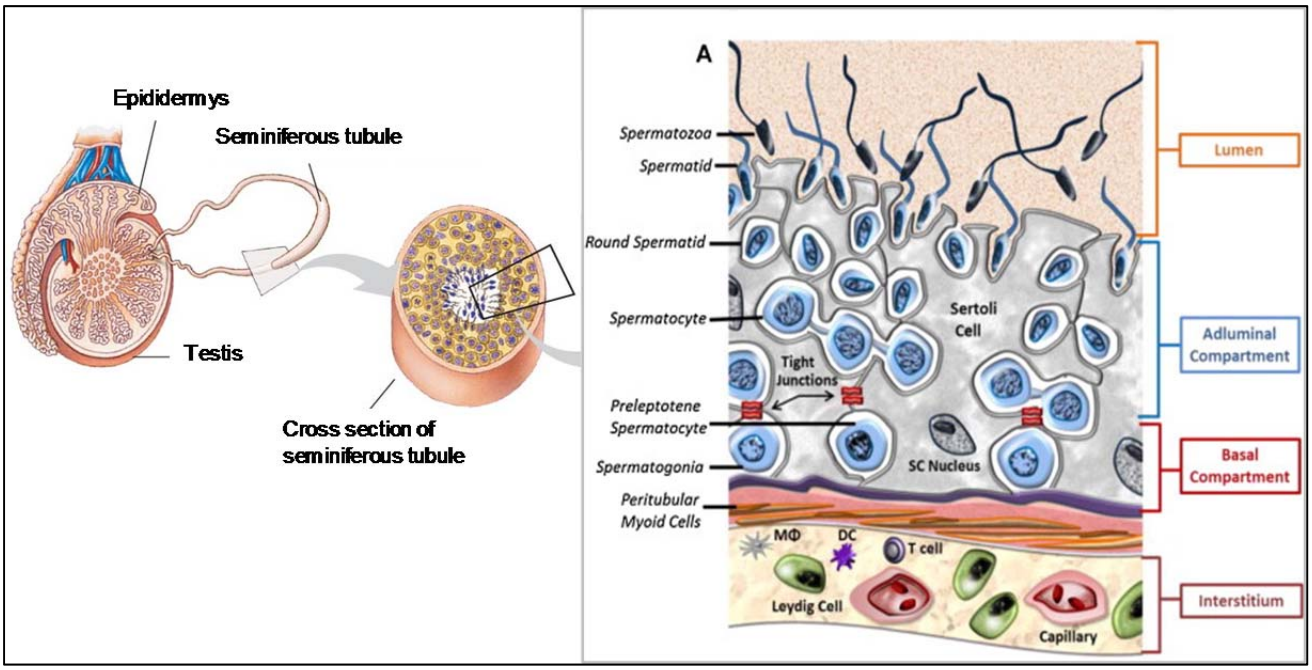


*Figure 8: Anatomical structures and cell types of the human testis (Picture modified from Pearson's Education Inc. and Kaur (Kaur et al., 2014), with permission from Elsevier*

## 4.4 Pathways involved in androgen formation – front-door versus back-door

The AR conducts pleiotropic physiological functions on one side, but also plays an important pathological role in various types of cancers such as breast or prostate cancer (Giovanni Luca *et al.*, 2013; Helsen *et al.*, 2014). The use of the AR as a therapeutic target against cancer is the focus of many research groups worldwide. To understand AR activation, it is crucial to elucidate the mechanisms and pathways by which its ligands, testosterone and DHT are formed. For decades, it was generally thought that the potent AR ligand DHT is formed by testosterone reduction catalyzed by 5α-reductase (Wilson and Walker, 1969). This hypothesis was mainly supported by the fact that 46, XY DSD (also referred to as *pseudo hermaphroditism*, a disorder where AR activation is strongly impaired) is caused by testicular 17β-HSD3 deficiency (Wilson, 1975). In patients with 17β-HSD3 deficiency, minimal testosterone is formed. However, studies using the human sebaceous gland cell line SZ95 and the human prostate cancer cell line DU-145 suggest that testosterone does not need to be the intermediate metabolite in the formation of DHT (M. Samson, 2010; Samson *et al.*, 2010). Additionally, the pathway of reducing androstenedione to androstanedione is thermodynamically more favorable, since 5α-reductase binds androstenedione with a higher affinity than it binds testosterone (Andersson and Russell, 1990). Auchus (Auchus, 2004) was the first to describe a pathway in which DHT is produced from the inactive precursor androstanediol in tammar wallybies (Wilson *et al.*, 2003). Auchus introduced the term “back-door” pathway to describe a new, alternative testosterone independent pathway to produce DHT. A lot of research has been carried out to validate the back-door pathways and there still remain many open questions. For example whether 5α-reduced C21 steroids are transformed to androsterone in humans (Attard *et al.*, 2012; Kamrath *et al.*, 2012) and whether 5α-androstenediol is produced from androstenedione or testosterone *in vivo* (Mahendroo *et al.*, 2004; Wilson *et al.*, 2003). Figure 9 (Luu-The, 2013) gives an overview of the front- and back-door pathways according the current knowledge.

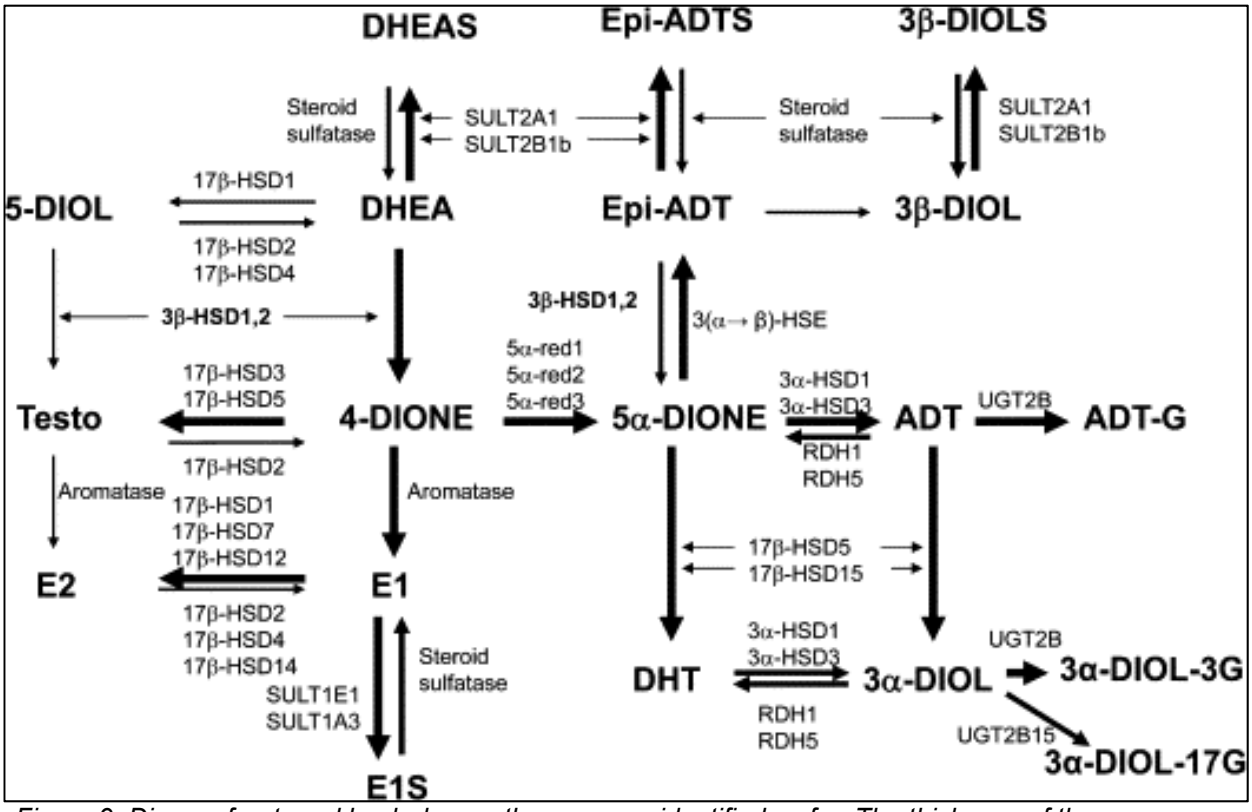


*Figure 9: Diverse front- and back-door pathways were identified so far. The thickness of the arrows represents the relative importance of the reaction. Figure used with the permission of Elsevier.*

(**3α-diol** : 5α-androstane-3α,17β-diol ; **3α-diol-G** : 5α-androstane-3α,17β-diol glucuronide ; **3α-HSD** : 3α-hydroxysteroid dehydrogenase, **3β-diol** : 5α-androstane-3β,17β-diol ;**3β-diol-G** : 5α-androstane-3β*17β-diol glucuronide ; **3β-HS** : 3β-hydroxysteroid dehydrogenase ; **4-dione**: 4-androstenedione; **5-diol**: 5-androstene-3β,17β-diol; **5α-red**: 5α-reductase; **17β-HSD**: 17β-hydroxysteroid dehydrogenase; **ADT**: androsterone; **ADT-G**: androsterone glucuronide ; **DHEA**: dehydroepiandrosterone ; **DHEAS**: dehydroepiandroserone sulfate ; **DHT**: dihydrotestosterone; **E1**: estrone ; **E1S**: estrone sulfate; **E2**: estradiol ; **epi-ADT**: epi-androsterone ; **epi-ADTS**: epi-androsterone sulfate; **SULT1E1**: estrogen sulfotransferase type 1; **SULT1A3**: phenol sulfotransferase type 3; **T**: testosterone; **UGT**: UDT-glucuronosyl transferase)

## 4.5 Leydig cell models in endocrine research

Cell-based *in vitro* bioassays are used on a routine basis in basic and applied research. A transformed cell line is an immortalized population of cells originating from a specific tissue within an organism that, due to mutations, undergo continuous growth and division. Cell lines can therefore be grown for extended periods of time. The major challenge in the development and maintenance of a cell line is to preserve the physiological phenotype (Rahman and Huhtaniemi, 2004). Leydig cell lines are often chosen in basic research to study testicular endocrine functions and to investigate the molecular basis for hormone actions, testicular cell interactions and tumor genesis. In applied research, Leydig cells are used as a model to study the mechanisms of endocrine disruptors. Fetal and adult Leydig cells differ markedly with respect to their physiology and morphology and have to be considered as two different cell types (Svechnikov *et al.*, 2010). Until now, to the best of my knowledge, no fetal type Leydig cell line exists, though such a cell line could be of great interest to study the special features of fetal-type Leydig cells. Adult-type Leydig cells appear in the mouse testis at around postnatal day 10 and increase markedly in number after postnatal day 15 (Saez, 1994).Primary Leydig cell cultures are the most physiological relevant cell model but are very difficult to culture, since these cells stop growing after the first trypinisation (Nagpal *et al.*, 1994).

Immortalized Leydig cell lines have been established by various methods such as spontaneous immortalization, like the TM3 cell line (Mather, 1980), primary cells transfected with viral oncogene (Nagpal *et al.*, 1994), by hybridization of freshly isolated murine Leydig cells with existing immortalized Leydig tumor cells (Finaz *et al.*, 1987) and from transgenic mouse Leydig cell tumors (Chalifour *et al.*, 1992). Ascoli and Puett generated the most frequently used Leydig cell line (MA-10) from a Leydig cell tumor originating from a C57BL/6 mouse (Ascoli, 1981; Ascoli and Puett, 1978). They generated the cells by first isolating them from a Leydig cell tumor (designated tumor M5480) and subsequently alternating their cultivation to and from *in vitro* and *in vivo* systems multiple times. Culture-derived tumors were cloned and clonal lines were isolated and characterized. Clones 10 and 12, later referred to as MA-10 and MA-12, respectively, were shown to have retained several functional characteristics of normal Leydig cells, such as gonadotropin binding, LH responsiveness and steroidogenic response (Ascoli, 1981; Ascoli and Puett, 1978; Lacroix *et al.*, 1979). However, later studies claimed that steroidogenesis in MA-10 cells is impaired, since it would not proceed further than the formation of progesterone (Finaz *et al.*, 1987). Another clonal Leydig cell line (mLTC1) derived from the same tumor M5480 was established and characterized by Rebois (Rebois, 1982). mLTC1 and MA-10/12 seem to obtain very similar characteristics (Rebois, 1982). MA-10 cells are by far the most widely used Leydig cell line in steroidogenesis field. However, controversy surrounds this cell line, specifically concerning its ability to produce steroids. More recently a new Leydig cell line (BLTK1) was established from a tumor of a transgenic inhα/tag mouse (Kananen *et al.*, 1996). BLTK-1 cells possess a large number of high-affinity LH receptors. They maintain their characteristics up to passage number 45 (Kananen *et al.*, 1996).

Immortalized cell lines are limited as models, but as mentioned before, animal as well as human primary Leydig cells are expensive and difficult to culture. In addition, even suing sophisticated methods, it is difficult to isolate a pure Leydig cell fraction from animal testes (Lai *et al.*, 2014). Also,

primary cells rapidly dedifferentiate upon cultivation and the duration of experiments is limited. Another major problem in studying steroidogenesis represents species dependent differences, therefore comparative analysis of hormone levels from different species should be taken with extreme caution. Furthermore, since the Leydig cells only form a small subpopulation of testicular tissue, a high number of animals are needed in order to carry out primary cell experiments, which raise ethical concerns and conflict with the 3R rules. Screening assays, requiring a high number of identically behaving cells would be complicated to perform. Another critical issue is the design of a stably transfected cell line. The establishment of stably transfected cell lines is only feasible in immortalized cells.

## 4.6 Aims of the Leydig cell characterization project

As described above, Leydig cell lines are very important tools for studying various aspects of steroidogenesis. However, caution must be taken when making conclusions using these models, since many enzymes involved in steroidogenesis are deregulated in cancer cells and importantly, Leydig cell lines derive from testicular cancers. Therefore, it is absolutely necessary to first characterize the cell lines to determine their suitability for planned assays. We have faced certain difficulties working with Leydig cell lines, such as the uncertainties about the predominant steroidogenic pathways or the abundance and formation of androgen metabolites. We therefore decided to characterize the mostly commonly used Leydig cell model, the MA-10 cells, and compare them with the newly generated BLTK-1 cell line. The validation of the testosterone production in the two cell lines was our focus, since a Leydig cell model with physiologically comparable basal testosterone production would be a significant improvement for endocrine research and help to facilitate our bioassay designs. A further goal was to validate existing methods of steroid measurements and to establish LC-MS/MS methods for the measurements of various endogenous steroids in cell media (supernatant). The quantification of steroids is challenging, since the results vary widely upon the method used (Rosner *et al.*, 2007).

