Genes and proteins of the alternative steroid backdoor pathway for dihydrotestosterone synthesis are expressed in the human ovary and seem enhanced in the polycystic ovary syndrome

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Abstract (148)

Recently, dihydrotestosterone biosynthesis through the backdoor pathway has been implicated for the human testis in addition to the classic pathway for testosterone (T) synthesis. In the human ovary, androgen precursors are crucial for estrogen synthesis and hyperandrogenism in pathologies such as the polycystic ovary syndrome is partially due to ovarian overproduction. However, a role for the backdoor pathway is only established for the testis and the adrenal, but not for the human ovary. To investigate whether the backdoor pathway exists in normal and PCOS ovaries, we performed specific gene and protein expression studies on ovarian tissues.

We found aldo-keto reductases (AKR1C1-1C4), 5 α -reductases (SRD5A1/2) and retinol dehydrogenase (RoDH) expressed in the human ovary, indicating that the ovary might produce dihydrotestosterone via the backdoor pathway. Immunohistochemical studies showed specific localization of these proteins to the theca cells. PCOS ovaries show enhanced expression, what may account for the hyperandrogenism.

Key words: dihydrotestosterone, androgen biosynthesis, classic and alternative backdoor pathway, human ovary, testis, adrenals, PCOS, androgens

1. Introduction

Androgens are produced from cholesterol in the human gonads and in the adrenal cortex. They are mainly known for their masculine effects, which are principally exerted through transcriptional regulation via androgen receptors at the cellular level. Androgen action is needed very early in fetal life for male sexual development, during adolescent years for pubertal development and in adulthood for male fertility and reproduction. Similar to males, the female development, fertility and reproduction are equally dependent on androgens. Not only are androgens essential precursors for all estrogen biosynthesis, but also crucial for the normal development of ovarian follicles as shown by knockout of the androgen receptor in mice (Hu et al., 2004; Shiina et al., 2006). In addition, hyperandrogenism in women is associated with disease states such as the polycystic ovary syndrome (PCOS). PCOS is defined by androgen excess, menstrual abnormalities and polycystic ovaries. Although as many as 10% of women are affected by the PCOS and large efforts in terms of clinical and basic studies are ongoing, its pathophysiology is still poorly understood and therapeutic options are scarce. As androgen biosynthesis plays a pivotal role in ovarian physiology, a better understanding of androgen biosynthesis and its regulation may help in understanding this reproductive disorder, which is the most frequent cause of anovulation and infertility.

Though the biochemistry and the genetics of testosterone and dihydrotestosterone (DHT) synthesis from cholesterol have been described decades ago, only recent work has revealed a second pathway for DHT biosynthesis, which is now known in the literature as the alternative, backdoor pathway, to distinguish it from the classic pathway (Auchus, 2004 and Figure 1). In contrast to the classical pathway, DHT synthesis through the backdoor pathway does not proceed through testosterone, but diverges off at the level of 17-hydroxyprogesterone (17OHP) and proceeds through 17OH-dihydroprogesterone (17-DHP), 17OH-allopregnanolone (17-Allo), androsterone and androstanediol (3 α Diol) to form the most potent androgen DHT. Enzymes involved in the backdoor pathway are 5 α -reductase type 1 (SRD5A1), aldo-keto reductase family 1 members C2/C4 (AKR1C2/C4), cytochrome P450c17 (CYP17A1), aldo-keto reductase family 1 C3 (AKR1C3) /17 β -hydroxysteroid dehydrogenase type 5 (HSD17B5) and retinol

dehydrogenase (RoDH)/17 β -hydroxysteroid dehydrogenase type 6 (HSD17B6) (Auchus, 2004, Miller and Auchus, 2011) (Table 1). Regarding CYP17A1, its enzyme activity in the backdoor pathway is in contrast to the classic pathway not dependent from cytochrome b5. In addition to the classic and alternative backdoor pathway, Fassnacht et al. showed a third, androstenedione-mediated route to DHT (Fassnacht et al., 2003). This route of DHT formation from androstenedione via 5 α -androstenedione is dominant in castration-resistant prostate cancer (Chang et al., 2011).

