Use of the androgen signalling pathway to identify ovarian cancer patients suitable for hormonal therapy

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Northern Institute for Cancer Research

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Statement of originality

The work presented in this thesis is original except where acknowledged in the references and was carried out between February 2012 until January 2015 at the at the Northern Institute for Cancer Research, Newcastle University and the Northern Gynaecological Oncology Centre, Gateshead.

It has not been, or will not be, submitted for any other qualification at any other university.

Abstract

Background

Despite confirmed AR expression in epithelial ovarian cancer (EOC), clinical response to anti-androgen treatment is poor. Stratification of susceptible individuals with a specific biomarker, such as the previously described Rab35, might enable more effective treatment strategies. Abiraterone, a steroid synthesis inhibitor, might be of therapeutic benefit in specific EOC subgroups.

Methods

Primary cell cultures (PCO) generated from ascites were used as a representative model for the heterogeneity of EOC. PCOs were examined for AR and Rab35 expression at mRNA and protein level and were stimulated with androgens to evaluate subsequent Rab35 expression.

CYP17 expression was measured in ovarian cancer cell lines and PCOs and the effect of abiraterone on proliferation was assessed in two ovarian cancer cell lines.

Results

The AR expression was widely different when examined with qRT-PCR, Western blotting and immunohistochemistry. No correlations were found between the modalities for either AR or Rab35 expression.

In contrast, AR and Rab35 expression showed a positive correlation at the protein and mRNA level. However, androgen treatment of PCOs showed >50% increase in Rab35 mRNA expression in only 40% of PCOs.

CYP17 expression was confirmed in all examined cell cultures and PCOs at both, the protein and mRNA level. Abiraterone treatment of the ovarian cancer cell lines led to significant inhibitory effects on proliferation. On protein level however, abiraterone exposure resulted in increased expression of AR and CYP17.

Conclusion

Although AR expression was confirmed in POCs, it remains unclear which technique would be most suitable to stratify for androgen expressing tumours.

Rab35 in PCOs appeared to be androgen-related and hence may not be a suitable biomarker in EOC for AR.

The inhibitory effect of abiraterone on proliferation that was observed in ovarian cultures is suggestive of a dual action of the compound. Response to abiraterone exposure in PCOs might help to determine potential treatment effects.

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List of Abbreviations

ACTION	Adjuvant ChemoTherapy in Ovarian Neoplasm
ACTH	Adrenocorticotropic hormone
AIB1	Amplified by breast 1
APS	Ammonium persulphate
AR	Androgen receptor
ARA 70	Androgen receptor-associated protein 70
ARE	Androgen response elements
AUC	Area under the curve
AURELIA	Avastin Use in Platinum-Restistant Epithelial Ovarian Cancer
BMI	Body mass index
BSA	Bovine serum albumin
BRAF	murine sarcoma viral proto-oncogene homolog B1
BRCA	Breast cancer antigen
BrdU	Bromodeoxyuridine
CA125	Cancer antigen 125
cDNA	complementary DNA
CHORUS	Chemotherapy or upfront surgery (trial)
COCP	Combined oral contraceptive pill
CORAL	Cancer of the OvaRy Abiraterone triaL
CR	Complete response
CRPC	Castrate resistant prostate cancer
CYP	Cytochrome P450
DAPI	4',6-diamidino-2-phenylindole stain
DBD	DNA binding domain
DCC	Dextran coated charcoal
DEPC	Diethylpyrocarbonate
DESKTOP	The Descriptive Evaluation of preoperative Selection KriTeria for OPerability in recurrent OVARian cancer
DHEA	Dehydroepiandrosterone
DHT	Dihydrotestosterone
DMSO	Dimethyl sulfoxide

DNA	Deoxyribonucleic acid
ECL	Enhanced Chemiluminescence
EDTA	Ethylene diamine tetraacetic acid
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
EOC	Epithelial ovarian cancer
EORTC	European organisation for Research and Treatment of Cancer
EPCaM	Epithelial cell adhesion molecule
ER	Oestrogen receptor
FCS	Fetal calf serum
FDA	U.S. Food and Drug Administration
FFPE	formalin-fixed, paraffin-embedded
FIGO	International Federation of Gynaecology and Obstetrics
FSH	Follicle stimulating hormone
GnRH	Gonadotropin-releasing hormone
GPCR	G-protein-coupled receptors
GR	Glucocorticoid receptor
HNPCC	Hereditary non-polyposis colorectal cancer
HPRT1	Hypoxanthine-guanine phosphoribosyltransferase 1
HRT	Hormone replacement therapy
ICON	International Collaborative Ovarian Neoplasm
IHC	Immunohistochemistry
KRAS	Kirsten rat sarcoma viral oncogene
LBD	Ligand binding domain
LH	Luteinizing hormone
MAPK	Mitogen-activated protein kinase
MR	Mineralocorticoid receptor
NICE	National Institute for Health and Clinical Excellence
NTD	N-terminal regulatory domain
OC	Oral contraceptive
OCEANS	Ovarian Cancer Study Comparing Efficacy and Safety of Chemotherapy and Anti-AngiogenicTherapy in Platinum-Sensitive Recurrent Disease
OSE	Ovarian surface epithelium

PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate buffered saline
PCO	Primary ovarian ascitic cancer cells
PCOS	Polycystic ovary syndrome
PFS	Progression free survival
PKA	Protein kinase A
PKC	Protein kinase C
PR	Progesterone receptor
PR	Partial response
PSA	Prostate-specific antigen
RNA	Ribonucleic acid
RECIST	Response Evaluation Criteria in Solid Tumours
ROC	Receiver operating characteristic
RPM	Revolutions per minute
SCOUT	secretory cell outgrowths
SD	Stable disease
SDM	Steroid depleted medium
SHBG	Sex hormone-binding globuline
SRB	Sulforhodamine B
STIC	serous tubal-intraepithelial carcinoma
TBS	Tris buffered saline
TCA	Tricholoracetic acid
TEMED	Tetramethylethylenediamine
TGF	Transforming growth factor
TILT	tubal intraepithelial lesions in transition
TMA	Tissue microarray
UKFOCCS	UK familial Ovarian Cancer Screening Study
UKCTOCS	UK Collaborative Trial of Ovarian Cancer Screening
UTR	Untranslated region
VEGF	Vascular epithelial growth factor

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1 Introduction

1.1 Ovarian cancer

1.1.1 Epidemiology of ovarian cancer

After endometrial cancer, ovarian cancer is the most common gynaecological malignancy, accounting for 5% of all cancers in women. In the UK alone, there are around 7300 new diagnoses each year, coupled with over 4200 deaths annually, making ovarian cancer the gynaecological malignancy with the highest mortality rate.

Epithelial ovarian cancers (EOC) are the most common group, accounting for approximately 90% of ovarian cancers, and shall be the focus of this study, with other, rarer subtypes not discussed further.

The majority of women diagnosed with ovarian cancer are postmenopausal, with over 80% of cases being diagnosed in women over 50 years of age. There is a steep increase in incidence after the usual age of the menopause, showing the highest age-specific incidence rates in women aged 75-79 years at diagnosis as shown in *Figure* **1-1** (Cancer Research UK, 2015).

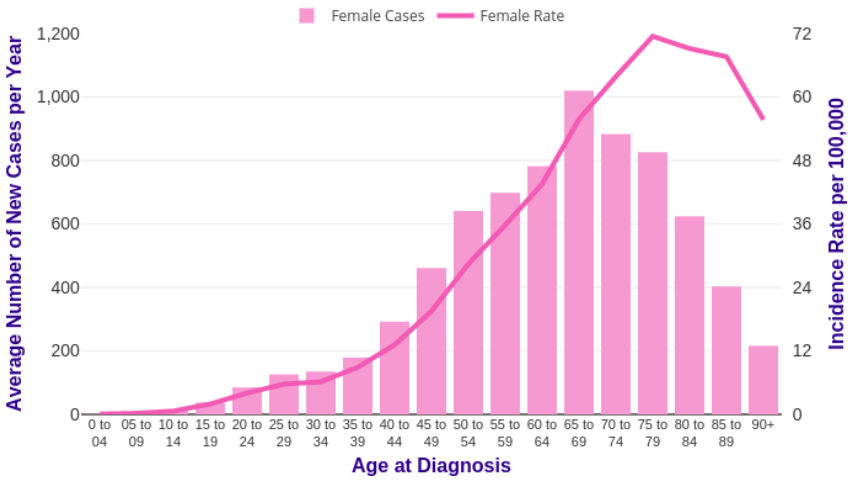


Figure 1-1: Ovarian cancer, average number of new cases per year and age-specific incidence rates, UK, 2013-2015: adapted from Cancer Research UK (Cancer Research UK, 2015).

Over the last ten years, the mortality rate of ovarian cancer has shown a small decline of 17% since the early 1970s for women in age groups 50-60, but have increased in females from the age of 70. The overall mortality rate of ovarian cancer appears stable over the last four decades (Cancer Research UK, 2015).

Since the 1980s and 1990s however, there has been an improvement in time-adjusted rates, possibly attributable to improved therapy options, including more widespread use of platinum based chemo-therapy and better primary treatment (Kitchener, 2008).

A major factor contributing to the poor prognosis associated with ovarian cancer is the detection of the disease at advanced stages, as a consequence of the lack of both specific symptoms, and availability of screening tests. Despite attempts to provide screening tools for earlier detection (Goff et al., 2007, Lim et al., 2012, Smith et al., 2005), 40% of women are diagnosed at stage III. 5-year survival rates in ovarian cancer decline markedly by stage, showing a 5-year survival rate of 90% in stage I, opposed to 19% in stage III and 4% in stage IV disease (Cancer Research UK, 2015). Furthermore, the prognosis is impaired by high recurrence rates, as 70- 80% of women with ovarian cancer will suffer from recurrence (Lorusso et al., 2012).

FIGO Stage	Definition
Stage I Cancer limited to the ovaries	IA: Growth limited to one ovary. Capsule intact. No tumour on surface. No malignant cells in ascites/washings. IB: Cancer in both ovaries. Capsule intact. No tumour on surface. No malignant ascites. IC: Stage IA or IB with capsule rupture or surface tumour and/or positive ascites/washings.
Stage II Cancer involving one or both ovaries with spread to other pelvic organs or surfaces	IIA: Extension to the uterus and/or fallopian tubes. Negative washings. IIB: Extension to other pelvic organs. Negative washings. IIC: Stage IIA or IIB with capsule rupture and/or positive ascites/washings.
Stage III Cancer spread into abdomen	IIIA - Tumour grossly limited to the pelvis with microscopic peritoneal/ omental metastases. Negative nodes. IIIB – Stage IIIA with macroscopic peritoneal/ omental metastases less than 2cm in size. Negative nodes. IIIC - Stage IIIA with peritoneal/ omental metastases > than 2cm or positive retroperitoneal or inguinal nodes.
Stage IV Liver or extra-peritoneal metastases	

Table 1-1: FIGO staging of ovarian cancer

1.2 Classification of ovarian cancer

1.2.1 Historical classification of ovarian cancer

Ovarian tumours have traditionally been classified by their originating tissue (Scully, 1975), and are subdivided by their histological resemblance of other tissues, with serous epithelial ovarian cancers being the most common subtype (see *Table* **1-2**).

Serous tumours have cells resembling those of the fallopian tube (Auersperg et al., 2001, Feeley and Wells, 2001), mucinous tumour cells resemble endocervical and intestinal epithelium, endometroid tumour cells resemble proliferative endometrium, clear cell tumour cells resemble gestational endometrium and transitional cell tumours resemble cells from the urogenital tract.

Surface epithelium	Germ cell	Mesenchyme/ Sex cord stromal
Serous	Dysgerminoma	Granulosa cell
Mucinous	Yolk sac	Thecoma
Endometroid	Embryonal carcinoma	Fibroma
Clear cell	Choriocarcinoma	Sertoli cell
Transitional cell (Brenner Tumours)	Teratoma	Sertoli-Leydig
		Steroid

Table 1-2: Ovarian cancer classification according to tissue of origin.

1.2.2 New classification of epithelial ovarian cancer

More recently, a new classification system based upon the tumours clinical and pathological behaviour combined with the underlying genetic mutations has been proposed (Shih Ie and Kurman, 2004, Vang et al., 2009) as shown in *Table* **1-3** below.

	Description	Morphological subtypes	Genetic mutations
Type I	Slow growing, diagnosed at lower grades. Identifiable precursor lesions.	Low grade serous Serous borderline Mucinous Endometrioid Clear cell Malignant Brenner tumours	•KRAS •BRAF •p53 •MMR pathway •PI3K pathway •WNT pathway
Type II	Rapid growth with early metastasis. No defined precursor lesions.	High grade serous carcinomas Undifferentiated carcinomas Malignant mixed mesodermal tumours	•p53 •AKT2 •p16 •PIK3CA

Table 1-3: Ovarian cancer classification adapted from Shih et al 2004, with common genetic mutations

1.3 Aetiology of ovarian cancer

Overall, the aetiology of ovarian cancer to date is poorly understood. However, factors associated with either increased or decreased risk of development of ovarian cancer have been identified.

Though histological subtypes and different grades of ovarian cancer are known, the disease is still treated with one standard treatment, comprising a combination of surgery and chemotherapy. Different responses to the treatment have been noted by clinicians which supports the theory that ovarian cancer might in fact be a more heterogeneous disease than it was thought of until now (Hennessy et al., 2008).

Historically epithelial ovarian cancer was understood as a disease derived from the mutation of single epithelial cells of the cuboid layer coating the ovary (Saad et al., 2010).

Fathalla proposed the theory of disruption of the ovarian surface epithelium through follicle rupture (Fathalla, 1971). The disrupted epithelium would form invaginations of the surface and could potentially be exposed to higher levels of hormones because of the proximity to surrounding parenchymal tissue (Hennessy et al., 2009). These ovarian inclusion cysts were suggested as a possible the source of epithelial ovarian cancer (Dubeau, 2008, Pothuri et al., 2010).

Other than a physiological insult, embryological and histological causes were considered as causes for ovarian tumorigenesis. Two theories- the coelomic metaplasia hypothesis and the Müllerian hypothesis- were developed.

The coelomic hypothesis postulates that ovarian cancer is derived from coelomic epithelium undergoing neoplastic transformation into Müllerian –like epithelium. The following neoplastic transformation is likely to occur in portions of the epithelium that are exposed to hormone-rich ovarian parenchyme, ie. cortical inclusion cysts (Dubeau, 2008).

The Müllerian theory speculates that primary peritoneum and structures derived from the Müllerian epithelium such as the lining of the fallopian tube, the endometrium and endocervix could be classified as “secondary Müllerian system”. Hence malignancies from the ovary, the fallopian tube and primary peritoneum could be regarded as a single entity rather than three different entities as the current classification suggests (Dubeau, 2008).

The Müllerian hypothesis is supported by the expression pattern of HOX genes. HOX genes are transcription factors that determine cellular identity during development (Kelly et al., 2011). They have also been implied in cell differentiation (Veraksa et al., 2000), and ovarian cancer

oncogenesis (Kelly et al., 2011), and associated with invasive characteristics of ovarian cancer cells (Yamashita et al., 2006).

As far as phenotypic determination is concerned, during embryological development, each HOX gene is expressed in a certain pattern along the anterior-posterior axis (Kelly et al., 2011, Veraksa et al., 2000). During the development of the female reproductive system, four HOX genes (HOXA9, HOXA10, HOXA11 and HOXA13) are expressed along the Müllerian duct axis (Taylor et al., 1997), whereas in the adult, the expression becomes organ specific. The fallopian tubes express HOXA9, the uterus HOXA10, the lower uterine segment and cervix express HOXA11 and HOXA13 is expressed in the upper vagina (Taylor et al., 1997).

In addition to that, Cheng *et al*. have found HOX gene expression in EOCs, but not in normal ovarian surface epithelium. The same set of HOX genes were found expressed in epithelial cells from normal fallopian tube, endometrium and endocervix as they were in serous, endometrioid and mucinous ovarian cancers, suggesting that the cancer originated from these structures (Cheng et al., 2005b).

Over the last decade, a paradigm shift has occurred towards the idea that ovarian cancer should be classified as a group of diseases from different cell types as cells of origin (including ovarian, fallopian and peritoneal cells) rather than one disease arising from one ovary (Moss et al., 2015). Shih and Kurman were the first to propose a dualistic classification model in view of the disease heterogeneity and based on clinical, histopathological and genetic findings (see *Table* **1-3**) (Shih Ie and Kurman, 2004). They proposed the distinction of type I cancers, characterised by possible pre-cursor lesions (serous cystadenomas or borderline tumours) versus type II cancers with no defined pre-cursor lesions, but instead *de novo* development from the ovarian surface or inclusion cysts. According to Shih and Kurman's observation, type I cancers would be characterised by slow disease progression, poor response to chemotherapy and a 5-year survival of 55%. In contrast, type II cancers- which account for 75% of the cancers- are much more aggressive, with quick progression and a 5-year survival of 30% (Shih Ie and Kurman, 2004).

Tying in with the Müllerian hypothesis and the recognition of the fallopian tube epithelium as the origin of high grade serous ovarian cancer (Vang et al., 2013) are precursor lesions in the tubal fimbrial end such as serous tubal-intraepithelial carcinoma (STIC) (Vang et al., 2013), secretory cell outgrowths (SCOUTs), and tubal intraepithelial lesions in transition (TILT)

(Nishida et al., 2016). Of these, STICs are the most examined and a mouse model has confirmed STICs as precursor cells of high-grade serous cancers driven by *BRCA*, *TP53* and *PTEN* genes (Perets et al., 2013).

In humans, STIC lesions have been confirmed to be found in both, asymptomatic low-risk populations negative for the *BRCA* gene defect (Chay et al., 2016), but more so in women with a known *BRCA* gene defect (Reitsma et al., 2013).

Interestingly, a recent study from Nishida *et al*. found SCOUTs in 21%, TILT 3.2% and no STIC cases in a population of 123 women with benign pelvic disease (Nishida et al., 2016). The predominant location for STIC was described in the fimbrial end of the fallopian tube (Przybycin et al., 2010).

Chay *et al* described a small cohort of non-*BRCA* carriers with STIC staging laparotomy and three out of the seven undergoing the procedure had to be upgraded to high grade serous cancer on histology and one more was tested positive for *BRCA1* mutation, suggesting, that staging surgery and *BRCA* gene testing should be considered in incidental findings of STIC (Chay et al., 2016).

