Steroidogenesis in Steroid Related Cancers

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Abstract

Prostate cancer growth is androgen dependent, but can bypass treatments inhibiting androgen production and continues to grow, probably utilising the adrenal alternative dihydrotestosterone (DHT) biosynthesis pathway. In contrast, the role of steroids in ovarian cancer is not clearly defined, although it is known that progesterone and oestrogens affect this cancer proliferation.

The thesis aims were to investigate steroid relation with prostate and ovarian cancers as well as characterise three adrenocortical carcinoma cell (ACC) lines derived from H295 cell line.

The steroidogenesis characterisation of three ACC cell line strains indicated that they have similar steroidogenic capability to the native adrenal cortex. Another ACC cell line, H295R1, was used to investigate whether CYP17A1, a key enzyme in androgen synthesis, inhibition was sufficient to cause a shift towards the alternative DHT biosynthesis pathway. Although knockdown was achieved and affected steroidogenesis, no shift towards the alternative pathway was demonstrated.

The ovarian cancer cell lines, A2780, COV434, CaOv-3 and OAW42, showed steroidogenic capabilities suggesting that the tumours are probably transformed with steroidogenic capability.

The results obtained indicate that the adrenal gland might have a role in prostate cancer resistance to treatment. They also demonstrate that ovarian cancer might be steroid-producing and/or dependent.
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**Abbreviations**

5α17HP – 17-Hydroxallopregnenolone

11OHA4 – 11-Hydroxyandrostenedione

17OHP – 17α-Hydroxyprogesterone

17OHPreg – 17α-Hydroxypregnenolone

ACC – Adrenocortical carcinoma

ACTH – Adrenocorticotropic hormone

AR – Androgen receptor

ATCC – The American Type Culture Collection

cAMP – Cyclic adenosine monophosphate

cDNA – complementary DNA

CRPC – Castration resistant prostate cancer

Ct – Threshold cycle

CYP – Cytochrome P450

DCC – Charcoal stripped serum

DHEA – Dehydroepiandrosterone

DHT – Dihydrotestosterone

DMEM – Dulbecco's modified eagle medium

DMSO – Dimethyl sulfoxide

DNA – Deoxyribonucleic acid

DOC – Deoxycorticosterone

dNTP – DNA nucleotide tri-phosphate

ER – Oestrogen receptor

FBS – Foetal bovine serum
FSH – Follicular stimulating hormone
FSHR – FSH receptor
FSK – Forskolin
GnRH – Gonadotropin releasing hormone
GR – Glucocorticoid receptor
HCl – Hydrochloric acid
HGS – High grade serous
HPA – Hypothalamus-pituitary-adrenal
HSD – Hydroxysteroid dehydrogenase
LC-MS/MS – Liquid chromatography-tandem mass spectrometry
LH – Luteinizing hormone
LHCGR – LH receptor
LGS – Low grade serous
M – Molar
µl – Microliter
mg – Milligram
ml – Millilitre
MR – mineralocorticoid receptor
mRNA–Messenger RNA
MTBE –Tert-Butylmethyl ether
n – Nano
NaCl – Sodium chloride
ng –Nanogram
OC – Ovarian cancer
OH - Hydroxy
POR – P450 oxidoreductase
PC – Prostate cancer
PCR – Polymerase chain reaction
PR – Progesterone receptor
P/S – Penicillin streptomycin
PSA – prostate specific antigen
qPCR – Quantitive polymerase chain reaction
RIPA buffer – Radioimmunoprecipitation assay buffer
RNA – Ribonucleic acid
rpm – Revolutions per minute
RPMI – Roswell Park Memorial Institute
RT – Reverse transcriptase
SCID – Severe combined immunodeficiency
siRNA – Short interfering RNA
1.0 General Introduction

Steroids are cholesterol-derived hormones essential for pre- and postnatal body function and development (Miller and Auchus, 2011), such as salt regulation, the inflammatory response, glucose metabolism, sex and brain development, pregnancy and lactation. Steroid hormones are all based on a 4-ring structure and all derived from cholesterol (Figure 1-1). They differ by the location of the double bond, which is either in ring A between carbon four and five (Δ4) or in ring B between carbon five and six (Δ5), and in the position of hydroxylation or redox modifications. The steroid hormones are mainly synthesised in the adrenal glands, in the cortex, that synthesises mineralocorticoids, glucocorticoids and androgen precursors, and in the gonads, that synthesise the sex hormones, androgens and oestrogens. The above hormones are synthesised in a multi-enzyme process called steroidogenesis. The steroidogenic enzymes synthesising the hormones are divided into two main groups, cytochrome P450 (CYP) enzymes, catalysing oxidative reactions, which are mostly irreversible (Miller and Auchus, 2011), and the hydroxysteroid dehydrogenase (HSD) enzymes catalyse redox reactions, which are mostly reversible (Miller and Auchus, 2011).

*De novo* steroid synthesis, accruing in the adrenal glands and gonads, starts when cholesterol is imported into the mitochondrion by steroidogenic acute regulatory protein (StAR), where the side-chain cleavage enzyme (CYP11A1) catalyses the synthesis of the first steroid, pregnenolone, by removing the side-chain at carbon 22, and carboxylation of carbon 20.

In this thesis, I will focus on adrenal and ovarian steroidogenesis.
1.1 Adrenal Anatomy and Steroidogenesis

The adrenal glands are positioned above each kidney, and are the main source of steroids in the body. Each adrenal gland has two functional sections, the medulla that synthesises catecholamines, which will not be further discussed, and the cortex which synthesises steroid hormones (Figure 1-2 A). The cortex contains three sections known as zones, the outer layer, zona glomerulosa, which synthesises mineralocorticoids; the middle layer, zona fasciculata, which synthesises glucocorticoids and the inner layer, zona reticularis, which synthesises androgens (Figure 1-2 B). The synthesis of the glucocorticoids and androgens is regulated by the hypothalamic-pituitary-adrenal (HPA) axis, while the mineralocorticoids are regulated by the kidney through renin secretion and fluctuations in renal blood pressure. The compartmentalisation of steroidogenesis in the adrenal cortex is achieved by enzyme expression patterns (Rainey, 1999, Rainey et al., 2002, Vinson, 2003, Nguyen and Conley, 2008). The zona glomerulosa does not express CYP17A1, preventing synthesis of any other steroid group other than mineralocorticoids (Miller and Auchus, 2011). The zona fasciculata, that synthesises glucocorticoids, expresses only CYP11B1, thereby preventing the conversion of corticosterone to aldosterone (Miller and Auchus, 2011, Pascoe et al., 1995, Rainey, 1999). The zona reticularis expresses the co-factor cytochrome b5 which is required for the 17,20-lyase activity of CYP17A1 (Rainey and Nakamura, 2008), and expresses little CYP21A2 and
CYP11B enzymes, therefore, synthesises only androgen precursors, and does not synthesise gluco- or mineralocorticoids.

Figure 1-2: The adrenal gland and cortex
A. The adrenal gland has a triangular shape, and contains three layers: a capsule, surrounding the gland, and the two functional sections the cortex and medulla, which produce steroids and catecholamines respectively. B. A cross-section of the adrenal gland, showing the different layers, capsule that surrounds it, the three zones of the cortex and the medulla.

Two key enzymes are involved in the steroidogenesis process determining the steroid route (3βHSD2) or family (CYP17A1) to be synthesised (Figure 1-3). The 3βHSD2, catalyses the shift between Δ5 and Δ4 (Andersen and Ezcurra, 2014, Simard et al., 2005), and is a bi-functional enzyme catalysing 3β-hydrogenation followed by 5 to 4 isomerization (Simard et al., 2005). It catalyses its five substrates, pregnenolone, 17α-hydroxypregnenolone (17OHPreg), dehydroepiandrosterone (DHEA) and androstenediol with the same efficiency (Lee et al., 1999, Miller and Auchus, 2011), and the products are the respective 4-ketosteroids, progesterone, 17α-hydroxyprogesterone (17OHP), androstenedione and testosterone (Simard et al., 2005). The affinity of 3βHSD2 for pregnenolone is much lower than of CYP17A1 (Km 5µM vs. 0.8µM respectively), favouring the Δ5 route (Miller and Auchus, 2011, Auchus et al., 1998). Nevertheless, the ratio between 3βHSD2 and CYP17A1 also plays a role in the determination as to which route, Δ5 or Δ4, will dominate.
CYP17A1 is an important bi-functional enzyme, catalysing the conversion of progestins to androgens. In the adrenal glands, it is located in the zona fasciculata and zona reticularis (Miller and Auchus, 2011, Suzuki et al., 2000) and in the ovaries it is located in the theca cells and in the extra-follicular parts of the ovaries (Smyth et al., 1993), although weak hydroxylation activity can be detected in granulosa cells (Moon et al., 1978, Tsang et al., 1980). CYP17A1 function depends on the electron transferase P450 oxidoreductase (POR) (Pandey and Miller, 2005, Auchus et al., 1998), which is also required for the function of other CYP enzymes such as CYP21A2 and CYP19A1 (Miller and Auchus, 2011, Pandey and Miller, 2005). The two functions of CYP17A1 enzyme, 17α-hydroxylation and 17,20-lyase activity, are performed in the same active site, and the modulation is post-transcriptional, by phosphorylation or with cytochrome b5 that supports the lyase activity (Pandey and Miller, 2005, Auchus et al., 1998). CYP17A1 low affinity towards 17OHP (Miller and Auchus, 2011, Auchus et al., 1998), prevents direct Δ4 androgen synthesis. Therefore, androstenedione and testosterone are synthesised through 3βHSD from DHEA and androstenediol.
Figure 1-3: The adrenal steroidogenesis
The three groups of steroids, mineralocorticoids (purple), glucocorticoids (blue) and androgens (red) are synthesised in the adrenal cortex. Each group is synthesised in a different zone the zona glomerulosa, fasciculata and reticularis respectively. POR – P450 oxidoreductase, an electron transferase is required for CYP17A1 and CYP21A2 enzyme activity. OH – hydroxy, DHEA – dehydroepiandrosterone. Adapted from J. Hofland

1.1.1 Mineralocorticoids

Mineralocorticoids, 21 carbon steroids, regulate salt and water balance in the body, affecting blood pressure. The mineralocorticoids, of which aldosterone is the most potent, act through a nuclear receptor, mineralocorticoid receptor (MR) (Williams and Williams, 2003, Nakamura et al., 2015), and mediate sodium/potassium adenosine tri-phosphate (ATPase) pumps in the kidney, resulting in an increase of sodium intake into the kidney’s nephron in exchange for
potassium, followed by an increase in water osmosis (Williams and Williams, 2003, Booth et al., 2002). This results in an increase in extra-cellular fluid and elevates the blood pressure (Williams and Williams, 2003, Booth et al., 2002). Mineralocorticoid synthesis occurs in the zona glomerulosa, where pregnenolone is converted to progesterone by 3βHSD2, and then is 21-hydroxylased to deoxycorticosterone (DOC) by CYP21A2. The last two stages are performed by CYP11B enzymes, CYP11B1 converts DOC to corticosterone and CYP11B2 converts it to aldosterone (Lisurek and Bernhardt, 2004, Suzuki et al., 2000) (Figure 1-3 Purple). Aldosterone synthesis is regulated through the renin-angiotensin system, and their secretion is affected by potassium concentrations (Clark et al., 1995, Nakamura et al., 2015, Quinn and Williams, 1988). Regulation may also include involvement of adrenocorticotropic hormone (ACTH) (Gallo-Payet et al., 1996, Quinn and Williams, 1988). Although aldosterone is the main mineralocorticoid, DOC and corticosterone also have mineralocorticoid activity (Williams and Williams, 2003, Sheppard and Funder, 1987).

1.1.2 Glucocorticoids

Glucocorticoids, 21 carbon steroids, are involved in many physiological functions including: energy metabolism – glucose regulation, cell function, reproduction, inflammation (Odermatt and Klusonova, 2015), birth (Liggins, 1994) and brain function (Miller, 2015) and are crucial for human survival, as they control glucose distribution in stress conditions. The glucocorticoids, primarily cortisol, are known as stress hormones, since stress increases their secretion, through the HPA axis (Sapolsky et al., 2000). They are synthesised in the middle zone of the cortex, the zona fasciculata. Pregnenolone is converted to 17OHP by 3βHSD2 and CYP17A1. The lack of cytochrome b5 in the zona fasciculata inhibits the 17,20-lyase activity of CYP17A1 thus preventing androgen synthesis (Suzuki et al., 2000, Miller and Auchus, 2011). CYP21A1 then converts the steroid to 11-deoxycortisol that is in turn converted to
cortisol by CYP11B1 (Figure 1-3 Blue). They affect their target cells through a nuclear receptor, glucocorticoid receptor (GR).

1.1.3 Androgen Synthesis

Androgens, 19 carbon steroids, are known as the male sex hormones, although they also affect females (Walters, 2015). Through androgen aromatisation, they are oestrogen precursors (Leung and Armstrong, 1980), and are needed for the normal development of oocytes (Hellbaum et al., 1956, Prizant et al., 2014, Walters et al., 2008). They are responsible for the manifestation of secondary sex characteristics, external genitalia development, muscle growth and brain development. In males, androgens have proliferative effects on prostate cells, which continue in prostate tumours and also in castration resistant prostate cancer (CRPC) (Tan et al., 2015, Heinlein and Chang, 2004, Ang et al., 2009). Androgens are synthesised in the adrenal glands and in the gonads. In the adrenal gland the synthesis takes place in the zona reticularis. In the ovaries androgens are synthesised in the theca cells, ending in androstenedione and testosterone (Abraham, 1974). In males, the testis synthesises steroids, mostly testosterone and dihydrotestosterone (DHT) de novo.

In females, about half of the androstenedione and testosterone is synthesised in the ovaries (Abraham, 1974, Judd and Yen, 1973), throughout the menstrual cycle, and their levels fluctuate with a peak during the luteinizing hormone (LH) surge (Abraham, 1974). Androgens affect their target cells via a nuclear receptor, androgen receptor (AR), that can be found in the theca and granulosa cells, indicating that androgens have a role in follicular development, as reviewed by Prizant et al. (2014) and Walters et al. (2008), and as can be seen in hyperandrogenism that causes polycystic ovaries. In this condition follicular development arrests before maturation, and there is accumulation of arrested follicles (Magoffin, 2002). It is noteworthy that most of the data concerning the androgens and the ovaries is derived from
animal studies and therefore their effects in women are not fully known. In the adrenal glands androgens can be synthesised by at least three pathways, and this multi-pathway synthesis might have a role in the development of prostate cancer resistance to the current treatments or other conditions evolved from androgen excess. The pathways will be presented in the following three sections.

1.1.3.1 **Classic Pathway**
The first androgen synthesised in this pathway is DHEA, which is converted from 17OHPreg by CYP17A1. DHEA can be converted to two other androgens, androstenedione and androstenediol, but probably due to enzyme specificity, is converted mostly to androstenedione (Miller and Auchus, 2011, Lasley *et al.*, 2013). No direct comparison could be found in the literature comparing their enzyme synthesis pathways. In the adrenal, the synthesis terminates with DHEA and androstenedione that are secreted into the circulation (Abraham, 1974). Some testosterone and DHT are also secreted by the adrenal gland (Abraham, 1974). The ovary synthesises androgens, androstenedione and testosterone *de novo* in the theca cells (Abraham, 1974, Tsang *et al.*, 1980), but most are aromatised to oestrogens, and a small amount is secreted into the circulation (Abraham, 1974, Smith and Ryan, 1961, Miller and Auchus, 2011). In the male, the testis synthesises DHT from testosterone by 5α-reductase type 1 (Miller and Auchus, 2011), while in the prostate gland DHT is synthesised from androstanedione (Luu-The *et al.*, 2008). Moreover, in studies performed to understand how prostate cancer becomes resistante to treatment it has been shown that DHT is synthesised through androstenedione rather than testosterone (Mohler *et al.*, 2011, Chang *et al.*, 2011) (Figure 1-3 Red).