## 4.7 Paper Draft

# Comparison of androgen synthesis in MA-10 and BLTK-1 mouse Leydig cells

### 4.7.1 Abstract

Leydig cell lines are essential tools for the analysis of androgen production and for mechanistic studies of steroidogenesis. There is some discrepancy in the literature about the steroidogenic enzymes expressed in different cell lines and a careful analysis of steroidogenic pathways is crucial for the appropriate interpretation of the results. In the search for a suitable cell model to study the impact of *xenobiotica* on testosterone synthesis, we compared the widely used mouse MA-10 with the more recently established BLTK-1 cells. We found important differences between the two cell models. MA-10 cells mainly produce testosterone via the well-known front-door pathway of 17β-HSD3-mediated reduction of androstenedione. In contrast, the BLTK-1 cell line produces only background levels of testosterone and predominately forms the inactive metabolite androsterone via 5α-reductase and subsequent 3α-HSD-induced metabolism of androstenedione. The differences may be explained by a higher expression of 17β-HSD3 and a lower expression of 3α-HSD1 in MA-10 compared with BLTK-1 cells. Furthermore, we provide an explanation for the misinterpretation of some results in earlier studies because of difficulties in measuring steroids in complex matrices where multiple metabolites are present. Our results emphasize the limitations of both classical thin layer chromatography (TLC) and enzyme linked immunoassays (EIA) for the quantification of steroids in medium from cultured Leydig cells. We present a validated liquid chromatography-mass spectrometry (LC-MS)-based method for the reliable quantification of steroids and propose to recognize LC-MS as a gold standard in the assessment of androgen metabolites in studies with Leydig cells.

### 4.7.2 Introduction

It is important to characterize the mechanisms involved in androgen pathways in order to understand androgen disruption-related diseases such as cancer. Steroidogenic enzymes are potential targets for drugs, chemicals, natural products and environmental contaminants (Sanderson, 2006; Whitehead and Rice, 2006). Disrupted steroidogenesis may result in problems and diseases concerning reproductive development and fertility but may also play key roles in the development and progression of cancer. Certain types of cancer are directly related to AR activation by deregulated AR ligands such as testosterone or/and 5α-dihydrotestosterone.

Leydig cells, which form a subpopulation of testicular cells, were identified in two distinct generations: fetal Leydig cell and adult Leydig cells (Svechnikov *et al.*, 2010). Fetal and adult Leydig cells markedly differ in their morphological and biochemical properties. Adult Leydig cells are not even derived from preexisting fetal Leydig cells; it is hypothesized that they are derived from undifferentiated peritubular stem cells (Ge *et al.*, 2006). However, fetal and adult Leydig cells share the principal function to produce androgens (Svechnikov *et al.*, 2010). For research focusing on the male steroid axis, Leydig cell are the best model. Studies focusing on the molecular pathways of androgen formation are often performed in rodent models (Li *et al.*, 2014; McNamara *et al.*, 2012). For mechanistic studies, primary

testis cells are used from a very diverse range of animals (Mankidy *et al.*, 2014; Morais *et al.*, 2013). The use of animal studies pose ethical concerns and, in addition, there are significant differences between animal and human pathways (Furstenberger *et al.*, 2012; Meyer *et al.*, 2013). Furthermore, animal experiments generally do not significantly contribute to the elucidation of mechanisms (Andersen and Krewski, 2009; Holsapple *et al.*, 2009). Primary human testis or prostate cells are generally not easy to obtain and culture conditions are difficult and expensive. Furthermore, since primary testis cells are difficult to keep in culture, they are not suited for screening assays requiring a multitude of conditions such as different time points or concentrations.

There are only a limited number of different Leydig cell lines available and their characterization in the literature often is controversial. Ascoli in the 1980's established a Leydig cell line from transplantable Leydig cell tumors (Ascoli, 1981). This cells line, the MA-10 cells, were shown to be gonadotropin receptor positive and able to produce basal levels of pregnenolone, progesterone, 20α-progesterone, 17α-hydroxyprogesterone, androstenedione and testosterone (Ascoli, 1981). However, it was later questioned whether the MA-10 cells express all of the key enzymes of steroidogenesis, especially with respect to production of testosterone (Midzak *et al.*, 2011; Payne, 1990). In 2012, Forgacs et al. (Forgacs *et al.*, 2012) introduced a new Leydig cell line, the BLTK-1, which was derived from cells that were isolated from a testicular tumor developed in a transgenic mouse expressing the mouse inhibin α promoter/ simian virus 40 T-antigen fusion gene (Rahman and Huhtaniemi, 2004). The BLTK-1 cell line was shown to produce progestines (Pregnenolone, 17α-hydroxypregnenolone, 17α-hydroxyprogesterone) and to further metabolize them into androgens (dehydroepiandrosterone (DHEA), androstenedione, testosterone, DHT) and estrogens (estrone and 17β-estradiol) (Forgacs *et al.*, 2012). Forgcas et al. based their conclusions on the mRNA expression and protein of steroidigenic enzymes and on steroidogenic end product measurement using EIA kits.

In the present study, we compared MA-10 and BLTK-1 mouse Leydig cell lines regarding their intrinsic steroidogenic pathways. The results are of special interest regarding the testicular "back-door pathway", a pathway first mentioned in 2000 (Shaw *et al.*, 2000; Wilson *et al.*, 2003) and leading to DHT formation by bypassing the intermediate testosterone. To our knowledge, this is the first study to compare androgen formation pathways for the quantification of androgens in different Leydig cell lines and to sub sequentially compare different methods for the quantification the androgens formed.

### 4.7.3 Materials and Methods

**Cell culture of MA-10 and BLTK-1 cells**

The mouse Leydig cell line MA-10 (kindly provided by Professor Mario Ascoli, University of Iowa, Iowa City, IA (Ascoli, 1981)) was cultivated as described previously (Legeza *et al.*, 2013). Briefly, cells were grown on 0.1% gelatin-coated cell culture dishes in DMEM/F12 medium (Invitrogen, Carlsbad, CA, USA) containing 20 mM HEPES (Gibco life technologies, Carlsbad, CA, USA), 15% horse serum (Gibco life technologies), and 50 µg/ml gentamicin (Sigma-Aldrich, St. Louis, MO, USA). The BLTK-1 mouse Leydig cell line (kindly provided by Professor Huhtaniemi and Dr. Rahman, University of Turku, Turku, Finland (Forgacs *et al.*, 2012)) was cultivated as described previously (Forgacs *et al.*, 2012). Briefly, cells were maintained in DMEM/F12 medium (Invitrogen) with 10 % fetal bovine serum (Gibco life technologies) 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich). Both cell lines were

incubated under standard conditions (5% $CO_2$, 37°C). For the LC-MS/MS samples, cells were cultivated in phenol red-free medium (Invitrogen).

**Determination of the mRNA levels of *SDR5A1*, *HSD17B3* and *HSD3A1***

Total RNA from MA-10 and BLTK-1 cells was extracted using Trizol reagent, followed by reverse transcription using the Superscript III reverse transcriptase (Invitrogen). The mRNA levels from different genes were analyzed using a Rotor-Gene 6000 light cycler (Corbett, Sydney, Australia). Briefly, reactions were performed in a total volume of 10 µl reaction buffer containing KAPA SYBR master mix (Kapasystems, Boston, MA, USA), 20 ng cDNA and specific oligonucleotide primers (Table 4). Relative gene expression compared with the internal control glycerinaldehyde-3-phosphate-dehydrogenase (GAPDH) was determined using the delta-delta-CT method.

| Gene | Oligonucleotide primer **forward** | Oligonucleotide primer **reverse** |
|---|---|---|
| *SDR5A1* | 5'-TCA-CCT-TTG-TCT-TGG-CCT-TC-3' | 5'-TTA-TCA-CCA-TGC-CCA-CTA-ACC-3' |
| *HSD17B3* | 5'-TGA-GTG-TGA-ATA-CAG-AGA-TAA-AGG-3' | 5'-TCC-GAT-CGT-GAC-ATA-TTT-CAA-GG-3' ' |
| *HSD3A1* | 5'-GGT-TTT-TGG-GGC-AGA-GGA-TCA-3' | 5'-GGT-ACT-GGG-TGT-CAA-GAA-TGT-CT-3' |

***Table 4: Oligonucleotide primers used for RT-PCR***

**Determination of the androstenedione metabolizing rate by thin layer chromatography**

The activity of androstenedione consumption was measured using a modified protocol from Legeza et al. (Legeza *et al.*, 2013). Briefly, cells were incubated in serum and steroid free DMEM/F12 medium containing 200 nM radiolabeled [$^{1, 2, 6, 7\text{-}3}$H]-androstenedione (GE Healthcare, Little Chalfont, UK) for 30 minutes (BLTK-1) and 4 hours (MA-10). The enzymatic reactions were terminated by adding unlabeled androstenedione and testosterone (Sigma-Aldrich) dissolved in methanol. The steroids were separated on UV-sensitive silica TLC plates (Macherey-Nagel, Oensingen, Switzerland) using a chloroform-methanol solvent system at a ratio of 9:1. Bands corresponding to the respective steroid were scraped off the TLC plate and transferred to tubes containing scintillation cocktail. Radioactive decay of androstenedione and corresponding metabolites were analyzed on a scintillation counter.

**Validation of the enzyme Immunoassay (EIA) kit for testosterone**

The EIA kit for testosterone (Cayman Chemical Company, Michigan, USA) was used according to the manufacturer's instructions. 5α- and 5β-dihydrotestosterone, androstendiol, 5α-androstanedione, androsterone, eticholanolone and dihydroepiandrosterone were purchased from Steraloids (Newport, RI, USA). All tested metabolites were dissolved in ethanol, diluted in the buffer provided with the kit and measured at three different concentrations (150 pg/ml, 75 pg/ml and 37.5 pg/ml) in duplicate. Cross-reactivity was calculated in percentage of the recovery rate of the corresponding metabolite.

**LC-MS/MS**

**Sample preparation**

MA-10 and BLTK-1 cells at confluence of 80% and passage 5-10 were washed with phosphate buffered saline (PBS) and incubated with DMEM/F12 containing 200 nM of androstenedione for 4 hours (MA-10 cells) or 30 min, 1 hour, 2 hours and 4 hours (BLTK.1 cells). Stock solutions were prepared in methanol for labeled internal standards (IS) (Steraloids) at a concentration of 10 mM. Thereafter, standards and deuterium labeled IS working solutions were prepared by mixing each individual stock solution to obtain a working concentration of 100 µM. Calibration curves were prepared by serial dilution of the working solutions of standards in DMEM/F12 phenol red-free medium in the range of 0.975 nM to 1000 nM. Cell supernatant was taken at the appropriate time point and IS at a final concentration of 0.1 µM in protein precipitation solution (zinc sulfate 0.8 M in water/methanol 50/50 v/v) were added. After shaking vigorously for 10 min at 4°C, the samples were centrifuged 10 min at 10'000 x g. Samples were transferred onto Oasis HBL SPE columns (Waters, Milford, MA, USA) that were preconditioned with methanol and water. Samples were washed 2 times with water, eluted with methanol and evaporated at a vacuum evaporator. Samples were reconstituted in 100 µl methanol and shacked vigorously for 30 min at 4°C.

**Separation, ionization and conditions for detection of androgens**

The samples were analysed by ultra-pressure liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). An Agilent 1290 UPLC coupled to an Agilent 6490 triple quadruple mass spectrometer equipped with an electrospray ionization (ESI) source was used (Agilent Technologies, Basel, Switzerland). Analytes were separated by using a reversed-phase column (ACQUITY UPLC BEH $C_{18}$, 1.7 µm, 2.1×150 mm, Waters, Wexford, Ireland) that was heated to 70ºC. Data acquisition and analysis was performed using Mass Hunter software (Agilent Technologies). The mobile phase consisted of water-acetonitrile-formic acid (A) (95/5/0.1; v/v/v) and (B) (5/95/0.1; v/v/v). The eluent gradients were set from 25 - 75% of B during 0- 20 min, and 100% of B at 22.min onwards. The run was stopped at 24 min, including washing and re-equilibration of the column. The flow rate was set to 0.650 mL/min. Ionization was performed using an ESI source operated in the positive ion modes (Table 5). Fragmentation was tuned for each compound using Optimizer software (Agilent Technologies) except 5β-DHT and androstenediol were quantified using the precursor ion. Optimal conditions are shown in Table 5. The source parameters were set to gas temperature 290°C, gas flow 14 L/min, nebulizer pressure 15 psi, sheath gas temperature 300ºC, sheath gas flow 11 L/min, capillary voltage 6000 V (positive), nozzle voltage 1500 V and cell accelerator voltage 4 V. Ion funnel parameters for positive and negative high pressure were set to 200 and 110 respectively.

| Steroid | RT [min] | Precursor Ion (*m/z*) | Product ion (*m/z*) | Collision energy (V) | Polarity | Internal Standard |
|---|---|---|---|---|---|---|
| Androstenediol | 7.4 | 255.2 | 255.2 | 0 | Positive | Testosterone-d2 |
| Androsterone | 18.1 | 273.2 | 255.2 | 12 | Positive | Testosterone-d2 |
| Testosterone | 8.1 | 289.2 | 97.1 | 28 | Positive | Testosterone-d2 |
| Androstenedione | 9.5 | 287.2 | 97.1 | 20 | Positive | Testosterone-d2 |
| 5α-DHT | 12.5 | 291.2 | 159.1 | 24 | Positive | Testosterone-d2 |
| 5ß-DHT | 13.8 | 255.1 | 255.1 | 0 | Positive | Testosterone-d2 |
| Testosterone-d2 | 8.1 | 291.5 | 111.1 | 24 | Positive | |

***Table 5**: Overview of androgens measured and mass spectrometer properties*

### 4.7.4 Results

**Expression of steroidogenic enzymes (mRNA) in MA-10 and BLTK-1 cells**

The mRNA levels of three the key steroidogenic enzymes, 17β-HSD3, 3α-HSD1 and 5α-reductase1 were measured using real-time polymerase chain reaction (RT-PCR) (Figure 10A and 10B). MA-10 and BLTK-1 express *HSD17B3* mRNA, encoding the key enzyme of the front-door pathway which catalyzes the activation of androstenedione to testosterone; however, the mean cycle threshold (ct) for *HSD17B3* values of 28.2 in MA-10 cells and 31.1 in BTK-1 cells indicate a higher expression in MA-10 cells. The 5α-reductase *SRD5A1* mRNA encoding a key enzyme of the back-door pathway (Luu-The, 2013) was highly expressed in both, BLTK-1 (ct value 18.8) and MA-10 cells (ct value 19.5). The 3α-HSDs also are an enzyme family of with important roles in the back-door pathway, catalyzing the reaction from the androstanedione to the androsterone. *HSD3A1*, an important isoform of the 3α-HSDs, is expressed in both cell lines, with a much higher expression (ct value of 17.6) in BLTK-1 than in the MA-10 cells (ct value of 25.3).