In women with *BRCA* mutation, the incidence of STIC has been described as 0.6% to 7% (Patrono et al., 2015). A literature review including 78 women with *BRCA* gene mutation undergoing prophylactic risk reducing salpingo-oophorectomy with confirmed STIC found only three women (4.5%) with subsequent primary peritoneal cancer at 43-72 months follow up (Patrono et al., 2015).

Above developments were also recognised by the International Federation of Gynecology and Obstetrics and led to the adjustment of the staging classification now including ovarian, fallopian tube, and primary peritoneal carcinoma (Prat, 2015). Furthermore, suggestions to rename the disease into pelvic or pertioneal cancer (Vaughan et al., 2011) or tubo-ovarian serous cancer (Moss et al., 2015) were made. However, new classifications systems were not implemented in order to avoid confusion for patients, clinician and potential bias in research (Moss et al., 2015).

To date the debate of the relative importance of ovarian cancer tumorigenesis (fallopian tube versus the ovarian surface epithelium) has not been resolved. It remains difficult to understand the molecular pathology of the disease, screening and prevention of the disease (Vaughan et al., 2011).

One of the dilemmas is the implication of the fallopian tube theory. Following the compelling evidence that the fallopian tube could be the origin of ovarian cancer, it was suggested that

prophylactic salpingectomies might reduce the disease burden (Tone et al., 2012). However, a systematic review on 11 studies evaluating the opportunistic salpingectomy in women undergoing hysterectomies found that the evidence to support risk reduction of ovarian cancer through prophylactic removal of the fallopian tube is lacking (Darelius et al., 2017).

In conclusion, whilst the involvement of fallopian epithelium and its precursor lesions are increasingly established in the pathogenesis of "ovarian" high-grade serous cancer, the implications on further investigations and management of incidentally detected precursor lesions remains unclear.

The understanding of the "fallopian tube theory" is however of importance, particularly for women with known *BRCA* gene mutation where risk reducing salpingo-oophorectomy is a recognized preventative option, reducing the risk for ovarian cancer by 80-95% (Domchek et al., 2006).

1.4 Risk factors for ovarian cancer

The predominant risk factor for ovarian cancer is increasing age with the majority of cases diagnosed in postmenopausal women, with a peak incidence at 80-84 years of age (UK, 2015). Hereditary factors have been associated with ovarian cancers. Ten percent of ovarian cancers are associated with germline mutations of susceptible genes, like *BRCA1* and *BRCA2* and further genetic mutations in combination with ovarian cancer have been implicated with hereditary nonpolyposis colorectal cancer (HNPCC) (Prat et al., 2005, Colombo et al., 2006) and Lynch syndrome II (Ketabi et al., 2011, Colombo et al., 2006).

Non-hereditary factors associated with increased risk for ovarian cancer are nulliparity (Beral et al., 2008) infertility (Kurian et al., 2005) or a raised body mass index (Reeves et al., 2007, Lahmann et al., 2010).

In addition to these, the inflammation of epithelial cells from such causes as the use of talcum powder (Huncharek et al., 2003, Ness and Cottreau, 1999), endometriosis and pelvic inflammatory disease have also been associated with increased risk for ovarian cancer (Ness and Cottreau, 1999).

Finally, ethnic background has been suggested as potentially influencing the risk of ovarian cancer, with higher incidence rates reported for Europe and Northern America compared to Asia or Africa (Sankaranarayanan and Ferlay, 2006).

1.4.1 Hormonal factors

As the ovary is a hormone producing organ underlying endocrine regulation via the hypothalamic- pituitary- gonadal axis, hormone related induction of carcinogenesis has been extensively investigated.

An examination of 200 hysterectomy and bilateral salpingo-oophorectomy specimens has shown that 92% of specimens with epithelial ovarian tumours demonstrating hyperplastic and metaplastic changes in the contralateral ovary, as opposed to only 22% of ovaries demonstrating these changes in the absence of disease. This suggests a hormonal stimulus could be involved in ovarian carcinogenesis (Resta et al., 1993).

1.4.1.1 Gonadotropins

Cramer and Welch postulated in 1983 that inclusion cysts would undergo proliferation and ultimately malignant transformation as consequence of excessive stimulation of ovarian tissue by pituitary gonadotropins (Cramer and Welch, 1983).

This hypothesis is supported by epidemiological findings, such as the protective effect of pregnancies and oral contraceptive use, both of which suppress gonadrotropin secretion (Riman et al., 1998). In addition, women with polycystic ovarian syndrome (PCOS) have raised serum luteinizing hormone (LH) serum levels and have been found to be at increased risk for ovarian cancer (Schildkraut et al., 1996).

The use of ovulation-stimulating drugs for infertility has been an opportunity for numerous, somewhat conflicting investigations, with some studies reporting elevated risk (Whittemore et al., 1992, Sanner et al., 2009), but most finding weak to no association between the use of infertility drugs and ovarian cancer risk (Jensen et al., 2009, Doyle et al., 2002, Brinton et al., 2004).

This trend is also reflected in the experimental data where some studies have shown a proliferative effect of gonadotropins (Kang et al., 2000, Syed et al., 2001, Edmondson et al., 2006, Zheng et al., 2000), coupled with anti-apoptotic activity, others failed to determine a proliferative effect of gonadotropin stimulation (Ivarsson et al., 2001) on ovarian cancer.

1.4.1.2 Oestrogens

The possibility of exogenous administration of hormones, such as use of hormone replacement therapy (HRT) influencing the development of ovarian cancer has also been investigated. Again, the data is conflicted, with some studies suggesting HRT may increase the risk for ovarian cancer (Negri et al., 1999, Rodriguez et al., 2001, Lacey et al., 2002, Anderson et al., 2003, Folsom et al., 2004), whereas others failed to show any association between HRT and ovarian cancer risk (Coughlin et al., 2000, Hempling et al., 1997). Despite these findings being consistent for both oestrogen-only and combined HRT, the increased risk of ovarian cancer has been reported as 22% with oestrogen only therapy and 10 % with combined HRT (Pearce et al., 2009).

One trial suggested that the mode of progesterone administration might influence the risk of ovarian cancer and suggested that sequentially administered progesterones increases the risk for ovarian cancer, whereas continuously administered progesterones might not (Riman et al., 2002).

1.4.1.3 Androgens

Androgens have also been implicated in the carcinogenesis of ovarian cancer (Risch, 1998), with epidemiologic evidence that hyper-androgenic states such as polycystic ovarian syndrome (Schildkraut et al., 1996), a high BMI (Lahmann et al., 2010, Yang et al., 2011), acne and hirsutism could be associated with an increased risk of ovarian cancer.

The polycystic ovarian syndrome (PCOS) is a condition resulting in a hyper-androgenic state. This is due to two underlying mechanisms: luteinising hormone (LH) stimulates the ovarian theca cells causing excess androgen production, and a decrease of sex hormone binding globulin (SHBG) that increases the free circulating and hence available testosterone. There is also an increase in 5-α-reductase activity is increased, leading to increased peripheral steroidogenesis. Some epidemiological evidence suggests that women with an ovarian cancer diagnosis were more likely than the control group to have a previous diagnosis of PCOS (Schildkraut et al., 1996). However, other investigations could not confirm a correlation between PCOS and raised epithelial ovarian cancer risk (Olsen et al., 2008). Histological examination of polycystic ovaries showed higher occurrence of inclusion cysts in women with

PCOS, and in 68% of PCOS patients, cells in these occlusion cysts had hyperplastic and metaplastic changes (Resta et al., 1989).

Central obesity can influence hormonally dependent tumours as a consequence of alterations in metabolism- a connection that has been demonstrated in both, breast and endometrial cancer (Parker and Folsom, 2003, Huang et al., 1997).

For ovarian cancer the evidence is less robust. In central obesity, SHBG is reduced, leading to an increase in free circulating androgens whilst peripheral steroidogenesis is increased. Several studies, amongst them two large prospective cohort studies have confirmed an increase in risk of ovarian cancer with increasing hip-to-waist ratio (Mink et al., 1996, Anderson et al., 2004, Lahmann et al., 2010, Engeland et al., 2003).

The effect of exogenous androgens was tested by Cottreau *et al* (Cottreau et al., 2003). In women with endometriosis, treatment aims to oppose androgen effects. In this study, the synthetic androgen danazol which binds to the androgen receptor was compared to two gonadotropin-releasing hormone analogues (Leuprolide and Nafarelin), with regard to their effect at suppressing the secretion of FSH and LH. Women taking the androgen were found to be at three-fold elevated risk of developing cancer compared to women taking GnRH analogues (Cottreau et al., 2003).

1.5 Protective factors for ovarian cancer

The suppression of ovulation seems to hold protective properties against developing ovarian cancer (Fathalla, 1971, Pike et al., 2004), irrespective of the means by which this is achieved. There has been identified, a decreased risk of developing ovarian cancer associated with the use of oral contraceptive (OC) pills or the experience of pregnancy and breastfeeding (Danforth et al., 2007, Chiaffarino et al., 2005b, Hennessy et al., 2009).

Contraception through combined oral contraceptives is based on the suppression of the mid-cycle gonadotropin surge and the inhibition of ovulation (Riman et al., 1998), but the protective effect could be enhanced by progestational effects (Risch, 1998). Whilst the used of progesterone-only contraceptives have not been extensively studied and require further evaluation (Riman et al., 1998), the use of combined oral contraceptive pills was found to reduce the risk for ovarian cancer in healthy women (Cramer et al., 1982, Rosenberg et al., 1994, La Vecchia, 2006, Ness et al., 2011), in addition to women who possess the *BRCA1* or 2 gene mutation (Narod et al., 1998, McLaughlin et al., 2007).

Longer duration of OC seems to increase the protection (Beral et al., 2008, Hankinson et al., 1992) with the risk of developing ovarian cancer reduced by 10-12% after only one year of OC use, with this decrease in risk rising to 50% after 5 years of use (Hankinson et al., 1992). Furthermore, the protective effect continues after the cessation of OC use (Riman et al., 1998). Risk reducing surgery, including removal of the ovary and fallopian tubes or tubes alone, has also been shown to have an effect in decreasing the risk of ovarian cancer with surgical measures such as hysterectomy and tubal sterilisation being shown to decrease the risk of developing ovarian cancer by approximately 30% (Parazzini et al., 1993, Chiaffarino et al., 2005a, Tworoger et al., 2007). The protective mechanisms in low risk populations are not fully understood, but might be explained by a reduction in blood supply and hence decreased ovarian steroidogenesis as well as interrupted retrograde transport of carcinogens (Riman et al., 1998). Tubal ligation has been shown to increase the protective effect in endometrial and clear cell cancer through the interruption of displaced malignant cells (Sieh et al., 2013). Furthermore, prophylactic salpingectomy has been suggested as an alternative treatment options with the additional benefit of removing a potential tumour site (Dietl, 2014) or tubal precursor lesions for cancer (Patrono et al., 2015), as well as interfering with retrograde transport. Prophylactic removal of the tube has shown to decrease the risk of developing ovarian cancer in high risk populations of *BRCA 1* or 2 germline mutation carriers (Rebbeck et al., 2002, Olivier et al., 2004).

Progesterone use also appears to be protective against ovarian cancer. Epidemiological studies suggest factors such as increased parity to reduce the ovarian cancer risk (Adami et al., 1994) and that oral contraceptives with high progestin potency might decrease the risk more than preparations with low progestin potency support this theory (Schildkraut et al., 2002).
In vitro studies have confirmed that progesterone inhibits proliferation in normal ovarian surface epithelium (Ivarsson et al., 2001), and ovarian cancer cell lines (Bu et al., 1997, Yu et al., 2001).

1.6 Screening in ovarian cancer

The aim in ovarian screening is to detect the disease at an early stage prior to spread into the abdominal cavity. There is, however, no reliable screening for early detection of ovarian cancer. This is in part, due to lack of specific, diagnostic biomarkers, with current practice relying on the use of ultrasound and serum CA125.

The pitfall with these investigative measures is the low specificity, as these investigations can be positive in non-malignant conditions. The sensitivity is low as the markers might be unaltered in some cases (Yu et al., 2001).

Nevertheless, the marked differences in survival rates for women in earlier cancer stages have triggered two large clinical trials in the UK to help develop screening tests for ovarian cancer.

The UK Collaborative Trial of Ovarian Cancer Screening (UKCTOCS) has assigned postmenopausal women in three trial arms: no investigation (control group), annual ultrasound, or annual multimodal screening (tumour marker CA125 and transvaginal ultrasound scan). Preliminary results based on 200.000 women showed that large scale screening is possible and ovarian cancer can be detected in probands without symptoms. Comparing CA125 to ultrasound scans, both measures have similar sensitivity, but CA125 has greater specificity (Menon et al., 2009). These findings led to an extension of the trial with further analysis expected in at the end of 2018.

The UK familial Ovarian Cancer Screening Study (UKFOCSS) has investigated women with a minimum of 10% lifetime risk for ovarian cancer with annual CA125 blood tests and ultrasound and found that screening in the year before diagnosis reduces the number of patients diagnosed with high grade disease (stage IIIc), but does not increase the number of women diagnosed with low grade disease (stage I) (Rosenthal et al., 2013). As the screening interval was delayed by a median of 88 days prior to detection of ovarian cancer, stricter adherence to the screening schedule was suggested and four monthly screening interval were proposed for a phase 2 trial (Rosenthal et al., 2013).

The lack of appropriate screening modalities needs to be taken into account as false positive results can potentially increase anxiety and lead to unnecessary surgery, whilst false-negative result can lead to false reassurance. A systematic review of ten randomised international trials suggested that ovarian screening so far failed to reduce overall mortality and was associated with unnecessary surgery (Reade et al., 2013). A better understanding of ovarian aetiology and the discovery of biomarkers could be helpful avoiding this.

1.7 Treatment in ovarian cancer

1.7.1 Treatment in primary disease

1.7.1.1 Management of early ovarian cancer (Stage I cancer)

In women with cancer confined to one ovary and who wish to conserve fertility, conservative surgery removing only one ovary can be considered. If both ovaries are affected, a total abdominal hysterectomy, bilateral salpingo-oophorectomy and infracolic omentectomy should be performed.

Either way, retro-peritoneal lymphnode assessment as part of optimal staging should be done (guideline, 2011).

Chemotherapy is not part of the standard treatment in stage I cancer, but can be considered in certain circumstances, such as poorly differentiated tumours or histological subtypes such as clear cell carcinomas (NICE, 2011).

Two large randomised phase III trials, ICON1 (International Collaborative Ovarian Neoplasm) and ACTION (Adjuvant Chemotherapy in Ovarian Neoplasm), have shown that platinum-based adjuvant chemotherapy after complete cytoreduction improves overall survival as well as recurrence-free survival at 5 years (Colombo et al., 2003, Trimbos et al., 2003).

1.7.1.2 Management of advanced ovarian cancer (Stage II-IV)

The current gold standard for treatment of advanced ovarian cancer is cytoreductive surgery in combination with platinum-based chemotherapy. Surgery aims to remove all macroscopic disease (Chi et al., 2006) and can be performed at two time points. Primary debulking surgery can be performed prior to administration of six cycles of adjuvant chemotherapy or interval debulking surgery can be performed after three cycles of neoadjuvant chemotherapy, followed by three further cycles of chemotherapy post operatively.

Two trials (EORCT 55971 and CHORUS) compared these treatment modalities using survival as outcome measure.

The CHORUS trial (Chemotherapy versus upfront surgery) was a phase 3, randomised, multi-centre trial conducted in 87 hospitals in the UK and New Zealand, randomly assigning 550 women with stage III/ IV ovarian cancer to either primary debulking or neoadjuvant chemotherapy. Measuring the overall survival as primary outcome, primary chemotherapy was deemed non-inferior to primary surgery (Kehoe et al., 2015).

The EORTC trial 55971 randomly assigned 670 patients with advanced ovarian cancer to either primary surgery or neoadjuvant chemotherapy, measuring the ten-year survival as primary outcome and found overall comparable survival rates. Further analysis though suggested increased survival for patients on stage IIIc disease with primary surgery and Stage IV disease with neoadjuvant chemotherapy (van Meurs et al., 2013)

1.7.2 Treatment of recurrent disease

The treatment of recurrent ovarian cancer is mainly based on chemotherapy and is used for palliative rather than curative intent (Hennessy et al., 2009).

The main prognostic factor for successful treatment is the disease-free interval from completion of chemotherapy until recurrence. If this time-interval is less than six months, the cancer is regarded as resistant to chemotherapy or platinum-refractory (Markman et al., 1991).

ICON 4 (International Collaborative Ovarian Neoplasm) has shown increased survival in recurrent ovarian cancer with use of combination chemotherapy of carboplatin and paclitaxel compared to single agent carboplatin (Parmar et al., 2003).

Surgery is not used routinely in recurrent ovarian cancer but is reserved for symptom relief or removal of resectable disease. However, secondary cytoreductive surgery has been suggested in a well- selected population but would need further evaluation in phase III trials (Lorusso et al., 2012).

The DESKTOP trial (The Descriptive Evaluation of preoperative Selection KriTeria for Operability in recurrent OVARian cancer) has suggested that patients with previous complete cytoreduction could potentially benefit from surgery post relapse (Harter et al., 2006).

1.7.3 Targeted therapy

High recurrence rates of ovarian cancer (of about 80%) despite aggressive surgical and systemic treatment, increased toxicity with recurrent chemotherapies and the development of resistance to chemotherapy remain a problem. This has led to extensive research into underlying pathological mechanisms and potential signalling pathways, with the aim to be able to exploit these for targeted treatments. The hope would be that specific therapies might prolong the disease-free interval with less toxicity and hence better quality of life.

Targeted therapies for ovarian cancer can broadly be grouped in hormonal and non-hormonal therapies and will be discussed below.

1.7.3.1 Non-hormonal therapies

Potential targets for non-hormonal treatment of EOC have been explored based on principles of tumorigenesis and vary widely- from DNA repair mechanisms to vascularisation (angiogenesis and epidermal growth factor receptors) and cell signalling (see *Figure* **1-2**).

A short overview is presented below, as the main focus of this thesis is based on androgen-related pathogenesis.