1.1.3.2 **Alternative Androgen Synthesis Pathway**
This pathway was first described in 2003 by Wilson and colleagues (Wilson *et al.*, 2003) in the tammar wallaby pouch young, and describes synthesis of DHT through androsterone (Wilson
et al., 2003, Arlt et al., 2004, Auchus, 2004) and not testosterone (Figure 1-4). According to Wilson, 17OHP is reduced by 5α-reductase to 5α-pregnan-17-ol-3,20-dione, that is then converted to 17-hydroxy-5α-pregnan-3α-ol-20one (17-OH-allopregnanolone) by 3βHSD and to androsterone by CYP17A1. Androsterone can be converted to DHT either through androstanediol or androstanedione. This pathway also functions in humans suffering from abnormalities in steroidogenesis (Miller and Auchus, 2011, Arlt et al., 2004, Turcu and Auchus, 2015). Children with the severe form of POR deficiency, a condition where P450 oxidoreductase, an electron transferase, which is needed for the correct function of CYP17A1 and CYP21A2, is mutant and not functioning, do not present the equivalent phenotype (Arlt et al., 2004), indicating that those patients bypass the block created by the lack of POR. Furthermore, these patients synthesise androgens through the alternative pathway, as delineated in the tammar wallaby (Arlt et al., 2004). Later, Attard and colleagues (Attard et al., 2012) demonstrated that this pathway is activated in adult patients treated with abiraterone against prostate cancer. Their results also showed that this pathway might be active in the adrenal gland. Although this pathway is considered to be an alternative it has been found that the metabolites of progesterone and 17OHP, allopregnanolone and 17-OH-allopregnanolone respectively are better CYP17A1 substrates than the “classic” steroids, namely, pregnenolone, progesterone and their 17α-hydroxylase forms (Gupta et al., 2003). Moreover, 17-OH-allopregnanolone is the most efficient 17,20-lyase substrate (Gupta et al., 2003). This high affinity of CYP17A1 to the alternative androgens means that even under inhibition the residual enzymatic activity is enough to metabolise steroids downstream to CYP17A1 that can then be metabolised to DHT.
In the zona reticularis of the adrenal gland DHT can be synthesised in an alternative pathway, shown here in blue. This pathway was discovered in 2003 (Wilson et al., 2003), and in humans it is active in pathological conditions. This pathway enables androgen synthesis even if CYP17A1 function is impaired, due to the high affinity of CYP17A1 to the alternative steroids, mostly to 17-OH-allopregnanolone. 17-OH-allopregnanolone is the most efficient 17,20-lyase substrate. DHT – dihydrotestosterone, OH – hydroxy. Adapted from J. Hofland
1.1.3.3 11β-Hydroxyandrostenedione Pathway

This pathway has been known for several decades (Bloch et al., 1957, Chang et al., 1963) but only recently recognised as an important source for adrenal androgens. Its products are secreted in higher levels than androstenedione (Schloms et al., 2012, Rege et al., 2013, Xing et al., 2011). In this pathway, CYP11B1 catalyses the hydroxylation of androstenedione to 11-hydroxyandrostenedione (11OHA4) (Schloms et al., 2012), a precursor for the active androgens 11-ketotestosterone and 11-hydroxyDHT (Figure 1-5) (Storbeck et al., 2013, Swart et al., 2013). This pathway was recently reviewed by Bloem (Bloem et al., 2013) and Swart (Swart and Storbeck, 2015), but is not discussed further here, and its products were not analysed in this dissertation.

![Figure 1-5: 11β-Hydroxyandrostenedione Pathway](image)

**Figure 1-5: 11β-Hydroxyandrostenedione Pathway**

Androstenedione is converted to 11-OH-androstenedione, which can be converted to two active 11-keto androgens, 11-ketotestosterone and 11-ketoDHT. This pathway occurs only in the adrenal gland.

DHT – dihydrotestosterone, OH – hydroxy Adapted from J. Hofland

1.2 The Ovary

The ovaries are the female gonads and are located in the abdominal cavity, on either side of the uterus, below the Fallopian tubes. They are loosely connected to the uterine wall by peritoneal ligaments. The ovaries are constituted of two functional areas; the medulla which contains the blood vessels and the cortex which holds the follicles. There are four types of cells: germ,
connective tissue, steroid producing and supporting cells. The latter three are somatic. The ovaries have a dual function, oocyte storage and assistance in their maturation, and during fertile years, synthesis and secretion of oestrogens. After ovulation, in the expectation of pregnancy the ovaries also secrete progesterone. The ovaries can synthesise oestrogens de novo or from androgen precursors derived from the adrenal gland. Ovarian steroidogenesis occurs mainly in the follicle cells surrounding the oocyte, the theca and the granulosa cells. The ovarian steroid levels change according to the menstrual cycle and the process is regulated by the hypothalamic-pituitary-gonadal axis. The steroidogenesis is considered to be divided between the two cell types of the follicle, in what is known as the two cells - two gonadotropin theory (Ryan and Petro, 1966, Ryan et al., 1968, Hillier et al., 1994, Short, 1962). According to this theory, luteinizing hormone (LH) regulates androgen synthesis in the theca cells and follicle-stimulating hormone (FSH) regulates the granulosa cells (Howles, 2000, Havelock et al., 2004, Meinhardt and Mullis, 2002). Gonadotropin releasing hormone (GnRH) is secreted from the hypothalamus stimulating secretion of the glycoprotein hormones LH and FSH from the anterior pituitary gland. The effects of LH and FSH are mediated through cyclic adenosine monophosphate (cAMP) (Conti, 2002), LH mediates CYP11A1 and aromatase activities (Andersen and Ezcurra, 2014), and androgen synthesis in theca cells (Fevold, 1941, Greep et al., 1942). FSH, as its name implies, facilitates follicle growth (Fevold, 1941, Greep et al., 1942), and it also mediates androgens, diffused from the theca cells, conversion to oestrogens in the granulosa cells aided by LH receptor expression (Andersen and Ezcurra, 2014, Wimalasena et al., 1991). Following ovulation, the granulosa cells of the follicle commence to secretion progesterone under the influence of human chorionic gonadotropin, an equivalent of LH (Figure 1-6).
Ovarian steroidogenesis takes place in the cells surrounding the oocyte: theca and granulosa cells. According to the two-cell-two-gonadotropin theory, theca cells synthesise androgens under the influence of luteinising hormone (LH) and the granulosa cells convert them to oestrogen under follicular stimulating hormone (FSH) regulation. The upper panel describes the steroidogenesis in the growing follicle, while the lower panel describes the steroidogenesis in the corpus luteum. DHEA – dehydroepiandrosterone, cAMP – cyclic adenosine mono phosphate, OH – hydroxy.
1.2.1 Progestins

Progestins are 21 carbon steroids that serve as precursors for all the other steroids. Their name is derived from the combination of pro and gestation (pregnancy), as the main steroid in this group progesterone is vital for pregnancy support. The progestins and their 17-hydroxy forms are not active steroids and only serve as precursors, with the exception of progesterone. In the adrenal gland progestins are synthesised only as precursors, whereas, in the corpus luteum, progesterone is synthesised in the granulosa cells (Figure 1-6, lower panel). Progesterone is the first functional hormone to be synthesised in the ovary, prepares the body for pregnancy, has a role in the prevention of ovarian cancer (Diep et al., 2015), serving as anti-proliferative (Yu et al., 2001, Syed et al., 2001, Keith Bechtel and Bonavida, 2001, Bu et al., 1997) and improving survival in a dose dependent manner (Sieh et al., 2013, Yu et al., 2001). It is metabolised directly from pregnenolone by 3βHSD2, and as described before, can be 17α-hydroxylated to 17OHP. Since 17,20-lyase has low affinity towards 17OHP the Δ5 pathway does not continue on to produce androgens (Andersen and Ezcurra, 2014). Progesterone secretion fluctuates during the menstrual cycle, being low in the beginning of the cycle and increases after the LH surge, about mid cycle (Abraham, 1974), during the luteal phase. Progesterone acts through a receptor, progesterone receptor (PR). There are two types of PR: type A (PR-A) is essential for ovarian function and its level decreases in ovarian cancer (Diep et al., 2015), and type B (PR-B), which on the other hand increases in ovarian cancer, and indicates a favourable prognosis (Diep et al., 2015).

1.2.2 Oestrogens

Oestrogens, 18 carbon steroids, are the main female sex hormones, and have a crucial role in female and male fertility (Stocco, 2008). Oestradiol is the most potent oestrogen in the female body and most of the data described here refers to it. Oestrogens are responsible for the
development of secondary sex characteristics in females, including pubic hair, breast
development, the menstrual cycle and the preparation of the female body for pregnancy and
birth. They also affect brain function (Hara et al., 2015, Kim et al., 2015, Nelson and Bulun,
2001). The oestrogens, oestrone and oestradiol, are synthesised in the ovaries, within the
granulosa cells under the regulation of FSH, or in target tissues, such as the breast, via
aromatisation of the A ring of either androstenedione or testosterone. The androstenedione
synthesised in the theca cells diffuses to the granulosa cells and is converted to oestrone, the
least potent oestrogen. The minority of the androstenedione is converted by 17βHSD3 to
testosterone, which is then aromatised to the most potent oestrogen, oestradiol. Oestradiol can
also be synthesised from oestrone by 17βHSD1 (Miller and Auchus, 2011). The oestradiol
levels vary during the menstrual cycle, being secreted mostly in the follicular phase (Abraham,
1974, Miller and Auchus, 2011), and decreases after the LH surge (Abraham, 1974). Oestriol
levels are very low except during pregnancy. GnRH and aromatase (CYP19A1) regulate
oestrogen synthesis, the former in the ovarian and brain production (Prange-Kiel et al., 2013),
and the latter in the peripheral tissues (Simpson, 2003). The role of ovarian oestrogens in
ovarian cancer (OC) is not clear. While one study suggests they have protective effect, as they
decrease cell proliferation (Wimalasena et al., 1992), another suggests the opposite (Li et al.,
2014).

1.3 Steroids and Cancer

1.3.1 Steroid Producing Cancer – Adrenocortical Carcinoma
Adrenocortical carcinoma (ACC) is a rare malignancy of the adrenal gland cortex. It affects
0.72 per million people in the USA (Else et al., 2014, Allolio and Fassnacht, 2006) with a low
survival rate (Kebebew et al., 2006, Allolio and Fassnacht, 2006). Adrenocortical carcinoma
affects females at higher rates than males, and there are two peaks, that accord in both genders:
in childhood and adults in their 40s and 50s (Allolio and Fassnacht, 2006, Wajchenberg et al., 2000, Wooten and King, 1993, Else et al., 2014, Luton et al., 1990). Most patients present with syndromes of adrenal hormone excess, either clinically or biochemically (Else et al., 2014, Allolio and Fassnacht, 2006). Functional ACC is mostly detected in children and women (Else et al., 2014, Wooten and King, 1993), and the functionality of the tumour correlates with adrenal steroidogenesis. Excess glucocorticoids will result in rapidly progressing Cushing’s syndrome sometimes with virilisation. In women, androgen excess will result in virilisation, male pattern balding and deepening of the voice. The male equivalent is gynaecomastia and testicular atrophy in oestrogen secreting ACC (Else et al., 2014, Luton et al., 1990). A third of the ACC cases present with tumour symptoms such as abdominal pain, weight loss and weakness (Luton et al., 1990, Else et al., 2014). A relatively large percentage of cases, up to 30%, are detected accidentally, during scans for unrelated conditions, known as incidentalomas (Else et al., 2014, Kebebew et al., 2006). The diagnosis of ACC is based on biochemistry, hormone level measurements, imaging using computerised tomography, magnetic resonance, positron emission tomography or ultrasound as well as pathology (Else et al., 2014, Allolio and Fassnacht, 2006, Fassnacht et al., 2004, Wajchenberg et al., 2000). Biochemical analysis includes measurements of ACTH, which is supressed, cortisol which is elevated, and a dexamethasone suppression test based on the Cushing’s test (Nieman et al., 2008, Else et al., 2014). Elevated aldosterone plasma levels and plasma renin activity will indicate the involvement of the zona glomerulosa in the ACC (Else et al., 2014, Allolio and Fassnacht, 2006). Since not all ACCs are functional, biochemistry analysis might not be sufficient to detect an ACC tumour, and therefore the diagnosis is also based on imaging (Else et al., 2014). Image detection is based on the size and texture of the tumour (Else et al., 2014). Adrenocortical carcinoma tumours are usually large, with necrosis and haemorrhage (Allolio and Fassnacht,
2006, Else et al., 2014). The treatment includes surgery, radiotherapy and chemotherapy. In some cases, mitotane is given alone or as an adjuvant with the other modalities, mostly with surgery (Else et al., 2014, Allolio and Fassnacht, 2006, Luton et al., 1990, Wajchenberg et al., 2000).

1.3.2 Steroid Dependent Cancer – Prostate Cancer

The prostate is an exocrine gland and part of the male reproductive system. Its physiological role is to help to create the semen by adding fluid to the sperm originated in the testis. It has no functional equivalent in female. The gland is located within the pelvis, below the bladder. The prostatic epithelial and stromal cells are androgen sensitive and depend on them for their growth. Due to the long-term exposure to androgen in the male, the prostate gland increases in size with age. Prostate cancer (PC) is the most prevalent cancer in men (Siegel et al., 2015, Cancer Research UK, 2014a), and the second most common cause of cancer-related death in men (Siegel et al., 2015, Cancer Research UK, 2014b). It affects mostly men over 50 years and the risk increases with age. The cancer development is divided into four stages, I-IV. Stages I and II are localised to the gland, and in the last two stages (III and IV) the cancer invades other organs. In stage III nearby organs or lymph nodes are invaded and in stage IV the cancer metastasises to remote organs. Each step has a subdivision allowing for a finer description of the tumour (American Cancer Society, 2015a), and PC survival rates vary according to the stage of detection; in the early stages, local and regional, the five-year survival rates approach 100%, while in the advanced stages the five-year survival drops to about 30% (Siegel et al., 2015, SEER program, 2014). The cancer is androgen dependent, utilizing adrenal and gonadal androgens to grow. Therefore, the first line of treatment is to deprive the tumour of androgens. The treatment strategy changes according to the stage. In the early stages, when the tumour is localised to the gland, some patients could choose non-active treatment, essentially wait, watch
and see [NICE (National Institute for Health and Care Excellence), 2014a, American Cancer Society, 2015b]. If an active treatment is chosen, the patient can choose to undergo a radical prostatectomy. If the tumour is not localised the first line of treatment is androgen deprivation (American Cancer Society, 2015b, NICE, 2014b), either surgically or pharmacologically, including orchidectomy, GnRH (agonist or antagonist) or other anti-androgen treatment. Treatment might also involve radio- or chemotherapy.

In later stages the treatment is not curative and most men will progress towards castration resistant PC (CRPC) after two to five years (Nussbaum et al., 2016, Leibowitz-Amit and Joshua, 2012, Harshman and Taplin, 2013), and most will die within three years after CRPC is detected (Nussbaum et al., 2016). Nevertheless, during the responsive period 70-80% of patients will benefit from the androgen deprivation treatment, resulting in reduction of symptoms, tumour regression and a decrease in prostate specific antigen (PSA) that serves as a biomarker (Damber and Aus, 2008, de Bono et al., 2011). Although CRPC continues to grow despite low androgen levels, it is still androgen dependent (Attard et al., 2008, Ang et al., 2009).