The backdoor pathway was first discovered in the testes of the tammar wallaby pouch young, where androstenediol is formed by a pathway involving 17OH-Allo as a key intermediate (Wilson et al., 2003). Accordingly, analysis of urine steroid profiles of patients presenting with features of hyperandrogenism due to cytochrome P450 oxidoreductase (POR) deficiency revealed steroid metabolites implicating the backdoor pathway in humans (Homma et al., 2006). Furthermore, human mutations in the aldo-keto reductase genes AKR1C2 and AKR1C4, both specific for the backdoor pathway, were found in 46,XY individuals with disordered sexual development, illustrating that both the classic and the backdoor pathways are needed for normal male fetal androgen biosynthesis in the testis (Flück et al., 2011). In addition, activity of the backdoor path was observed in patients with 21-hydroxylase deficiency, establishing a role of the backdoor pathway for the human adrenal cortex (Kamrath et al., 2012).

Overall, the steroid backdoor pathway for androgen biosynthesis plays a role in the normal human adrenal cortex and the testis, and it seems to play an important role in steroid disorders. However, its role in the human ovary remains elusive. Currently, circulating DHT in females is mostly assigned to peripheral conversion of precursor steroids (Miller and Auchus, 2011). The contribution of ovarian DHT production, either by stepwise conversion from cholesterol or by conversion of steroid metabolites circulating back from the periphery, is unknown. Therefore, this study aimed to establish the role of the backdoor androgen biosynthesis pathway for the human ovary itself. To illustrate the backdoor path in the female gonad, we performed gene and protein expression studies of the specific enzymes of the backdoor pathway in human ovarian tissues. Tissues of human adrenals and testes served as controls. In addition,

we compared ovarian tissues of healthy females with tissues of PCOS females. Using this approach we show that the human ovary expresses all genes necessary for DHT production via the backdoor pathway. Interestingly, alterations in the expression of alternative backdoor pathway genes were found in PCOS when compared to controls suggesting that androgen excess in PCOS may (at least in part or in some PCOS patients) originate from the backdoor pathway.

2. Materials and Methods

2.1 Tissue samples. Pathway analysis was performed on fresh frozen and formalin fixed and paraffin embedded (FFPE) tissue samples of human adrenals, testes and ovaries. Samples were obtained from the ethically approved biobank of the Institute of Pathology Bern and the Faculty of Medicine Bern (Bern, Switzerland). Samples were selected by histological evaluation, as well as evaluation of patients' medical data. PCOS samples were chosen according the Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Group and included tissue samples of women suffering from two of the following three criteria: one, clinical and/or biochemical hyperandrogenism, two, oligo- and/or anovulation and three, polycystic ovaries on ultrasound; other etiologies were excluded (Rotterdam ESHRE/ASRM-Sponsored PCOS consensus workshop group, 2004). In addition, only tissue samples of women aged 12 to 45 years, in the post pubertal and premenopausal age range were included in the study.

2.2 Quantitative real time PCR (qRT-PCR). Total RNA was isolated from fresh frozen paraffin embedded adrenal, testicular, normal ovarian and PCOS tissue samples using Ambion's RecoverAll™ kit according to the manufacturer's instruction (Ambion, Austin, TX, USA). For each tissue type, experiments were performed on three different samples, originating from different organs. Following isolation, reverse transcription to cDNA was performed using the Improm RNA transcriptase kit (Promega, Madison, WI). qRT-PCR analysis was performed on the 7500-Fast real-time PCR System

(Applied Biosystems, Foster City, CA, USA) using ABsolute SYBR Green Mix (ABgene; Thermo Fisher scientific, Waltham, MA, USA), specific primers (Microsynth, Balgach, Switzerland) and 50ng cDNA in a total volume of 25ul. GAPDH, cyclophilin A and ribosomal protein large P0 (RPLP0) genes were used as endogenous controls. Expression of the investigated genes was analyzed in relation to the geometric mean of the expression of endogenous control genes and calculated by the $2^{-\Delta Ct}$ method. Amplification curves and the mean cycle threshold (Ct) values were calculated with the help of the 7500 Fast System SDS software (Applied Biosystems).