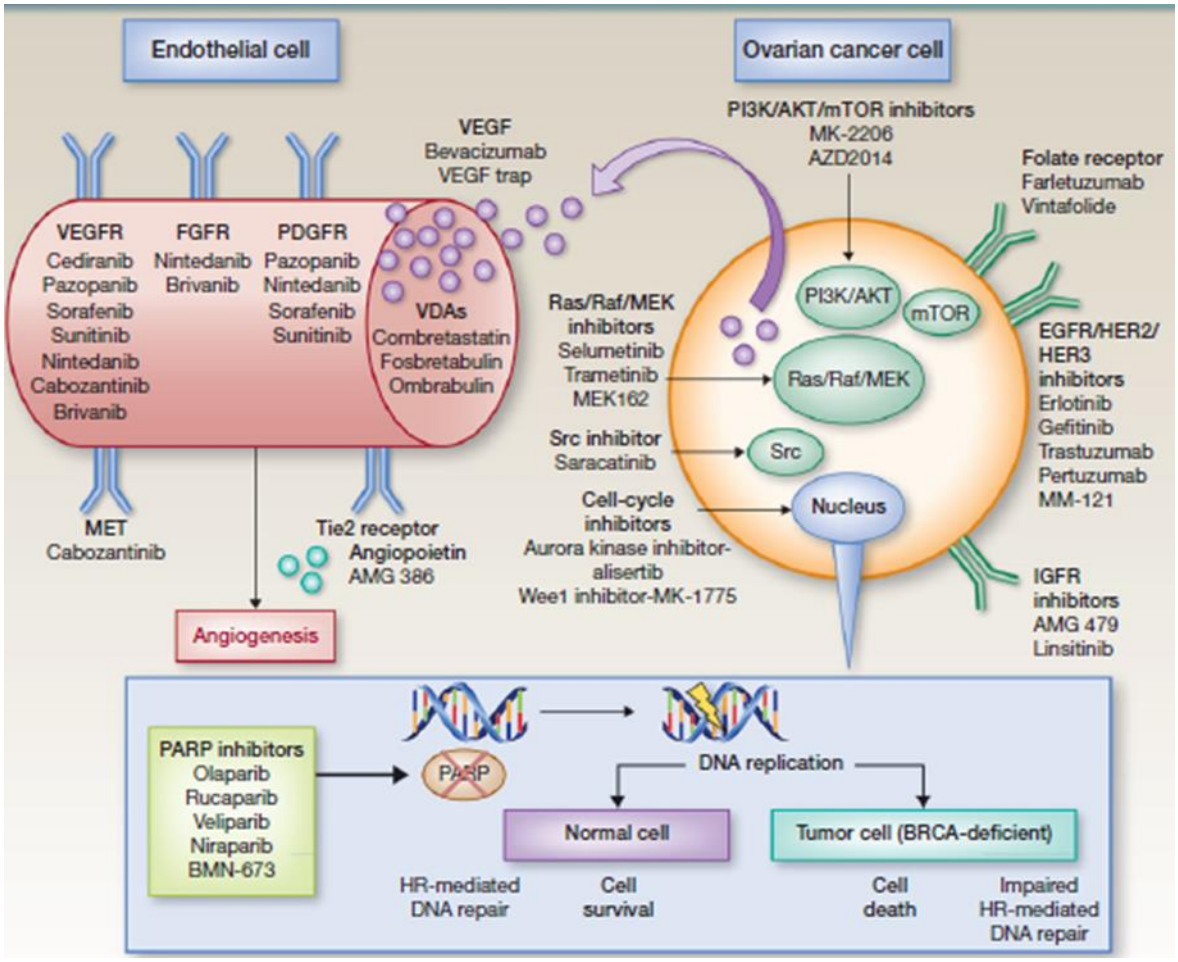


Figure 1-2: Schematic overview of non-hormonal treatment targets and treatment agents in ovarian cancer (adapted from Banerjee et al) (Banerjee and Kaye, 2013)

1.7.3.1.1 PARP Inhibitors

A hereditary component of EOC has been recognised- about 14 % of women with ovarian cancer are *BRCA* gene positive. Most of these women carry a germline mutation, but 7% have a somatic *BRCA* mutation (Alsop et al., 2012). Whilst the presence of this gene mutation predisposes to a higher risk of ovarian and breast cancer, it also increases the sensitivity for

Poly (ADP-ribose) polymerase (PARP) Inhibitors. Treatment with PARP inhibitors induces cancer cell death by a process of synthetic lethality by targeting the DNA repair mechanism to selectively kill the tumour cells. Clinical trials have shown that PARP inhibitors are well tolerated and had a high response rate in *BRCA1* and *BRCA2* mutation carriers who were either sensitive or resistance to platinum-based chemotherapy (Fong et al., 2010).

Olaparib has been approved in the US by the FDA back in 2014 and is licensed for patients with *BRCA* gene deficiency and previous three cycles of chemotherapy as well as monotherapy as maintenance in advanced ovarian cancer patients with *BRCA1/2* mutations (Lokadasan et al., 2016).

The approval of PARP inhibitors by the National Institute for Health and Care Excellence (NICE) in 2016 has markedly expanded the treatment options for women with platinum-sensitive high-grade serous cancer of the ovary, fallopian tubes or peritoneum in the UK. Olaparib was the first PARP inhibitor in use and was originally licensed for women with *BRAC1* or *BRCA2* mutations after relapse following the third course of platinum-sensitive chemotherapy (NICE, 2016). Further advances have recently been made, as since August 2019 olaparib can be offered much earlier in the disease course. It can now be given as maintenance treatment after response to first-line platinum-based chemotherapy in BRCA positive women (NICE, 2019). Even women without BRCA gene mutation can receive PARP inhibitors-niraparib is used after relapse following two courses of platinum-based chemotherapy in women with or without *BRCA* mutation (NICE, 2018).

1.7.3.1.2 Angiogenesis inhibitors

Angiogenesis inhibitors have had an increasing role in ovarian cancer treatment for advanced stage disease. Vascular endothelial growth factor (VEGF) is a key regulator of angiogenesis.

Bevacizumab (a humanised monoclonal antibody against VEGF) is the most investigated angiogenesis inhibitor and its benefit has been confirmed by four large randomised controlled phase 3 trials.

ICON 7 and GOG 218 have assessed bevacizumab as first line treatment alongside the standard chemotherapy (carboplatin and paclitaxel) and found that progression free survival was increased (Burger et al., 2011) as well as overall survival in patients with poor prognosis (Oza et al., 2015).

Two further phase 3 trials, OCEANS (Ovarian Cancer Study Comparing Efficacy and Safety of Chemotherapy and Anti-Angiogenic Therapy in Platinum-Sensitive Recurrent disease) and AURELIA (Avastin Use in Platinum-Resistant Epithelial Ovarian Cancer), both assessed bevacizumab in the second line setting and found an increase in progression free survival (Della Pepa and Banerjee, 2014, Pujade-Lauraine et al., 2014), but no increase in overall survival (Aghajanian et al., 2015, Pujade-Lauraine et al., 2014).

Cediranib, an oral antiangiogenic vascular endothelial growth factor receptor inhibitor, was trialled in ICON 6, a randomised, double-blind, placebo-controlled phase 3 trial in patients with relapsed platinum sensitive ovarian cancer and was found to increase progression free survival in women with recurrent platinum-sensitive disease (Ledermann et al., 2016).

70% of ovarian cancers show overexpression of epidermal growth factor receptor (EGFR). EGFR binding leads to subsequent cell proliferation and increased cell survival and has been associated with chemo-resistance. Clinical trials investigating EGFR tyrosine kinase inhibitors have failed to show significant benefit (Gui and Shen, 2012).

1.7.3.1.3 Cell signalling pathways

Interference with cell-cycle control is being trialled as treatment for ovarian cancer. During the cell cycle there are checkpoints which control the integrity of the dividing cell. Cyclin-dependent kinases control such checkpoints, as they halt the cell cycle in case of DNA damage to allow either repair or apoptosis. MK-1775 is an inhibitor of such a tyrosine kinase and is trialled in women with relapsed, platinum-sensitive disease (Banerjee and Kaye, 2013).

Inhibiting the Insulin-like Growth Factor (IGF), another tyrosine kinase receptor and targeting the folate receptor have been described, though clinical data are sparse (Banerjee and Kaye, 2013).

Certain histological subtypes of ovarian cancer have been found be correlated with distinct genetic abnormalities. Low-grade EOC harbour mutations in the Kirsten rat sarcoma viral oncogene (KRAS), the murine sarcoma viral proto-oncogene homolog B1 (BRAF) and express active mitogen-activated protein kinase (MAPK).

It was suggested that in patients with KRAS and BRAF mutations the ERK1/2 pathway influences tumour growth and survival when activated. Treatment with selumetinib, an MEK1/2 inhibitor, showed partial or complete response on 8/52 treated patients ((Banerjee and Kaye, 2013).

Treatment of ovarian cancer with multikinase inhibitors has been attempted. Unfortunately, clinical trials have not shown any benefit with multikinase inhibitors such as dasatinib which target the mitogen-activated protein kinase (MAPK)/ extracellular signal-regulated kinases (ERK) pathways (Bodnar et al., 2011). This type of therapy is associated with multiple unpleasant side effects such as nausea, diarrhoea and skin reactions (Ramasubbaiah et al., 2011). In clear cell cancer, a rare histological type of EOC with aggressive clinical behaviour and poor response to chemotherapy, the P13K/AKT pathway has been implied and associated with playing a role in cell cycle progression, survival and angiogenesis (Kotsopoulos et al., 2014).

1.7.3.2 Hormonal therapies

1.7.3.2.1 Anti-oestrogen therapy

As ovarian surface epithelium expresses oestrogen receptors, anti-oestrogen therapy with Tamoxifen was examined. However, the results were not very encouraging, showing complete responses in 10% and partial responses in 8% of the patients with platinum-refractory disease (Markman et al., 1996, Hatch et al., 1991).

A Cochrane review analysing 11 non-randomised trials, one randomised phase II study and two randomised trials showed an objective response in 9% of women to Tamoxifen. The variation between the studies was wide, ranging from 0-56%. Stable disease for four weeks or more, was observed in 32% of the women in eight studies (Williams, 2001).

1.7.3.2.2 Therapy with gonadotropin antagonists

The proposed mechanism of action of gonadotropin releasing hormone (GnRH) analogues is the desensitisation or down regulation of GnRH receptors in the pituitary, leading to a decrease of gonadotropin secretion and further decreased secretion of gonadal steroids (Kang et al., 2000).

1.7.3.2.3 Anti-androgen therapy

To date four phase II trials have been conducted to evaluate response of ovarian cancer to treatment with anti-androgens only (Thompson et al., 1991, Tumolo et al., 1994, Vassilomanolakis et al., 1997, van der Vange et al., 1995) as summarized in *Table* **1-4** and will be discussed in more detail in chapter 1.8.8.

1.8 Androgens and ovarian cancer

1.8.1 Androgens in the healthy female

Androgens are of physiological significance for bone and muscle growth and maintenance of cognitive function (Walters et al., 2008, Burger, 2002a), but also influence hair growth and libido. Androgen excess is associated with menstrual disturbances, virilisation, insulin resistance and glucose intolerance (Navarro et al., 2015).

In the female, androgens are produced in the ovaries and adrenal glands and are the sole precursors of oestrogens (see *Figure* **1-3**). The androgen secretion in the adrenal gland and ovary is stimulated through the pituitary secretion of adrenocorticotropic hormone (ACTH) and luteinising hormone (LH) respectively (Burger, 2002b).
In women, the three "pro-androgens" without androgenic activity- dehydroepiandrosterone sulphate (DHEAS), dehydroepiandrosterone (DHEA) and androstenedione- require enzymatic transformation by sulfatase and CY17A1 (17-20 lyase) to be converted into their biologically active forms testosterone and dihydrotestosterone (DHT) (Burger, 2002b) see *Figure* **1-3**. Testosterone and DHT exert their androgenic effects via the androgen receptor.

Whilst half the testosterone in women is synthesized in adrenal glands and ovaries (25% each) and the other half is synthesized from circulating, DHT is mainly derived from conversion of testosterone and only a small fraction is secreted by the adrenal gland (Burger, 2002b).

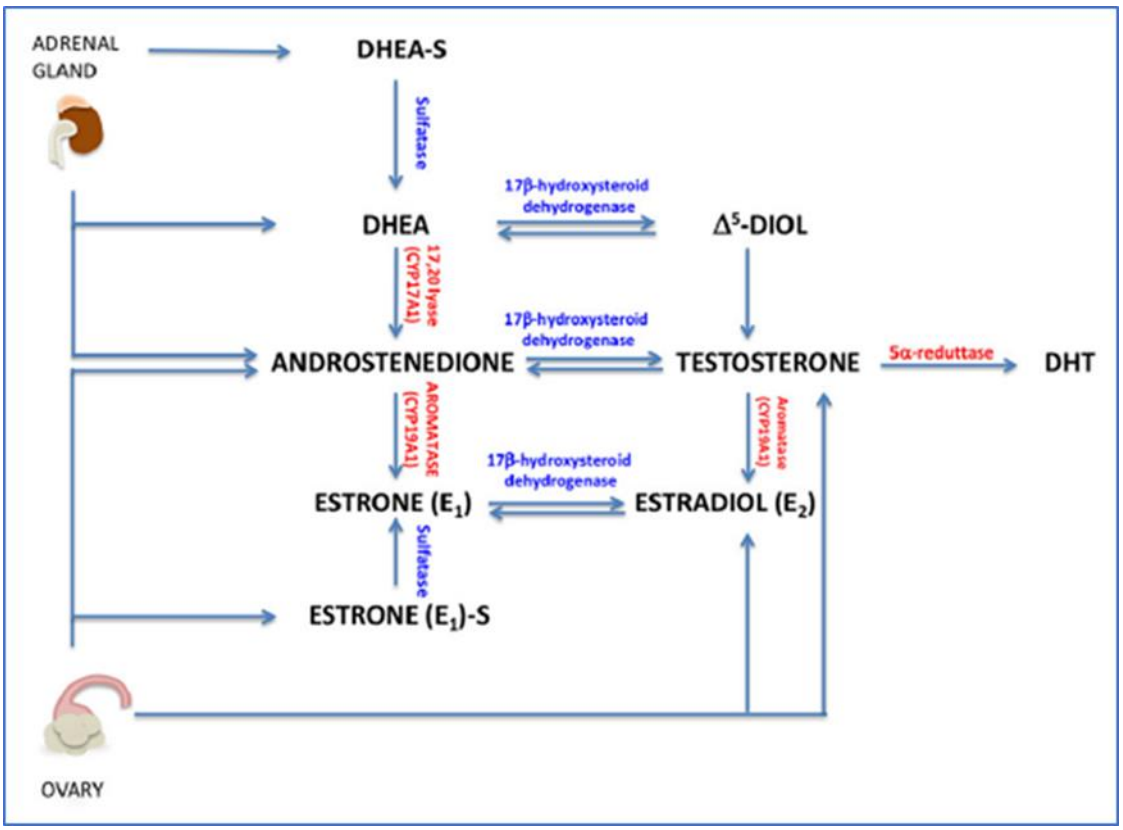


Figure 1-3: Schematic outline of androgen synthesis in women (adapted from Giovenalli et al (Giovannelli et al., 2018).

Most of the androgens in the serum are bound to plasma albumin and sex hormone-binding globulin (SHBG), leaving about 2-3% of the plasma androgens free. However, despite unbound proteins being the main active fraction, bound hormones may have the potential to enter target tissues (Pardridge, 1986).

Testosterone levels vary during the course of the day, with peaks in the early morning as well during the menstrual cycle. The lowest concentrations are found in the early follicular phase of the cycle, with a mid-cycle peak and a fall in the luteal phase (Burger, 2002b). Serum testosterone levels in women are in a range of 0.6–2.5nmol/L, with a daily production of 0.1–0.4 mg (Burger, 2002b).

Compared to oestrogens, plasma concentrations of androgens are greater in the healthy, non-pregnant women (Risch, 1998), in both, pre- and postmenopause (Helzlsouer et al., 1995). Moreover, androgen levels are higher than oestrogen levels even in the late follicular phase (Risch, 1998).

The ovary itself produces twice the amount of androgens compared to oestrogens (Mason et al., 1994). Ovarian androgens are found in the follicular fluid, with levels of androstenedione in follicles up to ten times higher than that of oestradiol (Mason et al., 1994).
Prior to ovulation, the follicular fluid in the primary ovulating follicle will become estrogenic through FSH stimulation of granulosa cell aromatase. The smaller, secondary follicles however, continue producing synthetisation of androgens after atresia.
Ovarian epithelial cells also express the enzyme 17β-hydroxysteroid dehydrogenase, converting androstenedione into the more potent androgen testosterone.

In the postmenopause, adrenal as well as ovarian production of androgens decrease (Davison et al., 2005). It was suggested that in the postmenopausal ovary, the androgen production decreases less than oestrogen production, making the postmenopausal ovary a relatively androgenic organ. A further factor for relative hyperandrogenicity in menopause is the significant reduction in SHBG levels resulting in increased serum androgen availability (Burger, 2002b).

1.8.2 Androgen receptor (AR)

1.8.2.1 Androgen receptor structure

The androgen receptor is a member of the super-family of ligand-activated nuclear hormone receptors (Mooradian et al., 1987). Other members of this superfamily are glucocorticoid receptors (GR), mineralocorticoid receptors (MR), progesterone receptors (PR) and oestrogen receptors α (ERα) and β (ERβ).
Two AR isoforms have been identified, the shorted AR-A (87 kDa), and the full length AR-B (110 kDa) (Wilson and McPhaul, 1994, Quigley et al., 1995, Li and Al-Azzawi, 2009).
The AR gene is located on the X chromosome, in the peri-centrometric region at Xq11-12 (Aimes and Quigley, 1995). It contains eight coding exons, encoding for the different functional protein domains. Those domains are the N-terminal regulatory domain (NTD), a DNA binding domain (DBD), a small hinge region and a ligand binding domain (LBD) (Li and Al-Azzawi, 2009).
The AR exerts its function stimulating target genes after ligand binding (Quigley et al., 1995).

1.8.2.2 Molecular mechanisms of androgen action

The native ligands for the AR, testosterone and its more potent metabolite 5α-dihydrotestosterone (DHT), both induce a conformational change upon binding, promoted by the dissociation of heatshock proteins. This allows the relocation of the receptor from the cytoplasm into the nucleus where it undergoes phosphorylation and dimerization. These modifications enable the binding of the AR to specific DNA sequences, termed androgen-response elements (ARE), which regulate the transcription of specific androgen-responsive genes (Quigley et al., 1995, Li and Al-Azzawi, 2009).