The resistance to the androgen ablation in the body is achieved by one or more of several mechanisms (Friedlander and Ryan, 2012, Karantanos et al., 2013, Agarwal et al., 2014, Egan et al., 2014). Interestingly, intra-tumoural androgen concentrations within the CRPC are similar to the concentrations before ablation (Titus et al., 2005), indicating intra-tumoural androgen synthesis (Ning et al., 2013, Yin et al., 2013), and these levels allow for androgen receptor (AR) activation. To counter the CRPC resulting from residual intra-tumoural androgens, gain of promiscuous AR function or AR overexpression, two new hormonal therapies were approved by the Food and Drug Administration in the last few years (Harshman and Taplin, 2013, Ning et al., 2013, Yin et al., 2013). These are enzalutamide, an AR antagonist and abiraterone, a cytochrome P450 (CYP) 17α-hydroxylase/17,20 lyase (CYP17A1) inhibitor (Tan and Haaland,
Abiraterone is a selective steroidal CYP17A inhibitor, derived from pregnenolone (Ferraldeschi et al., 2013). It was approved in the treatment for metastatic CRPC before and after chemotherapy (Kluetz et al., 2013, Thompson, 2011). The targeting of CYP17A1 is derived from the importance of the enzyme in the androgen biosynthesis pathway. It is a key enzyme in the synthesis process catalysing two steps, as described above. Theoretically, CYP17A1 inhibition should result in complete androgen abolition. Abiraterone is not 17,20-lyase selective and therefore results in cortisol inhibition and mineralocorticoid increase (Attard et al., 2012, Ryan et al., 2010). The treatment is not curative, although it has a beneficial effect on overall survival of four to five months on average (de Bono et al., 2011).

1.3.3 Ovarian Cancer

Ovarian cancer (OC) is the 7th most common and 7th most lethal cancer in women worldwide (Ferlay et al., 2010, Prat and Oncology, 2014). However, in the USA and Europe it is first and fifth most common cancer in women, respectively (Siegel et al., 2015, Ferlay et al., 2010, Prat and Oncology, 2014) and second and sixth most lethal cancer, respectively (Siegel et al., 2015). Furthermore, OC is the most lethal female reproductive tract cancer (Cho and Shih Ie, 2009). Its five-year survival rate is low and stands at around 45% (Siegel et al., 2015), although treatments have improved over the years. Survival rates vary according to the detection stage; the earlier the detection the better the prognosis and yet most cases are diagnosed only after the tumour has breached the ovary (79%) (SEER program, 2011).

Ovarian cancer has no distinctive symptoms and signs. The symptoms, include but not limited to, abdominal or back pain, abdominal bloating and nausea, which could have easily resulted from other conditions, and are only distinguished by the intensity and the duration of the symptoms. The effect of the lack of specific markers can be seen from the statistics. The earliest
stage, while the tumour is localised, has the highest five-year survival rate, but it accounts for only 15% of the cases. The regional stage accounts for 18% of cases, with about 72% five-year survival rate. The distant (metastatic disease) stage accounts for about 60% of cases but has the poorest survival rate of about 27% (Howlader N, 2014, SEER program, 2011). The late detection is partially due to the lack of biomarkers as symptoms and signs cannot be relied upon. Treatment options include surgery and chemotherapy. Women harbouring a BRCA gene mutation, have a higher lifetime risk of developing breast and/or ovarian cancer and are encouraged to have prophylactic mastectomy and/or uni-bilateral salpingo-oophorectomy, the removal of Fallopian tubes and ovaries. Those women are often diagnosed with Fallopian tube cancer while the ovaries are unremarkable (Callahan et al., 2007, Finch et al., 2006).

The progression of the cancer is divided into stages (Mutch and Prat, 2014, Prat and Oncology, 2014) according to locale spread, is further sub-divided based of various histological features (Mutch and Prat, 2014, Prat and Oncology, 2014, Meinhold-Heerlein et al., 2016).

- **Stage I** – The tumour is uni- or bilateral and confined to the ovary.
- **Stage II** – The tumour (uni- or bilateral) has breached the ovary but is confined to the pelvis. Cancer cells might be floating in the peritoneal cavity.
- **Stage III** – A uni- bilateral tumour that has spread outside the pelvis or to the lymph nodes.
- **Stage IV** – Tumour has metastasised to areas other than the peritoneum.

The ovarian cancer combines several distinct tumour types, each with unique clinical and pathological characteristics. They belong to three groups, with epithelial malignancies accounting for about 90%, malignant germ cell tumours account for about 3%, and malignant sex cord-stromal tumours for 1-2% (Mutch and Prat, 2014, Prat and Oncology, 2014).
(Figure 1-7 A). The division is mainly histological, and there is a therapeutic importance for correct identification and staging of the tumour (Prat and Oncology, 2014). The division is based on tumour cell origin and morphology (Cho and Shih Ie, 2009, Prat, 2012, Gilks and Prat, 2009), and today there are attempts to use genetics to identify the different tumour types (Domcke et al., 2013, Anglesio et al., 2013, Horlings et al., 2015). Although the tumours may present in the ovaries, the gathered body of evidence indicates that not all are of primary ovarian origin.

Epithelial malignancies are the most common OC (Cho and Shih Ie, 2009, Gilks and Prat, 2009, Mutch and Prat, 2014), and are divided to five sub-types, according to morphology, histology, molecular features and genetics (Gilks and Prat, 2009, Cho and Shih Ie, 2009, Prat, 2012, Jayson et al., 2014, Mutch and Prat, 2014) (Figure 1-7 B). Albeit grouped under the same title, these sub-types are in fact different diseases (Prat, 2012, Mutch and Prat, 2014, Gilks and Prat, 2009).

![Figure 1-7: A summary of ovarian cancer groups](image)

**Figure 1-7: A summary of ovarian cancer groups**

A. The groups of ovarian cancer.
B. The sub-divisions of the epithelial malignancies.
The most common sub-type is the serous malignancies (Seidman et al., 2004, Mutch and Prat, 2014, Prat and Oncology, 2014). The serous group is sub-divided into two. The high grade serous (HGS) is the most frequent (70%) ovarian carcinoma (Gilks and Prat, 2009, Prat, 2012, Seidman et al., 2004, Mutch and Prat, 2014, Prat and Oncology, 2014) and is considered to have originated in the Fallopian tubes (Prat, 2012, Diep et al., 2015, George and Shaw, 2014, Perets et al., 2013), and by the time of detection are mostly at an advanced stage (Prat, 2012, Kobel et al., 2008) resulting in an unfavourable prognosis. Recently a genetics based study (Domcke et al., 2013) comparing genetic features such as DNA copy-number, mutation and mRNA expression data, examined high grade serous malignancies and compared them to the genetics of cell lines that serve as supposed models for this type of cancer. They discovered that most of the cell lines in use as HGS models are actually not so. They also showed that about 10% of the HGS tumours and cell lines have a BRCA mutation; each mutation (BRCA 1 or 2) was identified in 15% of the tumours but only in about 10% of cell lines (Domcke et al., 2013), this data is in disagreement with data previously published (Kobel et al., 2008, Gilks and Prat, 2009). Breast cancer (BRCA) susceptibility genes, responsible for preventing DNA damage, are known for their role in breast cancer development. Mutations in either of the BRCA genes increase the risk for breast and ovarian cancers (King et al., 2003, Cancer Genome Atlas Research, 2011, Sogaard et al., 2006, Antoniou et al., 2003). In OC BRCA mutations can be found only in the epithelial cancer, and are serving as marker for HGS tumours (Domcke et al., 2013, George and Shaw, 2014). However, as mentioned above not all HGS cell lines have BRCA mutation (Domcke et al., 2013).

The second sub-type is the low grade serous (LGS) cancers, accounting for <5% of ovarian epithelial malignancies (Gilks and Prat, 2009). Their origin is uncertain, and their identification is supported by genetics (Gilks and Prat, 2009, Singer et al., 2003, Jayson et al., 2014).
The third epithelial tumour sub-type is the endometrioid malignancies which account for 10% of the OC cases (Gilks and Prat, 2009). They are similar to endometrial tumours, (Cho and Shih Ie, 2009), and at least some are related to the uterine endometrium (Sainz de la Cuesta et al., 1996, Catasus et al., 2004).

The fourth sub-type, clear cell tumours, cells with clear cytoplasm, account for 10% of the epithelial malignancy cases (Gilks and Prat, 2009). Their source is controversial but some consider it to be of endometrial origin (Diep et al., 2015, Kato et al., 2006, Tan and Kaye, 2007). Despite the fact that the disease is usually detected at an early stage, the prognosis is not favourable (Gilks and Prat, 2009), as they do not respond to commonly used chemotherapy (Goff et al., 1996). These cells are not limited to any one of the tumour types and therefore are difficult to diagnose (Cho and Shih Ie, 2009, Gilks and Prat, 2009). Nevertheless, there are several genetic / molecular markers that might allow for better identification (Kobel et al., 2009, Wiegand et al., 2010, Prat, 2012, Kato et al., 2006, Tsuchiya et al., 2003).

The fifth epithelial sub-type is the mucinous tumours which account for 10-15% of ovarian tumours, but only of 3% of ovarian carcinomas (Prat, 2012). They are usually detected in an early stage (Jayson et al., 2014), and are mostly non-malignant (Prat, 2012). Many are probably metastases from the lower intestinal tract (Diep et al., 2015).

The second main group of OC is the malignant germ cell tumour. Germ cell tumours as a group account for 20-25% of ovarian tumours (Smith et al., 2006, Pectasides et al., 2008), but only account for 3-5% (Prat and Oncology, 2014, Tewari et al., 2000) of malignancies, and most malignant cases are detected at an early stage (Pectasides et al., 2008). Those malignancies mostly occur in children and young women (Tewari et al., 2000, Park et al., 2015, Smith et al., 2006) and have a good prognosis. The germ cell malignancies are sensitive to chemotherapy and have good survival rates (Gershenson, 2012, Pectasides et al., 2008). According to the
World Health Organisation, there are five sub-types of germ cell cancers (Rijlaarsdam et al., 2015), and the three most common are dysgerminoma, yolk sac and immature teratomas accounting for more than 90% of malignant cases in this group.

The third and last OC group to be discussed is the sex cord-stromal malignant tumours, the rarest group, accounts for 1-2% of cases (Mutch and Prat, 2014). The commonest in the group is the granulosa tumour (Prat and Oncology, 2014), and most patients are adults (Kalfa et al., 2009). The granulosa cells in these tumours have the same gene expression as normal proliferating granulosa cells, indicating that they respond to FSH, and that their proliferation is related to its stimulation (Fuller et al., 2002). The other members of the family are thecoma-fibromas, Sertoli cell tumours, sex cord tumours with annular tubules, and gynandroblastomas (Kalfa et al., 2009). Most of the data regarding sex cord-stromal tumours has been gathered from testicular tumour counterparts.

Cell lines are used as models for cancer, and OC is no different, with cell lines for most if not all types of tumours. As the ovaries are steroidogenic tissues it could be expected that the steroidogenesis capabilities would have been examined in the cell lines. Unfortunately, such data is lacking, and in most cases the data is conflicting. Most of the information comes from granulosa cells and epithelial tissue (Zhang et al., 2000, Havelock et al., 2004). Moreover, the data collected is not from a systematic study but a result of the general use of the cells or tissues during a study. Nevertheless, in cancer related studies it was demonstrated that steroids and their receptors have a role in tumour development. Progesterone receptor expression varies between the different cancers; 67% of endometrioid tumours, 60% of low grade serous and in only 30% of the high grade serous tumours (Diep et al., 2013, Sieh et al., 2013). It would appear
that there is a correlation between expression and survival. As mentioned earlier in this section, progesterone has an anti-proliferative effect (Yu et al., 2001, Syed et al., 2001, Keith Bechtel and Bonavida, 2001, Bu et al., 1997) and progesterone receptor type B (PR-B) indicates a favourable prognosis (Diep et al., 2015). The effect of oestrogens is not clear, as some data suggest it has an anti-proliferative effect while other data suggest the opposite (Wimalasena et al., 1992, Li et al., 2014). Ovarian cancer is also affected by LH and FSH, which support cell proliferation (Mertens-Walker et al., 2012, Wimalasena et al., 1992, Choi et al., 2007), and decreases apoptosis (Choi et al., 2007). Their effect differs, while FSH increased proliferation in 16 of 17 cell lines tested in 20 studies, the LH effect is mixed, with different studies showing different results for the LH effect (Lukanova and Kaaks, 2005). It is nevertheless possible to say that FSH has a proliferative effect whereas the LH effect needs to be further investigated (Lukanova and Kaaks, 2005).

1.4 Summary

Steroid hormones are important to body function, regulating salt concentrations and glucose distribution, most importantly in stress conditions. They are mainly synthesised in the adrenal glands, ovaries and testes.

Three steroid related cancers have been discussed above. The adrenocortical carcinoma (ACC) a rare and lethal cancer of the adrenal cortex in the majority of cases affecting steroidogenesis. Prostate cancer is a common cancer and is androgen dependent. Late stage treatments are designed to inhibit the androgen activity in the tumour and treatments are not curative and the tumour continues to grow even when the body is deprived of androgens, utilising the alternative pathways to bypass the pharmaceutically induced inhibition. Better treatments are needed.
Ovarian cancer is known as the silent killer since in most cases there are no warning symptoms and signs until too late. The survival rates depend on the stage of detection but also on the cancer type as some are more responsive to treatment than others. The tumours are clearly hormone dependent, but no systematic steroid analysis has been performed. Moreover, research studies suggest that the OC, in some cases is actually a metastasis from another part of the reproductive tract and only the late discovery of it has classified it as OC. The most common tumour, serous, originates in the Fallopian tube. Data regarding steroidogenesis or steroid effects on tumours and cell lines is scarce, and often contradictory.

Based on the body of evidence detailed in this chapter, hypotheses about the role and source of steroids in two lethal cancers, prostate and ovarian, were raised. Another point that was examined was the steroidogenesis of three adrenocortical carcinoma cell lines.
2.0 Hypotheses and Aims

Steroids have a role in cancer development and progression. In this thesis adrenocortical carcinoma cell lines served as a model for adrenal steroidogenesis in order to determine if DHT alternative biosynthesis pathway androgens have a role in the transformation of prostate cancer to castration resistant. In the second section of this thesis ovarian cancer cell lines were used as a model to determine if ovarian cancer has the capability to synthesise steroids.

Characterisation of three adrenocortical carcinoma cell lines derived from the H295 cell line.

The adrenal gland can synthesise three groups of steroids and it is the largest source of steroids in the body. In section 4.0, the steroidogenesis capabilities of three distinct cell lines derived from the H295 cell line is analysed using mRNA and steroid measurements.

Alternative androgen synthesis in the adrenal gland may underpin castration resistant prostate cancer.

Prostate cancer is an androgen dependent cancer, yet in a large percentage of patients the tumour progresses even when deprived of androgen and evolves into castration resistant prostate cancer. According to previous research, it is possible that the adrenal gland bypasses the CYP17A1 inhibition. In chapter 5.0, an adrenocortical carcinoma cell line serves as a model and gene knockdown is used to inhibit gene activity in order to mimic the effects of the drug abiraterone. Knockdown efficiency is examined using mRNA analysis and steroid measurements.
We believe that ovarian steroid metabolism can serve as a biomarker to detect ovarian cancer.