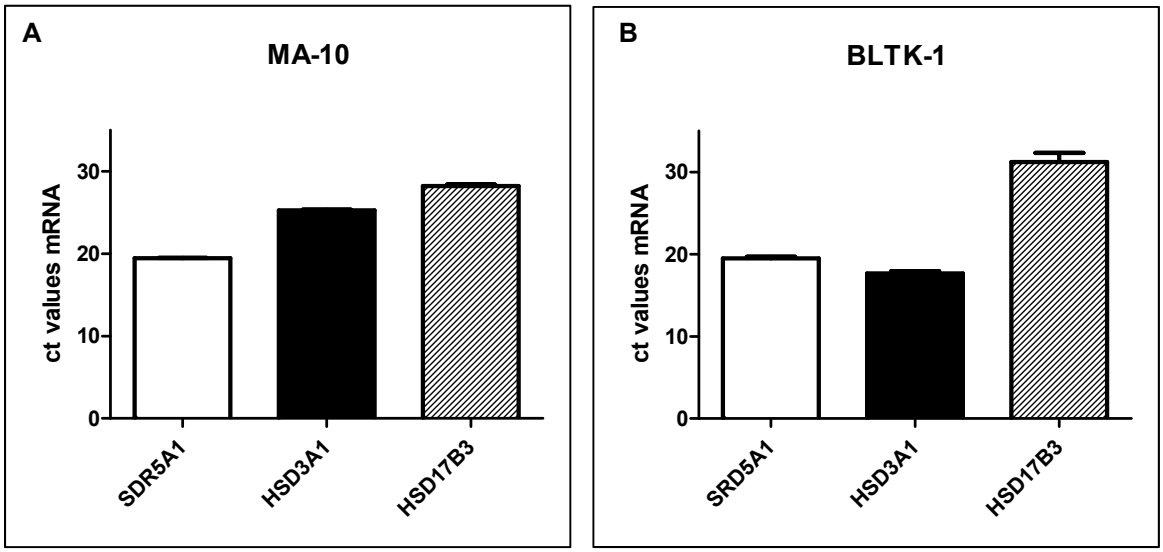


***Figure 10: mRNA expression in MA-10 (A) and BLTK-1(B) Leydig cells differ.** The expression of three key steroidogenic enzymes (SDR5A1, HSD3A1 and HSD17B3) and were measured in MA-10 and BLTK-1 Leydig cells. The data represent the mean ± S.D. of three independent measurements.*

**Metabolism of androstenedione in MA-10 and BLTK-1 cells measured by using thin layer chromatography**

In a first step, we compared the ability of MA-10 and BLTK-1 cells to metabolize androstenedione by determination of the conversion rate of radiolabeled androstenedione (scintillation counting), following separation of the steroids by TLC. The BLTK-1 cells showed a much higher rate of androstenedione disappearance than MA-10 cells (Figure 11). After only 30 min of incubation 46% of the initially supplied androstenedione was converted to a product with similar migration behavior on the TLC plate than the unlabeled testosterone that was added to the reaction termination buffer. In contrast, only 22% of the androstenedione supplied was converted by the MA-10 cells after 120 min. Thus, BLTK-1 cells displayed a 5-10 times higher efficiency to metabolize androstenedione than MA-10 cells, despite clearly lower *17BHSD3* mRNA levels.

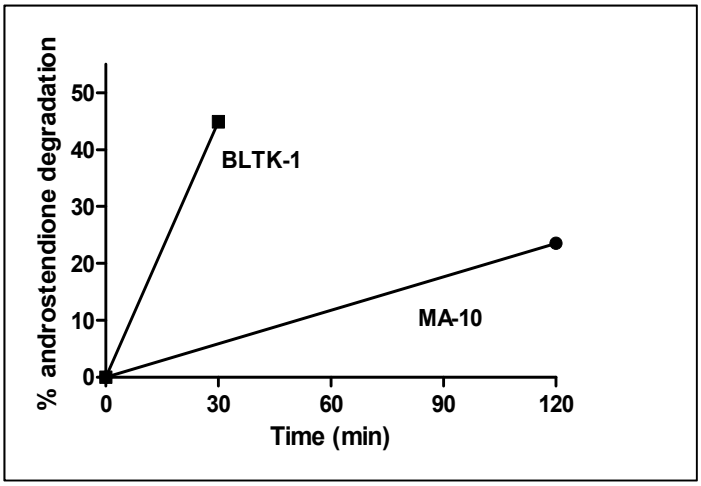


***Figure 11: Androstenedione degradation in the two different Leydig cell lines, MA-10 and BLTK-1****. Scintillation counting following TLC was performed after incubation with 200 nM radiolabeled androstenedione (incubation time was 30 min for the BLTK-1 cells and 2 hours for MA-10 cells). The data represent the mean ± S.D. of three independent experiments.*

After adjusting the thin layer chromatography protocol (extended run, excision of several sections per lane) it could be shown, that the main metabolite formed in BLTK-1 migrates slightly slower than the testosterone control on the TLC plate. The unknown metabolite was formed rapidly, showing a reverse pattern compared with androstenedione disappearance (Figure 12).

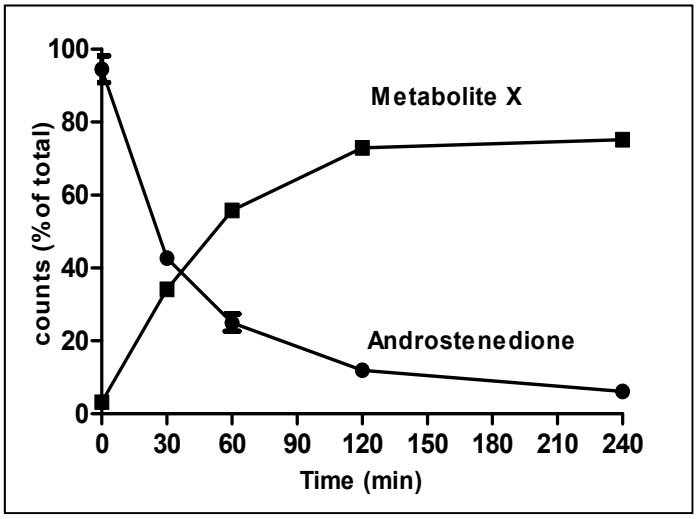


***Figure 12: The rapid metabolism of androstenedione follows a reverse pattern to the formation of a new metabolite in adjusted TLC separation****. Scintillation counting following adjusted TLC was performed after incubation with 200 nM radiolabeled androstenedione at 30 min, 1 hour, 2 hours and 4 hours. The data represent the mean ± S.D. of three independent experiments.*

**Assessment of the TLC results using LC-MS/MS**

In order to identify the metabolite predominately formed from androstenedione in BLTK-1 cells, we applied LC-MS/MS. Upon verification with an authentic standard we could show that not testosterone but androsterone is time-dependently formed from androstenedione in BLTK-1 cells (Figure 13). DHT and testosterone are produced in small quantities (less than 1% of total) in BLTK-1 cells.

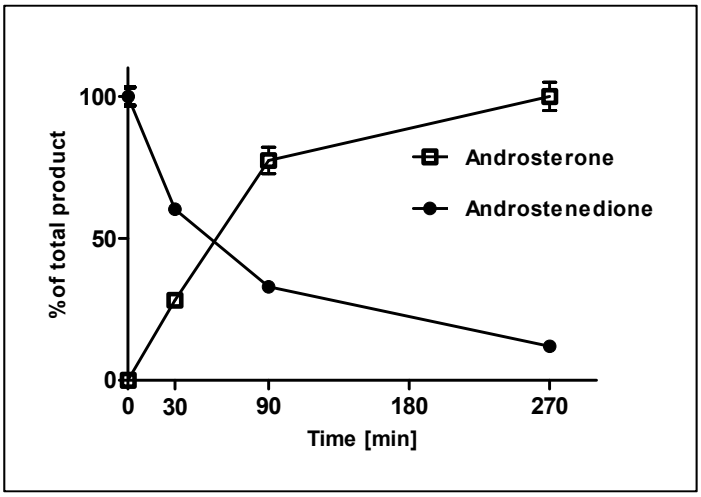


***Figure 13: LC-MS/MS measurements show that androstenedione is time dependently metabolized to androsterone in BLTK-1 cells****. The levels of androstenedione and androsterone were measured using LC-MS/MS after 30, 60 and 90 minutes. The data represent the mean ± S.D. of technical triplicates.*

Using LC-MS/MS measurements, we could show that MA-10 cells indeed produce testosterone following the incubation with androstenedione (Figure 14). DHT and androsterone were not produced at relevant levels.

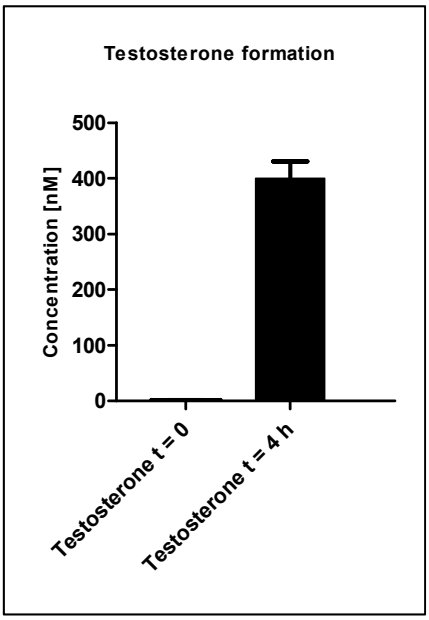


***Figure 14: Testosterone formation observed in LC-MS/MS measurements in MA-10****: The level of testosterone in cell supernatant of MA-10 cells which incubated with 1000 nM androstenedione for 4h were measured using LC-MS/MS. The data represent the mean ± S.D. of technical triplicates.*

**Validation of the testosterone enzyme immunoassay kit**

Previous studies by Forgacs et al. (Forgacs *et al.*, 2013; Forgacs *et al.*, 2012) reported the production of testosterone by BLTK-1 cells based on the results from a testosterone EIA kit (Cayman Chemical Company, Michigan, USA). The validation of the kit by the provider did not include several androgen metabolites, including androstenediol. Therefore, we validated the EIA kit for cross-reactivity towards several androgen metabolites (Table 6 and Figure 15).The validation resulted in the detection of multiple metabolites from the front-door as well as the back-door pathway, confirming a high cross-reactivity rate in this commercially available testosterone assay kit.

| | **mean recovery rate (cross-reactivity ) mean ± SD** |
|---|---|
| Testosterone | 98.3 ± 9.6 |
| 5α-Androstanedione | 20.6 ± 8.4 |
| 5α-Dihydrotestosterone | 58.6 ± 8.8 |
| 5β-Dihydrotestosterone | 28.5 ± 4.9 |
| Androstenedione | 25.4 ±6.2 |
| Androstenediol | 36.4 ± 2.8 |
| Androsterone | 16.4 ± 10.9 |
| Dihydroepiandrosterone | 5.5 ± 3.0 |
| Etiocholanolone | 18.3 ±0.6 |

***Table 6: The EIA kit validated shows marked cross-reactivity.*** *Different metabolites were tested in three concentrations (150 pg/ml, 75 pg/ml and 37.5 pg/ml) in duplicates. (The table shows the data in an alphabetical order) The data represent the mean values obtained within the three concentrations ± S.D.*

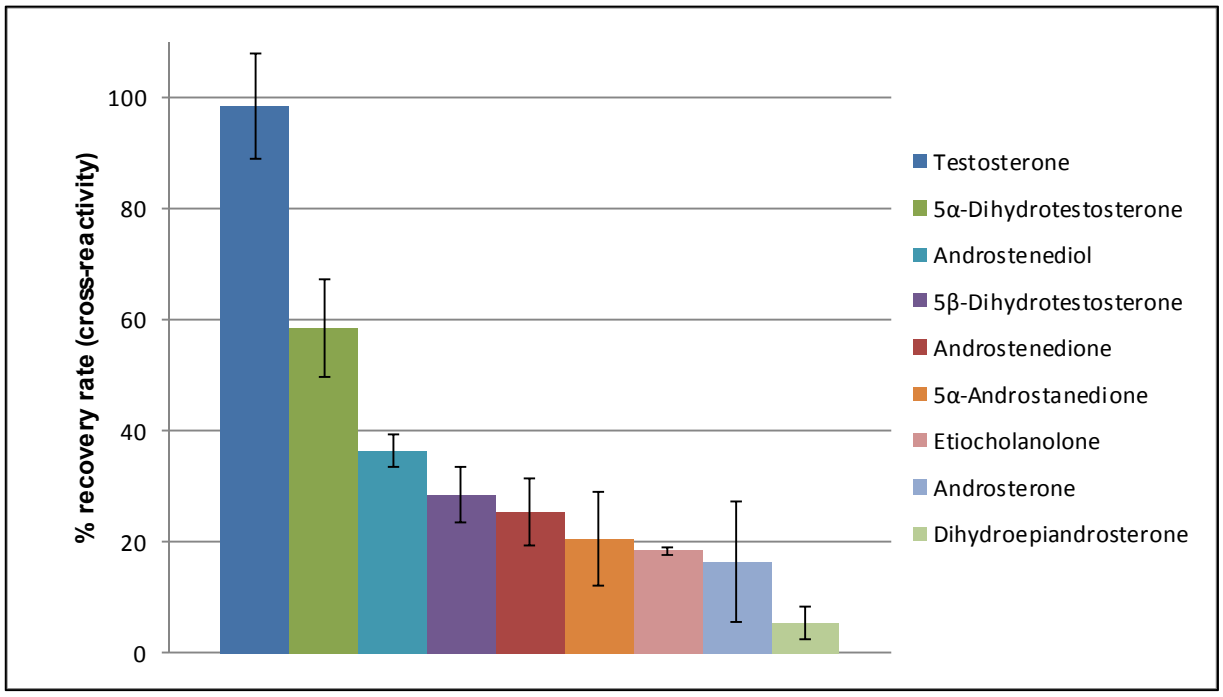


***Figure 15: Graphical representation of cross-reactivity of metabolites obtained with the EIA kit validated.*** *The different metabolites which were tested in three concentrations (150 pg/ml, 75 pg/ml and 37.5 pg/ml) in duplicates are shown in descending order. The data represent the mean values obtained within the three concentrations ± S.D.*