Ligand selectivity and DNA-binding capacity of AR is regulated by co-regulators, three of which have been identified. The first group directly regulates transcriptional control through physical interaction with general transcription factors and RNA polymerase II, whereas the other two groups either modify histones covalently or act through chromatin remodelling.

Androgens may also indirectly influence expression of genes without ARE interaction, using second messenger cascades such as activation of protein kinase A (PKA), protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) (Li and Al-Azzawi, 2009).

1.8.3 Androgen receptor signalling in ovarian cancer

Although androgen signalling and phenotypic effects of ovarian cancer are poorly understood, several mechanisms involved in the AR signalling in ovarian cancers have been identified (see *Figure* **1-4**).

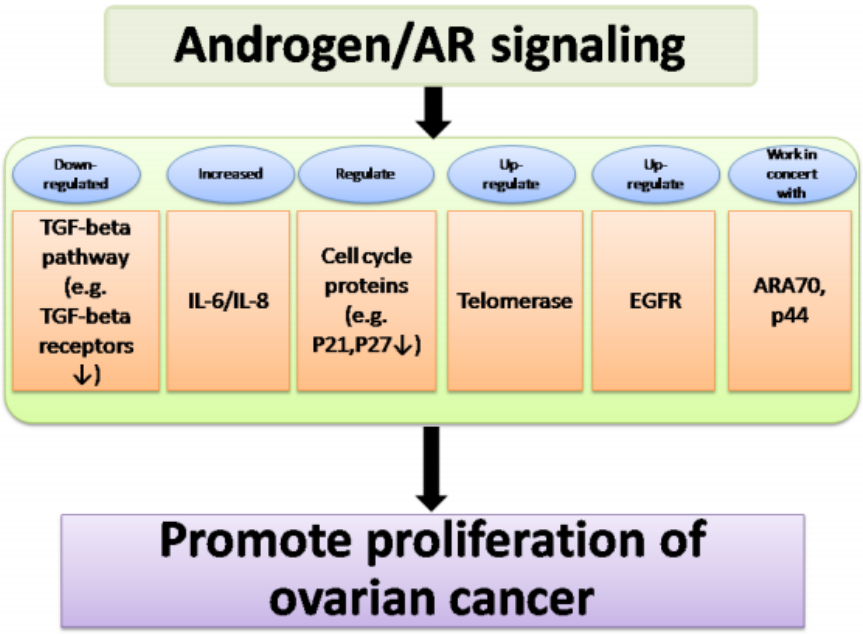


Figure 1-4: Schematic illustration of pathways influencing the AR and androgen signalling in ovarian cancer, adapted from Zhu et al. (Zhu et al., 2017)

1.8.3.1 Nuclear receptor co-activators

Nuclear receptor co-activators interact with steroid hormone receptors and enhance transcription.

Androgen receptor-associated protein 70 (ARA70) is an androgen receptor coactivator that enhances the transactivation potential of the androgen receptor up to ten-fold. Shaw *et al* have shown that though ARA 70 was only expressed in some normal surface epithelial cells or in inclusion cysts, ARA70 mRNA was detected in 85% of the ovarian carcinomas examined (Shaw et al., 2001).

A nuclear co-factor called "amplified by breast 1" (AIB1), was found to be amplified in 25% of EOCs and is associated with ER positivity (Tanner et al., 2000).

Both of these AR-associated proteins, AIB1 and ARA70 were found to be overexpressed in three breast-cancer cell lines and one ovarian cancer cell line (Anzick et al., 1997).

1.8.3.2 TGF-β

Transforming growth factor β (TGF-β) is known to be a potent inhibitor of proliferation in epithelial cells, thought the exact mechanisms of signal transduction are poorly understood (Havrilesky et al., 1995).

Involvement of TGF-β in ovarian carcinogenesis has been postulated and is supported by observations of TGF-β growth inhibition in malignant and non-malignant ovarian epithelial cancer cell lines (Evangelou et al., 2000) as well as primary epithelial cell cultures derived from patient's ascites (Havrilesky et al., 1995). Androgen treatment promotes the downregulation of TGF receptor expression, which leads to a loss of TGF-promoted growth inhibition on epithelial cells (Kohan-Ivani et al., 2016, Evangelou et al., 2000).

1.8.3.3 Epidermal growth factor receptor (EGFR)

Epidermal growth factor is a tyrosine kinase receptor, with considerable influence in signalling cascades governing cell growth and survival (Hudson et al., 2009), and as such, has been considered for investigation in some cancers.

One such cancer is prostate cancer where evidence of a potential a cross-talk between EGFR and AR (Traish and Morgentaler, 2009) has been suggested, supporting the consideration of EGFR as a therapeutic target for some human cancers (Okamoto et al., 2010).

With regard to epithelial ovarian cancer EGFR expression has been highly variable, with a range of expression between 10-70% (Hudson et al., 2009, Gui and Shen, 2012), however, a subset of EOC, namely those expressing AR have been shown to have higher EGFR expression than those not expressing AR (Ilekis et al., 1997).

1.8.3.4 Gene polymorphisms influencing androgen pathways

Gene polymorphisms can influence receptor signalling. Polymorphisms of CAG repeats and CYP17 have been suggested to interfere with AR signalling.

1.8.3.4.1 CAG repeats

CAG is a trinucleotide repeat on exon one of the AR gene with a normal variation of 8-31 repeats (Giovannucci et al., 1997). It is known from prostate cancer, a cancer which is dependent on AR signalling, that a change in number of CAG repeats influences the signalling. In vitro studies have shown that larger CAG repeats decrease the AR transcriptional activity (Chamberlain et al., 1994) and shorter CAG repeats relate to increased risk and aggressiveness for prostate cancer (Rodriguez-Gonzalez et al., 2009).

Studies investigating CAG repeats in ovarian cancer however, have shown conflicting results, with some studies reporting longer CAG repeats decrease the risk for ovarian cancer (Zhu et al., 2016, Meng et al., 2016) and its recurrence (Ludwig et al., 2009), whereas others associate an increased risk of ovarian cancer with greater number of CAG repeats (Terry et al., 2005). It has also been reported that shorter CAG repeats in women with EOC correlate with a significantly shorter overall survival time (Li et al., 2003, Meng et al., 2016). On contrary, in a recent meta-analysis Deng *et al* have not found an association in CAG length and ovarian cancer risk (Deng et al., 2017).

1.8.3.4.2 CYP17 A1 Polymorphisms

Though the influence on the AR and biological importance of polymorphisms of cytochrome P450 17A1 is not clear, they have been described to increase the risk for ovarian cancer by 1,8 fold (Garner et al., 2002).

1.8.4 Androgen Receptor expression in ovarian cancer

AR are known to be expressed by normal ovarian surface epithelium and by the fallopian tube (Edmondson et al., 2002) in addition to epithelial ovarian cancers (Lee et al., 2005, Nodin et al., 2010, Sheach et al., 2009).

Compared to other steroid hormones in ovarian surface epithelial cells, AR is the predominantly expressed steroid hormone receptor (90%) compared to oestrogen (55%)- and progesterone (52%) receptors (Lee et al., 2005).

This expression, however, is highly variable, with a different mean level of AR expression for each of the OEC histological subtypes, where the highest expression of AR is found in serous cancers (Pardridge, 1986) (Mason et al., 1994). However, despite this finding, studies have found no correlation between hormone receptor expression and disease stage (Lee et al., 2005).

Mendez et al compared the AR expression in pre- and postmenopausal women with concomitant pathology including cervical cancer, endometrioid adenocarcinoma and fibroids. In this study, no difference was seen when comparing the AR in view of the menopausal status, but women with cervical cancer showed higher AR expression in the ovary (Mendez et al., 2013).

At least one investigation using AR expression status as a prognostic factor have shown AR expression in serous EOC to be associated with prolonged disease free survival (Nodin et al., 2010), however, other studies have not be able to confirm this effect (Lee et al., 2005).

The AR was also implied to be related to improved 5 year survival if expressed in cancers also expressing the progesterone receptors (Jonsson et al., 2015).

AR expression might also be considered for the optimal timing of anti-androgen therapy, as the comparison or the AR expression in paired samples on immunohistochemistry before and after chemotherapy, samples post chemotherapy showed a lower expression of the androgen receptor, but no change in oestrogen or progesterone receptors (Elattar et al., 2012). Moreover, another study group found a decrease of 41% in the AR expression in recurrent ovarian cancer when compared with the paired sample at primary presentation (Feng et al., 2017).

1.8.5 Androgen receptor expression in ovarian cancer cell lines

Both, normal ovarian surface epithelium and ovarian cancer cell lines, have been shown to not only express the AR, but also be androgen responsive (Edmondson et al., 2002, Lee et al., 2005, Hamilton et al., 1983).

The OVCAR3 cell line is the most characterised ovarian cancer cell line regarding AR expression, where it is predominantly expressed in the nucleus, in keeping with its functional role (Sheach et al., 2009). This functionality of the AR in the OVCAR3 cell line was demonstrated by the proliferative effect of dihydrotestosterone (DHT) stimulation that could be inhibited using the AR-specific antagonist, casodex. (Sheach et al., 2009).

Evangelou *et al* postulated that the growth-stimulatory effect of androgen was mediated through decreased expression of transforming growth factor (TGF)-β receptors (TGF β) (Evangelou et al., 2003), an important cytokine in the mediation of growth in both malignant and non-malignant ovarian epithelial cells (Mason et al., 1994).

There is also the suggestion that the AR plays a role in cell survival within ovarian epithelial cells, with the administration of androgen being shown to inhibit cell apoptosis (Kang et al., 2000, Hamilton et al., 1983).

With increased cell proliferation and decrease in cell apoptosis being the hall marks of carcinogenesis, the above findings strengthen the hypothesis that androgen signalling has a significant involvement in ovarian cancer.

1.8.6 Androgen receptor expression data in primary cell cultures

Primary cultures, derived from ascites of patients with ovarian cancer, do express AR. Of the two isoforms identified on protein level, the shorter AR isoform was expressed in 75%, and the longer isoform in 100% of the primary cultures (Elattar et al., 2012).

DHT stimulation of the primary cultures led to an increase in percentage of cells in S-phase of the cell cycle in 54% of primary cell cultures stimulated (Elattar, 2010). Furthermore, in primary cell cultures showing an increase in S-phase post androgen treatment a significant correlation with AR protein expression on immunohistochemistry (IHC) was confirmed (Elattar et al., 2012). If these findings could be reproduced in larger numbers, IHC might be one way of patient stratification for anti-androgen therapy trials.

1.8.7 Epidemiologic evidence

As previously states, relative hyperandrogenic conditions such as polycystic ovary syndrome (PCOS), high body mass index (BMI), acne and hirsutism have been implicated as risk factors for ovarian cancer (Schildkraut et al., 2002, Parker and Folsom, 2003, Huang et al., 1997).

A study measuring androstenedione and dehydro-epiandrosterone in women found higher levels of these androgen-precursors in women who were subsequently diagnosed with ovarian cancer, compared to controls in dependent menopausal status (Helzlsouer et al., 1995).

Aiman *et al* confirmed increased androgen levels in ovarian veins draining the tumour in comparison with the androgen levels from the contra-lateral, non-malignant ovary in pre-and postmenopausal women (Aiman et al., 1986) and hence suggested the association of androgens and ovarian malignancy in the 1980s (Helzlsouer et al., 1995, Pardridge, 1986).

A further small study in a cohort of five postmenopausal women with ovarian malignancy measured reduced levels of the follicle stimulating hormone (FSH) and luteinizing hormone (LH) compared with levels of healthy controls (Mason et al., 1994).

In the same cohort of women, gonadotropin levels in the vein draining the tumour were lower than compared to FSH/ LH levels in peripheral blood (Mason et al., 1994).

This evidence, when combined with the negative feedback mechanism that steroids have on gonadotropins and the presence of elevated levels of oestrogen, progesterone and testosterone – where they were suggested as possible tumour markers – in the same women (Mason et al., 1994), suggests that ovarian tumours could be secreting steroid hormones.

This idea of ovarian tumours secreting steroid hormones has been confirmed by a different study that detected steroid hormone release from epithelial ovarian tumours themselves. However, testosterone was released in larger quantities from benign than malignant tumours or normal ovaries, whereas malignant tumours released more of the precursor androstenedione than either benign growths or normal ovaries (Hamilton et al., 1983), suggesting level of complexity with regard to steroid hormone secretion.

More recently, high serum levels of unbound testosterone have been shown to have a positive association with ovarian cancer risk in women below the age of 55. In women above this age, neither free testosterone nor SHBG have shown positive correlations to the ovarian cancer risk (Helzlsouer et al., 1995).

1.8.8 Anti-Androgen therapy in ovarian cancer

Taken together, the evidence outlined above suggests androgens may have a role in the aetiology of epithelial ovarian cancer. As recurrence rates in ovarian cancer are high despite aggressive treatment with surgery and platinum-based chemotherapy, hormonal treatment with anti-androgens is a promising area for further treatment options.

To date four phase II trials evaluating the response of ovarian cancer to treatment with anti-androgens as single therapy have been concluded (see *Table* **1-4**).

Author	Medication used	FIGO stage	Inclusion criteria	n	CR (%)	PR (%)	SD (%)
Thompson (1991)	Cyproterone acetate 100 mg, PO, TDS	I-1 II-6 III-38 IV-9 Unknown-8	Relapsed platinum resistant disease	56	0	7% (4 pts)	14% (8 pts)
			Too frail for chemotherapy	6			
Tumolo (1994)	Flutamide, 750 mg, PO, daily	III + IV	Platinum resistant disease	32	3% (1 pt)	3% (1 pt)	28% (9pts)
Vassilomanolakis (1997)	Flutamide, 100 mg, PO, TDS	1 pt unclassified III + IV	Progressive chemotherapy resistant disease.	23	0	4% (1 pt)	9% (2 pts)
Van der Vange (1995)	Flutamide, dose not specified	I, III, IV	Progressive chemotherapy resistant disease	12	0	0	7 % 1 pt
List of abbreviations: PO= oral administration, TDS=three times per day, n= number of patients include, CR= complete response, PR= partial response, SD= stable disease, PD= progressive disease, pts = patients, PFS = progression free survival, NA= not applicable							

Table 1-4: Summary of clinical trials using anti -androgens

Three of these trials have used flutamide, a competitive, non-steroidal anti-androgen (Thompson et al., 1991, Tumolo et al., 1994). The third trial used cyproterone acetate, a synthetic steroidal anti-androgen, which exerts its effects through competitive antagonism of the androgen receptor as well as the inhibition of enzymes in the androgen biosynthesis pathway (Vassilomanolakis et al., 1997).

Van der Vange *et al* have undertaken a trial testing four different hormonal agents. 12 of the patients were treated with Flutamide. One of these patients showed stabilization for eight months (van der Vange et al., 1995).

A further trial testing anti-androgen therapy was aiming to inhibit the androgen axis by using a combination of bicalutamide, a non-steroidal anti-androgen and goserelin, a GnRH analogue (Levine et al., 2007).

In summary the response rates of ovarian cancer to anti-androgens were low in all three studies, achieving complete response in only one patient, lasting 44 weeks.

Overall, it is difficult to reliably compare the response of ovarian cancers to anti-androgens in these trials due to a combination of factors, chief amongst these being inclusion criteria, agents used, dosages and cohort size.

Despite the lack of total success across the four studies, a partial response was achieved for 3-7% of the cases, with highly variable duration ranging from 10 to 72 weeks.

Percentages for stable disease lie between 9 and 28%, with a progression- free intervals of 2-11 months, depending on the study.

Overall, the patients included were heavily pre-treated with chemotherapy, allowing for two or more chemotherapy regimens in the Vassilomanolakis study. Tumolo *et al*, who had the only patient with a complete response, have not outlined pre-treatment of FIGO classification of the responding patient (Tumolo et al., 1994)

A difference in patient recruitment was also found in Thompson *et al's* trial, who had the highest partial response rate at 4 %. Whereas the other two trials collectively included patients with progressive disease following previous surgery and at least one cycle on platinum-based chemotherapy, Thompson *et al* also included patients considered too frail for platinum-based chemotherapy. Two of the four patients had never received platinum-based chemotherapy, however they had progressed on Chlorambucil, whilst the remaining two patients showing a partial response had previously experienced complete remission on Cisplatin for 5 and 2.5 years, respectively. The interval of partial response in these two patients was 17 and 18 months compared to 2.5 and 3 months in the two patients without platinum-based therapy.

The two patients with response to anti-androgen therapy in the Vassilomanolakis study were patients who had received one regimen of chemotherapy only. This could be put into context with Elattar *et al's* findings, where decreased AR expression post platinum-based chemotherapy in paired samples was seen (n=29) (Elattar et al., 2012). These results suggest that patients might get a higher therapeutic effect from administration of anti-androgen therapy prior to platinum-based chemotherapy due to higher AR expression. This suggest that refined patient selection could improve response to anti-androgen therapy in ovarian cancer. In order to support this, AR expression status would be helpful, but was not determined in any of the four studies.

Levine's study, using a combination treatment of anti-androgens and GnRH analogues, included patients in complete remission post platinum-based chemotherapy. Some patients were heavily pre-treated, with 8% having received one chemotherapy regimen only, 60% two, 28% three and 6% four regimens respectively (Levine et al., 2007).

A limitation of this approach is that the use of a combination treatment to inhibit the androgen axis does not allow conclusions as to which individual treatment is more beneficial, with the effect of both the AR and GnRH treatments decreasing the release of LH and FSH, and suppression of ovarian androgen and oestrogen secretion. Using an endpoint of progression-free survival, the results are promising with 21 months for patients in their first remission, and 11 months for patients in their second, third and fourth remission, however, they cannot be directly compared to outcomes of the other three studies.

Overall, the use of anti-androgen therapy in ovarian cancer shows only moderate response, but this fails to account for the fact that all the patients were heavily pre-treated with chemotherapy, likely reducing their tumour steroid hormone receptor expression, and had a poor prognosis. As such it is possible that an improved selection stratified using AR status would benefit some patients with a therapy that is non-toxic and generally well tolerated.