Due to the lack of literature about ovarian cancer steroidogenesis, in chapter 6.0, the steroidogenesis in four ovarian cell lines is examined using mRNA for enzymatic analysis and steroid measurements. In order to eliminate the problems of low level steroidogenesis, steroid precursors and forskolin are added to increase steroidogenesis.
3.0 General methods and materials

3.1 Cell Culture

3.1.1 General Principles

All cell lines were grown in T75 flasks (CellStar, Greiner, Frickenhausen, Germany) in 10-15ml of appropriate media. Before the experiments were commenced cells were detached from the flasks, using 2ml trypsin (TrypLE Express, Gibco, Life technologies—Thermo Fisher Scientific, Paisley, UK). The trypsin activity was then neutralised with 8ml of medium and the cells centrifuged at 1200rpm for 5 minutes. The medium was removed and the cells were re-suspended before counting, using Cells Fastread (Biosigma (ISC), Palgnton, UK) and then seeded into 6-well plate (CellStar, Greiner), see relevant chapters for details. The various cells were allowed to attach overnight in full culture medium before treatment the next day. Treatments were given in phenol-red free media (OAW42: Gibco 11880-028+ L-Glutamine PAA M11-044 (The cell culture company, Pasching, Austria), A2780: Gibco 11835-063 and COV434/CaOv3: Sigma P1145, H295R1/ H295R-S1 Gibco 21041m H295R-S2/S3 Gibco 11039-21). The use of phenol-red free medium was at the request of the mass spectrometry team due to the fact that the phenol-red blocks the LC-MS/MS column.

In order to increase steroidogenesis forskolin (FSK) was used. Forskolin connects directly to the enzyme adenylate cyclase (cAMP), which regulates many cell events including hormone activity and gene regulation, thus causing an increase in enzyme levels and activity creating a cascade of reactions ending in an increase in cell steroidogenesis (Williams-Ashman and Reddi, 1971, Hedin and Rosberg, 1983, Nakamura et al., 2016, Mangelis et al., 2016).
3.1.2 Adrenocortical Carcinoma Cell Line

All the adrenal cells discussed here were derived from the same parent cell line NCI-H295, derived from an adrenocortical carcinoma mass removed from the right adrenal of a 48 year old woman of black ethnicity from the Bahamas (Gazdar et al., 1990). The tumour cells were grown and pure floating cells were chosen to continue cell propagation (Gazdar et al., 1990). Cells were also grown in two types of media, HITES and Roswell Park Memorial Institute 1640 (RPMI1640); in the former, cells were free-floating, and in the latter, adherent. Since the original cell line had a long doubling time (>96 h) (Rainey et al., 1994) and were not growing in a monolayer, an attempt was made to receive monolayer cells by using a different medium resulting in shorter doubling time. After a three month selection period a monolayer growth was achieved (Rainey et al., 1994). This cell line was designated as H295R. Later modifications in the medium resulted in two other strains (Rainey et al., 2004, Wang and Rainey, 2012).

H295R1 cell line (H295R, ATCC no.CRL-2128). The H295R1 cells were received from Professor Enzo Lali’s laboratory (University of Nice, France). The cells were grown in Dulbecco's modified eagle medium/F12 (DMEM/F12) with L-Glutamine medium (Gibco) enriched with 2% Nu-Serum (Becton, Dickinson and Company, BD, Oxford, UK), 1% ITS+ (insulin, transferrin and selenium, BD) and 1% penicillin-streptomycin (P/S) (PAN BIOTECH, Aidenbach, Germany).

H295R-S1 cell line. The cells were derived from H295 (Wang and Rainey, 2012, Gazdar et al., 1990, Rainey et al., 1994, ATCC, 2014b) as described in the beginning of section 3.1.2. The cells were received from the William E Rainey’s laboratory (University of Michigan, MI, USA). They were grown in DMEM/F12 medium with HEPES ((4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)), 15nM (Gibco) enriched with 5% Nu-Serum, Gentamicin Reagent Solution (1ug/ml) (Gibco) and 1% P/S.
H295R-S2 cell line. The cells were derived from H295 (Wang and Rainey, 2012, Gazdar et al., 1990, Rainey et al., 1994, ATCC, 2014b) as described in the beginning of section 3.1.2. The cells were received from the William E Rainey’s laboratory. They were grown in DMEM/F12 medium with HEPES, 15nM (Gibco) enriched with 2.5% Ulstroser G (France, St-Germain-en-Laye, France, #15950-017), 1% ITS+, Gentamicin Reagent Solution (1ug/ml) and 1% P/S.

H295R-S3 cell line. The cells were derived from H295 (Wang and Rainey, 2012, Gazdar et al., 1990, Rainey et al., 1994, ATCC, 2014b) as described in the beginning of section 3.1.2. The cells were received from the William E Rainey’s laboratory. They were grown in DMEM/F12 medium with HEPES, 15nM enriched with 10% Cosmic Calf Serum (Hyclone, Thermo Fisher Scientific), Gentamicin Reagent Solution (1ug/ml) and 1% P/S.

3.1.3 Ovarian Cancer Cell Lines

Ovarian cell lines were chosen in order to cover a wide range of ovarian cancer subtypes based on use in literature (internal correspondence, not detailed), and secondly according to their commercial availability. Out of the variety of cells available, it was decided to use cell lines from three common ovarian cancer tumours and a cell line derived from steroidogenic cells.

A2780 cell line (Public Health England (PHE) no. 93112519). The cell line was derived from primary tumour of an untreated patient (Public Health England) and considered to have an epithelial morphology (Public Health England). The tumour was classified as an endometrioid tumour (Anglesio et al., 2013). Cells were grown in RPMI1640 medium (Gibco) enriched with 10% foetal bovine serum (FBS) (Sigma-Aldrich (Merck), St. Louis, MO, USA) and 1% P/S.

COV434 cell line (PHE no. 07071909). A granulosa cell line that was derived from a solid primary tumour (Public Health England). No further details could be obtained. The cells were grown in DMEM (Dulbecco's Modified Eagle's Medium) high glucose (Sigma-Aldrich) enriched with 10% FBS and 1% P/S.
CaOv-3 cell line (The American Type Culture collection (ATCC) no. HTB-75). The cell line originated from a 54 year old female with an ovarian adenocarcinoma (ATCC, 2014a). The cells were grown in DMEM high glucose enriched with 10% FBS and 1% P/S.

OAW42 cell line (PHE no. 85073102). The cell line was derived from a 46 year old patient with ascites due to an ovarian cystadenocarcinoma (Wilson, 1984), described as being of papillary serous type. The cells were grown in DMEM (Gibco) enriched with 10% FBS and 1% P/S.

### 3.2 Messenger RNA Expression Analysis

#### 3.2.1 RNA Isolation

At the end of the experimental period, usually 72h after treatment, the medium was removed and 1ml of Tri Reagent (Sigma-Aldrich) was added to the wells and the cells were collected into 1.5ml microfuge tubes and stored at -20ºC for future analysis. For RNA isolation, 200µl chloroform (all volumes are according to the use of 1ml Tri Reagent for cell collection) was added to the Tri Reagent. Samples were briefly vortexed and then incubated at room temperature for 10 minutes before centrifugation at 16,000xg for 10 minutes. Subsequently, the upper layer was transferred to a new tube and 0.5ml of isopropanol was added and vortexed prior to being incubated for 40-60 minutes at -20ºC for enhanced RNA yield. After precipitation, the samples were centrifuged for 10 min at 16,000xg. The supernatant was removed leaving behind a small pellet. One millilitre of 70% ethanol was added, as a washing step, and it was then vortexed before being centrifuged for 5 minutes at 16,000xg. The supernatant was removed and samples were left to dry for up to 15 minutes. Thereafter 20-25µl of nuclease free water (Promega, Madison, WI, USA) was added. The RNA concentration and
quality were measured with Nano-Drop ND-1000 photospectrometer (Labtech International, Uckfield, UK).

The RNA of the H295R-S1 cells was extracted by a colleague, Dr Vasileios Chortis from the Institute of Metabolism and Systems Research at the University of Birmingham, using Qiagen RNeasy mini kit (Hilden, Germany), according to the manufacturer’s protocol.

### 3.2.2 Reverse Transcriptase

RNA (1000ng) was added into a reverse transcriptase (RT) reaction mix. Each reaction sample contained 2µl of 10X RT buffer, 4.4µl of 25mM MgCl2, 4µl 10mM dNTPs (DNA nucleotides), 0.5µl 2.5µM oligo (dT), 0.5µl 2.5µM random hexamers, 0.4µl RNAse inhibitor and 0.5µl of 50u/µl multiscribe RT enzyme (TaqMan kit, Applied Biosystems – Thermo Fisher Scientific, Paisley, UK). A total of 20µl, containing 13µl of the above of the reaction mix, 7µl RNA and RNAse free water were loaded into a polymerase chain reaction (PCR) plate (Thermo Fisher Scientific) and amplified using a GeneAmp PCR system 2700 (Applied Biosystems – Thermo Fisher Scientific). The RT reaction was performed under the following cycle conditions: 25ºC for 10 minutes, 37ºC for 60 minutes, 48ºC for 30 minutes and 95ºC for 5 minutes. At the end of the reaction, 105µl of RNAse free water was added for a final concentration of 8ng/µl cDNA.

### 3.2.3 Real Time Quantitative PCR (qPCR)

The real time polymerase chain reaction (PCR) system is based on fluorescent light measurement to determine the amount of complementary DNA (cDNA) of a specific gene. The TaqMan real-time PCR technology is based on a pair of unlabelled primers and a labelled oligonucleotide probe. The probe has two dyes; a 5’ receptor and 3’ quencher, the quencher dye reducing the receptor fluorescent illumination. During the reaction, the primers and probe are annealed to their specific location on the cDNA template, and a Taq-polymerase synthesises a new strand based on the primers and template. During this stage, the 5’ dye is removed from
the probe and without the restriction of the 3’ quencher dye its signal increases. At the end of each cycle a measurement of the fluorescent illumination is performed. The more cycles passed the higher the signal (Figure 3-1). With this method, it is possible to find the first time a target is detectable, but not the target levels. The first cycle in which the target is detected is referred to as the threshold cycle (Ct) value, and the higher the number of target copies in the original sample the lower the Ct at which its fluorescent illumination is first detected. By comparing the target gene to housekeeping genes, a relative quantification of the different target genes is possible.

Each quantitative PCR (qPCR) sample contained 5μl TaqMan 2X PCR master mix (Applied Biosystems), 3μl primers and probe mix and 16ng cDNA in 2μl per sample, or 2μl RNAsé free water as control, totalling 10μl. Primer and probe mixes (TaqMan-on-demand) were ordered from Life Technologies or Sigma (see relevant chapters for genes names and catalogue numbers). The qPCR samples were loaded into a 384 well plate (Applied Biosystems) and ran in a 7900HT qPCR machine (Applied Biosystems) using the following programme: 50ºC for 2 minutes followed by 40 cycles of 95ºC for 15 seconds and then 60ºC for 1 minute. Their Ct value was used for the analysis. ΔCt (gene of interest Ct minus housekeeping gene Ct) and fold change \((2^{\Delta C_t})\) were calculated. These were used for statistical analysis. Ct values above 40 were considered as “no expression” and marked 40 for analysis purposes.
3.3 Protein Measurements

Proteins were collected for steroid analysis normalization. They were isolated from cells collected in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris hydrochloric acid (HCl) pH 8 (Sigma-Aldrich), 150 mM sodium chloride (NaCl, Sigma-Aldrich), 0.5% sodium deoxycholate (Sigma-Aldrich), 1% IGEPAL (octylphenoxypolyethoxyethanol) Ca-630 (Sigma-Aldrich), 1 mM ethylenediamine tetra-acetic acid, disodium salt dehydrate MW272.2, Sigma-Aldrich) and 0.1% sodium dodecyl sulfate (SDS, Fluka-Sigma-Aldrich) and protease inhibitors cocktail (PIC, Roche, Burgess Hill, UK). Proteins from H295R1 cells were isolated using ready-made RIPA buffer (Sigma). Samples were frozen in dry ice and then thawed at 37°C in two cycles before being centrifuged for 10 minutes at 16,000xg. The supernatant was collected and transferred to a 1.5 or 0.2 microfuge tube and stored at -20°C.

The Bradford assay was used to measure protein concentrations. Coomassie Brilliant Blue G-250 dye binds to the basic amino acids, arginine, lysine and histidine present in the protein. The binding causes the dye to change colour from red to blue. The measurements were done in triplicate with a Bradford kit (Bio-Rad, Hertfordshire, UK), according to manufacturer’s
instructions. Briefly, 5µl of sample and 225µl of Bradford mix were added to a 96 well plate. After five minutes incubation at room temperature on a plate shaker (~1000rpm), samples were measured at 690nm on a PerkinElmer VICTOR3 multilabel counter (Waltham, MA, USA) and analysed in comparison to a standard curve of bovine serum albumin (Sigma).

### 3.4 Short Interfering RNA (siRNA) Knockdown

H295R1 cells were seeded into a 6-well plate, 5x10⁵ cells/well and left to attach overnight before transfection by Viromer® (Lipocalyx GmbH, Halle (Saale), Germany). Viromer® is a high-tech polymer that mimics the influenza virus membrane fusion mechanism to insert short interfering RNA (siRNA) into cells. The polymer has fatty acids and alkyl areas mimicking two amino acids on the influenza virus that are needed for virus cell invasion, namely Glutamine and Alanine (respectively). The siRNA is attached to the polymer prior to the transfection (see below). The particles enter the cell in endosomes, and the pH change (from pH 7 to pH 5) causes them to release the siRNA enabling them to attach to the mRNA (Figure 3-2). A comparison of transfection efficiency was initially undertaken between the two Viromer products designed for siRNA transfection, Viromer BLUE or GREEN. Viromer BLUE was chosen. The transfection mix was prepared according to the manufacturer’s recommendation to the highest transfection levels (11µM siRNA and 5.5µl Viromer in Buffer F) and incubated at room temperature for 10 minutes. The cell media was replaced with 1ml growth medium, and the 150µl (1.5:10 ratio to media) Viromer™ mix was added to the medium in each well. Cells were incubated for 48 hours. Then the medium was replaced with 1ml phenol-red free experiment medium with 1µM FSK (Sigma-Aldrich) in dimethyl sulfoxide (DMSO, Sigma-Aldrich) for another 24 hours before terminating the experiment. The medium was then collected for steroid analysis and cells were collected in Tri Reagent or RIPA buffer for RNA isolation and protein measurement respectively.
Ovarian cells were seeded 5x10^5-1.25x10^5 cells/well in media, and left overnight to attach at 37°C with 5% CO_2. Thereafter, the medium was removed and replaced with the relevant phenol-red free medium, containing the relevant steroid substrate (pregnenolone, 17OHP or androstenedione), at concentrations of 200nM, 500nM or 1000nM. The cells were then incubated for 72h before the medium and cells were collected for steroid analysis and protein measurement.