### 4.7.5 Discussion

The present study confirms the complexity of steroidogenesis and demonstrates the importance of the characterization of appropriate cell models for studying steroidogenesis for translational purposes. In addition, appropriate methods for detection and quantification are required. Our study compared two Leydig cell models and revealed marked differences in their predominant steroidogenic pathways. We focused on the well-established and broadly used mouse Leydig cell line MA-10 and the more recently established cell line BLTK-1 (Forgacs *et al.*, 2012). The results indicate, that the MA-10 cell line follows the well characterized front-door pathway, where androstenedione is converted to testosterone by 17β-HSD3 as recently supported by Legeza et al. (Legeza *et al.*, 2013). However, our results show that the time of incubation for testosterone formation from androstenedione is relatively long in MA-10 cells, indicating a relatively modest expression of 17β-HSD3. Thus , the MA-10 cells partially lost the characteristics of primary Leydig cells, which have high expression of 17β-HSD3 (Luu-The, 2013). Although the mRNA expression of *HSD17B3* and the conversion rates are relatively low, in contradiction to previous studies (Midzak *et al.*, 2011; Rahman and Huhtaniemi, 2004), we demonstrate that MA-10 cells express functional 17β-HSD3 and produce testosterone from the inactive precursor androstenedione. Possible reasons for the discrepancy may include different passage numbers of the MA-10 cells used, culture conditions and/or different methods for detection and quantification that were applied in these studies.

The 17β-HSD3–dependent conversion of androstenedione to testosterone is referred to as the “front-door” pathway (Auchus, 2004), the common pathway by which testicular Leydig cells produces the AR ligand testosterone (Luu-The, 2013). However, under conditions of cellular stress such as in cancer, Leydig cells can undergo changes, and as a consequence favor the formation of the more potent AR ligand DHT by bypassing testosterone formation, a process known as the “back-door” pathway (Auchus, 2004; Luu-The, 2013). Several back-door pathways have been identified so far. Their common feature is that they utilize intermediate metabolites, which were previously considered as decomposition products of steroidogenesis (Luu-The, 2013), for the production of potent androgens. Our results suggest that the Leydig cell model BLTK-1 favors the metabolism of androstenedione through a back-door pathway. Our results suggest that upon incubation with androstenedione, 5α-steroid reductase1/2 (SDR5A1/2) catalyzes the formation of the intermediate 5α-androstanedione which is rapidly transformed to androsterone by 3α-HSD1/3. In the presence of both, 17β-HSD3 and 5α-reductase1/2, androstenedione favors the binding of 5α-reductase1/2 (Luu-The, 2013; M. Samson, 2010; Yamana, 2010). In tissues where high concentrations of testosterone are required, such as in the testis, *SDD5A1* mRNA is approximately 85% lower than *HSD17B3* mRNA (Luu-The, 2013). In tissue with 17β-HSD3 excess, testosterone is formed from androstenedione (Geissler *et al.*, 1994). In BLTK-1 cells, the mRNA expression of *SDR5A1* is much higher than the mRNA levels of *HSD17B3*. This finding is coherent with our observations that testosterone formation is limited to less than 1% of the total conversion products identified by LC-MS/MS. Surprisingly, *SDR5A1* mRNA is also highly expressed in MA-10 cells. However, LC-MS/MS and TLC measurements suggest that testosterone is the major metabolite in the MA-10 cell line. Further experiments are required to understand the reasons why MA-10 cells still favor the conversion of androstenedione to testosterone. The protein expression and the functionality of MA-10 5α-reductase1/2 have to be examined. The absence of the

conversion of androstenedione to 5α-androstanedione may be due to the absence of cofactors or low protein expression. 5α-androstanedione in BLTK-1 cells is rapidly transformed into androsterone. Androsterone is thought to be an inactive steroid that is found in the circulation at relatively high concentrations (around 1400 nM) in healthy men (Luu-The, 2013). It was reported by Labrie et al. that androsterone is the most abundant inactive precursor steroid in males aged of 20- 30 years (Labrie *et al.*, 1997a). Besides the formation of androsterone, very low amounts of DHT are formed in BLTK1-cells. The reasons why MA-10 cells predominately favor the front-door pathway whereas in BLTK-1 cells favor the back-door pathway remains unexplained so far. Both cell lines originate from cancer cells, though they were isolated using different methodologies (Rahman and Huhtaniemi, 2004).

Measurements of complex steroid matrices by simple laboratory techniques such as TLC are very difficult and prone to error. We propose that two dimensional TLC systems should be used for validated applications only and mass spectrometry based methods should be recognized as gold-standard. The immunoassay kit that was used in earlier studies (Forgacs *et al.*, 2013; Forgacs *et al.*, 2012) showed high cross-reactivity towards metabolites of the back-door pathway that have not all been included in the validation by the provider. Despite ability of the kit to accurately determine testosterone levels within a controlled sample, unfortunately, it also detected a wide variety of other androgen metabolites. This led to misinterpretation and overestimation of the testosterone levels in earlier studies (Forgacs *et al.*, 2013; Forgacs *et al.*, 2012). Testosterone-selective antibodies are very difficult to design, due to structural similarities to other metabolites of the androgen axis. The interpretation of the results of the recent study by Forgas et al (Forgacs *et al.*, 2013) need to be revisited. The data presented as testosterone concentrations may represent androsterone levels or a mixture of metabolites of the back-door pathway. Forgas et al. tested the impact of triazine herbicides on steroidogenesis. They proposed that in the cell supernatant of BLTK-1 cells incubated with several triazide herbicides testosterone may be up regulated, which may reflect androsterone instead. They also conducted experiments on the mRNA levels of several enzymes involved in steroidogenesis and showed that they are altered at high micromolar concentrations of the herbicides. Importantly, a direct link between the elevation of “testosterone” levels and the up regulation of 17β-HSD3 could not be concluded from their study, which is in line with our assumption that the elevated steroid may not be testosterone but rather androsterone. Herbicides were shown to induce the steroidogenic acute regulatory protein (StAR), which exerts its action on the entire steroidogenic pathway. This suggests that triazine herbicides may act as general disruptor of steroidogenesis and not via the induction of 17β-HSD3- mediated testosterone production.

In conclusion, the Leydig cell lines MA-10 and BLTK-1 both express key elements involved in steroidogenesis, yet their enzymatic profiles differ. Both models are equally suitable to study regulatory effects on the *HSD17B3* mRNA level since both cell lines possess functional 17β-HSD3. However, the lack of a selective antibody for 17β-HSD3 limits studies on the protein level. With respect to enzymatic activity assays, MA-10 cells and BLTK-1 cells differ markedly. For studies measuring the enzymatic activity of 17β-HSD3, MA-10 cells should be chosen, since upon incubation with androstenedione the preferred pathway is the 17β-HSD3-mediated reduction to testosterone. Our data suggest that BLTK-1 cells represent a good model to study effects on the back-door or

degradation pathways, since androstenedione is predominately metabolized to the inactive androgen androsterone. For future studies, the regulation of the key enzymes determining the back-door pathways in BLTK-1 cells, specifically 5α-reductase1/2 should be investigated in more detail. Such studies may unveil the mechanism by which 5α-reductase inhibitors induced 5α-reductase resistance in castration resistant prostate cancer

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# 5. Project 3: Transcriptional regulation of 17β-hydroxysteroid dehydrogenase

## 5.1 Introduction

The importance of a fully functional 17β-HSD3 is, as described in chapters above, essential for sexual differentiation and development as well as for multiple health aspects. It is generally accepted that reduced enzyme expression/function or events that results in enzymatic dysregulation, underlie the pathogenesis of multiple disorders. This chapter focuses on different aspects of 17β-HSD3 up regulation, including promoter activation, mRNA levels and enzymatic activity.

### 5.1.1 Possible consequences of 17β-hydroxysteroid dehydrogenase type 3 up regulation

17β-HSD3 up regulation directly results in higher testosterone levels. Dysregulated testosterone levels are associated with cancer progression, although its distinct mechanistic role remains unclear and needs further research. Older studies showed a direct association between testosterone levels and the development of various types of cancer, in addition to an increased risk of overall mortality (Gann *et al.*, 1996; Parsons *et al.*, 2005). More recently, there is increasing evidence that higher testosterone levels do not directly contribute to the onset of cancer (Eisenberg *et al.*, 2014; Orsted *et al.*, 2014). It was concluded by Orsted et al. (Orsted *et al.*, 2014) that increased plasma testosterone levels are associated with an 80% higher risk of early death following cancer diagnosis in both men and women, but do not alter the risk of developing cancer in the first place. It appears that high testosterone levels negatively influence the pathogenesis of cancer and therefore the pathophysiological mechanisms of testosterone need to be further evaluated. It was shown in two older studies, that testosterone stimulates the growth of lung and colon cancer cells *in vitro* and that this effect can be halted with anti-androgens (Maasberg *et al.*, 1989; Tutton and Barkla, 1982). In a recent review, focusing on molecular pathways of prostate cancer (Mazaris and Tsiotras, 2013), several mutations in various genes encoding for enzymes of the steroidogenesis such as in *HSD3B1/2* (Chang *et al.*, 2002) or in *CYP17* allele (Hughes *et al.*, 2005) were identified and are suspected to contribute to prostate cancer. The AR, the target nuclear receptor of testosterone, is widely accepted to play an important role in the initiation and growth of prostate cancer (PC) (Taplin and Balk, 2004).

PC is typically treated in the clinic with androgen deprivation therapy. Within the different stages and etiology of PC, different drug regimens are selected. Predominately in the early phases of the disease, inhibitors of steroidogenesis such as 5α-reductase inhibitors (e.g. finasteride or dutasteride) or the CYP17-inhibitors (e.g abiraterone) are administered (Fryczkowski *et al.*, 2014). Such enzyme inhibitory treatment results in castration-like low androgen levels. In locally advanced as well as in metastatic PC, AR inhibitors are used to induce chemical castration (Poutiainen *et al.*, 2014). About 85% of PCs (in terms of low prostate specific antigen (PSA), the clinical biomarker of prostate cancer) can be successfully treated (Taplin and Balk, 2004). However, there are a distinct set of PCs which develop resistance towards androgen deprivation therapy. This type of PC is referred to as hormone-independent PC. The AR is believed to play an important role in the shift from an androgen-dependent to androgen-independent cancer (Taplin and Balk, 2004). Furthermore, the facts that a majority of PCs

express high levels of AR and are dependent on testosterone for growth, underlie the importance of this receptor-ligand interaction for the development of PC (Cai and Balk, 2011). In addition to PC, AR activation plays an important role in the development of breast cancer. However, unlike in PC, AR expression and activation plays a more complex role in breast cancer growth and development, since beneficial effects have been demonstrated with both androgens and anti-androgen therapy, suggesting that multiple factors are involved (D'Abreo and Hindenburg, 2013). In summary, the role of testosterone in the development, progression and outcome of cancer remain to be fully understood. However, it is assumed that dysregulation of the processes that control testosterone action, either enzymatic or via target receptor activation, contribute to cancer development.

### 5.1.2 Inflammation, tumor necrosis factor-α and cancer

Tumor necrosis factor-α (TNF-α) was initially identified as “endotoxin-induced serum factor that causes necrosis of tumors” (Carswell *et al.*, 1975). Extensive research has been carried out on the effects of TNF-α, including cell cycle, cell death and survival (Locksley *et al.*, 2001). There was initial enthusiasm that TNF-α could be used as an anticancer cytokine. However, this faded when it was shown that TNF-α and its down-stream targets are in fact major mediators of cancer-related inflammation (Balkwill, 2009; Pikarsky *et al.*, 2004; Sethi *et al.*, 2008). Besides its roles in cancer, TNF-α was identified as a master regulator of inflammation, resulting in TNF-α antagonists being developed to treat rheumatoid arthritis and other inflammatory diseases (Feldmann, 2002; Sands *et al.*, 2004; Tracey *et al.*, 2008). Pro-tumor actions of TNF-α are mainly mediated via the tumor necrosis factor receptor 1 (TNFR1) (Harrison *et al.*, 2007). *Tnfr1*$^{-/-}$ mice show attenuated development of primary cancers and metastases compared to wild-type mice (Arnott *et al.*, 2004; Popivanova *et al.*, 2008). The link between TNF-α, inflammation and cancer is well established, however, it is still debated whether TNF-α is a therapeutic target, a treatment or both. Efforts still persist to use the TNF-α tumor-destructive activity as therapy (Daniel and Wilson, 2008).

TNF-α exerts its action via multiple pathways, which are still being investigated. However, the most commonly known and best characterized pathway of TNF-α action is the activation of nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF-κB). Briefly, TNF-α activates the cytoplasmic protein I kappa B-Kinase (IKK), which phosphorylates the inhibitory I kappa Bα-protein *(Mercurio et al., 1997).* This phosphorylation results in the dissociation of IκBα from NF-κB, which then translocates into the nucleus and activates the expression of target genes. NF-κB interacts with the glucocorticoid receptor (GR), such that GR activation antagonizes NF-κB induction (Rao *et al.*, 2011). Another known TNF-α signalling pathway is mediated through the mitogen activated protein kinases (MAPKs) (Sabio and Davis, 2014). TNF-α-dependent receptor activation ultimately results in a transcriptional up regulation through a cascade starting with the activation of MAPK kinase kinase (MAP3K) which phosphorylates MAPK kinase (MAP2K) which in turn phosphorylates MAPK, which finally activates downstream transcription factors, such as AP-1 or ATF-2 (Sabio and Davis, 2014).
Three MAPK cascades have been defined: ERK (extracellular signal-regulated kinase), JNK (c-Jun N-terminal kinase) and p38 MAPK (Sabio and Davis, 2014).