The key challenge to using AR targeted therapies is to identify the potential beneficiaries, as the previously mentioned studies have identified a small cohort with the potential to respond to androgen-based treatments despite the limitations of each. Timing of anti-androgen therapy might also be crucial and further trials would be needed as to whether it might be useful as treatment of ovarian cancer.

The most recent trial attempting to answer this question is currently ongoing in the United States with expectant completion in 2019. This phase II trial (NCT01974765) is designed to examine the effect of the anti-androgen enzalutamide on women with AR receptor positive malignant disease, including ovarian, primary peritoneal or fallopian tube cancer. It is aimed at patients who had previously been treated with at least one cycle of platinum-containing chemotherapy regimen. The primary outcome measures are complete or partial response to treatment at six months and secondary outcomes will evaluate adverse events and AR positivity on IHC (ClinicalTrials.Gov, 2018).

1.9 GTPases

GTPases, or G-proteins (Guanine nucleotide-binding proteins), are a superfamily of proteins involved in signal transduction that transduce extracellular signals into intracellular changes via secondary messenger cascades predominantly involving transmembrane-domain G-protein-coupled receptors (GPCRs), but can also utilise non- GPCR receptors (Patel, 2004) or signalling events that do not employ transmembrane receptors at all (Hampoelz and Knoblich, 2004).

Two groups of G-proteins can be identified: the larger heterotrimeric forms, and smaller monomeric form. The large GTPases, consisting of three subunits, are located close to the cell surface adjacent to the GPCR. Upon ligand binding, the inactive guanosine diphosphate on the α subunit (GDP) is phosphorylated to the activated guanosine triphosphate (GTP) (Oldham and Hamm, 2006), promoting phosphorylation of downstream proteins that have effects on cell differentiation, proliferation and survival, gene expression, and signal transduction, in addition to vesicular trafficking and movement (Vigil et al., 2010).

The small, monomeric GTPases, comprise the Ras superfamily of small GTPases and consist of over 150 members (Vigil et al., 2010, Konstantinopoulos et al., 2007) divided into five subgroups: Rho, Ras, Rab, Arf and Rab. (Vigil et al., 2010, Etienne-Manneville and Hall, 2002, Pfeffer and Aivazian, 2004, Rocks et al., 2006, Quimby and Dasso, 2003).

The significance of these proteins in cancer have been partially investigated, with Ras genes being found to possess oncogenic potential when mutated. Ras gene mutations can be found in adenocarcinomas of the pancreas (90%), the colon (50%), and the lung (30%), as well as thyroid tumours (50%) and myeloid leukaemia (30%) (Bos, 1989).

1.9.1 Rab subfamily

The Rab family is the biggest of the small GTPases with approximately 60 human Rab genes being encoded in the human genome and additional Rab proteins being generated by alternative splicing. Through regulation of endocytic trafficking, they interact with multiple signalling pathways that are involved in cell proliferation, apoptosis and migration (Konstantinopoulos et al., 2007).

Dysfunction of Rab GTPases has been associated with diseases including Griscelli syndrome type 2, retinal degeneration, Hermansky-Pudlak syndrome and X-linked non-specific mental retardation, and two of the Rab GTPase family – Rab25 and Rab35 – have both been associated with ovarian cancer (Cheng et al., 2005a).

1.9.1.1 Rab25

Rab25 is expressed in epithelial cells (Goldenring et al., 1993, Calhoun et al., 1998) and is involved in vesicular transport.

Rab25 is located on chromosome 1q22, a region associated with amplification in a range of cancers including, potentially. 54% of epithelial ovarian cancers and 47% breast cancers, suggesting that genes encoded within this region could be involved in pathogenesis of ovarian and epithelial cancer (Cheng et al., 2004).

In ovarian cancer patients with amplified 1q22 a decrease in disease-free interval post treatment was observed, suggesting that genes in this region are potentially oncogenes for ovarian cancer and an indicator for the aggressiveness of the disease (Cheng et al., 2004, Schaner et al., 2003).

This is further supported by examination of knockdown of Rab25 in mice, which has shown to reduce Rab25 expression, decreasing cell proliferation and increasing apoptosis (Fan et al., 2006). The underlying mechanism is unclear, but does not seem to be due to Rab25 mutation (Cheng et al., 2005a).

mRNA overexpression of Rab25 was also seen in testicular germ cell tumours (Korkola et al., 2008) transitional cell tumours of the bladder and prostate cancer (Calvo et al., 2002). Of the expressed genes, Rab25 was significantly increased at mRNA level by 89% in ovarian cancer, and DNA was also amplified.

Therefore, specific down-regulation of Rab25 might be a potential therapeutic strategy against human ovarian cancer.

Rab25 has also been found to be related to cisplatin resistance as it is overexpressed in the cisplatin resistant ovarian cancer cell line SKOV3, and cisplatin sensitivity was increased when silencing Rab25 in SKOV3 cells (Fan et al., 2015).

1.9.1.2 Rab35

Rab35, also known as Rab1c or Ray (Abe et al., 2006) has an essential role in cell cytokinesis (Kouranti et al., 2006, Jean and Kiger, 2012) and regulation of cell recycling pathways. (Patino-Lopez et al., 2008, Jean and Kiger, 2012).
Potential oncogenic effects were found in mouse cell lines, leading to neurite outgrowth (Chevallier et al., 2009). In the human, interactions of Rab35 with p53-related protein kinase (PRPK) with subsequent transcription suppression were suggested (Abe et al., 2006).

Rab35 in ovarian cancer is poorly described and has mainly been investigated by the Newcastle ovarian cancer group as outlined below.
Microarray studies investigating the acute response of the ovarian cancer cell line OVCAR3 to dihydrotestosterone (DHT) stimulation have shown an upregulation of G-proteins of which Rab35 was the most up-regulated gene of 138 upregulated genes, even including Rab25- a result that was validated with qRT-PCR. The marginal upregulation of Rab25 was surprising as androgen response elements have been described in the Rab25 gene, which would suggest an upregulation as response to androgen exposure. It was hypothesised that this marginal response could have been caused through an indirect mechanism, such as TGFβ receptors which have been found to have inhibitory effect on EOC (Evangelou et al., 2000).
Further examination of Rab35 protein expression using IHC confirmed the presence of Rab35 protein in 95% of the examined ovarian cancer specimens and showed a positive correlation with AR protein expression in the same patient cohort (Sheach et al., 2009).
These findings were further supported by an independent investigation showing Rab35 gene up-regulation after DHT stimulation in OVCAR3 cells. As the up-regulation occurred two hours after DHT treatment, the change was deemed to be likely due to the direct effect of DHT

on the AR via AR-dependent pathways. Moreover, this effect was abrogated after AR silencing (SooHoo, 2013).

This upregulation of Rab35 gene was also extended by Rab35 protein expression, with the results confirmed by Western blotting (SooHoo, 2013).

Upon investigation of the phenotypic effects of Rab35, the silencing of Rab35 resulted in a significant reduction in S-phase of OVCAR3 cells and decreased proliferation in addition to increased cell apoptosis. However, no effect on cell migration was detected via wound healing assay when Rab35 was silenced.

In view of the above it was hypothesised that Rab35 gene over-expression could be associated with increased cell proliferation and decreased cell apoptosis, suggesting that Rab35 has oncogenic potential (SooHoo, 2013).

To determine whether the observed phenotypic effects were androgen dependent, the experiments were repeated in different conditions. Whereas S-phase changes and cell proliferation did not differ in full medium, basal medium or after stimulation with DHT, cell apoptosis was found to be potentially androgen dependent (SooHoo, 2013).

Following this, a panel of 14 primary ovarian cultures derived from ascites of ovarian cancer patients were examined for AR and Rab35 expression using qRT-PCR. AR and Rab35 expression were shown at the mRNA level in all 14 cultures, however widely varying levels were observed. Positive correlation for AR and Rab35 was shown in all primary cultures (SooHoo, 2013).

These findings support the hypothesis that Rab35 is an androgen-regulated gene and could be potentially be a biomarker for androgen expressing and regulated epithelial ovarian cancer.

This study aims to further investigate the expression of Rab35 in primary ovarian cancer cells and to assess if Rab35 could be confirmed as androgen-dependent downstream product of the AR.

1.10 Cytochrome P450 17α (CYP17)

A different approach in treating AR dependent cancers other than direct receptor inhibition is targeting the hypothalamic-pituitary-androgen axis with antagonists such as cytochrome P450 17α (CYP17) inhibitors (Papadatos-Pastos et al., 2011).

CYP17 is a key enzyme in the generation of androgens and oestrogens, encoded by a single gene on chromosome 10 (Picado-Leonard and Miller, 1987). It is located in the endoplasmic reticulum in Leydig cells, adrenal glands and the ovaries (Ang et al., 2009).

In the ovaries it is located in the theca interna (Murayama et al., 2012), which is the inner layer coating the ovarian follicles and responsible for the production of androstenedione.

CYP17 catalyses two independent steroid reactions in the androgen biosynthesis pathway, involving the 17α-hydroxylase which converts pregnenolone to 17α-hydroxypregnenolone and progesterone and the C17, 20-lyase which converts 17α-hydroxypregnenolone to dehydroepiandrosterone (DHEA) (Miller et al., 1997). Ultimately, CYP17 is essential for the synthesis of testosterone and DHT from cholesterol.

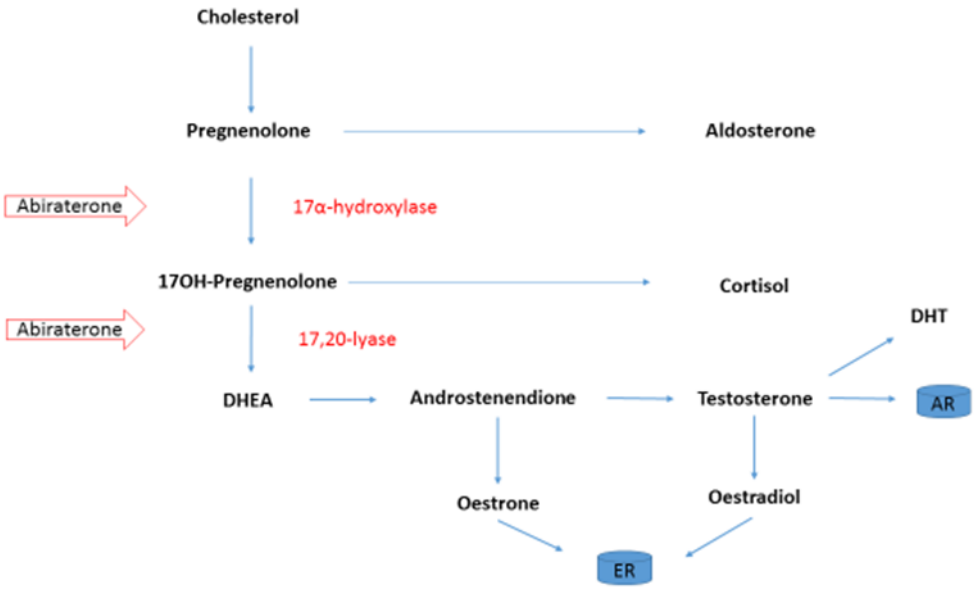


Figure 1-5:Steroid synthesis pathway. Adapted from Ang et al (Ang et al., 2009). DHEA= dihydroepiandrosterone, DHT= dihydrotestosterone, AR= androgen receptor, ER= oestrogen receptor.

1.10.1 Congenital 17-hydroxylase/ 17,20 deficiency

17-hydroxylase and 17, 20-lyase deficiency interferes with cortisol, testosterone and oestradiol production which, in affected females presents with sexual infantilism whereas males will have female external genitalia (Biglieri et al., 1966, Yanase, 1995).

The synthesis of corticosterone however, is preserved. As corticosterone is only a weak glucocorticoid, in treatment with CYP17 antagonists the reactive raised excretion of adrenocorticotropic hormone (ACTH) excretion is needed to reach a new equilibrium. This leads to secondary mineralocorticoid excess and clinical symptoms such as fluid overload, hypertension and hypokalaemia which can be treated with mineralocorticoid antagonists (Ang et al., 2009, Attard et al., 2005).

1.10.2 CYP17 and ovarian cancer

To date CYP17 involvement in the aetiology of ovarian cancer has not been well studied. Most of the evidence of CYP17 being linked to ovarian cancer is based on gene polymorphisms that influence receptor signalling.

One polymorphism in CYP17, known as A2, is a single base substitution of Thymidine to cytosine in the 5'promotor region creating a modified promoter sequence site (Spurdle et al., 2000) and introducing a new restriction site for MspA1 (Haiman et al., 1999).

This variant of the A2 CYP17 polymorphism has been hypothesized to alter the promoter activity, possibly increasing CYP17 transcription and theoretically increasing oestrogen or androgen production. It has extensively been examined in steroid hormone dependent breast cancer (Dunning et al., 2004). Women with breast cancer who are homozygous for the A2 allele of CYP17 have been found to have increased steroid hormone levels, amongst them testosterone, androstenedione and dehydroepiandrosterone, but the presence the A2 allele alone cannot be used as an independent risk factor for breast cancer (Dunning et al., 2004)

The A2 CYP17 polymorphism has also been associated with polycystic ovarian syndrome (PCOS) (Carey et al., 1994), a condition with dysfunctional ovarian steroidogenesis, elevated androgen levels and potential ovarian cancer malignancy risk. Though this mutation alone cannot explain the aetiology of the disease, individuals with an A2 allele in CYP17 may be affected by an alteration of the phenotype with a more severe clinical picture that unaffected controls (Carey et al., 1994). An increase in death hazard rate of 30% has been suggested in ovarian cancer in women with CYP17 polymorphisms (Nagle et al., 2007).

The overall risk for ovarian cancer for women with the CYP17 polymorphism was deemed to be increased for both, heterozygotes and homozygotes for the A2 variant by Garner *et al* (Garner et al., 2002). In their study the polymorphism increased the risk for developing EOC by a factor of 1.86, without putting homozygotes at higher risk when compared to heterozygotes. These date could not be confirmed by a large Australian study, examining 319 ovarian cancer patients (Spurdle et al., 2000).

The biological importance of CYP17 polymorphisms in combination with AR signalling is unclear, as its presence is not always associated with higher levels of circulating androgens (Dunning et al., 2004).

1.10.3 Abiraterone (CB7598)

1.10.3.1 Abiraterone in Vitro

The blockade of CYP17 suppresses the synthesis of androgens and oestrogens in adrenals and potentially androgen-expressing tumours (Stein et al., 2012, Ang et al., 2009) and has so far shown promising results in clinical response in castrate resistant prostate cancer (CRPC).

The molecular mechanisms are not fully understood, but suggest a con-comittant direct anti-androgenic effect. Treatment of bovine theca cells with abiraterone has shown a decrease in androgen secretion (Glister et al., 2013).

In the human AR dependent prostate cancer cell line LNCaP treatment with abiraterone led to a dose dependant decrease in AR expression at the protein level (Soifer et al., 2012). The same study suggests that abiraterone treatment deceases androgen-induced AR transcription (Soifer et al., 2012). The decrease in AR transcription was observed with abiraterone treatment alone, but was more pronounced with the compound TOK-001, a steroid used in castration resistant prostate cancer acting as both, AR receptor antagonist and CYP17 inhibitor (Soifer et al., 2012).

Results from Richards *et al* demonstrated the downregulation of AR regulated genes (PSA and TMPRSS2) in LNCaP cells after stimulation with DHT and treatment with abiraterone. This would suggest abiraterone to not only have enzymatic inhibition properties, but also an element of direct AR antagonism (Richards et al., 2012).

1.10.3.2 Clinical data on Abiraterone

Abiraterone is a potent, selective and irreversible inhibitor of CYP17 (Rowlands et al., 1995, Chan et al., 1996, Attard et al., 2008) and was developed as part of a series of potent, inhibitory steroids in order to treat castrate resistant prostate cancer (Barrie et al., 1994, Potter et al., 1995,

Rowlands et al., 1995). For oral use, its prodrug, abiraterone acetate (CB7630) is used due to its good bioavailability (Ang et al., 2009, Attard et al., 2008) . Abiraterone treatment is generally well tolerated (de Bono et al., 2011) with few side effects. Toxicities are predominantly symptoms of secondary mineralocorticoid excess and can be counteracted with synchronous administration of corticosteroids (Attard et al., 2008).

To date 28 trials are registered in the US, testing CYP17 inhibitors either a s single agent or in conjunction with anti-androgen therapy mainly for castrate resistant prostate cancer, and two phase I/II study in metastatic oestrogen receptor positive metastatic relapsed breast cancer (ClinicalTrials.Gov, 2018).

A recent phase III trial has not only confirmed the efficacy of abiraterone, but also shown increased survival rates in patients with metastatic castration-resistant prostate cancer and previous chemotherapy (de Bono et al., 2011).

For ovarian cancer, there is only one trial investigating the effects of abiraterone conducted in the UK. The CORAL (Cancer of the OvaRy Abiraterone trial) is a phase II trial, investigating the effects of Abiraterone in women with ovarian, fallopian tubal and primary peritoneal cancer with disease relapse within one year of standard therapy as well as aiming to identify biomarkers of abiraterone sensitivity and to evaluate the molecular impact of abiraterone. The trial had found low response rates as only one of the 42 recruited patients responded to abiraterone with a disease- free interval of 47 months. A subgroup of patients had some benefit, as 26% had disease stabilisation after three months and 14% had stable disease six months after treatment (Banerjee et al., 2016).

Hypothesis

- Primary cancer cells would be a potential model to examine AR expression and Rab35 as its downstream transcriptional target.

- Abiraterone could have an inhibitory effect on ovarian cancer cells.

Project Objectives

1. To examine AR and Rab35 expression on primary ovarian cancer cells.
2. To assess which methodology is the most representative to evaluate AR and Rab35 expression.
3. To evaluate if Rab35 could be used as androgen dependent biomarker in primary ovarian cancer cell lines.
4. To examine CYP17 expression in ovarian cell lines and primary cancer cell lines in order assess if CYP17 antagonists would be a target in ovarian cancer.
5. To assess if abiraterone exerts anti-androgenic or inhibitory effects on ovarian cancer.