### 3.6 Steroid Extraction and LC-MS/MS

A Xevo liquid chromatography-tandem mass spectrometer (LC-MS/MS) with Acquity uPLC system (Waters, Elstree, UK) was used to measure steroid concentrations in the cell media. The conditions for the LC-MS/MS electrospray ionization source were 4kV capillary, source temperature 150°C and desolvation temperature of 500°C (Figure 3-3). The LC-MS/MS allows for accurate measurement of the steroids based on their mass/charge ratio. The steroids extracted into a liquid phase, are loaded onto a column containing a solid stationary phase. The steroids bind to the solid phase, and by changing of the liquid phase polarity, the steroids elute into the mass spectrometer. The steroid identity is determined based on the retention time (the
time it took the steroid to travel through the column), steroid mass/charge ratio (m/z) and the ions created when fragmented. For steroid extraction, 20µl of an internal standard solution containing deuterated steroids (Steraloids, Newport, RI, USA or Sigma-Aldrich, St. Louis, MO, USA) in LC-MS/MS grade methanol (Greyhound Chromatography, Merseyside, UK) was added to 1ml of cell medium that was used for the incubation and was then vortexed. Three millilitre MTBE (tert-butyldimethyl ether, Sigma-Aldrich, St. Louis, MO, USA) was added, and samples were vortexed and stored for at least an hour and up to 24h at -20ºC. The upper phase containing the steroids was transferred into a 96 well LC-MS/MS plate (Thermo Fisher Scientific, Paisley, UK or Porvair Sciences Limited, Wrexham, UK), and was evaporated at 60ºC under nitrogen. Once all the MTBE was collected and evaporated, 125µl of 1:1 methanol /water (Fisher) (LC-MS/MS grade) was added to each well and the plate was stored at -20ºC for LC-MS/MS measurement and analysis. Concentrations were quantified relative to a linear standard curve containing the steroids of interest in known concentrations ranging from 0.5 to 500 mg/ml.

Figure 3-3: LC-MS/MS schematic
A mixture of steroids is injected into the machine. Each steroid is fragmented to two ions that can be detected and identified. According to the retention time (time to reach the mass spectrometer), mass and ions relative to authentic steroid standards the identity of the different steroids can be determined. Adapted from internal department datasheets by Dr A. Taylor.
3.7 Statistical Analysis

Statistical analysis was performed using Prism 6 (GraphPad, La Jolla, CA, USA). QPCR results were transformed using $Y=\log(Y)$ and LC-MS/MS results were analysed directly. Analysis was performed using the non-parametric unpaired two-tailed t-test for single variant analysis (Mann-Whitney test) or one-way non-parametric ANOVA with multiple comparisons corrected by the Bonferroni test for multi-variant results.
4.0 Steroidogenesis in Adrenocortical Carcinoma (ACC) Cell Lines

4.1 Introduction

The adrenocortical cell line (H295) has the capability to synthesise all adrenal steroids (Gazdar et al., 1990). NCI-H295R (H295R) and the three strains H295R-S1, -S2 and -S3 were derived from the same parent cell line (Rainey et al., 2004, Rainey et al., 1994, Wang and Rainey, 2012), created under different growing conditions (see Method section 3.1.2). Adrenal steroidogenesis is a compartmentalised process, each of the three cortical zones are responsible for different types of steroid, as described before (Section 1.1). Briefly, the zona glomerulosa synthesises mineralocorticoids, zona fasciculata synthesises glucocorticoids and zona reticularis synthesises androgen precursors. In a clinical trial by Attard and colleagues (Attard et al., 2012), alternative pathway steroid metabolites were measured in castrated patients with elevated androgens prior to abiraterone treatment. In order to examine the possibility that adrenal cells contribute to prostate cancer growth by the alternative pathway, their steroidogenesis first needed to be characterised. The above adrenal cell lines, chosen as an in vitro model of adrenal steroidogenesis were examined using qPCR and LC-MS/MS analysis in order to characterise their steroidogenesis capability.

4.2 Methods

4.2.1 Cell Culture

Cells were seeded in 6-well plates, 5x10^5 cells/well, in the appropriate medium, and allowed to attach overnight at 37°C with 5% CO₂. Their density was based on confluence experiments. The cells were seeded at different concentrations for 96 hours to determine the cell concentration that will give the best confluence for the duration of an experiment, and was the same for all H295R cell lines. Thereafter, the medium was removed and replaced with the
equivalent phenol-red medium, with or without 1μM forskolin (FSK). The cells were incubated at 37°C with 5% CO₂ for 72 hours, based on a time course experiment designed to determine the best time point for steroid measurements. Media, for steroid analysis, and cells, for mRNA analysis and protein, were collected as described before (section 3.0), at the end of each experiment.

4.2.2 Steroidogenesis Characterization

The adrenal cell lines H295R-S1, S2 and S3 were grown in flasks in their respective medium, as described in section 3.0, to a 75-90% confluence, and then detached as described in section 3.1, and seeded in 6-well plates. After an overnight attachment period, the medium was replaced with a phenol-red free including the relevant supplements as described previously (section 3.0) and 1μM FSK was added to the experiment wells while the control wells had media with no added FSK. Later, the experiment was repeated using media with charcoal stripped serum, serum treated with charcoal to reduce steroid concentrations in serum, to examine if the steroids in the serum or in the serum supplements affected steroidogenesis.

4.2.3 QPCR (Quantitative Polymerase Chain Reaction)

Quantitative polymerase chain reaction (qPCR) was performed as mentioned in the general methods (section 3.2.3) using two housekeeping genes and steroidogenesis related target genes, see Table 1 for details.

Table 1: The genes used for qPCR analysis of H295R-S1, -S2 and -S3 steroidogenesis characterizations

<table>
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<tr>
<th>Gene name</th>
<th>Enzyme</th>
<th>Catalogue number</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase (housekeeping)</td>
<td>Custom order</td>
</tr>
<tr>
<td>PROBE</td>
<td>[6FAM]CGCCCAATACGACCAAATCCGTGCAC[TAM]</td>
<td></td>
</tr>
<tr>
<td>FOWARD</td>
<td>ATGGGAAGGGTGAGGTTCG</td>
<td></td>
</tr>
<tr>
<td>REVERSE</td>
<td>TAAAAAGCACGCCCTGGTGACC</td>
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</tr>
</tbody>
</table>
Table 1 (cont.): The genes used for qPCR analysis of H295R-S1, -S2 and -S3 steroidogenesis characterizations

<table>
<thead>
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<th>Gene name</th>
<th>Enzyme</th>
<th>Catalogue number</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPRT1</td>
<td>Hypoxanthine phosphoribosyl transferase 1 (housekeeping)</td>
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<tr>
<td>PROBE</td>
<td>[6FAM]CAAGCTTGCGACCTTGACCATCTTTGGA[TAM]</td>
<td>Custom order</td>
</tr>
<tr>
<td>FORWARD</td>
<td>TGCTTTCTTGTCAGGCGATAT</td>
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<tr>
<td>REVERSE</td>
<td>TCAAATCCAAACAGTCTGGCTATATC</td>
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<td>CYP11A1</td>
<td>Cytochrome P450 family 11 subfamily A member 1</td>
<td>Hs00167986</td>
</tr>
<tr>
<td>HSD3β1</td>
<td>Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-</td>
<td>Hs04194787</td>
</tr>
<tr>
<td></td>
<td>isomerase 1</td>
<td></td>
</tr>
<tr>
<td>HSD3β2</td>
<td>Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-</td>
<td>Hs00605123</td>
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<td>isomerase 2</td>
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<td>CYP11B2</td>
<td>Cytochrome P450 family 11 subfamily B member 2</td>
<td>Hs00167984</td>
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<tr>
<td>POR</td>
<td>P450 (cytochrome) oxidoreductase (electron transferase)</td>
<td>Hs00287016</td>
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<td>17βHSD1</td>
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<td>Hs00166219</td>
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<td>17βHSD3</td>
<td>Hydroxysteroid (17-beta) dehydrogenase 3</td>
<td>Hs00970002</td>
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<td>17βHSD10</td>
<td>Hydroxysteroid (17-beta) dehydrogenase 10</td>
<td>Hs00189576</td>
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<td>Aldo-keto reductase family 1, member C1</td>
<td>Hs00413886</td>
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<td>AKR1C3</td>
<td>Aldo-keto reductase family 1, member C3</td>
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<td>RDH5</td>
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<td>Hs00161263</td>
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<td>Retinol dehydrogenase 16</td>
<td>Hs00559712</td>
</tr>
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<td>Hs00602694</td>
</tr>
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<td>steroid delta 4-dehydrogenase alpha 1)</td>
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<tr>
<td>SRD5A2</td>
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<td></td>
<td>steroid delta 4-dehydrogenase alpha 2)</td>
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</table>
4.3 Results

4.3.1 Steroidogenic Profile

H295R cell lines were derived from one parent cell line (Wang and Rainey, 2012, Gazdar et al., 1990, Rainey et al., 1994), and thus their steroidogenic gene expression was similar to each other. The results presented here will show that although having similar steroidogenic gene expression, the steroid production varied between the cell lines. Gene analysis of 16 steroidogenic enzymes (Figure 4-1) showed that the three cell lines characterised (H295R-S1, -S2 and S3) expressed the examined steroidogenic genes demonstrating that the cells had the potential to synthesise mineralocorticoids, glucocorticoids and sex hormones (expression level in H295R-S1 20-40 Ct, H295R-S2 22-37 Ct and for H295R-S3 20-39 Ct). The steroidogenic gene expression profile is very similar between the cell lines. All the cell lines expressed \textit{CYP11A1}, \textit{CYP21A2}, \textit{CYP17A1}, \textit{SRD5A2}, \textit{HSD17B10} and \textit{AKR1C3} at high levels, and the other genes expression pattern is similar. The variations in expression levels affect steroid production, since mRNA is translated to proteins, higher levels of mRNA may indicate higher levels of enzymes which could affect the steroidogenic metabolism (Figure 4-1 and Figure 4-3). In H295R-S1 (Figure 4-1 A) the \textit{CYP11B} levels were relatively low compared to the other cell lines, suggesting that this cell line was less able to synthesise gluco- and mineralocorticoids, which was supported by steroid analysis. However, the low n number (N=1), prevents this data from being conclusive. Treatment with 1µM FSK did not cause any significant affect in H295R-S1 cells, although both H295R-S2 and S3 were significantly affected in gene expression (Figure 4-2 A and B) and steroid levels (Figure 4-3 B and C).
4.3.1.1 H295R-S1

The H295R-S1 (S1) cell line was derived from the parent cell line by serum supplement modification as describe by Rainey et al. (Rainey et al., 2004, Wang and Rainey, 2012). In brief, the cells were grown in a medium containing a serum replacement, Nu-Serum. After 48h incubation, with or without FSK, 10 steroids were detected in the medium (Figure 4-3 A), but only androstenedione showed significant difference between the controls (left bar) and the FSK treatment (right bar). The combination of the steroid and gene analysis indicates that these cells synthesise mostly androgens, though less testosterone than other androgens. The results suggest that their mineralo- and glucocorticoid synthesis were also limited due to low CYP11B enzymes levels.
Figure 4-1: Steroidogenesis gene profile in H295R-S1, H295R-S2 and H295R-S3 cell lines
The baseline gene expressions of H295R-S1 (A), -S2 (B) and -S3 (C) cell lines demonstrate that the cells have the potential to synthesise all of the steroid groups synthesised by the adrenal cortex. The results indicate that the cells express the same genes but at different levels. H295R-S1 has the lowest expression of CYP11B genes, however low n numbers prevent data from being conclusive. The expression levels of the genes in H295R-S2 and S3 are similar. Suggesting that the cells are able to synthesise androgens more easily than gluco- or mineralocorticoids. H295R-S1 experiments were performed in serum-free media 48h, N=2, except CYP11B2 N=1. H295R-S2 and H295R-S3 experiments were performed in full media, incubation time is 72h. N=3. Results shown as Mean±SEM.
**Figure 4-2: Forskolin effects on steroidogenic enzymes in H295R-S2 and -S3 cells**

H295R-S2 (A) and H295R-S3 (B) were exposed to 1µM FSK for 72h and mRNA levels were analysed. Of 16 genes analysed, only seven (S2) or three (S3) genes were significantly affected by the FSK. Expression of all the genes, except AKR1C3, was increased under the FSK treatment.

The cells were treated with 1µM FSK for 72h. Significance relative to the control level *=P<0.05, **=P<0.01.
Figure 4-3: Steroidogenesis in H295R-S1, -S2 and -S3 cell lines
H295R-S1 (A), H295R-S2 (B) and H295R-S3 (C) were derived from the same parent cell, nevertheless, their steroidogenesis profiles differ. H295R-S2 and -S3 synthesise the same steroids, while H295R-S1 does not synthesise measurable levels of progesterone, 17-OH-pregnenolone and androstanedione. The steroid concentrations vary between the cell lines, but all cell lines present the ability to synthesise all the adrenal steroids. DOC – deoxycorticosterone, 17OHP – 17-hydroxyprogesterone, 17OHpreg – 17-hydroxypregnenolone, DHEA- dehydroepiandrosterone.

Left bar: control; Right bar: 1µM FSK. H295R-S1 – 48h, H295R-S2 and S3 – 72h Mean±SEM
N.D – non detected*=P<0.05, **=P<0.01, ***=P<0.005. H295R-S1 48h incubation, N=3 H295R-S2 72h N=3, H295R-S3 72h, N=3
4.3.1.2 H295R-S2

The H295R-S2 (S2) cell line was derived from the parent cell line by serum supplement modification as describe by Rainey et al. (Rainey et al., 2004, Wang and Rainey, 2012). Briefly, the cells were grown in Ultroser-G supplement, which is known to preserve steroidogenesis and used to increase cell growth (Rainey et al., 2004). The levels of expression of steroidogenic genes varied between 22-37Ct values (Figure 4-1B) and FSK caused a decrease of 1-2 Ct values in responding genes, indicating that FSK caused an increase in gene expression. Of the 16 genes analysed only seven were affected significantly by the FSK treatment, six of them increased, and one, AKR1C3, was significantly decreased by the treatment (Figure 4-2 A). The effect of the FSK is supported in the literature, except for AKR1C3 and RDH16 for which no article involving FSK treatment could be found (Schwartz and Roy, 2000, Xing et al., 2010, Kayani et al., 2009). Steroidogenic analysis (Figure 4-3 B) identified 13 steroids out of the 30 detected in the parent cell line (Rainey et al., 1994). Steroids belong to the three groups of adrenal (mineralocorticoids, glucocorticoids and androgen precursors) were detected and their concentrations range between 0.2-66 ng/mg protein, yet the levels of progesterone and testosterone were very low. It is noteworthy that three of the steroids; deoxycorticosterone (DOC), 11-deoxycortisol and androstanedione (Figure 4-3 B) were decreased with FSK (right bar) compared to the control (left bar). This, however, did not correlate with the increase in gene expression. When the cells were treated with stripped serum to determine as to whether the Nu-serum supplement affected steroidogenesis, the gene expression was found to be similar to that expressed in full medium, indicating that the medium did not affect the gene levels (Data not shown). In another experiment, performed as a pilot, after 72h incubation three steroids of the alternative androgen synthesis pathway, 3α5α17HP (3α,5α,17-hydroxypregnenolone), androsterone and androstanediol (concentrations at 72h 0.2ng/mg protein 0.17ng/mg protein
and 0.72 ng/mg protein respectively) were detected (Figure 4-4 A). The alternative pathway steroid 5α-pregnan-17-ol-3,20-dione (10 µg/L) was previously detected by Kamrath et al. in controls of a congenital adrenal hyperplasia study (Kamrath et al., 2012), but no other studies supporting this could be found.