Li et al. identified the p38 MAPK pathway (Li *et al.*, 2005). This pathway involves the recruitment of receptor interacting protein (RIP) by TNFR-associated death domain (TRADD). RIP recruits MAP3K which in turn phosphorylates the MAPK kinase 3 and 6 (MAPKK3 and MAPKK 6 ), which leads to p38 MAPK activation (Aggarwal, 2003). The JNK MAPK pathway involves recruitment of the MAPK kinases 4 and 7 (MKK4 and MKK7); the ERK MAPK pathway is mediated by MAPK kinases 1, 2 and 5 (MKK1/2 and MKK5) (Sabio and Davis, 2014). Figure 16 provides an overview on the major signalling pathways of TNF-α, NF-κB and the three major MAPK pathways.

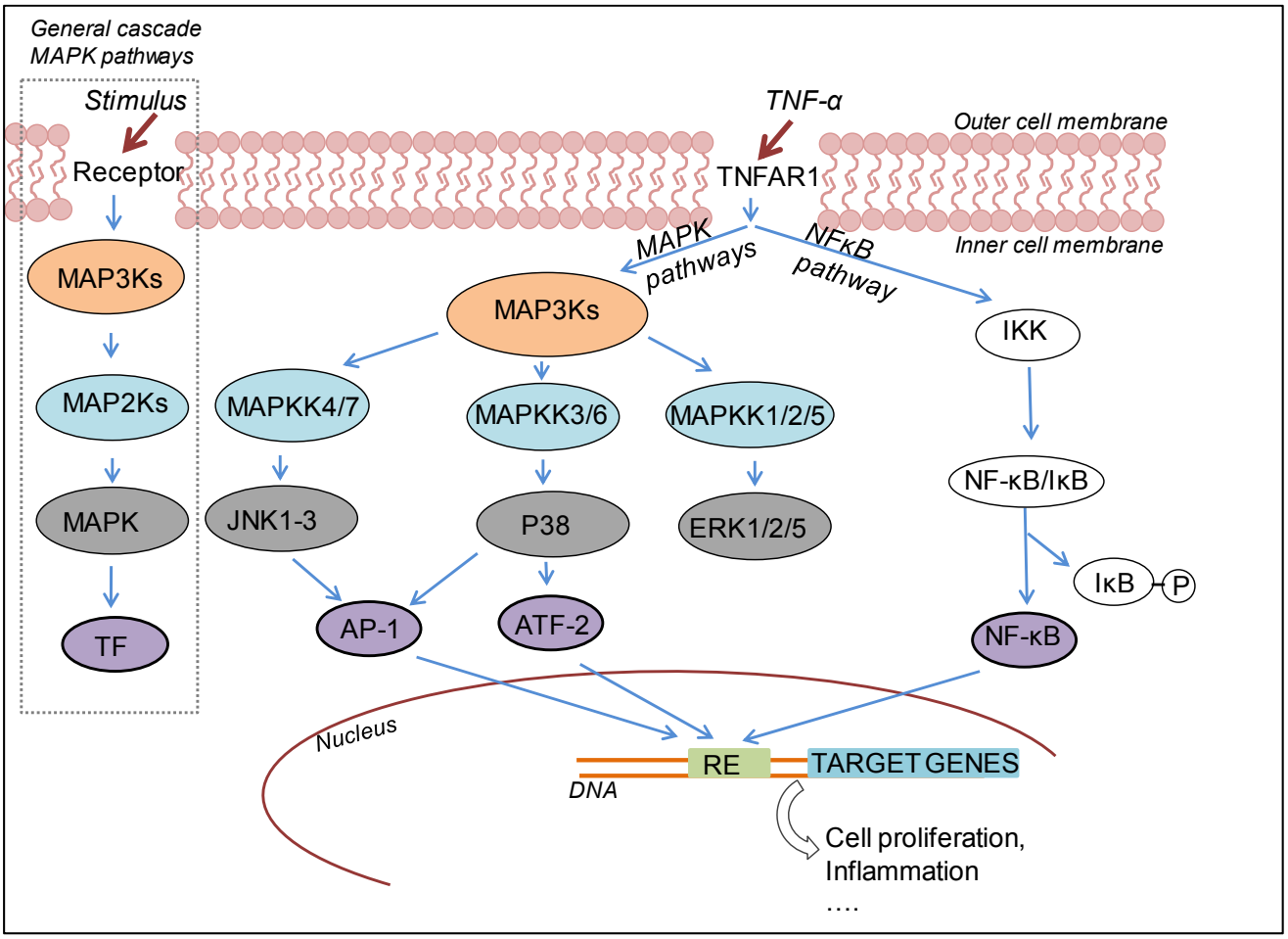


*Figure 16: Overview of the main TNF-α signalling pathways.*

## 5.2 Our approach: Study the transcriptional regulation of 17β-hydroxysteroid dehydrogenase 3

Steroidogenesis and steroidogenic effects can be studied at different levels. Since most research groups focus on nuclear receptors, we decided to focus on the enzymes responsible for the interconversion of active and inactive hormones. In our studies, we addressed the dysregulation of 17β-HSD3, the key enzyme converting androstenedione to testosterone in the testis in Leydig cells. We believe that dysregulation of 17β-HSD3 is an important contributor to the pathologies discussed above. By stably transfecting the MA-10 mouse Leydig tumor cells with a 2.8 kb sequence containing the putative human *HSD17B3* promoter and a downstream luciferase reporter, we created a tool to screen for androgen disruption. This approach, to the best of my knowledge, is a novel tool for studying steroidogenesis.

## 5.3 Focus part A: The inflammatory cytokine TNF-α activates the promoter of 17β-hydroxysteroid dehydrogenase 3

Within the course of our studies, we observed that the inflammatory cytokine TNF-α activates the *HSD17B3* promoter. Inflammatory processes mediated by TNF-α are strongly associated with cancer development and progression (Balkwill, 2009). Taken together, we hypothesize that *HSD17B3* promoter dysregulation could have an impact on inflammation mediated carcinogenesis, e.g. tumors which chronically secrete TNF-α could contribute to higher testosterone levels due to increased 17β-HSD3 activity, which would in turn promote the growth of androgen sensitive tumors.

## 5.4 Part A manuscript draft: TNF-α stimulates testosterone production by p38 MAPK-dependent activation of 17β-hydroxysteroid dehydrogenase 3 promoter

### 5.4.1 Abstract

Androgens play a key role in physiological and pathophysiological processes. They play a critical role in male sexual development as well as in growth and progression of testicular and prostate cancer In the testis the reduction of the weak androgen Δ4-androstene-3, 17-dione (androstenedione) to the potent androgen testosterone is catalysed by the short-chain dehydrogenase/reductase 17β-hydroxysteroid dehydrogenase type 3 (17β-HSD3). Thus, impaired regulation of testosterone production by this enzyme is likely to contribute to androgen-dependent diseases. A risk factor closely linked with cancer is inflammation. The pro-inflammatory cytokine TNF-α plays important roles in the pathogenesis of chronic inflammatory diseases and cancer. Cancer-related inflammation reflects a state where cells and mediators of inflammation, such as TNF-α, form an important part of the tumor microenvironment. Such conditions may promote the pathogenesis of cancer and lead to further growth of the tumor. In the present study, we characterized the effects of the inflammatory cytokine TNF-α on the regulation and expression of 17β-HSD3. Performing transactivation assays in MA-10 mouse Leydig cells stably expressing a luciferase reporter under the control of the *HSD17B3* promoter, we observed an activation of the reporter gene expression upon incubation with TNF-α. The results were confirmed in BLTK-1 mouse Leydig cells transiently transfected with the reporter construct. To confirm the results obtained from the reporter gene assays, we measured *HSD17B3* mRNA levels by real-time RT-PCR. Furthermore, we showed that TNF-α induced 17β-HSD3 enzyme activity. We suggest that TNF-induced *17BHSD3* promoter activation and the subsequently enhanced testosterone production plays an important role within the interaction of inflammation, elevated testosterone concentrations and cancer pathogenesis.

### 5.4.2 Introduction

17β-HSD3 is a key steroidogenic enzyme in humans and animals. It converts the inactive precursor steroid androstenedione to the potent androgen receptor (AR) ligand testosterone. Dysregulated testosterone levels have been associated with cancer. Previous studies showed a direct association between testosterone levels and the development of various types of cancer as well as an increased risk of overall mortality (Gann *et al.*, 1996; Parsons *et al.*, 2005). It was shown in two older studies, that testosterone stimulates the growth of lung and colon cancer cells *in vitro* and that this effect can be halted with anti-androgens (Maasberg *et al.*, 1989; Tutton and Barkla, 1982). However, there is increasing evidence that higher testosterone levels do not directly contribute to the development of cancer (Eisenberg *et al.*, 2014; Orsted *et al.*, 2014) but that increased plasma testosterone levels are associated with an up to 80% higher risk of early death following cancer diagnosis in both men and women. It appears that high testosterone levels negatively influence the pathogenesis of cancer. Such pathophysiological mechanisms involving testosterone need to be further validated. However, it has been observed that a majority of prostate tumors express high levels of the testosterone target

receptor, the AR and that increased expression of AR in cancer cells is dependent on testosterone (Cai and Balk, 2011). These observations emphasize the importance of both testosterone and the AR in PC. In summary, elevated testosterone levels are assumed to play a role in the development, progression and outcome of cancer. However, the role of testosterone in cancer has yet to be fully understood.

The link between cancer and inflammation has been clearly established and is well accepted. Controlled inflammation is essential for the survival of an organism. However, when inflammation is unresolved it leads to enhanced formation of reactive oxygen species that might contribute to many acute and chronic pathologies including cancer (Balkwill and Mantovani, 2001; Coussens and Werb, 2001). Cytokines mediate a broad range of processes involved in the pathogenesis of cancer (Candido and Hagemann, 2013). The prototype among the proinflammatory cytokines is the tumor necrosis factor alpha (TNF-α). Its critical role in chronic inflammatory states is well known (Feldmann and Maini, 2008). As its name suggests TNF-α was initially shown to be toxic for tumor cells at high concentrations (Carswell *et al.*, 1975). However, the tumor promoting function of TNF-α has been clearly demonstrated in mice (Moore *et al.*, 1999). Cancer, in both men and women, is the second most common cause of death (after cardiovascular diseases) in economically developed countries and the leading cause of death in developing countries (Jemal *et al.*, 2011). In males, prostate cancer is the cancer type with the highest incidence (Siegel *et al.*, 2014), about 1-2 men in every 10 are affected (lifetime prevalence) (Siegel *et al.*, 2014). Approximately 85% of all PCs can be successfully (in terms of low prostate specific antigen (PSA), the clinical biomarker prostate cancer) treated with inhibitors of steroidogenesis, such as; 5α-reductase inhibitors, CYP17-inhibitors or AR antagonists (Fryczkowski *et al.*, 2014; Poutiainen *et al.*, 2014). However, there are distinct forms of PC, which develop resistance towards androgen deprivation therapy during their progression. This type of PC is referred to as hormone-resistant PC. The AR is suggested to play an important role in this shift from androgen-dependent to androgen-independent cancer (Taplin and Balk, 2004).

To study the promoter regulation of *HSD17B3*, we designed a novel tool. Mouse Leydig cells (MA-10 cells) derived from a Leydig cell tumor stably expressing a luciferase reporter gene under the control of a 2.8 kb sequence of the human *HSD17B3* promoter. This cell line can be used in screening assays to identify potential disrupters of the testosterone formation. Furthermore, this tool may help to identify upstream factors (such as transcription factors or nuclear receptors) involved in the *HSD17B3* promoter regulation.

### 5.4.3 Materials and Methods

**Cultivation of MA-10 and BLTK1 cells**

The mouse Leydig cell line MA-10 (kindly provided by Professor Mario Ascoli, University of Iowa, Iowa City, IA (Ascoli, 1981)) was cultivated as described previously (Legeza *et al.*, 2013). Briefly, cells were grown on 0.1% gelatin-coated cell culture dishes in in DMEM/F12 medium (Invitrogen, Carlsbad, CA, USA) containing 20 mM HEPES (Gibco life technologies, Carlsbad, CA, USA ), 15% horse serum (Gibco life technologies, Carlsbad, CA, USA )), and 50 µg/ml gentamicin (Sigma-Aldrich, St. Louis, MO, USA). The BLTK-1 mouse Leydig cell line (kindly provided by Professor Huhtaniemi and Dr. Rahman, University of Turku, Turku, Finland (Forgacs *et al.*, 2012)) was cultivated as described previously (Forgacs *et al.*, 2012). In brief, cells were maintained in DMEM/F12 medium (Invitrogen) containing 10% fetal bovine serum (Sigma-Aldrich) 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich). Both cell lines were incubated under standard conditions (5% $CO_2$, at 37°C).