2.3 Immunohistochemical studies (IHC).

Antibodies were tested for specificity by using cell lines with known positive (H295R, HepG2) and negative (HEK293) immunoreactivity. Four tissue samples originating from four different normal and four PCOS ovaries, respectively, were investigated. Tissues were fixed in a 10% formaldehyde solution and embedded in paraffin. We used one paraffin embedded tissue block per patient, which was cut in serial sections. The samples were cut at 3 μ m thickness and dried at 60° for 30 min. Tissues were investigated by expert pathologists (AP, CT, MT) and an area representing the whole specimen was chosen for quantification and figure presentation. The automated system BOND RX (Leica Biosystems, Newcastle, UK) was used to carry out the IHC detection of proteins. The following conditions were used for IHC: Antigen retrieval was done with citrate buffer solution (pH 6.5) at 95° for 20 min., then endogenous peroxidase was blocked with H₂O₂ solution for 4 minutes. All samples were incubated with primary antibodies described in Table 2 at room temperature for 30 minutes. The following secondary antibodies were used: rabbit anti-chicken antibody (ab97136, AbCam, Cambridge, UK.) at 1:2000 dilution, goat anti-rabbit (E0466, Dako, Glostrup, Denmark) at 1:400 dilution. The slides were visualized with the Bond Polymer Refine Kit (with 3-3'-Diaminobenzidine-DAB as chromogen) (Leica Biosystems). Samples were counterstained with hematoxylin and mounted in Aquatex (Merck,

Darmstadt, Germany). Finally, all slides were scanned and photographed in a Pannoramic P250 scanner (3DHistech, Hungary).

2.4 Assessment of immunohistochemistry. Results were scored by assessing two parameters, one, the percentage of positive cells (P; 0%-100%) and two, the intensity of the staining (I; 0-1-2-3). This assessment is based on the H-score (Ishibashi et al., 2003). Our final score (Q) was calculated by multiplying the two parameters ($Q = P \times I$; minimum = 0, maximum = 300) as described (Galván et al., 2013). In addition, the samples were blinded before being assessed by five independent examiners (NM, JAG, AP, CEF, JM) and the results of all examiners were combined in a final Q-score for analysis.

2.5 Statistics. Quantitative data of qRT-PCR represent the mean $\pm$ SD of three independent experiments. Statistical analysis was performed using two-tailed Student's t-test (Excel; Microsoft Corp., Redmond, WA). Significance was set at * $p < 0.05$ and ** $p < 0.01$. For semi-quantitative analyses of IHC data the mean of five independent assessments was calculated and the scatter blots and median for four different tissues are given.

3. Results

3.1 Backdoor pathway genes are expressed in normal human adrenal, testis and ovary tissues. To get a full picture of the expression of the backdoor pathway genes in human androgen synthesizing organs, we assessed mRNA expression of HSD3B2, RoDH, SRD5A1/2 and AKR1C1/2/3/4 in the normal human adult adrenals, the testes and in the ovaries (Figure 2). Overall, the genes of interest were expressed in all three organs but at a significantly lower level when compared to the endogenous control genes. Relative expression of the backdoor pathway genes was ten (AKR1C3) to hundred times (RoDH, SRD5A1/2 and AKR1C1/C2) lower; and AKR1C4 was even too low to be reliably quantified. High

levels were only found for the HSD3B2 gene, which also served as a control for a specific steroid biosynthetic gene that is essential for androgen production in both the classic and the backdoor pathway. The in between organ comparison revealed that the testicular expression of HSD3B2 was significantly lower than the adrenal expression. In contrast, the testes seemed to express the genes for RoDH, SRD5A2 and AKR1C2 at higher levels than the adrenals and the ovaries. SRD5A1, known as the gate keeper enzyme to the backdoor pathway (Auchus, 2004), was found in all three organs studied, but was expressed highest in the ovaries.

3.2 Immunohistochemical studies identifying backdoor pathway proteins in the normal human ovary. Faced with limitations using quantitative RT-PCR for obviously low expressed genes of the backdoor pathway in the androgen producing organs, we switched to a more qualitative approach and used specific IHC staining. This approach allows not only a semi-quantitative measure of a specific protein, but also gives its distribution and localization in the tissue. In pilot experiments specificity of used antibodies was tested and confirmed (see section 2.3). Specific staining for HSD3B2, RoDH, CYP17A1, SRD5A1 and AKR1C2/3/4 revealed a highly differential expression of the backdoor pathway related proteins when comparing adrenal, testicular and ovarian tissues (Figure 3). The adrenal cortex showed a zone specific protein expression pattern. In the zona glomerulosa (ZG), where the mineralocorticoid production takes place, we found a high expression of HSD3B2 and AKR1C3. HSD3B2 and CYP17A1 were found to be expressed at high levels in the zona fasciculata (ZF), where both enzymes are needed for cortisol biosynthesis. In contrast to its essential role in aldosterone and cortisol production, HSD3B2 is inversely correlated with DHEA production (Manson et al., 1993). This is illustrated by the low HSD3B2 immunoreactivity in the androgen producing zona reticularis (ZR). In the ZR however, we found expression of RoDH, SRD5A1 and AKR1C2-4, indicating that the backdoor androgen biosynthesis pathway may be functional in this zone. As published before, we found a higher expression of AKR1C3 in the ZR than in the ZF (Nakamura et al., 2008). Similar to our observations in