Figure 4-4: Steroidogenesis in H295R-S2 and H295R-S3
H295R-S2 (A) and S3 (B) cells were grown for 72h in appropriate media, and steroidogenesis was measured. Seventeen steroids were detected, three of them are alternative pathway steroids. This pathway bypasses the regular DHT through testosterone synthesis, resulting in DHT synthesis without testosterone as a precursor. The alternative pathway is known to be active in pathologic conditions, as in P450 oxidoreductase deficiency and in castration resistant prostate cancer patients treated with abiraterone. In the graph the three alternative steroids together with 17OHP, their precursor can be seen. DOC – deoxycorticosterone, 17OHP – 17-hydroxyprogesterone, 3α5α17HP – 3α,5α,17-hydroxyprogrenolone. N=1

4.3.1.3 H295R-S3
The H295R-S3 (S3) cell line was derived from the parent cell line by serum supplement modification as describe by Rainey et al. (Rainey et al., 2004, Wang and Rainey, 2012). In brief, cells were grown in cosmic calf serum. Gene expression (Figure 4-1 C) indicated that the cells express all 16 genes examined, and that the cells had the ability to synthesise all of the adrenal steroids, glucocorticoids, mineralocorticoids and androgen precursors. However, only
three of the 16 genes examined (Figure 4-2 B) were affected significantly by FSK (right bar) compare to the control (left bar), and as mentioned in section 4.3.1.2, H295R-S2, these results are supported in the literature (Kayani et al., 2009, Xing et al., 2010). In LC-MS/MS steroid analysis 13 steroids were measured (Figure 4-3C), the same as in the H295R-S2. However, the steroid concentrations, 0.2-147 ng/mg protein, significantly differed between the cell lines (Data not shown). Stripped serum was used to examine if the cosmic calf serum steroid concentrations affected the results. No differences were detected between the full and stripped sera medium (Data not shown). In another experiment, done as a pilot, after 72h incubation three alternative androgen synthesis pathway steroids, 3α5α17HP, androsterone and androstane diol (concentrations at 72h 3.9ng/mg protein 1.2ng/mg protein and 0.99ng/mg protein respectively) were measurable (Figure 4-4 B). As explained in section 4.3.1.2 the alternative pathway steroid 5α-pregnan-17-ol-3,20-dione was measured in healthy controls (Kamrath et al., 2012).

4.4 Summary

H295R cell lines are the only human adrenal cancer cell lines, with steroidogenesis capabilities, widely used. Their ability to synthesise all of the adrenal steroids, mineralocorticoids, glucocorticoids and androgen precursors, makes them a useful tool in adrenal and steroidogenesis research. The H295R strains S1, S2 and S3 were derived from the same parent cell line using different serum replacements which created three cell lines that present similar steroidogenic gene expressions, but differ in their steroidogenesis, secreting the same steroids but at different concentrations. The decreased secretion of DOC, 11-deoxycortisol and androstenedione seen in H295R-S2 cells treated by FSK, did not correlate with the gene expression levels. Yet, it might be explained as a shift between substrate and metabolite, while DOC and 11-deoxycortisol significantly decreased, their metabolites corticosterone and
11-dexoycortisol, respectively, significantly increased. A similar thing happens with androstenedione and its precursor DHEA, while the former decreased, the latter increased, suggesting that the FSK affected the metabolism rate unequally.

The fact that the S2 and S3 cell lines can synthesise alternative pathway steroids needs to be further investigated since this may indicate that these cell lines could serve as a good model for conditions involving inhibition of the “classic” adrenal pathways. The results also indicate that the H295R cells can serve as a good cell line model for the adrenal cortex. Since cell steroidogenesis is sensitive to their growth medium make-up, the growth medium in steroidogenic experiments is of great importance. The characterization was a side project during a larger project to investigate the possibility that the adrenal gland has a role in prostate cancer resistance to castration using adrenocortical carcinoma cell lines as an \textit{in vitro} model. H295R-S1, -S2 and -S3 were never characterised before, and this is the first time a full steroidogenesis characterisation was performed on them. To-date no normal range of alternative pathway steroids is available in either healthy individuals or in diseased. This study was not designed to determine if the alternative pathway should be targeted as therapeutic target but only to investigate if it has a role in CRPC development. The adrenal cancer cells were used only as a model, the role of steroids in adrenocortical cancer was not examined here.
Figure 4-5: Steroidogenesis in H295R-S2 and H295R-S3
H295R-S2 (A) and-S3 (B) cells were grown for 72h in appropriate media, and steroidogenesis was measured. Seventeen steroids were detected, three of them are alternative pathway steroids. This pathway bypasses the regular DHT through testosterone synthesis, resulting in DHT synthesis without testosterone as a precursor. The alternative pathway is known to be active in pathologic conditions, as in P450 oxidoreductase deficiency and in castration resistant prostate cancer patients treated with abiraterone. In the graph the three alternative steroids together with 17OHP, their precursor can be seen. DOC – deoxycorticosterone, 17OHP – 17-hydroxyprogesterone, 3a5a17HP – 3α,5α,17-hydroxypregnenolone. N=1
5.0 SiRNA manipulation to reveal cell alternative pathway activity

5.1 Introduction

The adrenal gland is a major steroidogenesis organ responsible for the synthesis of glucocorticoids, mineralocorticoids and androgen precursors. In 2012, Attard and colleagues (Attard et al., 2012) showed that the adrenal glands might have a role in the resistance of prostate cancer to abiraterone treatment. Patients treated with abiraterone have a progress free period (de Bono et al., 2011, Fizazi et al., 2012, Ryan et al., 2013) but after several months the disease continues to progress. Since prostate cancer is androgen dependent (Attard et al., 2008), this progress indicates that the abiraterone inhibition is no longer affective and androgens are synthesised. The alternative DHT biosynthesis pathway products, serves as better substrates for CYP17A1, and 17-OH-allopregnanolone is the most efficient 17,20-lyase substrate (Gupta et al., 2003). Therefore, this pathway allows for DHT synthesis even when CYP17A1 is in limited availability due to the inhibition. In this project, the possibility that CYP17A1 knockdown in the adrenal will result in a shift towards the alternative pathway resulting in androgen or androgen precursor synthesis was examined. In order to knockdown the genes CYP17A1, CYP21A2 and POR (P450 oxidoreductase) commercial siRNAs were used, and transfection was performed using a relatively new method known as Viromer, a high-tech polymer that mimics the influenza virus.

5.2 Methods

5.2.1 Transfection

A Viromer-siRNA mix, for transfection of very resistant cells, was prepared according to manufacturer’s instructions. Briefly, 18µl of 11µM siRNA (See Table 2 for details) in Buffer F were mixed with 5.5µl Viromer in 495µl buffer F. This mix was chosen following an
experiment preformed to determine the best combination of Viromer and siRNAs and the time point resulting in the best transfection rates.

Table 2: Silence interfering RNA used for gene knockdown

<table>
<thead>
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<th>Catalogue number</th>
<th>Company</th>
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<td>4390844</td>
<td></td>
<td>Ambion (TermoFisher)</td>
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<td>CYP17A1</td>
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<td>HSS102587</td>
<td>Invitrogen</td>
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<td>SiRNA-I</td>
<td>HSS141782</td>
<td>Invitrogen</td>
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<tr>
<td></td>
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<td>HSS141783</td>
<td>Invitrogen</td>
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<td>SiRNA-I</td>
<td>HSS108264</td>
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<td></td>
<td>SiRAN-II</td>
<td>HSS182524</td>
<td>Invitrogen</td>
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</table>

5.2.2 Quantitative PCR

Following the knockdown, its effect on the mRNA levels of the relevant gene was examined according to the methods in section 3.2.3. GAPDH and HPRT1 served as housekeeping genes and CYP17A1, CYP21A2 and POR as target genes (for details Table 1).

5.3 Results

5.3.1 Viromer Gene Knockdown

The knockdown experiments were performed as part of a study investigating the possibility that the adrenal glands are the source of androgen precursors for prostate cancer when patients are treated with the CYP17A1 inhibitor abiraterone. The assumption that an alternative androgen synthesis pathway, known from other conditions, is utilised, was examined. In order to investigate the possibility that the adrenal bypasses the CYP17A1 inhibition induced by abiraterone, H295R1 cells were treated with siRNAs. Based on data from previous research
(Attard et al., 2012, Arlt et al., 2004), three types of siRNAs were used; namely, anti-CYP17A1, anti-CYP21A2 and anti-POR. Gene expression analysis demonstrated that the siRNAs were very effective, with 80-98% knockdown of the relevant gene (Figure 5-1). The effect on the mRNA was further evaluated with steroid analysis.

5.3.2 CYP17A1 Knockdown

CYP17A1 knockdown was achieved using two siRNAs, resulting in a significant (P<0.001) and more than 90% knockdown (Figure 5-1 left, si-I 97.5% and si-II 94%). This represented almost total knockdown of the enzyme, similar to the effect of abiraterone treatment in patients. However, in-vitro abiraterone did not cause a shift towards the alternative pathway (data not shown), although affecting at least three genes at once (Ferraldeschi et al., 2013, Attard et al., 2012, Huang et al., 2016, Udhane et al., 2016, Li et al., 2012). The siRNAs were used in an attempt to cause a shift towards the alternative pathway and possibly determine if the inhibition of CYP17A1 is sufficient to cause the shift seen in patients or if it is a combination of two or more of the enzymes blocked by abiraterone. Thereafter, the effect on the steroidogenesis was examined, and the results were as hypothesised (Figure 5-3). All of CYP17A1 precursors were elevated, 5-10 fold higher than control. The products of the 17α-hydroxylase were reduced by 30-40% compared to scramble (control), depending on the siRNA used. Androgen synthesis
was reduced by 90% or more to almost undetectable levels. Yet this almost complete knockdown, of more than 90%, indicating that almost no mRNA copies of the gene were available for transcription, did not result in a detectable shift to the alternative pathway, as occurs in castration resistant prostate cancer. The inhibition of the pathway resulted in a shift to the glucocorticoid and mineralocorticoid pathways mediated by CYP21A2. Deoxycorticosterone (DOC) was elevated 5-8 fold above control and corticosterone was 5-9 fold higher than in control. Nonetheless, cortisol, a glucocorticoid, which is synthesised through 17-OH-hydroxylase action, was decreased. Those results are similar to the effect measured in patients with congenital inhibition of CYP17A1 (Auchus, 2016, Carvalho et al., 2016, Han et al., 2016) or in abiraterone treated patients (Attard et al., 2012).

**CYP21A2 Knockdown**

Some studies indicate (Attard et al., 2012, Huang et al., 2016, Udhane et al., 2016), that abiraterone also inhibits CYP21A2, and since CYP17A1 and CYP21A2 pathways have
common precursors, the knockdown effect of \textit{CYP21A2} was examined. Similar to the situation with \textit{CYP17A1}, \textit{CYP21A2} siRNAs caused significant knockdown (Figure 5-1 B; si-I 82%, si-II 90%, P<0.001), which should translate into an increase in androgens and a decrease in glucocorticoids, as the enzyme inhibition would result in a shift to androgen synthesis, as happens in 21-hydroxylase deficient patients (Turcu and Auchus, 2015). However, the knockdown resulted in a decrease in most but not all mineralo- and glucocorticoids, and androgen levels were significantly increased only with si-II. The effect on the steroids also differed between si-I and si-II (Figure 5-3). SiRNA-I caused, as anticipated, a decrease in pregnenolone, the mineralocorticoids DOC and corticosterone, and the glucocorticoids 11-deoxycortisol and cortisol, and 17OHP were increased by 8-10 fold compared to control. Noteworthy, is the 80% decrease of androstenedione. SiRNA-II, had no effect on pregnenolone, and its effect on the mineralocorticoids and glucocorticoids is less than siRNA-I. However, the androgens, DHEA and androstenedione, are increased 2-3 fold. The profile received indicates that although the knockdown effect was significant and above 80% reduction of the mRNA, the downstream effect varies. There seems to be no correlation between the mRNA knockdown effects to the steroid concentrations.
Figure 5-3: Steroidogenesis in CYP21A2 knockdown cells
H295R cells were transfected with siRNAs against CYP21A2 for 48h and then replaced with a medium containing 1µM FSk for another 24h. The natural deficiency of CYP21A2 results in a 17OHP and androgen increase and a decrease in gluco- and mineralocorticoids. Here 17OHP was significantly increased, and the same is true for the mineralocorticoids, except of 11-deoxycortisol that was not effected with siRNA3. The androgens were increased with the siRNA3 knockdown, but reduced when cells were treated with siRNA2. However no compensation by other steroids could be detected. Progesterone was not detectable in the control, but both knockdowns increased it significantly. DOC –deoxycorticosterone, 17OHP –17-hydroxyprogesterone, DHEA –dehydroepiandrosterone. *=P<0.05, **=P<0.01, ***=P<0.005, signification compares the specific steroid of interest in siRNA-I and siRNA-II to the scramble control steroid of interest. N=3 Mean±SEM. N.D – none detected

5.3.4 POR Knockdown

No alternative pathway steroids were detected when knocking down either CYP17A1 or CYP21A2, as the steroids shifted between the androgen and glucocorticoid steroidogenesis pathways. POR is needed for the function of both enzymes (Miller, 2005). The assumption was that by inhibiting POR and mimicking a known disease, and the iatrogenic condition caused by abiraterone treatment, there will be a detectable shift toward the alternative pathway. The mRNA knockdown was effective, as with the other siRNAs. Yet in this case the siRNA-II effect had high variability (Figure 5-1 Right; si-I 91.2% P<0.001, si-II 56.6%, P<0.05). The steroid analysis indicated that CYP21A2 is less sensitive to the decrease in POR. The knockdown did cause a significant rise of 17OHP, and DHEA was decreased by both siRNAs. The other steroids were either unaffected or the effect varied, as can be seen in androsterone, pregnenolone, DOC and corticosterone that were down regulated by one siRNA.
and elevated or not affected by the other (Figure 5-4). Nonetheless, no alternative pathway steroids were detected.

Figure 5-4: Steroidogenesis in POR knockdown cells
H295R cells were transfected with siRNAs against POR for 48h and then replaced with a medium containing 1µM FSk for another 24h. POR is electron transferase needed for the function of both CYP17A1 and CYP21A2, therefore its knockdown should be equivalent to double knockdown of those genes. Results indicate that CYP17A1 is more sensitive than CYP21A2 to the POR knockdown, as the androgens were more affected than the mineralo- or glucocorticoids, which are CYP21A2 products.

DOC – deoxycorticosterone, 17OHP – 17-hydroxyprogesterone, DHEA – dehydroepiandrosterone. *=P<0.05, **=P<0.01, ***=P<0.005, signification compares the specific steroid of interest in siRNA-I and siRNA-II to the scramble control steroid of interest. N=3 Mean±SEM.

5.4 Summary
The NCI-H295R cell line was treated with siRNAs in an attempt to mimic the effect of abiraterone on the adrenal gland as shown by Attard and colleagues (Attard et al., 2012). Short interfering RNAs were used to knockdown CYP17A1, CYP21A2 and POR. The aim was to investigate the possibility that the resistance of prostate cancer tumour in patients treated with abiraterone that inhibits CYP17A1 body-wide, is a result of the bypassing of the obstruction, most likely in the adrenal gland, and shifting to an alternative pathway (Attard et al., 2012, Arlt et al., 2004). The siRNAs achieved good knockdown, of more than 80%. However, no alternative pathway steroids could be detected, although the alteration in steroid profiles indicated the knockdown affected steroidogenesis. There are several possible reasons for the failure to detect the alternative pathway steroids. It is possible that residual enzymatic activity prevented the shift. Since no Western blots were performed, this option cannot be ruled out.
This option is also supported by the steroidogenesis results. The experiment was performed without the addition of external steroids (precursors), and it is possible that a shift did occur but the concentrations were too low for detection. It is possible that a longer incubation time is required, to eliminate residual enzymatic activity, although stable or chronic knockdowns should be considered.
6.0 Steroidogenesis in Ovarian Cancer Cell Lines

6.1 Introduction

Ovarian cancer (OC) is one of the most lethal cancers in women (Ferlay et al., 2010, Prat and Oncology, 2014, Cancer research UK, 2014c). The ovaries contain an endocrine tissue, the follicles stored in the cortex, responsible for the synthesis and secretion of the female sex hormones, progesterone, oestrone and oestradiol. This project’s aim was to investigate whether ovarian cancer tumours have steroidogenesis capabilities using cell lines representing four types of cancer, low grade serous (OAW42), high grade serous (CaOv-3), a granulosa cells tumour (COV434) and a possible endometrioid cancer (A2780). The aim of the study was to define the steroidogenesis capabilities of the different types of ovarian cell lines, representing different sources and types of ovarian cancer, as there is no previous data available in the area. The hypothesis driving the study was that the steroidogenesis in OC might be of use for early detection.