**Generation of a reporter luciferase construct under the control of a 2.8 kb upstream sequence of the human *HSD17B3* gene**

Primers to amplify the 2,800 base pairs (bp) upstream region of the *HSD17B3* promoter were designed using the Primer-BLAST program and synthesized by Microsynth AG (Balgach, Switzerland). The sequences of the primers are listed in Table 7.

| 2.8kb promoter HSD17B3 oligonucleotide primer **forward** | 2.8kb promoter HSD17B3 oligonucleotide primer **reverse** |
|---|---|
| 5'-GGT-ACC-GCA-GCA-ATA-CAC-AGA-GGT-AAA-TTG-AG-3' | 5'-GCT-AGA-ATG-CCT-CCT-GGG-ACC-ACG-CTG-CTC-T-3' |

***Table 7: Oligonucleotide primers used for the amplification of the 2.8kb sequence of the HSD17B3 promoter***

The sequence in the putative promoter region of the human *HSD17B3* was amplified with PCR under standard conditions using human genomic DNA as a template. The PCR product was loaded onto a 1.5% agarose gel. The amplicon was excised from the gel and purified using the Wizard SV Gel and PCR Clean-Up System (Promega. Madison, WI, USA). For convenient cloning in an intermediate vector, TA cloning was performed using the pGEM-T Easy vector (Promega). For this purpose, a stretch of poly-A nucleotides was added to the purified product by TaqPolymerase (Invitrogen). Subsequently, the product was purified again and ligated into the pGEM-T Easy vector (Promega). For all ligations the following formula was used to calculate the amount of insert and vector in the ligation reaction: (50 ng vector x kb insert/kb vector) whereas the ratio kb insert:kb vector was 3:1. The ligation product was transformed into competent E.coli cells of the DH5a strain. Several colonies were picked and plasmid DNA was isolated using the GenElute Plasmid Miniprep Kit (Sigma-Aldrich). The plasmid DNA was then digested with the restriction enzymes KpnI (recognition site 5' GGTACC) and NheI (recognition site 5' GCTAGC), (New England Biolabs, Ipswich, MA, USA). Recognition sequences for these sites were included at the 5'ends of the forward and reverse primer, respectively. Colonies positive for the pGEM-T easy vector containing the insert were selected and sequence verified. For ligation to the pGL4.17 vector, the insert was digested from the pGEM-T easy vector with the KpnI and

NheI restriction enzymes. The vector was subjected to the same digestions and was additionally dephosphorylated using alkaline phosphatase (Roche, Basel, Switzerland) to prevent linearized plasmid from self-ligating. The ligation of the vector with the insert was performed using the T4 ligase (Promega). The ligation reaction was transformed into competent bacteria and several colonies were tested for the presence of the vector containing the insert.

**Generation of MA-10 cells stably expressing the *HSD17B3* promoter-luciferase reporter**

MA-10 cells (kindly obtained from Professor Mario Ascoli, University of Iowa, Iowa City, IA (Ascoli, 1981)) at passage 5 were transfected using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA) with the *HSD17B3* promoter-luciferase plasmid (described above) containing a neomycin resistance gene. Transfected cells were screened upon exposure to 200 µg/ml G418 (Invitrogen) over two weeks. Single clones stably expressing the plasmid were selected from neomycin resistant MA-10 cells. Isolated single clones were verified for their luciferase activity in transactivation assays measuring the light signal from luciferin production at 470 nm on a Spectramax L (Molecular Devices, Sunnyvale, CA, USA). Cell clones expressing medium to high levels of luciferase were further sub-cultivated for two weeks with DMEM/F12 medium containing 200 µg/ml G418 to maintain selective pressure. The single clone designated as MA-10C2 was used for our experiments.

***HSD17B3* promoter transactivation assays**

MA-10C2 cells were seeded on 24-well plates at a confluence of 60%. The following day, they were incubated with TNF-α (Sigma-Aldrich) at a concentration of 10 ng/µl, dexamethasone (Sigma-Aldrich) at a concentration of 100 nM or SB202/190 (Sigma-Aldrich) at concentration of 20 µM for 24 hours at standard conditions ( 37°C, 5% $CO_2$). After incubation, cells were washed with phosphate buffered saline (PBS), lysed with Tropix lysis buffer (Applied Biosystems, Foster City, CA) supplemented with 0.5 mM dithiothreitol, and frozen at -80°C following a 10 minutes incubation at room temperature. Lysates were analyzed for luciferase activity using a luciferine-solution (Rebuffat *et al.*, 2004) at 470 nm on a Spectramax L (Molecular Devices). Levels were normalized to DMSO control treatments.

**Measurements of *HSD17B3* mRNA with RT-PCR**

BLTK-1 cells were used in our assays between passages 10-15. BLTK-1 cells were seeded in 6 well plates at 60% confluence in DMEM/F12 for 12 h. Cells were washed once with PBS and incubated for 24 h in culture medium containing TNF-α (Sigma-Aldrich) at a concentration of 10 ng/µl, dexamethasone (Sigma-Aldrich) at a concentration of 100 nM or SB202/190 (Sigma-Aldrich) at concentration of 20 µM (or combined treatments) for 24 hours at standard conditions (37°C, 5% $CO_2$). DMSO at a concentration of 0.01% was used as solvent control. Total mRNA was extracted using the Trizol method (Invitrogen). Total mRNA (2 µg) was reverse transcribed into cDNA using the Superscript-III First-Strand Synthesis System and oligo-dTs (Invitrogen). Relative quantification of mRNA expression levels was performed by RT-PCR on a RotorGene 6000 (Corbett, Sydney, Australia) using the KAPA SYBR FAST qPCR Kit (Kapasystems, Boston, MA, USA) with specific oligonucleotide primers for *Hsd17B3 (*forward primer*:* 5'-TGAGTGTGAATACAGAGATAAAGG-3'; reverse primer: 5'-TCCGATCGTGACATATTTCAAGG-3'). Relative gene expression was normalized

to the internal control gene glycerinaldehyde-3-phosphate-dehydrogenase (GAPDH) using the delta-delta-CT method.

**17β-hydroxysteroid dehydrogenase activity assay**

The enzymatic activity of 17β-HSD3 was measured using a modified protocol from Legeza et al. (Legeza *et al.*, 2013). Briefly, 12 hours following the seeding of MA-10 cells at 60% confluence in 96 well plates, cells were washed once with PBS and incubated in DMEM/F12 medium containing either TNF-α at a concentration of 10 ng/ul or DMSO as solvent control (0.01 %) and incubated at standard conditions for 24 h (37°C, 5% $CO_2$). Cells were then washed once with PBS and incubated in steroid free DMEM/F12 medium containing 200 nM radiolabeled [$^{1, 2, 6, 7-3}$H]-androstenedione (GE Healthcare, Little Chalfont, UK) for 4 hours. Cells treated with the 17β-HSD3 inhibitor benzophenone-1 (BP-1) (Sigma-Aldrich) were pre-treated with 10 µM BP1 for 20 min allowing BP1 to enter the cells and to access 17β-HSD3 prior to addition of androstenedione. The enzymatic reactions were terminated by the addition of unlabeled androstenedione and testosterone dissolved in methanol. The steroids were separated on UV-sensitive silica TLC plates (Macherey-Nagel, Oensingen, Switzerland) using a chloroform-methanol solvent system at a ratio of 9:1. Bands corresponding to the respective steroid were scraped off the TLC plate and transferred to tubes containing scintillation cocktail. Radioactive decay of androstenedione and corresponding metabolites were analyzed on a scintillation counter.

**Promoter analysis by *TFSearch***

The 2.8 kilo base sequence of the putative promoter of *HSD17B3* was analyzed using the *in silico* tool TF search (*http://www.cbrc.jp/research/db/TFSEARCH.html)* programmed by Y. Akiyama, Kyoto University, Japan. Threshold score for matches was set at 85%.

**Statistical analysis**

Data are expressed as the mean ± standard deviation (S.D.) of at least three independent experiments. The significance of differences within the data was assessed by one-way analysis of variance (ANOVA), with Tukey post-test for multiple comparisons.

### 5.4.4 Results

**Activation of 17β-hydroxysteroid dehydrogenase type 3 promoter by TNF-α in MA-10 cells**

In order to test our hypothesis that TNF-α can induce *HSD17B3* promoter activity, we treated MA-10C2 cells for 24 h with TNF-α. We observed an 8-fold up regulation in promoter activity (Figure 17). To determine the pathway by which TNF-α exerts its action, we treated the cells with a selective p38 mitogen activated protein kinase-(MAPK) inhibitor (SB202/190) and the GR agonist dexamethasone. Dexamethasone was not able to reverse the effect of TNF-α on the *HSD17B3* promoter. In contrast, SB202/190 was able to suppress the TNF-mediated *HSD17B3* promoter activation, indicating the involvement of p38 MAPK.

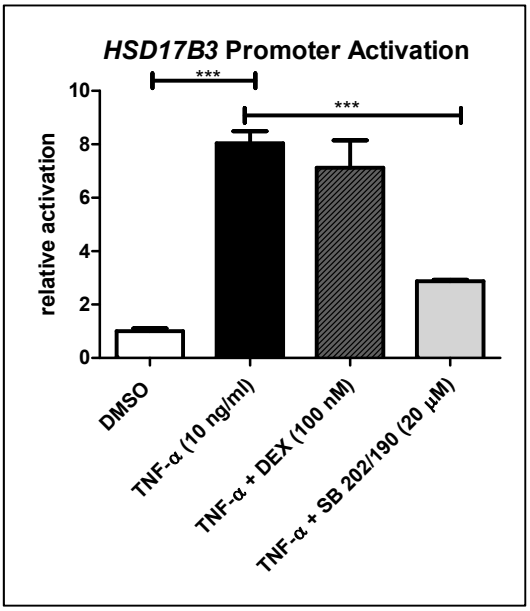


***Figure 17: TNF-α potently activates the HSD17B3 promoter through p83 MAPK****. MA-10C2 cells stably expressing a luciferase reporter gene under the control of the human HSD17B3 promoter (clone MA-10C2) were treated with 10 ng/ µl TNF-α. A significant activation of the HSD17B3 promoter could be observed. Simultaneously treatment with dexamethasone at 100 nM was not able to reverse the effects of TNF-α. Upon co-incubation with TNF-α and 20 µM of the selective p38 MAPK inhibitor SB 202/190, the activation of the promoter was suppressed. The data represent the mean ± S.D. of three independent experiments performed in triplicate. ***p ≤ 0.001.*

In order to confirm these effects in a second cell line, the expression plasmid for the luciferase reporter under the control of the human HSD17B3 promoter was transiently transfected into mouse BLTK-1 Leydig cells. Similar to the results above, TNF-α induced *HSD17B3* expression (data not shown).

**Elevated 17β-hydroxysteroid dehydrogenase type 3 enzyme activity upon treatment of MA-10 cells with TNF-α**

In order to confirm that increased *HSD17B3* promoter activity translated to an increase in the conversion of androstenedione to testosterone, we measured the enzymatic activity of 17β-HSD3 in MA-10 cells. We observed a two-fold increased 17β-HSD3 activity following TNF-α treatment. Upon inhibition of 17β-HSD3 with benzophenone-1 (BP1), a chemical UV-filter shown to inhibit 17β-HSD3 (Nashev *et al.*, 2010), the effect could be blocked (Figure 18).

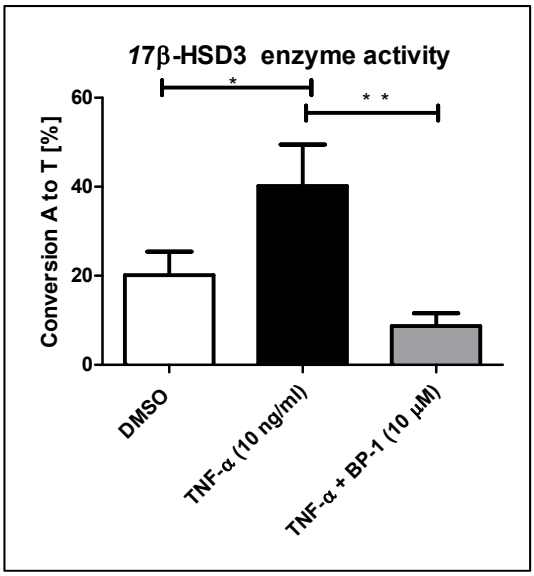


***Figure 18: 17β-HSD3 enzyme activity is significantly increased in MA-10 cells upon treatment with TNF-α.*** *MA-10 cells treated with 10 ng/µl of TNF-α showed enhanced 17β-HSD3 enzyme activity. Upon co-incubation with TNF-α and BP-1, a 17β-HSD3 inhibitor, the conversion from androstenedione to testosterone was inhibited. The data represent the mean ± S.D. of three independent experiments performed in triplicates. *p ≤ 0.05,***p ≤ 0.001.*

**17β-hydroxysteroid dehydrogenase type 3 mRNA levels are elevated in TNF-α treated BLTK-1 cells**

In order to confirm that the increased 17β-HSD3 activity upon treatment with TNF-α resulted in enhanced transcription of the endogenous *HSD17B3* gene we measured the mRNA level in BLTK-1 mouse Leydig cells, following treatment with TNF-α. In correlation with the promoter activity, we observed an 8 fold increase in *HSD17B3* mRNA levels which could by suppressed by co-treatment with SB202/190 (Figure 19).