the ZR of the adrenals, we found all proteins involved for the biosynthesis of DHT via the backdoor pathway expressed in the androgen producing testicular Leydig cells and the ovarian theca cells (Figure 3). While both Leydig and theca cells are known to have high immunoreactivity for CYP17A1, we also found high immunoreactivity for RoDH and AKR1C3 in the Leydig cells and for RoDH in theca cells. Only weak immunoreactivity was detected for AKR1C4 in all investigated tissues.

3.3 Immunohistochemical studies suggest altered expression of backdoor pathway proteins in PCOS. After having demonstrated that the backdoor pathway for DHT synthesis exists in the normal human ovary, we hypothesized about its possible role for androgen production in the hyperandrogenic disorder of PCOS. In a first approach, we tried to assess the expression of the backdoor pathway genes by quantitative RT-PCR. However, as those genes were found to be expressed at extremely low levels and as the number of available samples was very limited, no conclusive results for comparing PCOS with normal tissues were achieved. We therefore switched to IHC to perform pilot experiments on the few available PCOS tissue sections. Specific IHC staining of PCOS ovarian tissues revealed the expression of all backdoor pathway proteins at different intensities and also showed differences when compared to normal ovarian tissues (Figure 4). We found a strong immunostaining for SRD5A1, AKR1C2 and RoDH in the assessed PCOS tissues. Moreover, HSD3B2, CYP17A1 and AKR1C3, which are involved in the DHT production through the classic and the backdoor pathway, were also well expressed. Comparing normal and PCOS tissues, we observed a higher immunoreactivity for HSD3B2, RoDH, SRD5A1 and AKR1C2 in PCOS samples, indicating that there might be a higher activity of these enzymes in PCOS (Figure 4).

To confirm these observations, IHC stained tissue samples from normal ovaries (n = 4) and PCOS ovaries (n= 4) were assessed for the expression of HSD3B2, RoDH, SRD5A1 and AKR1C2-C4 using the Q-score (see section 2.4) (Galván et al., 2013). As shown in Figure 5, all investigated proteins seemed to be more strongly expressed in PCOS than in normal ovaries. The biggest difference by the Q-score was observed

for the gate-keeper enzyme to the backdoor pathway, SRD5A1 ($p=0.08$). For all other proteins the variability between the samples within the two groups was too high to reach statistical significance.

4. Discussion

In this study, we showed that all genes of the alternative, backdoor pathway for androgen biosynthesis are expressed in the human ovary (Figure 1 and Table 1). In previous studies, we found a similar expression profile of genes of the backdoor pathway for the human testis and the adrenals (Biaison-Lauber et al., 2013; Fluck et al., 2011). While SRD5A1 was found in the ovary, testis and adrenal, SRD5A2 was only found expressed in the testis, where it is thought to convert testosterone to DHT via the classic pathway. The exact role of the AKR1C1-4 in the backdoor pathway remains unclear. Overall, the enzymes of this group seem very promiscuous (Miller and Auchus, 2011). AKR1C2 and AKR1C4 share the ability to oxidize 3 α Diol to DHT *in vitro* (Penning et al., 2000). AKR1C3 (also known as HSD17B5) is expressed in steroidogenic and non-steroidogenic tissues and converts androstenedione to testosterone in the testis, the adrenals and in the peripheral organs (Miller and Auchus, 2011). Unlike AKR1C2, we found no expression of AKR1C4 in the ovary.