Project outline

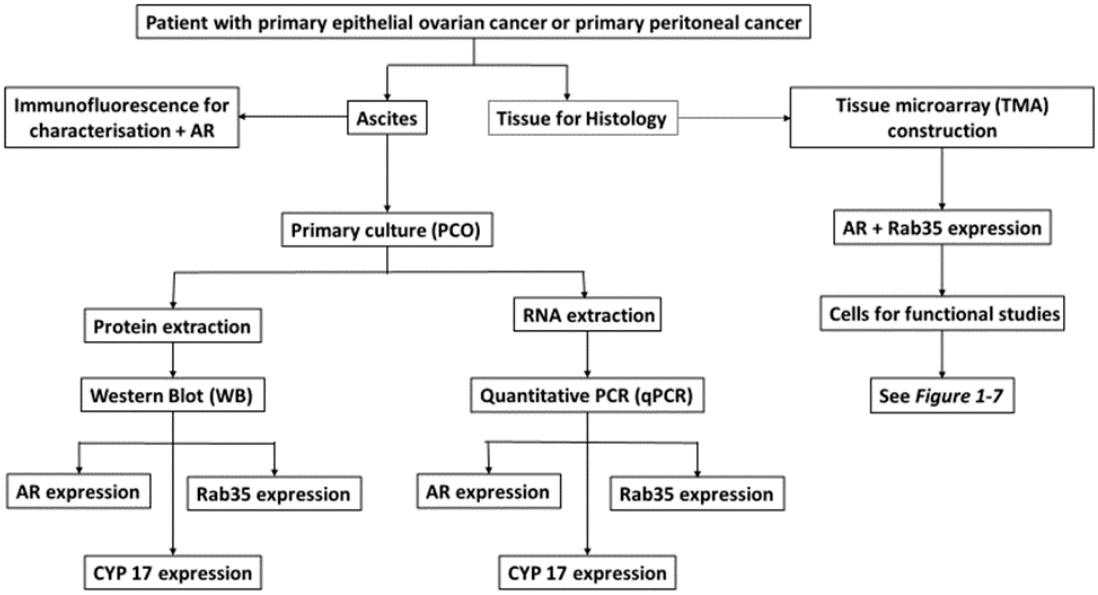


Figure 1-6: Summary of examinations for expression data in PCOs in this project.

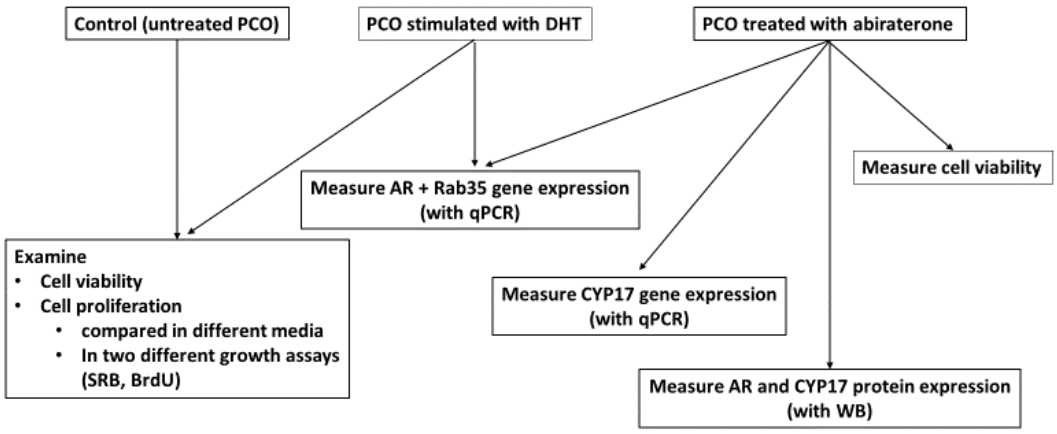


Figure 1-7: Summary of functional studies with PCOs in this project.

2 Materials and Methods

2.1 General Laboratory Practice

All experiments were performed to university standards for safe working with chemical substances in laboratories, which comply with the Control of Substances Hazardous to Health Regulations 2002 (COSHH) and Biological COSHH (BioCOSHH).

2.2 Tissue culture

2.2.1 Cell lines

Experiments were carried out with cell lines listed in *Table* **2-1**. Most cell lines were obtained from the American Type Culture Collection (ATTC©) and tested regularly for mycoplasma with the Mycoalert Mycoplasma detection kit (Cambrex).

Cell line	Derivatives
OVCAR 3	Human ovarian adenocarcinoma, established from malignant ascites after combination chemotherapy with cyclophosphamide, adriamycin, and cisplatin.
LNCaP	Human prostate adenocarcinoma, established from a lymphnode metastasis.
PC3	Human prostatic adenocarcinoma, established from a bone metastasis.
HeLa	Human adenocarcinoma of the cervix.
PEO1	Human poorly differentiated serous adenocarcinoma, derived from ascites, after previous treatment with cisplatin, 5-fluorouracil and chlorambucil. Derived from the same patient as PEO4.
PEO4	Human serous adenocarcinoma, derived from the same patient as PEO1. Derived from ascites collected after the development of resistance to chemotherapy. Oestrogen receptor positive.
PEO14	Human well differentiated serous adenocarcinoma, established from ascites, collected prior to treatment. Oestrogen receptor negative.
IGROV1	Human ovarian adenocarcinoma.
A2780	Human ovarian carcinoma, cisplatin sensitive.
CP70	Human ovarian carcinoma, derived from the A2780 cell line, cisplatin resistant.

Table 2-1: List of cell lines used

2.2.2 Cell culture maintenance

Cell culture of established cell lines and primary ovarian ascitic cancer cells (PCO) was performed with aseptic technique in a containment level II laminar flow microbiological safety cabinet.

Cells were seeded out in tissue culture flasks (Corning-Costar) in full medium. Each cell line or primary culture was handled separately with their own reagent. All Media were stored at 4°C and warmed in a water bath to 37°C prior to use. *Table* **2-2** summarises all media used.

The cells were incubated at 37°C (5% CO_2 and 95% humidified air) and allowed to grow to a confluence of 80% to enable exponential growth. The medium was changed every 3-5 days.

For routine passaging, the medium was aspirated and cells were washed with warm sterile phosphate buffered saline (PBS). Following this, cells were treated with 5 ml 0.25% trypsin-ethylene diamine tetra acetic acid (EDTA) for 5 minutes at 37ºC in order to detach the cells. To neutralise the trypsin, 5 ml of medium were added and the cell suspension was centrifuged for 5 minutes at 1500 rpm. The supernatant was discarded, the cell pellet re-suspended in media and seeded out into flasks as required.

Media	Components
Full medium (FM)	RMPI 1640 with 25mM HEPES modification, with 20 % heat inactivated foetal calf serum (FCS), 20 mM L-glutamine and 1% penicillin and streptomycin (all Sigma-Aldrich)
Serum free medium **(= basal medium, BM)**	RMPI 1640 with 25mM HEPES modification, with 20mM L-glutamine, 1% penicillin and streptomycin and and tissue culture tested bovine serum albumin (250µg/ml) (all Sigma Aldrich)
Freezing medium	RPMI 1640 with 10% FCS and 10% DMSO
Steroid depleted medium (SDM)	RPMI 1640 with 25mM HEPES modification, with 10% DCC treated serum, 20 mM L-glutamine and 1% penicillin and streptomycin (all Sigma-Aldrich)

Table 2-2: List of media

2.2.3 Primary cultures (PCO)

Ethical approval was granted by the local ethics committee (12/NW/0202) and specimens registered in accordance with the Human Tissue Act 2004.

Patients undergoing primary debulking or interval surgery or attending the ward for drainage of ascites at the Queen Elizabeth Hospital, Gateshead were consented for participation in research (consent form see in *Appendix 3*) and the ascitic fluid and ovarian tissue was collected. Transport of the samples from the hospital to the lab was done immediately and in compliance with UK Category B regulations UN3373.

Each sample was registered on the NICR central tissue resource database and given the specific notation "PCO" followed by a serial number to allow identification at a later stage.

Cell culture was performed with aseptic technique in a containment level II laminar flow microbiological safety cabinet.

Several procedures have previously been described for establishing cultures of primary ovarian cancer cells from ascites, all of which involve several steps (Auersperg et al., 1984, Kruk et al., 1990, Hirte et al., 1994). Simpler and reliable culturing methods for epithelial cell cultures have been establishes since, using a 1:1 mixture of medium and ascites (Dunfield et al., 2002, Shepherd et al., 2006). Other contents of ascites, such as red cells, lymphocytes, mesothelial cells, fibroblast-like cells and fatty tissue do not interfere with the growth of epithelial cancer cells and are removed with debris through the media changes.

Using the latter technique, 20 ml of ascitic fluid were transferred into T75 flasks and 20 ml of full medium was added.

Cells were then incubated at 37°C (5% CO_2 and 95% humidified air) and left undisturbed for 3-7 days. Once confluence of 80% was reached, cells were washed with PBS once and routine passaging was performed as described above in the chapter 2.2.2.

Primary cultures from ascites tended to survive until passage two to five.

Morphological features were studies with an inverted microscope (Olympus CK40) at 10x magnification. The images were captured using Visicam software.

2.2.4 Cell culture storage

In order to allow use at a later date, established cell lines and primary cultures were frozen at early passages.

Once sufficiently confluent (80%), cells were washed with PBS once and incubated with trypsin-EDTA for cell detachment. To neutralise the EDTA full medium was added and the suspension was centrifuged at 1500 rpm for 5 minutes. The supernatant was discarded, the cell pellet was resuspended in 1 ml of freezing medium (see *Table* **2-2**) and the cell suspension was stored in a cryovial at -80°C.

2.2.5 Thawing of cell cultures

The cryovial was removed from the -80°C freezer and placed in the incubator for a few minutes, until the suspension was liquefied. Four ml of full medium were added following which the suspension was centrifuged at 1500 rpm for 5 minutes. The cell pellet was then re-suspended in 5 ml of full media and incubated in a T25 flask at 37°C.

2.3 Immunofluorescence

Ascites is often contaminated with red cells, mucin or fatty tissue. In order to confirm the epithelial origin of the obtained PCO samples, pancytokeratin staining was performed. If more than 90% of cells stained positive for pancytokeratin, the sample was considered epithelial, likely ovarian in origin and used for further experiments.

In order to further characterize the epithelial cells and determine their specificity for ovarian cancer, Cancer antigen 125 (CA125) and the epithelial adhesion molecule (EPCaM) were examined with immunofluorescence. Vimentin, a mesenchymal marker, was investigated as a negative control (see *Table* **2-3**).

For the investigation of the whole antibody panel, ascites derived cells were seeded onto a sterilised glass coverslip at a concentration of $5x10^5$ cells/ well and incubated for 24 hours to allow adherence. Cells were fixed with ice cold methanol (-20°C) for at least ten minutes. Rehydration with 2 washes of PBS for 20 minutes performed prior to antibody incubation in the concentration as in *Table* **2-3**. Generally, 100-150 µl of antibody solution were used per slide. Following three washes with PBS, the glass slips were mounted onto a glass slide with 4 µl Vectashield (Vector laboratories) mounting medium, containing 4',6-diamidino-2-phenylindole (DAPI) as nuclear stain.

Cells were then examined with a fluorescence microscope (Leica DMR, Leica Microsystems, UK). Images were captured with the SPOT advanced software and stored as Tiff/bitmap files.

Antibody used	Species	Concentration
Pancytokeratin FITC conjugated (Sigma-Aldrich)	Mouse monoclonal	1:100
CA125 (Abcam)	Mouse monoclonal	1:100
Alexa Fluor 546 (ThermoFisher)	Goat anti mouse IgG	1:1.000
EpCAM (Alexa Fluor® 488 anti-human CD326. Cambridge Bioscience)	Mouse IgG	1:100
Vimentin (Abcam)	Rabbit monoclonal	1:100
AR: N20 (Santa Cruz)	Rabbit polyclonal	1:250
Alexa Fluor 594 (Invitrogen)	Mouse monoclonal	1:100

Table 2-3: Antibodies used for immunofluorescence.

2.3.1 Pancytokeratin and EPCaM

For Pancytokeratin and the epithelial cell adhesion molecule (EPCaM) FITC- conjugated anti-pancytokeratin antibodies were used as described in chapter 6.3 at a concentration of 1:100 (see *Table* **2-3**). 150 µl of the FITC-conjugated antibody was applied for one hour at room temperature after the rehydration. Following this, three further 15 minute washes with PBS were done and the glass slips were mounted onto each glass slide with 4 µl DAPI mounting media.

2.3.2 CA125 and Vimentin

Incubation for the Cancer Antigen 125 (CA 125) and Vimentin with the primary antibody was either done for one hour at room temperature or overnight at 4°C. After three further washes with PBS, cells were incubated with the secondary antibody for one hour at room temperature avoiding light exposure.

Alexa Fluor 546 and Rad51 448 were used for CA125 and Vimentin, respectively. Following three further PBS washes, the coverslips were mounted on DAPI.

2.3.3 Androgen receptor

For the detection of the AR by immunofluorescence (IF), cells were seeded onto 22 x 22 mm coverslips at the desired concentration and left to adhere for 24 hours. After a wash with PBS, cells would be quiesced with serum free (basal) medium for 24 hours and treated with DHT if required.

Fixation was done with 2% paraformaldehyde for at least two hours at 4°C. Cells were washed with PBS for 15 minutes three times and each coverslip was incubated with 250 µl 0.1% Triton X-100 (BDH, Poole, England) dissolved in PBS. Following this, incubation with a block solution made of 0.1% Triton in 1% bovine serum albumin (BSA) was done for 1 hour at room temperature. The primary antibody incubation (see *Table* **2-3**) was performed overnight at 4°C. After three further 15 minute washes with PBS, the secondary antibody (Alexa Fluor 594) was applied for 1 hour at room temperature. After a further 3 PBS washes, the coverslips were mounted on Vectashield ® mounting medium with DAPI (Vector laboratories).
Cells without primary antibody application were used as negative control.

2.4 Proliferation assay

2.4.1 Sulforhodamine B (SRB) assay

The sulforhodamine B assay determines cell density by measuring the cellular protein content. Dye is bound to basic amino acids of cellular proteins, causing a colour change that is quantified by a spectrometer. The SRB assay was used to calculate doubling times of cell lines and primary ovarian cultures.

2.4.1.1 SRB staining

Cell lines were seeded out at the desired density per well in 96 well plates in full medium and incubated for 24 hours to allow cell adherence.
For establishment of doubling times, cells were fixed with 25 µl of 50% trichloracetic acid (TCA) in 24 hourly intervals for 10 days, labelling the first 24 hours after seeding out day 1, 48 hours, day 2 and so on.

For DHT stimulation experiments without quiescing, cells were seeded out in full medium in 96 well plates at desired concentrations and allowed 24 hours to adhere. A separate plate was used for each day and each treatment condition was loaded in six repeats.
Following this, one plate of cells was fixed with 25 µl of 50% TCA on day 0. The remaining cells were washed twice with PBS and treated with full, serum free medium or DHT (10 nM, 50 nM or 100 nM). These plates were fixed at desired time points (day 1, day 2 and so on) and stored at 4°C.

For experiments with DHT stimulation after quiescing, cells were seeded out in 96 well plates in full medium and allowed to adhere for 24 hours. Following this, the cells were washed

with PBS twice and treated with serum-free medium for 24 hours. Thereafter, one plate was fixed on day 0, and the remaining plates were washed twice with PBS and treated with full, serum-free medium or DHT (10 nM, 50 nM, 100 nM).

Fixation with 25 µl of 50% trichloracetic acid (TCA) at the desired times points and storage at 4°C was exactly the same for quiesced and non-quiesced cells.

Cells incubated in full medium were used as positive control and cells in serum-free medium as negative control.

At least an hour after the last fixation, plates were washed with distilled water and dried. Once dry, 100 µl of 0.4% SRB solution (4g SRB in 1 litre of 1% acetic acid) was added per well for 30 minutes at room temperature prior to five washes in 1% acetic acid. Following this wash and after drying, 100 µl of 10mM Tris (pH 10.5) were added to each well to dissolve bound SRB. The absorbance was read at 570nm using a Spectra Max 250 plate reader (Molecular devices).

2.4.1.2 SRB data analysis

Measured absorbance data was saved and analysed with Microsoft Excel software. The mean and standard deviations of the measured absorbance at the determined time points were calculated and displayed as line graphs.

2.4.2 Bromodeoxyuridine (BrdU) ELISA

This enzyme linked immunosorbent assay (ELISA) enables the colorimetric assessment of cell proliferation. Bromodeoxyuridine (BrdU) is incorporated in the DNA of proliferating cells instead of the pyrimidine deoxynucleoside, thymidine. Cells were then fixed and the DNA was denatured in order to allow the antibody binding of the incorporated DNA. An anti-BrdU antibody probe was then allowed to bind the newly synthesised cellular DNA. Formed immune complexes can then undergo a colorimetric reaction and their absorbance was measured with a spectrometer. The amount of colorimetric reaction and absorbance values correlate directly to the DNA synthesis and therefore with the number of proliferating cells.

OVCAR3 cells and primary ovarian culture cells were seeded out in 96 well plates in full medium at $5x10^3$ cells/ well and allowed to adhere for 24 hours. On day two, the cells were washed with PBS twice and quiesced with serum free medium for 24 hours. After two further washes on day three, cells were treated with the desired treatment medium (full medium, 10

nM DHT, 100 nM DHT and serum free medium as negative control). Time points of measuring the absorbance were either at 24 or 96 hours or 10 days after treatment.

The Cell Proliferation ELISA BrdU kit (Roche, 11647229001) was used as per manufacturer's protocol. 10 µl BrdU labelling solution (1:100, diluted with PBS) was added to the medium in each well 24 hours prior to the measuring time point, and cells were re-incubated for a further 24 hours at 37°C. For assessing proliferation at 24 hours, the BrdU labelling solution was added at the time of treatment, for measurement at 96 hours it was added at 72 hours, for 240 hours it was added at 216 hours. At the time of measurement, the medium with the labelling solution was suctioned off and the 96-well plate was dabbed dry. 200 µl of FixDenat solution as per manufacturer's protocol was added and cells were incubated for 30 minutes at room temperature. The fixing and denaturing solution was then removed and cells were treated with 100 µl/ well with anti-BrdU labelling solution (1:100) and incubated at room temperature for 90 minutes. The antibody conjugate was then flicked off and the wells were washed with 200 µl PBS three times for 5 minutes on a shaker. Following this, 100 µl substrate solution / well were added and incubated for 5- 30 minutes until colour developed. At this point 25 µl 1 M sulphuric acid was added as stop solution to each well and the plate was incubated at room temperature for 1 minute on a shaker to allow good mixing. Absorbance was then measured at 450 nM with the Spectra Max 250 plate reader, (Molecular devices). Data were transferred to and analysed with Microsoft Excel.