6.2 Methods

6.2.1 Cell Culture

After cells were detached as described in the general methods section (section 3.5), cells were seeded in 6-well plates. COV434 cells were seeded 1.5x10^6 cells/well and the other cell lines, CaOv-3, A2780 and AOW42, at 5x10^5 cells/well, and were allowed to attach overnight. Concentrations were decided based on confluence experiments; the cells were seeded at different concentrations for 96h to determine the cell concentration that will give the best confluence for the duration of an experiment. After the attachment period all the media were replaced with a phenol-red free version of the growth medium, including the relevant supplements as described previously (section 3.0) and 1µM forskolin (FSK) was added while
control wells had only medium. In the steroid conversion experiments, no FSK was used. Cells were treated with androstenedione, pregnenolone or 17OHP at 500nM, or medium alone as control. Cells were incubated at 37º with 5% CO₂ for 72 hours.

6.2.2 Quantitative PCR

Gene expression was analysed in the base line experiments, using the method described in the general methods section 3.2.3. The housekeeping gene was RPLP0, chosen following a recommendation from a colleague, Doctor Lorna Gilligan from the Institute of Metabolism and Systems Research at the University of Birmingham, in view of the fact GAPDH and HPRT1 that were used for the adrenal cells are not suitable for the ovarian cells (Jacob et al., 2013, Li et al., 2009, Sadek et al., 2012). RPLP0 was recommended due to its Ct values and repetitive results both in cell lines and primary cells. The target genes were chosen due to their function in the steroidogenesis (Table 3).

Table 3: The genes used for qPCR analysis of ovarian cell lines steroidogenesis.

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<th>Enzyme</th>
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<td>4310879E</td>
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<td>HSD3β1</td>
<td>Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1</td>
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<td>HSD3β2</td>
<td>Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 2</td>
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Table 3 (cont.): The genes used for qPCR analysis of ovarian cell lines steroidogenesis.

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<td><em>SULT1E1</em></td>
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<td>Follicle stimulating hormone receptor</td>
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<td>Luteinizing hormone/choriogonadotropin receptor</td>
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<td>Nuclear receptor subfamily 3 group C member 1</td>
<td>Hs00353740</td>
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6.2.3 Steroid Conversion

A2780 ovarian cancer cells were exposed to three concentrations of steroids, 200nM, 500nM and 1000nM, for 24-96h. After initial analysis, it was decided to continue with 500nM for 72h for future experiments. This combination was chosen because it was the first to allow measurement of all the converted steroids. Three steroids were examined: pregnenolone—the first steroid synthesised from cholesterol, 17OHP—a gateway steroid, an important steroid that can be converted to androgens (classic and alternative), mineralo- or glucocorticoids. The last
steroid tested was androstenedione – which can be converted to testosterone, and both of them can be aromatised, which enables the examination of the aromatisation capabilities of the cell lines.

6.3 Results

6.3.1 Baseline Steroidogenesis

In this set of experiments, ovarian cells were seeded and treated with media with or without 1µM FSK in order to investigate their steroidogenesis (Figure 6-1). No significant FSK effect could be detected. Although gene expression indicated that cells have the ability to synthesise steroids, none could be detected.

6.3.1.1 A2780 Cell Line

The cell line was derived from an endometrioid tumour (Anglesio et al., 2013). Gene expression analysis (Figure 6-1) revealed that the cells have CYP11A1, although in low levels, suggesting the cells should be able to synthesise steroids de novo. However, they lack HSD3B enzymes, and therefore can only synthesise Δ4 steroids (pregnenolone, 17OHPreg, DHEA and androstenediol). The qPCR results also showed that the FSK did not affected the cells gene expression (data not shown).

6.3.1.2 OAW42 Cell Line

The cell line is considered to be of low grade serous type and likely to be of Fallopian tube origin (Li et al., 2011). Gene expression revealed CYP11A1 in relatively high levels (Figure 6-1). It has HSD3B1 but not HSD3B2 or CYP17A1. According to the gene expression, these cells have the mRNA required to express CYP19A1 (aromatase) which aromatise androstenedione and testosterone.
6.3.1.3 CaOv-3 Cell Line

This high grade serous cell line, derived from a tumour originated in the Fallopian tube (Perets et al., 2013), expresses all the genes needed to synthesis oestrogens de novo (Figure 6-1), CYP11A1, CYP17A2, HSD3B2, 17BHSD1 and CYP19A1.

6.3.1.1 COV434 Cell Line

A granulosa cell line (Public Health England, Zhang et al., 2000, van den Berg-Bakker et al., 1993) is the only cell line originated from cells that are known to have steroidogenesis function. The gene expression (Figure 6-1) indicates the cells have the potential to synthesise oestrogens when given pregnenolone or other downstream steroids, as they lack CYP11A1.
Figure 6-1: Steroidogenic gene expression in 4 ovarian cancer cells
Ovarian cell lines were examined for steroidogenesis potential at 72h. The cells have different gene profiles, which could be expected since they originate from different tissues. However, they have some common traits, no expression of CYP11B1, CYP11B2 that synthesis mineralocorticoids and no HSD17B4. All of the cell lines express HSD17B3, which is normally expressed only in the testis, CYP21A2, SRD5A1, AKR1C3, CYP19A1, SULT1E1 and STS the last three are related to the oestrogen synthesis. The results indicate that the cells, including the granulosa cell line, cannot synthesis steroids de novo but can utilise precursors to synthesis oestrogens. Housekeeping gene was RPLP0 (Ribosomal protein, large, P0). Results shown as Mean±SEM, A2780 N=3, except SRD5A2 N=1, CaOv-3 N=2, except SRD5A2 N=1, COV434 and OAW42 N=1.
### 6.3.2 Ovarian Cancer Cells’ Receptor Expression

The expression levels of the receptors for LH (LHCR), FSH (FSHR) and several steroids were analysed. The LHCR and FSHR mediate their ligand activity in the follicle cells, LH regulates androgen synthesis and FSH regulates oestrogen synthesis, yet each of the cell lines examined expressed only one of them, three of the cell lines expressed FSHR while CaOv-3 expresses only LHCR. The steroid receptors tested for were expressed in all cell line except for ESR1 (oestrogen receptor α) and PGR (progesterone receptor) which were not expressed in OAW42 and/or A2780. All cell lines expressed high levels of mineralo- and glucocorticoid receptors that are not required for ovarian steroidogenesis regulation (Figure 6-2).

![Figure 6-2: Receptor expression in ovarian cell lines](image)

The expression levels of LH and FSH receptors, which regulate follicular steroidogenesis, and of different steroid receptors were measured. The LH and FSH receptors were not expressed in all the cell lines, with the LHCR expressed only in the HGS cell line CaOv-3. Each steroid receptor was expressed in all cell lines, except for two ESR1 and PGR. It is noteworthy that all cell lines expressed relatively high levels of mineralo- and glucocorticoid receptors, to the best of my knowledge. These steroids have no known physiological role in the follicles.

LHCR – luteinizing hormone receptor, FSHR – follicle stimulating hormone receptor, ESR – oestrogen receptor, PGER – G-coupled oestrogen receptor, PGR – progesterone receptor, MR – mineralocorticoid receptor, GR – glucocorticoid receptor, AR – androgen receptor. Results shown as mean±SEM, N=3
6.3.3 Steroid Conversion in Ovarian Cells

Following the baseline experiments, indicating that the cell lines express the genes needed for oestrogens synthesis, mostly through precursors, an experiment was set up to examine the steroidogenesis when the cells were treated with key steroid precursors. Pregnenolone, 17OHP or androstenedione, 500nM each, were added to the cell media and steroids were measured using LC-MS/MS as described before (section 3.5). The addition of pregnenolone or 17OHP, resulted in little to no measurable conversion in all the cell lines (Data not shown).

6.3.3.1 A2780 Androstenedione Conversion

When cells were co-incubated with 500nM androstenedione some downstream conversion was detected, as can be seen in Figure 6-3 A. Androstenedione was converted to androstanedione but also to andosterone which belongs to the alternative DHT biosynthesis pathway. No testosterone or DHT were detected, although AKR1C3 was expressed.

6.3.3.2 OAW42 Androstenedione Conversion

In this cell line, the androstenedione conversion resulted in both measurable testosterone and DHT. It seems that some of the androstenedione was converted directly to testosterone, but some was converted to the alternative pathway steroids androsterone and androstanediol (Figure 6-3 B).

6.3.3.3 CaOv-3 Androstenedione Conversion

Although this cell line expresses the genes to convert androstenedione to androstanedione, testosterone, DHT and oestrogens, only low concentrations of androstanedione were detectable (Figure 6-3 C). It is possible that the rest of the androstenedione was converted to oestrogens. However, the oestrogens levels were undetectable.
6.3.3.4 COV434 Androstenedione Conversion

COV434 cells express all the genes needed for oestrogens synthesis when given pregnenolone or other steroids. Yet, only conversion to androstanedione could be detected (Figure 6-3 D). Oestrogens could not be measured due to low concentrations, below the LC-MS/MS level of sensitivity, about 30nM, it is impossible to know if any of the androstenedione was converted to them.

Figure 6-3: Androstenedione conversion in ovarian cell lines

The ovarian cell lines showed no steroidogenesis at control conditions, therefore key steroids were added to the media to examine their steroidogenesis, as mRNA analysis indicated they have steroidogenic genes. A2780, endomiatriotic cell line (A), OAW42 low grade serous cell line (B), CaOv-3, high grade serous cell line (C) and COV434, granulosa cell line (D), were used. Five hundred nano molar androstenedione were added to the cell media and cells were allowed to grow. The results show conversion of the Androstenedione to the downstream steroids, up to DHT, OAW42 seems to be the most steroidogenic from this point, although androgen synthesis was detected, no oestrogen synthesis could be detected. The break in the Y axis and androstenedione is due to the high concentration of the steroid. Without it the other steroids will be too low to see. DHT – dihydrotestosterone. Mean±SEM. N=1
6.4 Summary

The ovaries are endocrine tissues, and according to the results described above, the cell lines express steroidogenic genes allowing them to synthesise oestrogens either *de novo* or through precursors. No measurable steroids were detected when cells were not co-incubated with steroid substrate. When given 500nM of steroid precursors, pregnenolone, 17OHP and androstenedione, only the latter showed conversion. Detection limitation prevented the measuring of oestrogens in the cells, and the fact that only free steroids, and not modified, were measured may explain why less than 500nM of metabolites were detected.

Although OC cell lines are derived from different sources and only one of the cell lines used, COV434, was derived from a steroidogenic cells (granulosa), yet all cell lines expressed steroid enzymes and under stimulation produced detectable levels of androgens from both classic and alternative pathways. As no non-cancerous cells were available, the question arises as to whether this steroidogenic capability is cancer related or not. If so, could it be used for early cancer detection? If the OC tumours secret steroids the increase in steroid concentrations could be measured in the blood or urine and assist with early detection.
7.0 Discussion and Conclusions / Future Directions

7.1 Discussion and Conclusion
Steroid hormones are essential for body development and function, being responsible for regulating blood pressure, metabolism, the inflammatory response, brain and reproductive system function, pregnancy and birth (Williams and Williams, 2003, Booth et al., 2002, Odermatt and Klusonova, 2015, Liggins, 1994, Miller, 2015, Walters, 2015).

In this project, the steroidogenesis in cell lines derived from two cancers was examined. Adrenocortical carcinoma is a cancer that can secrete steroids (Else et al., 2014, Allolio and Fassnacht, 2006). Ovarian cancer is a cancer of an endocrine tissue, and research showed that its growth might be affected by steroids. Progesterone through progesterone receptor type B (PR-B) has a positive effect, but it is not clear if oestrogens have pro- or anti-proliferative affect (Diep et al., 2015, Wimalasena et al., 1992, Li et al., 2014, Jeon et al., 2016). Its growth might be FSH and/or LH dependent (Mertens-Walker et al., 2012, Wimalasena et al., 1992, Choi et al., 2007), and although these specific hormones are not steroids they regulate follicular steroidogenesis.

The growth of other cancers such as prostate, discussed in the introduction, breast and uterine are known to be affected by steroids (Diep et al., 2015, Capper et al., 2016). With the progress in cancer research other cancers may in future be found to be steroid sensitive.

During the study I examined the role of steroidogenesis in ovarian cancer and examined a hypothesis regarding the role of the adrenal gland in the development of castration resistant prostate cancer, and characterised three adrenocortical carcinoma cell lines derived from H295R cell line from Dr William Rainey’s laboratory.
In the first section, the H295R strains S1, S2 and S3 were characterised for their steroidogenic capabilities, and the results indicate that all three cell lines can synthesise mineralocorticoids, glucocorticoids and androgens, although gene expression and steroid concentrations vary between the cell lines. Since the cell lines were derived from the same parent cell line it is logical to assume that growth media supplements play a role in the steroidogenesis capabilities of the cells (Wang and Rainey, 2012). On one occasion, alternative DHT biosynthesis pathway steroids could be detected in H295R-S2 and -S3 that were treated with growth media alone. Those results support the data collected by Attard and colleagues (Attard et al., 2008), suggesting that the adrenal glands might be the source for androgen in abiraterone treated patients. The alternative pathway steroids were synthesised without external manipulation, and therefore might indicate that the adrenal glands have a role in the development of androgen dependent conditions such as polycystic ovary and prostate cancer. Literature search did not reveal any direct link between the alternative pathway androgens and the growth of CRPC. However, based on neonatal study (Fluck et al., 2011) it is possible to assume that the steroidogenic tissue in males can utilise those steroids as DHT precursors. Moreover, it was shown in controls and patients of polycystic ovary syndrome study that they secrete alternative pathway androgens and also that there is a correlation between them and the DHT levels (Saito et al., 2016). A congenital adrenal hyperplasia study (Kamrath et al., 2012) also demonstrated that alternative pathway androgens can be measured in controls and at higher levels in patients from both sexes, therefore it is logical to assume that a similar thing occurs in males, both healthy and with prostate cancer. It is difficult to compare results to other studies due to the different techniques, however the alternative pathway steroid concentrations measured are similar to the “classic” pathway steroids measured in those cells (Figure 4-3 B and C, Figure 4-4), therefore it is possible to assume that the adrenal cells secrete high concentrations of those
steroids. It is difficult to determine why the alternative steroids were detected only in one experiment out of four performed. Yet, several options are possible, the steroid concentrations are actually lower and the experiment that they were measured in is the anomaly; it is also possible that extraction or detection problems were responsible for the lack of detection, especially if the steroid concentrations were low. Lack of internal standards for those steroids make it hard to determine the reason for this situation. Future analysis is needed in order to determine if the H295R cell lines can serve as model for alternative DHT biosynthesis pathway inhibitors. To the best of my knowledge this is the first time the steroidogenesis of those adrenal cell lines is characterised both by genetics and by steroid levels in those cell lines. In literature it is possible to find steroid analysis, mostly from patients (Kamrath et al., 2012, Saito et al., 2016, Auchus, 2004) but not complete analysis that includes genes and steroids nor one that includes all the steroids in the pathway. Due to methodology differences it is not possible to compare the published data with the data gathered in this study. The active field of adrenal steroidogenesis research requires cell lines that mimic the adrenal physiology and pathology (Wang and Rainey, 2012). These cells can serve as adrenal steriodogenic models, synthesising mineralocorticoids, glucocorticoids and androgen precursors, adding to the research armamentarium. They may also serve as models for the study of the alternative DHT biosynthesis pathway, and might help in the understanding of androgen excess conditions.
Figure 0-1: A suggested pathway showing how adrenal androgens can affect CRPC cell growth

A possible explanation to how alternative androgens from the adrenal glands can cause CRPC cells to grow. The adrenal gland synthesis androgens through the alternative pathway, those steroids are secreted to the blood stream and reach the prostate cancer cells were it metabolite to DHT. This pathway, based on a clinical study al., 2008), and the results shown in this thesis about the possibility that the adrenal can synthesis androgens even under CYP17A1 inhibition. This pathway suggestion need to be examined in the future.