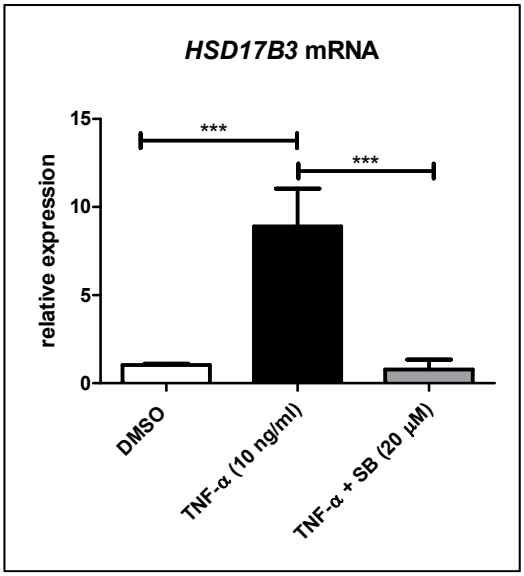


***Figure 19: HSD17B3: mRNA levels of are markedly elevated in TNF-α treated BLTK-1 cells.*** *BLTK-1 cells treated with 10 ng/µl TNF-α showed elevated HSD17B3 mRNA levels. Upon co-incubation of TNF-α and 20 µM SB 202/190, the mRNA up regulation was reversed. The data represent the mean ± S.D. of three independent experiments performed in triplicates. ***p ≤ 0.001.*

**Promoter analysis for potential transcription factor binding sites**

The 2.8 kb *HSD17B3* promoter sequence was analyzed for the identification of potential transcription factor binding sites using the *TFSearch* software (Y. Akiyama, Kyoto University, Japan). Setting the threshold value at a minimum of 80% coherence to known consensus response elements, 49 putative transcription factors able to bind to the DNA sequence were identified. Putative transcription factor binding sites were identified to be present in different repetitions. Table 8 provides an overview of all the putative transcription factors identified and the number of their consensus motifs within the 2.8 kb. The transcription factors associated with the MAPK pathways (Sabio and Davis, 2014) are highlighted in Table 8. AP-1 is the only transcription factor that can be directly associated with p38 MAPK pathway, assuming that AP-1 is the down-steam target of p38 MAPK in the TNF-α mediated *HSD17B3* promoter activation.

| Transcription factor binding site | Number of repetitions |
|---|---|
| CdxA | 55 |
| GATA-1 | 22 |
| SRY | 17 |
| **C/EBPβ** | **16** |
| **C/EBP** | **16** |
| Ik-2 | 15 |
| USF | 13 |
| HSF2 | 10 |
| **CREBP** | **10** |
| Evi-1 | 10 |
| AML-1a | 9 |
| **AP-1** | **9** |
| GATA-2 | 8 |
| GATA-3 | 8 |
| Nkx-2. | 8 |
| MZF1 | 7 |
| Lyf-1 | 7 |
| RORalp | 6 |
| deltaE | 6 |
| S8 | 6 |
| **CREB** | **6** |
| HSF1 | 5 |
| GATA-X | 5 |
| Oct-1 | 5 |
| HFH-2 | 5 |

| Transcription factor binding site | Number of repetitions |
|---|---|
| c-Ets- | 4 |
| N-Myc | 4 |
| SREBP- | 3 |
| HNF-3b | 3 |
| Sox-5 | 3 |
| Tst-1 | 3 |
| c-Rel | 2 |
| c-Myc | 2 |
| E4BP4 | 2 |
| VBP | 2 |
| HLF | 2 |
| Elk-1 | 1 |
| NRF-2 | 1 |
| Brn-2 | 1 |
| c-Myb | 1 |
| v-Myb | 1 |
| Pbx-1 | 1 |
| STATx | 1 |
| MyoD | 1 |
| Tal-1a | 1 |
| XFD-1 | 1 |
| E2F | 1 |
| CP2 | 1 |
| TATA | 1 |

***Table 8: Overview of the putative transcription factors that are able to bind to the 2.8 kb sequence of the HSD17B3 promoter analyzed.*** *Transcription factors involved in the TNF-α signaling pathways are highlighted.*

### 5.4.5 Discussion

Steroid research in cancer, especially in PC predominately focuses on the AR, with respect to its expression, mode of action and inhibition (Miyahira *et al.*, 2014). Although the AR is indisputably a very important player in the pathogenesis of cancer, upstream mechanisms determining the availability of AR ligands also need to be considered. In our study, we present novel data on *HSD17B3* promoter activation, which may be important for understanding the dysregulation of androgen production in diseases characterized by chronic inflammation, such as cancer. We are the first to investigate the *HSD17B3* promoter regulation. We constructed a Leydig cell model stably expressing a luciferase reporter under the control of the human *HSD17B3* promoter. We used this cell line as a tool to identify compounds, which may activate the *HSD17B3* promoter and subsequently enhance testosterone formation. We validated our new cell model with traditional, well-established methods such as RT-PCR and enzyme activity assays. The key finding of our study was the ability of TNF-α to induce the expression and activity of 17β-HSD3.

TNF-α is a pleiotropic cytokine. It plays major roles in the pathogenesis of various diseases. TNF-α is mainly secreted by macrophages for sequential roles in inflammation (Manderson *et al.*, 2007). Inflammation and inflammatory diseases, acute or chronic represent a heterogeneous group of conditions that can affect any organ or system (Sozzani *et al.*, 2014). In many diseases, such as various autoimmune diseases, inflammatory processes represent cause and effect of the disease. However, in a wide variety of diseases, inflammation occurs subliminal as a second effect of the primary disease (Cox, 2012; Kaur, 2014). In such cases, chronic inflammation often develops, which propagates into overall systemic inflammation associated with co-morbidities. Inflammatory processes can promote or possibly even initiate malignant diseases (Balkwill et al., 2005). A recent review (Balkwill, 2006) summarizes the evidence for a link between cancer and inflammations. Many chronic diseases are associated with an increased risk of cancer. Cancers often arise at sites of chronic inflammation because of the increased presence of reactive oxygen and nitrogen species. Chemical mediators and nanoparticles (such as asbestos) are found in many cancers, and anti-inflammatory agents reduce the risk of mortality from some cancers (Balkwill et al., 2005; Balkwill and Mantovani, 2001; Coussens and Werb, 2002). We suggest that *HSD17B3* promoter dysregulation could have an impact on inflammation-mediated carcinogenesis and chronically inflamed tumors could contribute to higher testosterone levels indirectly through excessive secretion of TNF-α, which would in turn promote the growth of the tumor by AR activation.

Furthermore, our observations may help to understand the development and progression of other forms of cancer in inflammatory diseases. In particular, the metabolic syndrome is well known for its underlying chronic inflammatory states (Trayhurn and Wood, 2004). In obesity, blood supply to adipocytes may be reduced, which may lead to hypoxia-induced cell damage and an overproduction of cytokines such as TNF-α or IL-6 (Cinti *et al.*, 2005; Lau *et al.*, 2005). Furthermore, adipocytes are known to independently express and secrete TNF-α. The prevalence of polycystic ovary syndrome, a metabolic disorder resulting in higher androgen levels, is markedly higher in obese women compared to woman of normal weight (Alvarez-Blasco *et al.*, 2006), suggesting a relationship of inflammation and androgen levels. Adipocytes were identified early to act as an “endocrine organ” and to produce

steroids such as androgens (Siiteri, 1987). In premenopausal women, adipocytes contribute to the production of up to 50% of circulating testosterone (Belanger *et al.*, 2002; Meseguer *et al.*, 2002). It was shown, that different adipose tissues express 17β-HSD3. However, the total serum testosterone in obese men is lowered when compared to the levels of normal weight men. Additionally, testosterone treatment in obese man improved obesity parameters (body weight, waist circumference, and BMI) and lowered total cholesterol, LDL cholesterol, triglycerides, fasting blood glucose, $HbA_{1c}$, and blood pressure in a 5-year study (Yassin *et al.*, 2014). This discrepancy of higher androgen production within adipocytes and at the same time lowered systemic androgen levels is not yet fully understood and need to be studied further. However, it is known that the prevalence for multiple cancer types is elevated in obese populations (De Pergola and Silvestris, 2013). Prostate cancer also occurs more often in obese men compared to normal weight men (De Pergola and Silvestris, 2013). We suggest that the TNF-α-induced activation of the *HSD17B3* promoter and subsequent up regulation of testosterone may play a role in the mechanisms by which obesity impacts on cancer development and/or progression.

The establish links between cancer, inflammation and obesity are indisputable. In our study, we designed a novel tool, where mouse Leydig cells (MA-10 cells) derived from a Leydig cell tumor, stably expressed a luciferase reporter gene under control of a 2.8 kb sequence of the human *HSD17B3* promoter. Using this tool and combining it with assays in MA-10 cells and BLTK-1 Leydig mouse cells, which both endogenously express 17β-HSD3, we could demonstrate that the prototypic inflammatory cytokine TNF-α strongly activates the *HSD17B3* promoter, which further leads to increased mRNA levels and enzymatic activity of 17β-HSD3. The *HSD17B3*-promoter activation suggests a novel pathway resulting in increased testosterone levels. We suggest that chronic inflammatory states, such as in cancer, may contribute to higher testosterone levels, which would in return promote growth of the tumor by AR activation. Moreover, other diseases states which chronic inflammation, such as the metabolic syndrome, could be adversely affected by higher androgen levels. Therefore, we investigated the pathway by which TNF-α exerts its action. We observed that the activation of the *HSD17B3* promoter by TNF-α was mediated through the p38 MAPK and possibly the transcription factor AP-1. Another important finding of our study is that the TNF-α mediated action cannot be reversed by the potent and widely used anti-inflammatory agent dexamethasone. We are the first to suggest a pathway by which inflammation my directly lead to higher testosterone levels, which may contribute to the vicious circle of cancer progression, inflammation and elevated steroids level.

With our study, we unveil several important observations which may contribute to the understanding of the mechanism underlying related inflammation, inflammation in adipocytes and altered testosterone levels.

## 5.5 Outlook for part A: TNF-α dependent activation of 17β-hydroxysteroid dehydrogenase type 3 promoter

- **Investigate the mechanism of TNF-α activation of p38 MAPK by inducing point mutations in the putative AP-1 consensus sequences within the promoter of *HSD17B3***

The promoter analysis with the software *TF-search* suggests that p38 MAPK may activate AP-1. By point mutation of the putative AP-1 binding sites on the 2.8 kb promoter sequence, this hypothesis can be investigated. If TNF-α no longer is able to activate the mutated *HSD17B3* promoter, it can be assumed that AP-1 is the nuclear factor responsible for the TNF-α-mediated activation. There were 9 putative AP-1 binding sites predicted by the *TFsearch*, all of which may be involved. I propose to study first point mutations on the side closest to the start codon of the gene. In addition to its position, this site also showed the highest similarity to the putative AP-1 consensus binding sequence in the *TFsearch*.

- **Investigate the transcription factor involved in p38 MAPK downstream activation of the *HSD17B3* promoter using CHIP-assays**

It is very unlikely that TNF-α, being known to provoke whole cascades of actions, only acts via one single nuclear factor. It can be expected, that a multitude of nuclear factors and/or nuclear receptors play a role in the TNF-α induced *HSD17B3* promoter activation. Using Chromatin Immunoprecipitation (CHIP) assays we will try to identify proteins associated with the *HSD17B3* promoter activation.

- **Measuring *HSD17B3* mRNA levels in MA-10 cells at different time points**

The observation that the *HSD17B3* promoter in MA-10 cells is activated and that the enzymatic activity of 17β-HSD3 is enhanced suggests that also the *HSD17B3* mRNA of MA-10 cells is up regulated. However, we were so far not able to show such an up regulation in MA-10 cells. The mRNA is usually isolated 24 hours after the treatment. If the up regulation was transient and taking place very quickly after TNF-α incubation, it is possible that its expression levels reversed to normal after 24 hours. Shorter incubation time points will be tested (e.g. RNA isolation after 6 or 12 hours).

- **Verify the enhanced enzyme activity with mass spectrometry**

Although we showed in previous chapters that MA-10 cells produce testosterone as their predominant metabolite upon incubation with androstenedione, I suggest confirming the enhanced enzyme activity by LC-MS/MS. In addition, I also suggest measuring 17β-HSD3 enzyme activity after multiple time points (12 h, 24 h, and 48 h), since the stability of 17β-HSD3 protein has yet to be elucidated.

## 5.6 Focus Part B: Organotins activate the 17β-hydroxysteroid dehydrogenase type 3 promoter

Organotins are very potent environmental toxins. Their toxicity manifests itself in very diverse organs and systems affecting a wide spectrum of species. Especially aquatic organisms suffer from organotin toxicity, since organotins persists in the water. We observed that organotins, especially tributyltin (TBT) and triphenyltin (TPT) result in an activation of the *HSD17B3* promoter in the low nanomolar range. It is known that organotins can exert at least part of their action via the nuclear retinoid X receptor (RXR) (Grun and Blumberg, 2006; Nishikawa *et al.*, 2004). If is further know, that organotins can cause imposex in mollusks. Imposex is a disorder caused by pollutants, such as organotins (Harrison *et al.*, 2007), where affected female mollusks, develop male sex gonads, in addition to their female genital tract. Different pathways leading to imposex have been suggested. One of which involves RXR-signaling, since it was shown that 9-cis retinoic acid is able to promote imposex in sea shells (Nishikawa *et al.*, 2004). We focused on the question, by which mechanism TBT and TPT exert their androgenic effects and whether the activation of the *HSD17B3* promoter may provide an explanation for the research on organotin-mediated imposex.

## 5.7 Part B manuscript draft: Tributyltin- and triphenyltin-dependent up regulation of 17β-hydroxysteroid dehydrogenase

### 5.7.1 Abstract

Organotins exert a wide range of toxic effects. They were shown to act as endocrine disruptors in adipocytes as well as to cause reprotoxicity in both males and females in a variety of species. The molecular mechanism underlying theses effects are largely unknown. The organotins tributyltin (TBT) and triphenyltin (TPT) are known to induce imposex in female mollusks. Marine animals are predominately affected since organotins persist in aqueous environments. Female organisms affected by imposex develop male sex organs such as a penis, in addition to their fully developed female genital tract. The currently debated mechanisms by which imposex is driven include: high levels of testosterone, steroidogenic inhibition (to lower estrogen levels) and the activation of the nuclear receptor retinoid X receptor (RXR). Using a cell model stably expressing a luciferase reporter under the control of the promoter sequence of *HSD17B3*, the key enzyme in the androgen steroidogenesis catalyzing the formation of testosterone, we could show that TBT and TPT both potently up regulate the *HSD17B3* promoter. The results demonstrate the pro-androgenic effects of TBT and TPT in a mouse/human Leydig cell system. Furthermore, we could show that the well characterized RXR ligand, 9-cis retinoic acid (9-cis RA), activates the *HSD17B3* promoter. Our data provide evidence, that the TBT- and TPT-mediated up regulation of the *HSD17B3* promoter is dependent on RXR activation. Future experiments need to test whether TBT- and TPT-dependent RXR activation also up regulates 17β-HSD3 expression and activity in mollusks and fish and whether an enhanced 17β-HSD3 activity might play a role in the development of imposex in aquatic species.

### 5.7.2 Introduction

Organotin is a collective term for metal organic compounds containing one or more tin-carbon bonds. Their collective chemical formula is $R_nSnX_m$ , where R refers to a carbon group, and X represents any other chemical group such as a halogen or hydroxyl group (Graf, 1996). The first organotin synthesis dates back to 1853 (Frankland, 1835). Until the 1990s, the worldwide annual production of organotins amounted to about 40‘000 tons. Three quarters of them were used as polyvinylchloride stabilizers. Other important applications included their use as biocides in anti-fouling coatings on ships to reduce encrustations by barnacles, algae, mussels, and other marine invertebrates (Choi *et al.*, 2009) and as fungicides in agriculture (Graf, 1996). It took more than 100 years to figure out that the versatility of these chemicals is dramatically counterbalanced by its toxicity to biota (Appel, 2004; Fent, 1996; Nicholson, 1989). Goldberg and Maguire even stated that the organotin TBT is the most toxic substance that was ever deliberately introduced into the environment (Goldberg, 1986; Maguire, 1987). One of the most surprising feature of organotin toxicity is the wide spectrum of responsive species, and the similarity of the responses in very different cell types and species (Pagliarani *et al.*, 2013). Organotin use today is restricted by law, but because of their harmful persistence in water environments (B. Eklund, 2008) , their continuous illegal use and their leaching from old paints make the organotins a persisting threat, even for future generations (Antizar-Ladislao, 2008; Fent, 1996).