We confirmed by IHC the gene expression results. Furthermore, we were able to localize the proteins of the backdoor pathway in the human ovary, the testis and the adrenal cortex. In the ovary, HSD3B2, SRD5A1, AKR1C2, AKR1C3, CYP17A1 and RoDH are specifically expressed in the theca cells. Similarly, these proteins are expressed in the testicular Leydig cells. In line with published data, we found HSD3B2 highly expressed in the zona glomerulosa and fasciculata, and CYP17A1 in the zona fasciculata and reticularis of the adrenal cortex (Nakamura et al., 2008). By contrast, expression of the characteristic proteins of the backdoor pathway was seen predominantly in the zona reticularis. Thus, our IHC analysis show for the first time that all known androgen producing steroid cells express the enzymes needed for the production of DHT through the backdoor pathway.

IHC studies of normal and PCOS tissue samples suggested enhanced expression of HSD3B2, SRD5A1, AKR1C2 and AKR1C3 in PCOS. SRD5A1 scored highest in PCOS compared to controls. This result is in line with several studies showing increased 5 α -reductase activity in PCOS (Fassnacht et al., 2003; Goodarzi et al., 2006; Torchen et al., 2015). Enzyme kinetic analyses have demonstrated an approximatively 4-fold higher 5 α -reductase activity in PCOS follicles than in control follicles (Jakimiuk et al., 1999). Urine steroid profiling in PCOS women and their daughters (Torchen et al., 2015) have shown evidence for higher 5 α -reductase activity from the steroid metabolome. Similarly, higher activity of HSD3B2 and CYP17A1 has been reported for PCOS theca cells when compared to normal cells (Nelson et al., 2003). In the same study, AKR1C3 was expressed in human theca cells, but not altered in PCOS. Expression profiles of AKR1C2 and RoDH have not been reported previously for either the normal or PCOS human ovary. In our tissue samples AKR1C2 and AKR1C3 were both expressed at low levels in normal ovaries, but seemed slightly enhanced with PCOS, while RoDH showed no difference.

Increased expression and activity of HSD3B2 leads to 17-OH-Prog, which is the substrate for the gatekeeper enzyme SRD5A1 for entering the backdoor androgen biosynthesis pathway (Figure 1). The next step involves AKR1C2, which is also slightly upregulated with PCOS, and produces 17OH-Allo. Further, 17OH-Allo is more efficiently metabolized by CYP17A1 than 17OH-Preg (Miller and Auchus, 2011), even in the absence of cytochrome *b5* (Gupta et al., 2003). Therefore, the flux for androgen production may rather flow through alternate pathways to DHT than through the classic pathway. Alternate flux may follow the backdoor pathway or go through androstenedione linking to the backdoor path via SRD5A1 (Figure 1). In support of this hypothesis are the following findings: First, PCOS women have higher serum 17OH-Prog levels than normal women (Keefe et al., 2014), and high 17OH-Prog levels have been shown to promote androgen production through the backdoor pathway (Kamrath et al., 2012; Homma et al., 2006); second, PCOS women have higher 5 α -reductase activity (Fassnacht et al., 2003; Jakimiuk et al., 1999; Nelson et al., 2003); third, elevated serum DHT levels have been described in some PCOS patients (Munzker et al., 2015), but DHT is only rarely assessed in routine clinical steroid

measurements. Finally, regularly assessed serum androgens in the laboratory work-up for PCOS reflect only the classic pathway and are often not elevated even though clinical signs of virilization are clearly evident. Thus, it might be necessary to include backdoor steroid metabolites in future clinical work-up of PCOS.

Major limitations of our study are the lack of actual backdoor androgen measurements from tissues, and the low sample numbers for both control and PCOS ovaries. But studies of PCOS have always been hampered by tissue availability for obvious reasons. No woman in her childbearing years donates tissue for studies and surgical procedures are not indicated for the diagnosis or treatment of PCOS. Rarely, a larger cyst leads to an ovarian torsion and the removal of an ovary, which can then be further investigated. Accordingly, we were only able to retrieve few tissue samples from our biobank, and these were paraffin embedded and thus not suitable for enzyme kinetic experiments or tissue steroid measurements. Additional studies are clearly needed to confirm our pilot findings and establish the role of the backdoor pathway for androgen biosynthesis in the ovary at the functional level. This should be feasible in the future for normal human ovarian tissues using novel chromatographic, mass-spectrometric methods for androgen profiling. But it certainly remains difficult for PCOS ovaries, as tissues are lacking. .