In the second section, based on the results of a clinical trial (Attard et al., 2012) H295R1 cells were used in knockdown experiments using siRNAs against CYP17A1, CYP21A2 and POR. These experiments were part of a project designed to investigate if alternative pathway steroids from the adrenals could be converted to DHT in the prostate cancer cells (Figure 7-1). The siRNAs caused a significant knockdown of the target genes which manifested in steroid concentrations. The steroidogenesis did not correspond, however, with the hypothesised effect based on known human diseases (Arlt et al., 2004, Conference, 1968, Turcu and Auchus, 2015, Miller and Auchus, 2011). The results might be explained based on residual gene expression, and since proteins were not measured, residual protein activity cannot be ignored. Moreover, in CYP21A2 and POR knockdowns, the differing siRNAs produced different results. This could indicate a non-specific binding as has been suspected in the CYP21A2 knockout, in an attempt to explain the decrease in androgen with siRNA-I, or residual enzymatic activity because of protein turnover time. This could explain both situations (Figure 7-2). Moreover, in an experiment performed by Dr Hofland previously from the Arlt group in the University of
Birmingham, H295R1 cells were exposed to abiraterone but no alternative pathway androgens could be detected. On the other hand preliminary results with the VCaP prostate cancer cell line transplanted into orchidectomised mice and treated with abiraterone did show detectable concentrations of alternative steroids. The hypothesis that abiraterone treatment (CYP17A1 inhibitor) caused a shift towards the alternative DHT biosynthesis pathway androgens could not be confirmed. Data not shown here hinted that the shift occurred but at very low concentrations and sporadic measurements prevented the data from being reliable.

Figure 7-2: Summary of steroidogenesis due to siRNAs knockdown
Knockdown of CYP17A1 (A) and CYP21A2 (B) affected the steroid concentrations of precursors and products of the knockdown enzymes. The CYP17A1 knockdown caused a decrease of the enzyme metabolites and increased its precursors. The CYP21A2 knockdown affect the cells in less conclusive, as the androgens downstream to the enzyme were affected differently by the siRNAs. Orange arrows indicate effect direction, horizontal arrow effect not clear.

The third section examined ovarian cancer cell line steroidogenesis. The aim was to examine whether tumour steroidogenesis can be used for early cancer detection. Ovarian cancer has no
distinctive signs and is usually detected in late stages (Howlader N, 2014, SEER program, 2011), resulting in low survival rates (Siegel et al., 2015). All the cell lines, three of them (A2780, OAW42 and CaOv-3) from non-steroidogenic tissues, expressed steroidogenic enzymes enabling them to synthesise different steroids when given precursors and all expressed the genes needed for oestrogenesis from androstenedione. Nonetheless, LC-MS/MS analysis did not detect any steroids without the addition of precursors, probably since the steroid concentrations were below the detection limit of the LC/MS-MS system that was used. Since addition of precursors did result in detectable androgen synthesis it is possible to assume that the cells are not capable of synthesising high concentration of the steroids de novo, yet no literature could be found to determine if this assumption is correct or not. Moreover, and as mentioned before, steroidogenesis is not usually examined in ovarian cancer cells Oestrogens are measured in picograms (Xu and Veenstra, 2012) and the lower detection limit of the technique used is 30nM. In order to measure the oestrogen a different extraction technique is required and there will be a need for larger amount of cells, possibly tens of millions. When cells were given precursors (pregnenolone, 17OHP and androstenedione), only downstream conversion products of androstenedione were measured. The measurements of pregnenolone and 17OHP could not be properly analysed due to a probable faulty batch of the extraction solvent, Tert-butylmethyl ether (MTBE), affecting steroid extraction, causing poor recovery. This acquirement of steroidogenesis capabilities might have a role in the cancer development and should be studied farther. The ovarian cancer cells ability to synthesise alternative DHT biosynthesis pathway steroids and their expression of CYP21A2, MR and GR genes mRNA are not, to the best of my knowledge, a feature of normal ovarian steroidogenesis. These results suggest that the ovarian cancer tumours become steroidogenic, able to metabolise certain steroids, mainly androstenedione, regardless of their tissue of origin. Although not shown in
this study, it can be assumed that those cell lines can synthesis oestrogens (Xu and Veenstra, 2012, Zhang et al., 2000), which are likely pro-proliferative (Wimalasena et al., 1992, Li et al., 2014, Jeon et al., 2016), but this is yet to be determined (Xu and Veenstra, 2012, Zhang et al., 2000). The change in steroidogenesis might also serve to detect cancer due to a surge or change in oestrogens or androgens in the blood or urine, especially of the alternative DHT biosynthesis pathway androgens. The alternative pathway steroids are not aromatizable, and therefore will not affect oestrogen production. However, depending on their levels, they could cause symptoms of androgen excess, especially if causing an increase in DHT. This possibility, if proven true, should be considered when treating women showing androgen excess symptoms. Those androgens, however, probably have no role in tumour growth since androgens have no known role in ovarian development. Therefore, those androgens are most likely to have a role as biomarkers for ovarian cancer rather than affecting its progress. Their receptors expression pattern should be compared to that of other fast growing tissues as potential targets for specific chemotherapy administration. Their expression pattern might also allow for molecular differentiation between normal and cancerous cells. The ovarian cancer cells express either LH or FSH receptors. However, they did not respond to 1µM FSK treatment, that activates cAMP, although the FSH and LH affect the cells through cAMP and therefore direct stimulation is supposed to cause an increase mRNA expression of the effected genes such as CYP17A1, CYP19A1, CYP21A2 and the 17/3βHSD. The lack of effect might indicate that the cells are already expressing these genes at their limit, or that the FSK stimulation did not affect the cells for another reason. Both options need to be further evaluated. No oestrogens could be detected in the cell lines, although it was shown previously (Ren et al., 2015, Xu and Veenstra, 2012). This was probably due to sensitivity level (detection level 30nM)
of the LC-MS/MS machine, or since they were modified (sulphated or glycosylated). During the study only free steroids were measured.

This is the first time that ovarian cancer cells steroidogenic capabilities were examined, although the effects of selected steroid and their receptors were previously examined (Jonsson et al., 2015, Jeon et al., 2016), and the results show that this field should be further investigated. The results of the H295R cells suggest that H295R-S1, -S2 and S3 are good models for the adrenal steroidogenesis. The knockdown experiments however did not confirm the hypothesis that inhibition of CYP17A1 and/or CYP21A2 by abiraterone cause a shift to the alternative DHT biosynthesis pathway as a source for androgens.

The experiments in these studies were performed using only cell lines derived from cancer tumours and therefore need to be validated and repeated in primary cells, derived from both healthy and malignant tissues. The use of benign tumours might also prove useful, maybe enabling the development of a tool to differentiate benign from malignant. No protein results were obtained regarding the knockdown effects, preventing the option to determine if the unexpected results in the steroidogenesis of the knockdown cells were due to residual enzymatic activity or for other reasons. The LC-MS/MS used also limited the data, since its sensitivity levels might have limited steroid detection. Moreover, only the free form of the steroid was measured as representing the steroidogenesis. Yet, they are only a fraction of the total steroids produced. This might explain why in some cases steroids could not be detected or why there was difference between the concentrations measured in steroid conversion experiments.

7.2 Future Directions

Synthesis of alternative DHT biosynthesis pathway androgens in the adrenal cortex.

The results of the cell lines characterisation supported by previous studies (Saito et al., 2016,
Kamrath et al., 2012), indicate that the adrenal cortex is capable of synthesising the alternative DHT biosynthesis pathway androgens, even under basal conditions. This possibility should be further investigated, including at the protein level. Since steroid concentrations might be too low for detection the use of precursors should be considered, to increase concentrations of the steroids of interest and allow detection. This trait, the synthesis of alternative DHT biosynthesis pathway androgens, if found reliable, could serve as diagnostic and possibly therapeutic targets for steroid related carcinomas and also as a tool for the understanding of androgen excess conditions.

**Determining how abiraterone treatment causes shift toward the alternative pathway.** Since the experiments to determine if CYP17A1 inhibition by abiraterone is sufficient to cause a shift towards the alternative pathway did not result in the hypnotised effect, nor CYP21A2 or POR inhibitions, this set of experiments should be revised and repeated. The original knockdown experiments did cause more than 80% reduction of relevant mRNA after 72h incubation, yet it did not translate to a repeated detectable steroid secretion. The repeat experiment should examine protein levels and the possibility that residual enzymatic activity affected the results. Depending on the protein levels detected a longer incubation period should be considered in order to achieve significant decline in enzymatic levels, hopefully to undetectable levels. Steroid concentrations should be take into account and the use of precursors for the examined enzymes might be needed to increase metabolites to detectable concentrations. The repeat experiment is needed to determine how the knockdown affects the enzymes levels and also consider the use of precursors to increase steroid concentrations to a detectable level.
**Stable knockdown of CYP17A1, CYP21A2 and POR**

The abiraterone treatment is a long-term treatment and a resistant is developed only after several months (de Bono *et al*., 2011, Fizazi *et al*., 2012, Ryan *et al*., 2013). Knockdown experiments using siRNAs are very short in comparison. It is therefore possible that the shift towards the alternative pathway needs long inhibition of the relevant enzyme or enzymes. It is therefore recommended to try a stable knockdown in order to mimic the drug treatment. This technique, stable knockdown, might even prove to be a better model due to the long-term treatment with abiraterone.

**In vivo studies**

Preliminary study by Dr Hofland showed that a prostate cancer cell line xylograph in orchidectomy mice caused an increase in circulating alternative pathway steroids. This supports the hypothesis that the adrenal gland supports the prostate tumour growth in CRPC patients. This indicates that *in vivo* studies should be strongly considered. Since *in vitro* experiments were unable to detect alternative pathway steroids when cells were treated with siRNAs in an attempt to better understand how abiraterone inhibition causes shift towards the alternative pathway in patients, *in vivo* knockdown experiments might prove more effective. Literature search reveals that *CYP17A1* and *POR* knockout mice are already in use (Liu *et al*., 2005, Ribes *et al*., 2007) but no *CYP21A2* knockout mice were found. Since each of those knockouts has a known human disease equivalent the knockout effects are already known. However, animal models will allow researchers to investigate the effect of different therapeutics on androgen synthesis both in the classic and alternative pathways. It is likely that double and perhaps triple knockout animals will be needed in order to mimic the effect of certain drugs as
is the case with abiraterone that inhibits CYP17A1, CYP21A2 and 3βHSD activities (Attard et al., 2012, Huang et al., 2016, Udhane et al., 2016, Ryan et al., 2010, O'Donnell et al., 2004).

**Congenital adrenal hyperplasia patients as clinical model for Alternative DHT biosynthesis pathway**

Studies with congenital adrenal hyperplasia patients should be considered favourably as those patients are nature created models for steroid studies and can be proven important armamentarium for understanding the role of alternative pathway steroids in health and pathologic conditions.

Alternative DHT biosynthesis pathway androgens were already measured in patients with CYP21A2 or POR deficiency (Reisch et al., 2013, Arlt et al., 2004, Kamrath et al., 2012) but no data regarding alternative pathway in CYP17A1 deficiency patients could be found. CYP21A2 and POR deficiency patients might contribute to studies determining the effects and roles of the alternative pathway androgens in the body, including prostate cancer. Patients with CYP17A1 deficiency have very low to undetectable levels of androgens (Carvalho et al., 2016, Camats et al., 2015, Kim and Rhee, 2015), with a similar steroidogenesis profile as abiraterone treated prostate cancer patients, although CYP17A1 deficient patients are not castrated, as in both cases CYP17A1 enzyme function is impaired. Therefore, those patients could be a valuable model for the effects of abiraterone on CYP17A1 and alternative pathway androgens due to CYP17A1 inhibition. With that said, CYP17A1 deficiency is rare and the patients vary regarding 17-hydroxylase and 17,20 lyase deficiency combinations. Therefore it will be difficult to obtain a large enough patient cohort for a comparison study with abiraterone patients. If possible a pilot study should be conducted to investigate the effect of CYP17A1 deficiency on alternative androgen synthesis in comparison to abiraterone. Serum samples
could also be used to investigate prostate cancer development when given to cells as supplement.

**Prostate tumour transplantation in severe combined immunodeficiency mice**

Severe combined immunodeficiency (SCID) mice may be used to determine alternative androgen concentrations in sera in humans by transplanting them with human adrenal cortex cells or tissue, as a pre-clinical stage, to calibrate and standardise measuring methods, prior to clinical measurements in humans. This is needed since today there is not enough data on those steroids to allow clinical use. The SCID mice, transplanted with prostate cancer tumours can serves as a model to examine how alternative androgens affect their development, utilising knockout and knockdown techniques to create enzymatic deficiency. These models will allow the determination of the role of the alternative pathway androgens in prostate cancer development and serve as a model for drug development targeting the alternative pathway.

**Ovarian cancer steroidogenesis**

The ovarian cancer experiments were aimed to examine the possibility that steroidogenesis can serve as a biomarker for early ovarian cancer detection. A similar study regarding early detection of ACC based on urine steroids is currently being conducted (Arlt *et al.*, 2011). Results were unexpected, as it was found that tumour originating from non-steroidogenic tissue presented with a wide steroidogenic enzyme profile. Moreover, all the examined cell lines expressed mineralo- and glucocorticoid receptors, and *CYP21A2*. None, to my knowledge, is related to ovarian steroidogenesis. These results need to be further examined in primary tumours, and healthy tissues, and if found to be unique and common to the tumours might be useful in clinical practice. The fact that the cell lines have steroidogenesis capability might be useful for early cancer detection based on steroid concentrations in blood or urine.
Animal Models

Animal models should be considered, SCID mice with CYP17A1, CYP21A2 or POR knockout, implanted with ovarian cancer cells will allow the investigation of the role of steroids, namely androgen and oestrogen in OC development. It will also allow the measurement of the concentration of steroids secreted by the tumours to determine if the change in steroidogenesis can serve as a biomarker. Steroids and steroidogenesis need to be considered as possible biomarkers and therapeutic targets in ovarian cancer research.

To conclude, in this research it was shown that ovarian cancer cells have steroidogenesis capabilities, regardless of their origins. This fact, not reported before, should be further investigated as a means of detecting ovarian cancer in early stage and as pharmacological targets. H295R steroid secretion is affected by the media supplement used in the growth media, and therefore it is very important to grow the cells in their original media since change of supplement will probably change the results. It was also shown that adrenal cell lines are capable of secreting alternative androgens making them an in vitro model for alternative androgen studies. Alternative androgens can be found in healthy people (Kamrath et al., 2012), and a comprehensive study is needed to be conducted in order to set a normal range which will enable the use of androgens to detect pathologies.
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