2.4.3 Cell count

Primary ovarian culture cells were seeded in 12 well plates at a density of 10^5 cells/ well in full medium and let to adhere for 24 hours. After a wash with PBS, they were quiesced with serum free medium for 24 hours. A further wash with PBS was done and cells were incubated with either full medium, 10 nM DHT, 100 nM DHT or serum free medium as negative control. Cells were then trypsinized at 1, 3, 6, 10 days and counted using a haemocytometer. The cell suspension was mixed 1:1 with 0.4% trypan blue, a vital stain, in order to assure that only live cells would be counted. For this cell suspension mix, 10 µl were administered into the counting chamber and the non-stained viable cells were counted with a light microscope. The number of cells counted were multiplied by 10^4 to give the concentration of cells per millilitre.

2.5 Western Blotting

Western blots were performed to examine protein expression of AR and Rab35.

Cancer cell lines (OVCAR3, LNCaP, HeLa) were seeded out in 6 well plates (as in section 6.2.2.) in the desired quantity and incubated for 24 hours. The culture medium was then removed and the cells washed once with PBS. The cells were lysed with 200 µl of SDS lysis buffer (SDS sample buffer with 10% β-mercaptoethanol). The lysates were stored at -20°C. Polyacrylamide gels were set up, comprising a resolving and a stacking gel (see *Table* **2-4**).

	Buffer A (ml)	Buffer B (ml)	Water (ml)	Acrylamide, 30 % (ml)	APS, 10 % (µl)	TEMED (µl)
Resolving gel 10%	5	0	1.66	3.32	100	20
Stacking gel 10%	0	2.5	1.66	0.83	50	10
Resolving gel 15%	5	0	0	5	100	20
Stacking gel 15%	0	2.5	1.25	1.25	50	10

Table 2-4 Polyacrylamide gels.

The lysates were denatured at 100°C for 5 minutes before loading on the acrylamide gel. The gels were resolved in a Protean III Cell System (Bio-Rad). In order to assess the protein size, seablue protein ladders (4 kDa to 250 kDa, Invitrogen) were resolved alongside protein samples (4 kDa to 250 kDa).

For analysis of AR protein expression (110 kDa) in OVCAR3 cells, a 10% resolving acrylamide gel was used and 10µl of protein lysate were loaded into each well. The gels were then electrophoresed at 200 mV for 45 minutes in the reservoir buffer (77.9% glycine, 16.6% tris-base, 5.48% SDS).

For analysis of Rab35 protein expression (25 kDa) in OVCAR3, a 15% acrylamide gel was used and 15µl of protein lysate were loaded into each well. The gels were then electrophoresed at 200 mV for 35 minutes in the reservoir buffer.

Proteins from the gel were transferred onto a nitrocellulose membrane (Hybond C-extra, Amersham, Biosciences) using electrophoresis at 100 mV for one hour in transfer buffer. The membranes were then blocked for one hour in a solution containing 5% non-fat milk powder (Marvel) in tris buffered saline (TBS). Thereafter the membranes were washed twice in TTBS (TBS solution supplemented with 0.05% tween) for 10 minutes. The membranes were treated with primary antibody in diluent (1% Marvel in TTBS) for either one hour at room temperature

or overnight at 4°C. After 2 further 10 minute washes with TTBS, the membranes were then treated with HRP-conjugated secondary antibody in 1% Marvel in TTBS as diluent for one hour at room temperature. All antibodies were used in the concentrations as listed in *Table* **2-5**. Enhanced Chemiluminescence (ECL) (Amersham, Biosciences) was used to detect protein band intensity by developing film (Kodak) on an automated developer (Mediphot 937).

Primary Antibody	Species	Concentration
SC 441 (Santa Cruz)	Mouse monoclonal	1:1000
Androgen receptor (BD Pharmingen)	Mouse monoclonal	1:1000
C19 (Santa Cruz)	Rabbit polyclonal	1:1000
N20 (Santa Cruz)	Rabbit polyclonal	1:1000
Rab35 (Abcam)	Rabbit Polyclonal	1:500
Rab35 (Proteintech group)	Rabbit Polyclonal	1:500
Rab35 (Sigma Aldrich)	Rabbit polyclonal	1:500
CYP 17 (Santa Cruz)	Goat polyclonal	1:100
Alpha tubulin (Sigma Aldrich)	Mouse monoclonal	1:4000
GAPDH (Santa Cruz)	Rabbit polyclonal, HRP conjugated	1:1000

Table 2-5: List of antibodies used for Western blotting.

2.5.1 Protein quantification

Protein quantification was performed using a colorimetric assay (RC DC Protein assay, Bio-Rad) as per manufacturer's protocol.

The DC assay is based on detergent solubilisation following which protein reacts with alkaline copper tartrate solution and Folin agent. In order to ensure compatibility with β-mercaptoethanol it is supplemented with the special reagents to be reducing agent compatible (RC) as well as detergent compatible (DC).

A standard curve of mixtures of stock solution (5mg BSA/ 1 ml SDS sample buffer) and SDS sample buffer was generated using concentrations 2.5, 2. 1.5, 1, 0.75, 0.5, 0.25, 0.125, 0.025 and buffer only.

The lysate samples and standard curve samples were treated the same and after adding the reagents as per protocol, absorbance was read at 655nm.

A correlation coefficient above 0.98 was considered sufficient. The measured concentrations (µg/µl) were converted in volume (µl) of lysate needed for loading of the western blot gels. Western Blots were quantified with densitometry using the Fuji LAS-300 Image Analyser System (Fuji film).

2.6 Quantitative Real Time PCR (qRT-PCR)

2.6.1 RNA extraction and quantification

RNA was extracted with the Qiagen RNeasy©Mini Kit (250) as per manufacturer's protocol and is outlined below.

Cells were either lysed from the frozen cell pellets or directly from 6 well plates.

For cell pellets, cells were seeded out in T75 flasks and passaged as in section number. In order to detach the cells, they were treated with 5 ml 0.25% trypsin-EDTA for 5 minutes at 37°C and spun down at 1500 rpm for 5 minutes. The cell pellet was kept at -80°C until RNA extraction was performed. Prior to RNA extraction the pellet was thawed, then centrifuged and excess fluid aspirated.

If cells were lysed from the 6 well plates, cells were seeded out in 6 well plates, incubated for 24 hours to allow adherence, then washed with PBS once and lysed as per protocol.

Cells were harvested from both, the cell pellets and the cells in the vessel by lysis with 350 µl of RTL buffer (Qiagen) and vortexed for 15 seconds. The lysate was then passed through a 20 gauge needle and 1 ml syringe in order to homogenise and then transferred into Eppendorf tubes. One equal volume (350 µl) of 75 % ethanol was added and the suspension well mixed by pipetting, before being transferred into a spin column within a 2 ml collection tube. The mixture was centrifuged for 15 seconds at 8000 g and the flow-through liquid was discarded. 700 µl Buffer RW1 was added, the mixture centrifuged for 15 seconds at 8000 g and the flow through liquid was discarded. 500 µl Buffer RPE was added twice, first centrifuged for 15 seconds, then for 2 minutes, both times at 8000g. After the last wash, the spin column was transferred into a new 2 ml collection tube and 50 µl diethylpyrocarbonate (DEPC) treated water was added onto the column membrane. The column was then centrifuged for 1 minute at 8000 g in order to elute RNA. As the expected RNA yield was > 30 µg, a repeat elution with another 50 µl of RNAase free water was performed.

The extracted RNA was quantified with the Nanodrop ND-1000 (labtech, International). RNA with a 260/280 O.D. ratio of 1.6 to 1.9 was considered to be of adequate quality and was used for qRT-PCR.

RNA was stored at -20°C up until a week, prior to reverse transcription into complementary DNA (cDNA).

2.6.2 Reverse Transcription

For use in quantitative real-time PCR, RNA was transcribed into double-stranded complementary DNA (cDNA) with the Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV RT) kit (Promega).

1 µg of RNA was made up to a volume of 12.7 µl using DEPC-treated water. This RNA solution was the incubated at 65°C for 5 minutes to remove any secondary structures within the RNA template and then cooled rapidly to 4°C to prevent secondary structures from reforming. To anneal the primer, 7.3 µl of MMLV reverse transcriptase reaction cocktail (see *Table* **2-6**) was added to 12.7 µl RNA and DEPC water mixture. This complete mix of RNA, DEPC-treated water and MMLV reverse transcriptase reaction cocktail (see *Table* **2-6**), was vortexed and incubated at 37°C for 60 minutes. Thereafter, the mixture was incubated at 100°C for 5 minutes to inactivate the reverse transcription enzyme.

The cDNA samples were stored at -20°C for later use in quantitative real-time PCR.

Substance	Volume in µl
5x Moloney Murine Leukaemia Virus (MMLV) RT Buffer	4
4 mM dNTPs	2
Oligo dT15 (50 µM)	1
MMLV Reverse Transcriptase	0.3

Table 2-6: MMLV Reverse Transcriptase Reaction cocktail per reaction

2.6.3 Quantitative Real-Time PCR

Androgen receptor (AR) primers and Rab35 primers were previously designed by the ovarian study group at the NICR using Primer Express 2.0 (Applied Biosystems, Warrington, UK) and purchased from SIGMA. A summary of primer sequences used is listed in *Table* **2-7**. The HPRT1 primers used as a housekeeping control were was also purchased from SIGMA.

Gene examined	Primer sequence
AR (Exon 3)	Fwd: CATGTGGAAGCTGCAAGGTCT
	Rev: TCTGTTTCCCTTCAGCGGC
AR UTR	Fwd: GAGTTCATGGGTGGCAAAG
	Rev: GCAAAGCCTAAAGCCAGA
Rab35	Fwd: CGTGGAAGAGATGTTCAACTG
	Rev: TTCTTTGCTCGGAGGACCAG
HPRT1	Fwd: TTGCTTTCCTTGGTCAGGCA
	Rev: AGCTTGCGACCTTGACCATCT
CYP 17	Fwd: CCGTAAGGGTATCGCCTTCG
	Rev: CCATCCTTGAACAGGGCAAAG

Table 2-7: Primer sequences used for qRT-PCR

These primers were used to analyse mRNA expression through amplification of cDNA using SYBR green. SYBR green is a fluorescent dye that binds to double stranded DNA and upon binding its fluorescence increases more than a thousand fold, emitting more fluorescence with increased DNA yield (Tajadini et al., 2014). The absolute quantification was measured with the ABI 7900 sequence detection system.

Purchased primers were diluted to a concentration of 1 µg/µl and stored at -20°C. For use in qRT-PCR, the primers were used at a dilution of 1:40 with DEPC-treated water.

PCR mastermix was prepared as per protocol (see *Table* **2-8**) and 8 µl of the PCR mastermix and 2 µl of individual cDNA were loaded in each well of a 384 well plate. All samples were loaded in triplicate.

Reagent	Volume in µl
SYBR Green	5
DEPC-treated Water	2.2
Forward Primer	0.4
Reverse Primer	0.4

Table 2-8: qRT-PCR mastermix per reaction.

A standard curve for absolute quantification was generated. This was done through serial dilution of cDNA expressing the gene of interest. The dilutions used were: neat cDNA, 1:10, 1:20, 1:50, 1:100, 1:200. DEPC-treated water was used as negative control and was loaded at 2 µl with 8 µl of PCR mastermix. In order to obtain the relative gene expression of the gene of interest, a housekeeping gene was used. Here the housekeeping gene was HPRT1 (Hypoxanthine-guanine phosphoribosyltransferase 1).

qRT-PCR Reactions were carried out on an ABI Prism 7900 Sequence Detection System platform (Applied Biosystems, Foster City, CA).

The PCR cycle conditions used were 10 minutes at 95°C, followed by 40 cycles of denaturing at 95°C for 15 seconds and annealing plus primer extension at 60°C for 60 seconds.

The obtained data were analysed with SDS 2.3 software (Applied Biosystems).

The standard curve was assessed by correlation coefficient (R^2) value, with the minimum of 0.97 to be considered for analysis.

The data was transferred and interpreted using Microsoft Excel software. The mean and standard deviation of relative gene expression to the housekeeping gene, were calculated from the three replicates in every experiment and the figures were displayed as bar graphs.

Final data were represented the mean of three experimental repeats with the standard error of mean.

CYP17 primers	Primer sequence
Primer set 1 (from (Cai et al., 2011)	Fwd: GCTGACTCTGGCGCACAT
	Rev: TTGAACAGGGCAAAGGTGG
Primer set 2	Fwd: GCTGCTTACCCTAGCTTATTTGT
	Rev: ACCGAATAGATGGGGCCATATTT
Primer set 3	Fwd: TATGGCCCCATCTATTCGGTT
	Rev: GCGATACCCTTACGGTTGTTG
Primer set 4	Fwd: CCGTAAGGGTATCGCCTTCG
	Rev: CCATCCTTGAACAGGGCAAAG

Table 2-9: CYP17 primers used during optimisation. Primer set 4 was used for final evaluation

2.7 Immunohistochemistry

Immunohistochemistry (IHC) on FFPE describes the detection of antigens in biological tissue fixed and embedded in paraffin.

Formalin fixation of tissues can lead to crosslinking of proteins, which might lead to antigens not being available for antibody binding. Heat treatment of formalin fixed tissue sections in retrieval buffer is used to partially reverse the crosslinking in order to increase the binding of the primary antibody.

IHC optimisation was aimed at finding the best antigen retrieval method and antibody concentration to allow for accurate staining and scoring.

AR expressing control tissues including testis, breast and uterus were identified with the Human Protein Atlas and the nuclear AR antibody expression was confirmed as described below.

4 µm thick sections were cut by Dr Peter Donoghue and dewaxed in xylene for 5 minutes prior to hydration in graded ethanol (99%, 95%, 70% and 50%). In order to establish the best antigen retrieval technique, four possible combinations of heat application and buffers were examined (see *Table* **2-10**).

The optimal method for antigen retrieval was found to be using the decloaker with citrate buffer. A decloaker is a sealed chamber used for heat induced antigen retrieval through the combination of heat and pressure in conjunction with exposure of the de-paraffinized fixed tissues to the appropriate buffer solutions.

Method	Buffer	Time
Microwave	Citrate	10 mins
Microwave	Tris	10 mins
Decloaker	Citrate	30 seconds at 125°
Decloaker	Tris	30 seconds at 125°

Table 2-10: Antigen retrieval techniques used for optimisation of AR

After the antigen retrieval, 3% hydrogen peroxide was applied for 10 minutes at room temperature in order to decrease endogenous peroxidase activity.

Slides were washed and incubated with a range of antibody concentrations (1:250, 1:500, 1:750) at 4°C overnight.

The antibody was made up in TTBS. Incubation with TTBS only without the addition of primary antibody was used as negative control.

Post incubation with primary antibody (see *Table* **2-11**), slides were washed twice with TTBS for 5 minutes to remove unbound antibody. Using the Menapath X-Cell detection kit (Menarini

Diagnostics, Berkshire, UK) as per manufacturer's protocol, HRP-Polymer was applied for 25 minutes at room temperature. Following this, the slides were rinsed in tap water for 10 minutes to remove any excess of the probe and placed into TTBS for 2 minutes. Then, 150 µl DAB solution was applied for 10 minutes. After a further wash in tap water, counterstaining in Gills II haematoxylin for 10 seconds, followed by blueing in Scott's tap water was performed. Any excess was washed off with tap water and the slides were dehydrated through graded alcohols and cleared in Xylene. Excess of the probe was washed twice for 5 minutes with TTBS and followed by incubation of a HRP-Polymer for half an hour.

Primary antibody	Species	Concentration used
AR: N20 (Santa Cruz, sc-816)	Rabbit polyclonal	1:500
Rab35 (Proteintech: 11329-2-AP)	Rabbit polyclonal	1:20

Table 2-11: Primary antibodies used for immunohistochemistry

For Rab35 staining, antigen retrieval was obtained with decloaker and citrate buffer, and the primary antibody was used at a concentration of 1:20, as optimised by a previous MRes student of the ovarian group, Katherine Warburton.

Previous AR staining on paraffin embedded tissue was done within the ovarian group (A Elattar and S SooHoo) with the same antibody that was used in this project for detection of AR with Western Blotting (SC441 antibody from Santa Cruz, concentration 1:20, see *Table* **2-11**), but could not successfully be replicated by current members of the team.

Another antibody for AR (see *Table* **2-11**), showed good nuclear staining and was used in different concentrations during optimisation (1:100, 1:500, 1:750). 1:500 was the concentration showing best staining and was hence used for the tissue microarrays (TMAs).

Tissue microarrays created from primary ovarian cancer tissue, were kindly created by Dr Peter Donoghue (see *Figure* **2-1**). Tissue cores were taken from ovarian tumour, omentum or peritoneal tissue from biopsies obtained from patients with epithelial ovarian cancer. The PCOs evaluated were chosen to match primary cell cultures generated from ascites to allow correlations and are outlined in *Figure* **2-1**. Tissue cores that were not marked in the figures below have not been used either as they had no matching PCO sample material derived from ascites to be compared with or due to loss of tissue cores during the process of TMA construction. Tissues from non-cancerous ovaries, uterus, testis, kidney and tonsil were used as normal tissue controls.

PCO 137	PCO 140	PCO 141	***PCO 143***	PCO 145	PCO 147	PCO 148
PCO 149	PCO 151	PCO 152	PCO 153	PCO 156	***PCO 157***	PCO 158
PCO 161	***PCO 162***	PCO 163	***PCO 168***	PCO 179	PCO 181	PCO 180
PCO 183	PCO 184	PCO 185	PCO 186	***PCO 187***	PCO 193	***PCO 197***
PCO 199	PCO 200	PCO 202	***PCO 209***	***PCO 210***	***PCO 211***	PCO 212
PCO 214	PCO 219				Uterus	Ovary
Breast	ER alpha	Testis	Kidney	Tonsil	Stomach	Bowel

PCO 224	***PCO 225***	***PCO 227***	***PCO 229***	PCO 231	***PCO 233***
PCO 234	***PCO 238***	PCO 239	***PCO 242***	***PCO 245***	***PCO 247***
PCO 249	***PCO 250***	***PCO 251***	***PCO 252***	***PCO 253***	***PCO 210***
PCO 211		***PCO 226***	***PCO 230***	***PCO 221***	
Uterus	Testis	Ovary	Kidney	Tonsil	Placenta

Figure 2-1: Layout of the primary ovarian culture TMAs. Marked in red are the PCOs evaluated for AR, and in italics are the PCOs scored for Rab35.