In summary, the human ovarian theca cells express all proteins needed for the synthesis of DHT through the backdoor pathway. In PCOS, theca cells show higher expression of specific enzymes of the backdoor pathway, suggesting that androgen production in PCOS may occur through the backdoor pathway to a greater extent when compared to normal ovarian theca cells. However, enzyme kinetic studies or steroid measurements from tissues are needed to provide ultimate proof of the functionality of the backdoor pathway in the human ovary.

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Figure Legends

Figure 1. The classic and the alternative backdoor pathway for androgen biosynthesis.

Dihydrotestosterone (DHT) is produced from cholesterol by two pathways, which diverge at the level of 17-hydroxyprogesterone (17OHProg). The diagram illustrates the two distinct routes depicting the intermediates and enzymes involved in both pathways. In addition, the diagram shows a third pathway from DHEA to DHT, the so-called non-conventional androstenedione-mediated pathway (17). See also Table 1. Preg, pregnenolone; Prog, progesterone; 17OHPreg, 17 α -hydroxypregnenolone; 17OH-DHP, 17-hydroxy-dihydroprogesterone; 17OH-Allo, 17-hydroxy-allopregnanolone; DHT, dihydrotestosterone; CYP11A1, cholesterol side-chain cleavage enzyme, P450_{scc}; StAR, steroidogenic acute regulatory protein; CYP17A1, 17 α -hydroxylase/17,20-lyase; HSD3B2, 3 β -hydroxysteroid dehydrogenase, type 2; HSD17B3/5, 17 β -hydroxysteroid dehydrogenase, type 3/5; SRD5A1/2, 5 α -reductase, type 1/2; AKR1C2/3/4, aldo-keto reductase family 1, member C2/C3/C4; RoDH, retinol dehydrogenase / 17 β -hydroxysteroid dehydrogenase, type 6; CYB5, cytochrome b5.

Figure 2. Expression of genes found in the backdoor pathway in the normal human adrenal, testis and ovary. Relative mRNA expression profiles were assessed by quantitative RT-PCR and illustrate the differential expression of the investigated genes comparing the three different tissues of interest. Overall, we found low expression of aldo-keto- and steroid-reductase genes with high variability. Gene expression is shown in relation to the geometric mean of the endogenous control genes GAPDH, cyclophilin A and RPLP0 and was analyzed using the $2^{-\Delta\text{CT}}$ method. Data are the mean $\pm$ SD from three independent experiments using samples from three different organs. HSD3B2, 3 β -hydroxysteroid dehydrogenase, type 2; RoDH, retinol dehydrogenase / 17 β -hydroxysteroid dehydrogenase type 6; SRD5A1/2, 5 α -reductase, type 1/2; AKR1C2/3/4, aldo-keto reductase family 1, member C2/C3/C4.

Figure 3. Immunohistochemistry for human adrenal, testis and ovary tissues investigating the expression and localization of proteins characteristic for the backdoor pathway. Normal human tissue samples were used to perform IHC experiments with specific antibodies characterizing the backdoor pathway. Hematoxylin and eosin staining show the histology of the assessed samples, depicting the androgen producing structures of each organ; the adrenal zona reticularis (ZR), the testicular Leydig cells (LC) and the ovarian theca cells (TC). Note that all three organs show immunoreactivity for all enzymes involved in the backdoor DHT production. The representative pictures are given at a magnification of 10x (scale bar = 200µm). The detail section of the normal ovary is given at a magnification of 40x (scale bar = 50µm). ZG, zona glomerulosa; ZF, zona fasciculata; H&E, hematoxylin and eosin; HSD3B2, 3β-hydroxysteroid dehydrogenase, type 2; RoDH, retinol dehydrogenase / 17β-hydroxysteroid dehydrogenase type 6; CYP17A1, 17α-hydroxylase/17,20-lyase; SRD5A1/2, 5α-reductase, type 1/2; AKR1C2/3/4, aldo-keto reductase family 1, member C2/C3/C4.