The toxicological effects of organotins are wide-ranging and include reprotoxicity shown by a multitude of studies in male (Chen *et al.*, 2008; Omura *et al.*, 2001) and female rats (Grote *et al.*, 2006; Harazono *et al.*, 1996). Organotins also act as obesogenes (chemicals that promote obesity by increasing the number of fat cells or the storage of fat in existing fat cells (Grun and Blumberg, 2009; Grun and Blumberg, 2006)). According to the literature, these lipid changes are less pronounced (or perhaps less well understood) in invertebrates compared to vertebrates (Pagliarani *et al.*, 2013). Exerting other toxic effects, organotins in particular are harmful to water-bound species, due to their persistence in water. During their early life, organisms are particularly sensitive to pollution and exposure can result in genital defects, abnormal gamete development, infertility or sex reversal (Jobling *et al.*, 2002). A study in danio rerio (zebrafish) suggested, that fish exposed to TBT show a population with more males than the control group (McAllister and Kime, 2003). TBT was furthermore shown to induce imposex, the development of male sex organs in female snail, in addition to their complete female genital tract (Fent, 1996; Oberdorster and McClellan-Green, 2000; Smith, 1981). It is known, that organotins inhibit the aromatase (cytochrome P450 19A1) that converts testosterone into estradiol (Oberdorster and McClellan-Green, 2000) though it remains controversial whether the imposex phenomenon is predominately caused through this mechanism. Another hypothesis proposes the involvement of the RXR signaling pathway. It was shown that the organotins, TBT and TPT are potent agonists of the RXR (Grun and Blumberg, 2006; Nakanishi *et al.*, 2005; Nishikawa *et al.*, 2004). An injection of the RXR ligand 9 cis-retinoic acid into female snails induced imposex (Castro *et al.*, 2007; Horiguchi *et al.*, 2008). These results strongly suggest the involvement of the RXR pathway in the development of imposex. Other mechanism have also been suggested such as whether increased testosterone levels caused by decreased metabolic clearance of testosterone leads to the development of imposex (Spooner N, 1991). This hypothesis is underlined by the observation that the AR agonist cyproterone acetate was able to inhibit imposex induction through TBT (Bettin, 1996).

In the present study, we could show that the organotins TBT and TPT within a low nanomolar range potently activate the human promoter of *HSD17B3,* the key enzyme for the formation of testosterone in Leydig cells. The RXR ligand 9-cis retinoic acid also potently activated the promoter, which strongly suggested an involvement of the nuclear receptor RXR. We therefore propose that at least part of the organotin-mediated masculinization effects are mediated by RXR-dependent up regulation of 17β-HSD3 and subsequent increase in testosterone synthesis. Since the mode of action in imposex formation is still debated, we further suggest that there might be a relationship between RXR signaling, elevated testosterone levels and the development of imposex.

### 5.7.3 Materials and Methods

**Cell culture**

The mouse Leydig cell clone MA-10C2, derived from the MA-10 cell line upon stably expressing a luciferase reporter under the control of a 2.8 kb sequence of the human *HSD17B3* promoter (design described in chapters above) was cultivated as described previously for the MA-10 cells (Legeza *et al.*, 2013). Briefly, cells were grown on 0.1% gelatin-coated cell culture dishes in in DMEM/F12 medium (Invitrogen, Carlsbad, CA, USA) containing 20 mM HEPES (Gibco life technologies, Carlsbad, CA, USA ), 15% horse serum (Gibco life technologies), and 50 µg/ml gentamicin (Sigma-Aldrich, St. Louis, MO, USA) under standard conditions (5% $CO_2$, 37°C). Organotins and 9-cis retinoic acid were obtained from Sigma-Aldrich at the highest grade available.

***HSD17B3* transactivation assay**

MA-10C2 cells were seeded on 24 well plates at a confluence of 60%, followed by incubation with medium containg TBT, TPT or 9-cis RA at the desired concentrations (500 pm, 1 nM, 10 nM, 50 nM for TBT; 1 nM, 10 nM, 50 nM for TPT and 10 µM for 9-cis RA) for 24 hours at standard conditions (37°C, 5% $CO_2$). After incubation, cells were washed with phosphate buffered saline (PBS), lysed with Tropix lysis buffer (Applied Biosystems, Foster City, CA) supplemented with 0.5 mM dithiothreitol, and frozen at -80°C following 10 minutes incubation at room temperature. Lysates were analyzed for luciferase activity at 470 nM on a Spectramex L (Molecular Devices, Sunnyvale, CA, USA ) using a luciferine-solution (Rebuffat *et al.*, 2004). Levels were normalized to DMSO control treatments.

**Statistical analysis**

Data are expressed as the mean ± standard deviation (S.D.) of at least three independent experiments. The significance of differences within the data was assessed by one-way analysis of variance (ANOVA), with Tukey post-test for multiple comparisons.

### 5.7.4 Results

**Induction of the *HSD17B3* promoter by TBT and TPT**

To investigate whether TBT and TPT activate the *HSD17B3* promoter within environmentally relevant, low nanomolar concentrations, we treated MA-10C2 cells with increasing concentrations of TBT (Figure 20A) and TPT (Figure 20B). TBT and TPT both dose-dependently activated the *HSD17B3* promoter, however, TBT showed a more potent effect with promoter activation detected at concentrations as low as 500 pm.

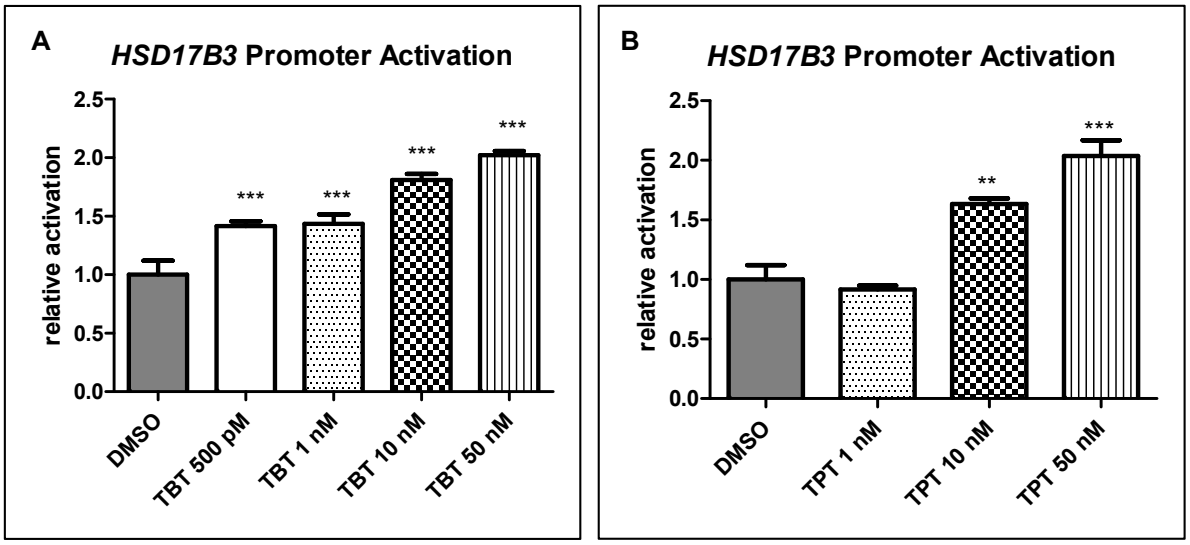


***Figure 20: TBT and TPT potently activate the HSD17B3 promoter.*** *In MA-10C2 cells treated for 24 h with TBT at 500 pm, 1 nM, 10 nM and 50 nM or TPT at 1 nm, 10 nM and 50 nM, a dose dependent activation of the HSD17B3 promoter was observed. The data represent the mean ± S.D. of three independent experiments. ***p ≤ 0.001, **p ≤ 0.01.*

**Induction of the *HSD17B3* promoter by the RXR ligand 9-cis retinoic acid**

To underline our hypothesis that the organotins tested act via RXR, we tested whether 9-cis RA activates the *HSD17B3* promoter. We observed a threefold increase in the *HSD17B3* promoter activation upon incubation with 9-cis RA (Figure 21), which is an endogenous RXR ligand in humans.

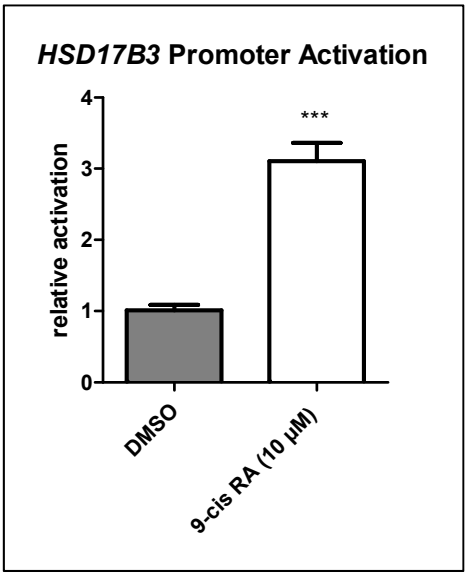


***Figure 21: The endogenous RXR ligand 9-cis RA, activates the HSD17B3 promoter*** *In MA-10C2 cells treated with 10 µM 9-cis RA for 24 h, a significant activation of the HSD17B3 promoter was observed The data represent the mean ± S.D. of three independent experiments.***p ≤ 0.001.*

### 5.7.5 Discussion

It has been shown that organotin-induced disturbances (especially due to TBT and TPT exposure), such as imposex or lipid dysbalance are mediated by RXR pathways (Castro *et al.*, 2007; Grun and Blumberg, 2006; Nakanishi *et al.*, 2005; Nishikawa *et al.*, 2004). Previous reports implicated aromatase inhibition in the development of imposex (Bettin, 1996; Spooner N, 1991). Based on further observations, it was concluded that higher testosterone levels due to inhibition of its degradation might lead to the development of imposex (Spooner N, 1991), or that imposex develops due to an imbalance of testosterone and estradiol (Schulte-Oehlmann *et al.*, 1995). In a recent review based in on all available data on imposex, the authors selected key experiments, which they repeated and they designed new experiments which supported the theory of RXR pathway involvement in imposex (Stange *et al.*, 2012). It was shown that TBT, HX630, a RXR agonist designed by Umemiya (Umemiya *et al.*, 1997), but not 9-cis RA, were able to induce imposex in the marine snail *Nucella lapillus*. The AR antagonist cyproterone acetate led to a suppression of TBT-induced imposex, while the RXR antagonist HX531 (Umemiya *et al.*, 1997) could not antagonize TBT effects (Stange *et al.*, 2012). Strange et al. concluded that aromatase inhibition and RXR signalling pathways may be cross interacting in the development of imposex. In another recent study, RXR isoforms from the sea snail *Thais clavigera* were cloned and expressed in a mammalian cell line (Urushitani *et al.*, 2011). Transcriptional activation of the isoform TC-RXR-1 was observed following treatment with 9-cis RA, TBT-Cl and TPT-Cl. However, cis-docosahexaenoic acid, an RXR activator in vertebrates (de Urquiza *et al.*, 2000), did not activate RXR isoforms. RXR gene transcription in different tissues of *Nucella lapillus* was studied by Lima et.al (Lima *et al.*, 2011). They observed that RXR transcription was down regulated in the central nervous system of females before and after imposex initiation by TBT. Higher transcription levels were observed in the male penis and the penis of females affected by imposex. Lima et al. suggested that different RXR signalling pathways are influenced by TBT. The action of RXR cannot be described conclusively, since RXR, depending on its dimerization partner and its ligand, can act as repressor or as co-activator. Upon dimerizing with the AR, RXR can either act as a repressor following the binding of 9-cis RA or as co-activator when unbound (Chuang *et al.*, 2005). RXR can furthermore form heterodimers with various nuclear receptors, e.g. RAR, thyroid hormone receptors or vitamin D receptors (Gronemeyer *et al.*, 2004). The existing data is partly conflicting since an activation of RXR by a synthetic agonist resulted in imposex formation, whilst a synthetic antagonist of RXR did not prevent imposex development (Stange *et al.*, 2012).

In summary, the mechanisms leading to imposex are not yet fully understood, but the available data strongly suggest that RXR signalling is involved. We hypothesize that RXR signalling plays an important role in the activation of the *HSD17B3* promoter. *HSD17B3* is a gene that has not been considered yet in previous studies of imposex. We show that the *HSD117B3* promoter is up regulated by nanomolar concentrations of TBT and TPT, which could possibly lead to higher expression of 17β-HSD3 *in vivo* and therefore, may result in elevated testosterone levels. Elevated testosterone levels may contribute to the development of imposex, as suggested by Spooner et al. in 1991 (Spooner N, 1991). We further suggest, that the organotin mediated activation of the *HSD17B3* promoter might be an important mechanism involved in androgen disruption and masculinisation in animals affected by disorders of sexual development caused by organotins.

## 5.8 Outlook for Part B: Tributyltin- and triphenyltin-dependent up regulation of 17β-hydroxysteroid dehydrogenase

The above findings strongly suggest that RXR is involved in the mechanism of *HSD17B3* promoter up regulation. Elevated testosterone levels *and* RXR mediated signaling may play a role within the development of imposex. I suggest conducting further experiments to support this hypothesis.

- **Measure *17BHSD3* mRNA levels in tissues from TBT-or TPT-exposed animals**

*HSD17B3* levels are a key measure of our hypothesis. Tissues of relevant animal models (snails) exposed to TBT or TPT should be analyzed by RT-PCR for elevated *HSD17B3* levels. Since it is a mechanistic study, other animal models (e.g. mice) may also be used.

- **Analyze the *HSD17B3* promoter for potential RXR binding sites with *Nubiscan*, followed by point mutations for functional confirmation**

Gaining further evidence for the involvement of RXR could be achieved by the analysis of the 2.8 kb sequence for putative nuclear receptor binding sites. I suggest using the *in silico* tool *Nubiscan (*designed by M. Podvinec, University of Basel, Basel) for such an analysis. Once putative RXR binding sites are identified, the functional relevance of these sites could be tested by deletions or point mutations.

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