Figure 4. Immunohistochemistry for healthy ovarian tissues and ovarian tissues of PCOS. Normal (n=4) and PCOS (n=4) tissues were stained with specific antibodies (Table x). Representative samples are shown. The androgen producing theca cells are highlighted by their high immunoreactivity for CYP17A1. Note the higher percentage of positive cells and the more intense specific staining for HSD3B2, RoDH, SRD5A1 and AKR1C2/4 in PCOS compared to normal ovarian tissues. The representative pictures are given at a magnification of 40x (scale bar = 50µm). H&E, hematoxylin and eosin; HSD3B2, 3β-hydroxysteroid dehydrogenase, type 2; RoDH, retinol dehydrogenase / 17β-hydroxysteroid dehydrogenase type 6; CYP17A1, 17α-hydroxylase/17,20-lyase; SRD5A1/2, 5α-reductase, type 1/2; AKR1C2/3/4, aldo-keto reductase family 1, member C2/C3/C4; TC, theca cells

Figure 5. Semi-quantitative analysis of backdoor pathway protein expression comparing normal and PCOS ovaries. For comparison of controls versus PCOS, a semi-quantitative analysis was

performed assessing two parameters, the intensity of the staining (0-1-2-3) and the percentage of positive cells (0-100%). The product of both parameters gives the Q-score for each case (normal n=4, PCOS n=4). The final Q-scores depicted in the diagram represent the mean for each case, assessed by five independent investigators. The median Q-score of the four cases is depicted by the black bar. Overall, there is a trend towards higher expression of the backdoor pathway enzymes in PCOS. HSD3B2, 3 β -hydroxysteroid dehydrogenase, type 2; RoDH, retinol dehydrogenase / 17 β -hydroxysteroid dehydrogenase type 6; SRD5A1/2, 5 α -reductase, type 1/2; AKR1C2/3/4, aldo-keto reductase family 1, member C2/C3/C4.

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Table 1. Overview of steroidogenic enzymes in the classical and the backdoor pathway.

Gene	Protein	Enzyme	Major reaction
HSD3B2	3 β HSD2	3 β -hydroxysteroid dehydrogenase type 2	3 β -dehydrogenase, $\Delta^{5/4}$ -isomerase
RoDH (HSD17B6)	RoDH (17 β HSD6)	Oxidative 3 α -HSD; Retinol dehydrogenase (17 β -hydroxysteroid dehydrogenase type 6)	3 α -hydroxysteroid dehydrogenase, 3 α -, 3 β -, and 17 β -ketosteroid reductase
CYP17A1	P450c17	17 α -hydroxylase, 17/20 lyase	17 α -hydroxylase, 17,20-lyase
SRD5A1	5 α -reductase 1	5 α -reductase type 1	5 α -reductase
SRD5A2	5 α -reductase 2	5 α -reductase type 2	5 α -reductase
AKR1C1	AKR1C1	Reductive 3 α -HSD; 3 α -hydroxysteroid dehydrogenase type 4	3 α -ketosteroid reductase, 20 α -ketosteroid reductase
AKR1C2	AKR1C2	Reductive 3 α -HSD; 3 α -hydroxysteroid dehydrogenase type 3	3 α -ketosteroid reductase
AKR1C3 (HSD17B5)	AKR1C3 (17 β HSD5)	Reductiv 3 α -HSD; 3 α -hydroxysteroid dehydrogenase type 2 (17 β -hydroxysteroid dehydrogenase type 5)	3 α -ketosteroid reductase, 17 β -ketosteroid reductase
AKR1C4	AKR1C4	Reductive 3 α -HSD; 3 α -hydroxysteroid dehydrogenase type 1	3 α -ketosteroid reductase

(Miller WL and Auchus, 2011)

Table 2. Primary antibodies used in immunohistochemical analysis.

Primary antibodies	Host	Clonality	Commercial reference	Dilution
AKR1C2	Chicken	monoclonal	Custom made	1:2000
AKR1C3	Mouse	monoclonal	A6229, Sigma Aldrich, MO, USA	1:5000
AKR1C4	Chicken	monoclonal	Custom made	1:3000
CYP17A1	Chicken	monoclonal	Custom made	1:15000
HSD17b6	Rabbit	polyclonal	HPA059141, Sigma Aldrich, MO, USA	1:100
HSD3b2	Chicken	monoclonal	Custom made	1:20000
SRD5A1	Goat	polyclonal	Ab110123, Abcam, Cambridge, UK	1:100

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

The ovary produces androgens through the backdoor pathway, which may be overactive in PCOS