2.7.1 IHC/ TMA scoring

Image slides were scanned with an automated digital scanner (Aperio Technologies, Bristol, UK). The images were accessed for scoring using the Spectrum^{TM} image management software. The scoring was done by a modified H score by two independent people (Angelika Kaufmann and James Murray). Initially, a range of intensities from zero to three was defined across the TMA by each scorer (see *Table* **2-12**). The intensities scored for AR were nuclear staining, and for Rab35 cytoplasmic staining.

Score	Staining intensity
0	None
1	Weak
2	Moderate
3	Strong

Table 2-12: Staining intensities and scores used for the modified H score.

The intensities were put into proportion to the surface of the core scored, dividing the score into six areas. The number of areas scored for each intensity were multiplied by the numeric equivalent of the intensity. The final score was then calculated from the total sum of the intensities identified for all six areas and lies between 0 and 18. Cores containing mainly stroma were excluded from the analysis.

Both scorers were blinded to each other's scores or any other data. The scores were then compared after preliminary analysis was complete. The correlation of inter-observer agreement was calculated with the Intraclass Correlation Coefficient in SPSS (see *Table* **2-13**). If the H score differed by 2 or more points, the cores would be reviewed together and after discussion a definite score would be agreed upon.

	Intraclass Correlation Coefficient (95% confidence intervals)	
Primary Antibody	**Raw scores**	**Post Discussion**
AR	0.988 (0.974- 0.995)	0.988 (0.974- 0.995)
Rab35	0.953 (0.897-0.978)	0.973 (0.941-0.987)

Table 2-13: Intraclass Correlation Coefficient for scoring PCO TMAs for AR and Rab35

2.8 Androgen stimulation experiments

Dihydrotestosterone (DHT) was used for androgen stimulation experiments, as it is not aromatisable. Testosterone can be aromatised into oestradiol and might hence exert function not only via the AR, but also the Oestrogen (ER) receptor, hence results might not have been conclusive. DHT has furthermore been found to be more effective than testosterone in stimulating human ovarian surface epithelium cell lines (Syed et al., 2001).

DHT (Sigma, catalogue number D5027) was kindly provided by Dr Kelly Coffey (Solid Tumour Target Discovery Group) and a stock concentration of 10mM in ethanol solvent was stored at -80°C.

The proliferative effect of DHT on PCOs was assessed using SRB and BrdU assays and cell counts.

2.8.1 DHT stimulation and SRB

For SRB assays PCO cells were seeded out in full medium in a concentration of $5x10^3$ cells/ well in 96 well plates with six replicates per treatment condition. The cells were then incubated and left to adhere for 24 hours. Following this, the full medium was taken off, and cell were washed with PBS twice prior to treatment with basal medium (see *Table* **2-2**) for 24 hours. After the quiescing, cells were again washed with PBS twice and incubated with the desired treatment conditions (10 nM DHT or 100 nM DHT in basal medium) Full medium was used as positive and basal medium as negative control, respectively. Both control treatments were corrected for with ethanol to ensure comparability of observed results.

For treatments with SDM medium, the steps were identical to the ones described above, with SDM medium being used instead of basal medium. Cells would also be quiesced with basal medium. SDM was used instead of basal medium for treatments (10 nM or 100 nM DHT in SDM medium) and as negative control.

Cells were then incubated at 37°C and fixed at 24 hour intervals. After fixation, the plates were stored at 4°C and the SRB assay was performed as described above in chapter 2.4.3.

2.8.2 BrdU and DHT

For BrdU assays, PCO cells were seeded out in full medium at a concentration of $5x10^3$ cells/ well in 96 well plates with 3 replicates per treatment condition. Cells were left to adhere for 24 hours, before being washed twice with PBS and quiesced for 24 hours with basal medium. Following this, cells were incubated for 24 or 96 hours or 10 days in different treatment conditions (10 nM DHT or 100 nM DHT in basal medium). Full medium was used as positive and basal medium was used as negative control with ethanol correction.

For treatments with SDM, cells were seeded out in full medium, left to adhere for 24 hours and quiesced in basal medium for 24 hours. The treatments (10 nM DHT or 100 nM DHT) were then applied in SDM medium for the same length of time as described above. Full medium was used as positive and SDM medium as negative control.

The BrdU assay was then performed as per manufacturer's protocol and described above in chapter 2.4.4.

2.8.3 qRT-PCR

For qRT-PCR, cells were seeded out at $6x10^5$ cells/ well in 6 well plates in full medium. Cells were incubated at 37^{o}C for 24 hours for cells to adhere. Cells were then washed with PBS twice before incubation with 10 nM DHT in basal medium for 0, 2, 4, 8 or 12 hours. After the desired treatment time with DHT, cells were detached with trypsin and spun at 1500 rpm for 5 minutes. The supernatant was discarded and the cell pellet was used for RNA extraction as described in chapter 2.6.

2.9 Treatment with Abiraterone

Abiraterone was obtained from Selleckchem (catalogue number S1123) and stored as a stock concentration of 10 mM in DMSO at -80°C.

2.9.1 Western Blot and qRT-PCR and Abiraterone treatment

OVCAR3 or PEO4 cells were seeded out at $6x10^5$ cells/ well in 6 well plates in SDM medium and allowed to adhere for 24 hours. They were then washed once with PBS. Cells were then incubated with SDM medium and the required abiraterone concentration (0.1 µM, 0.5 µM, 1 µM, 5 µM or 10 µM). Cells treated with SDM alone and DMSO for correction were used as negative control.

For Western Blots cells were treated for 2, 3 or 4 days. The medium was then taken off and cells were washed with PBS once. Cells were lysed with 200 µl SDS lysis buffer and Western blots run as described in chapter 2.5.

For qRT-PCR cells were detached with trypsin after a treatment duration of 2, 5 or 8 hours. Trypsin was neutralised with SDM medium and cells were spun down at 1500 rpm for 5 minutes. The supernatant was discarded and the cell pellet used for RNA extraction as described in chapter 2.6.1.

2.9.2 Proliferation assay

OVCAR3 or PEO4 cells were seeded out at a concentration of $5x10^3$ cells/ well in 96 well plates in SDM medium with six replicates per treatment condition. After incubation for 24 hours to allow for incubation, and a single wash of PBS, cells were treated with different abiraterone concentrations (0.1 µM, 0.5 µM, 1 µM, 5 µM or 10 µM). Cells were fixed at intervals of 24 hours with TCA over a ten-day period and subsequently stored at 4°C until further processing. Staining with SRB and reading of the absorbance was done as described in chapter 2.4.

2.10 Statistical analysis

Statistical analysis for correlation data, survival statistics and the ROC curve was done with IMB SPSS ver22.

2.10.1 Correlation of expression data (Spearman rho)

The Spearman's rank correlation coefficient (Spearman rho) was used to analyse the statistical dependence of two non-parametric values. The test was done with SPSS and significant differences were set at a p-value of <0.05.

2.10.2 Survival statistics

Progression free (PFS) and overall survival (OS) were evaluated with Kaplan-Meier survival analyses. Differences between cohorts were examined with univariate analysis using the log rank test (Mantel Cox).

2.10.3 ROC curve

A receiver operating characteristic (ROC) curve, is a graphical plot which allows to assess how accurately a numerical score can predict a binomial outcome and can be used for diagnostic test evaluation. Sensitivity (true positive rate) and specificity (false positive rate) are plotted for different cut off points of a parameter. For each examined value, a table of sensitivity and specificity is created to generate the ROC curve. This allows assessment of the area under the curve (AUC) which measures how well the test discriminates between the two outcomes. The perfect test is represented by an area of 1, a worthless test by an area of 0.5. The ROC curve in this project was generated with IMBSPSS ver22.

2.10.4 Power and sample size calculations

Power calculations are used to determine the sample size required to confidently observe an anticipated effect or to determine if there is sufficient power to detect a meaningful difference in a given sample size.

Calculations were made to evaluate what sample size that would be required to reach statistical significance with a (p=0.05) and a power of 80% using an online calculator (https://www.stat.ubc.ca/~rollin/stats/ssize/n2.html).

3 The expression of AR and Rab35 in primary ovarian cancer cells

3.1 Introduction

Androgens have been implicated to play a role in ovarian carcinogenesis (Risch, 1998) and exert their effect via receptors. Androgen receptor (AR) expression has been confirmed in both, the normal ovary (Edmondson et al., 2002) and ovarian cancers (Lee et al., 2005, Nodin et al., 2010).

In ovarian cancer the AR has been described to play different roles. Other than acting as a ligand-binding transcription factor it has also been proposed as a potential biomarker for androgen sensitivity (Elattar et al., 2012). Furthermore, high AR expression has been outlined as a predictive marker for clinical outcomes, suggesting increased survival times (Nodin et al., 2010, Jonsson et al., 2015). One study group even attempted to use glucocorticoid hormones as predictors for peripheral metastases in women with EOC and suggest that low level AR expression is associated with a 9.5 increased chance to develop brain metastasis when compared to individuals expressing AR in higher quantities (Mittica et al., 2017).

Measurements of the AR in ovarian cancer can be done in different ways, using methods for abundance of protein with immunohistochemistry (IHC) or Western blotting (WB) or measuring mRNA expression via quantitative PCR (qRT-PCR).

The majority of published work investigating AR presence uses immunohistochemistry on tumour tissue (Lee et al., 2005, Nodin et al., 2010, SooHoo, 2013, Elattar, 2010, Cardillo et al., 1998, Chadha et al., 1993). Little work has been done exploring other methods of quantification of the AR, such as Western blotting in ovarian cancer cell lines (Fisher, 2010**)** and in primary cancer cell lines derived from ascites (Elattar et al., 2012). Investigations of gene expression level abundance of mRNA have been used in cancer cell lines (Lau et al., 1999) and primary ovarian cancer cell lines (PCO) (SooHoo, 2013, Elattar, 2010).

So far it has not been determined if any of the above methods is more accurate than another in determining AR presence and if these measurements are correlated.

Hence, in this chapter AR expression was measured with all three methods- IHC, WB and qRT-PCR- in a panel of primary ovarian cancer cell lines and the results were correlated.

However, receptor expression per se might not give sufficient information regarding the receptor functionality and measuring a down-stream “product” might be more representative for receptor function.

Rab35, a small GTPase, has been confirmed as an androgen dependent protein in an ovarian cancer cell line and furthermore suggested to be a proto-oncogene and a potential biomarker for the AR (Sheach et al., 2009, SooHoo, 2013).

So far, only limited investigations have been done on Rab35 in ovarian cancer. This includes the expression of Rab35 on tissue micro arrays of ovarian cancer as well as protein and mRNA expression in the ovarian cancer cell line OVCAR3 and in a small cohort of primary ovarian cultures (n=14) (SooHoo, 2013).

This study aims to further expand the investigation of Rab35 expression at mRNA and protein level and its correlation with the AR in primary cell cultures.

3.2 Aims

The aims of this chapter are to examine a panel of primary cell cultures (PCOs) for the following as listed below.

- To quantify mRNA encoding the androgen receptor (AR) and Rab35 using qRT-PCR
- To examine and quantify the protein expression of AR and Rab35 in a panel of primary ovarian cancer cells using Western blotting
- To assess the abundance of AR and Rab35 protein in ovarian cancer tissue using immunohistochemistry (IHC)
- To develop a rapid screening test for AR protein expression with immunofluorescence (IF)
- To examine the effect of cell culture passaging on the AR expression of mRNA and protein level

- To examine the following correlations
    - AR protein expression measured by WB and IHC
    - AR gene expression and AR protein expression
    - Rab35 gene expression and Rab35 protein expression
    - AR mRNA and Rab35 gene expression
    - AR and Rab35 protein expression
    - AR and Rab35 expression (on protein and mRNA) with progression free survival (PFS) and overall survival (OS)

3.3 Characterisation of primary culture cells

Primary cell cultures are a desirable model used in translational research as they have the advantage to represent tumour heterogeneity better than cancer cell lines.

In this project, primary ovarian cell cultures were generated from ascites of patients suffering with epithelial ovarian cancer. The advantage of this model is that ascites is easily obtained with minimally invasive procedures and is not difficult to culture. Primary ovarian cultures have been successfully used within the ovarian cancer study group Newcastle (Asima Mukhopadhyay, Ahmed Elattar, Sandra SooHoo) for several years.

As ascites is a multicellular fluid, comprised of epithelial cells, lymphocytes, mucin, fat and mesenchymal cells and cells can undergo epithelial-mesenchymal transition, it was important to ensure cells examined were of epithelial origin.

The original panel of primary cell cultures was examined with immunofluorescence for pancytokeratin only, but was extended by Rachel O'Donnell to form a more robust framework (RL et al., 2014). All cultures used in this project were examined by immunofluorescence for the epithelial markers cytokeratin, cancer antigen 125 (CA125), EpCAM and the mesenchymal marker vimentin to exclude mesenchymal cells (see *Table* **2-3**).

3.3.1 Methods

3.3.1.1 PCO collection

PCO cell lines were established from ascites of women suffering from epithelial ovarian cancer. All these patients were treated at the North of England Cancer Network (NGOC) at the Queen Elizabeth Hospital, Gateshead for epithelial ovarian cancer and consented for participation in research with the consent form shown in Appendix 3.

Surgical samples obtained were examined for formal histological diagnosis and ascites for cytological diagnosis of ovarian cancer by the pathologists at the NGOC and classified according to the World Health Organisation (WHO) and FIGO criteria (Prat and Oncology, 2014). This process was independent and blinded to any data obtained in this study. Clinical and demographic data were obtained from the hospital database.

Samples used for experiments in this study had to satisfy all characteristics of epithelial ovarian cancer and were cross-referenced with the histological classification from the NGOC pathologists. Cultures with results inconsistent with epithelial origin were discarded.

Between February 2012 and January 2015, 84 ascitic samples were collected and 72 (86%) of these were successfully cultured as primary cell cultures (PCOs). 69 samples (82%) were obtained at the time of surgery (n=55 at primary and n=14 at interval debulking surgery) and 15 (18%) through drainage of ascites for symptom relief.

Samples were collected by Rachel O'Donnell and Angelika Kaufmann and maintained and characterised with the help of Aiste McCormick, Michelle Dixon and James Murray.

61/84 cultures were deemed suitable for further experiments, as three samples became infected, three did not satisfy the epithelial characterisation criteria and five samples excluded due to non-ovarian cancer pathology (breast and gastrointestinal malignancy), leaving 61 cultures that were used in further experiments.

In this study, a total of 57 samples were examined. Of these, 13 samples were collected by previous members of the ovarian group (Ahmed Elattar, Sandra SooHoo, Rachel O'Donnell and Aiste McCormick) prior to the start of this project. Either protein lysates or mRNA samples derived from these PCO samples were used in this study to gain expression data (summary of PCO list see Appendix 1).

3.3.1.2 Immunofluorescence methodology

Primary culture cells were cultured on glass cover slips in full medium and left to adhere. When confluence of 60-80% was reached, cells were fixed with ice cold methanol and stored at -4°C for a maximum of two weeks. After two washes with PBS, coverslips were incubated with antibodies as shown in *Table* **2-3** for one hour. After two further PBS washes cells were incubated with the appropriate secondary antibody for a further hour before mounting on a glass slide with 4 µl DAPI mounting media.

Images were captured using a Leica DMR fluorescent microscope and RT SE6 Slider Camera Spot advanced software version 3.408. Antigen expression was scored as absent, patchy or strong and compared to reference images from positive and negative cell line controls. In cases of controversy, a further reviewer was asked to validate the result.

3.3.2 Results

A majority of the patients were diagnosed with advanced disease (FIGO III or IV) with high grade serous cancer as the most common subtype. The patient characteristics are summarised

in Appendix 1. The full results of data collection including demographic and expression data are summarized in Appendix 1 and 2.

Demographic		Median (range)/ n(%)
Age at presentation (years)		63 (41-85)
Histology	High grade serous carcinoma (HGCS)	47 (77)
	Clear cell	2 (3.3)
	Endometrioid	1 (1.6)
	Other	11 (18)
Time of collection	Pre-chemotherapy	43 (70.5)
	Post-chemotherapy	18 (29.5)
FIGO stage	Stage I	2 (3.3)
	Stage II	2 (3.3)
	Stage III	46 (75.4)
	Stage IV	9 (14.8)
	No staging	2 (3.3)

Table 3-1: Demographic data of patients donating ascites for primary cell cultures

A majority of the cultures (80%) showed a morphological cobble stone appearance (see *Figure* **3-1**) and 20% showed mesenchymal appearance.

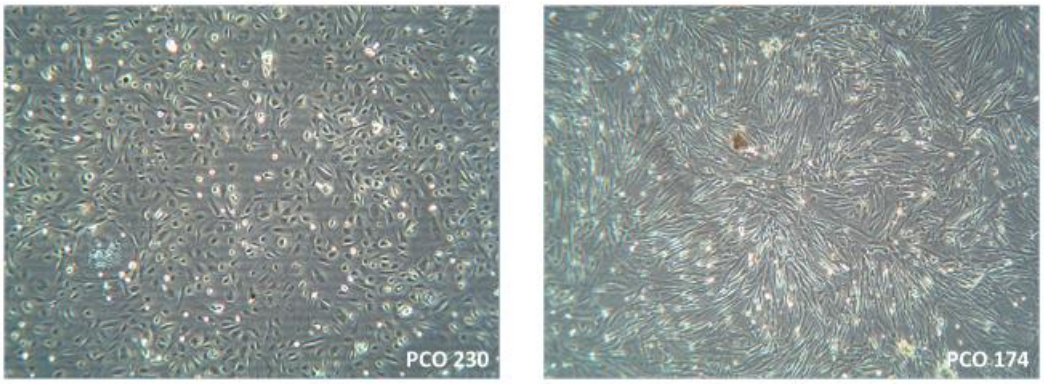


Figure 3-1: Morphology of primary ovarian cell culture samples. Images of PCO 230 and PCO 174 are examples for the cobblestone monolayer and mesenchymal appearance respectively.

A representative sample for characterisation of primary ovarian cultures in this project is shown in *Figure* **3-2**.

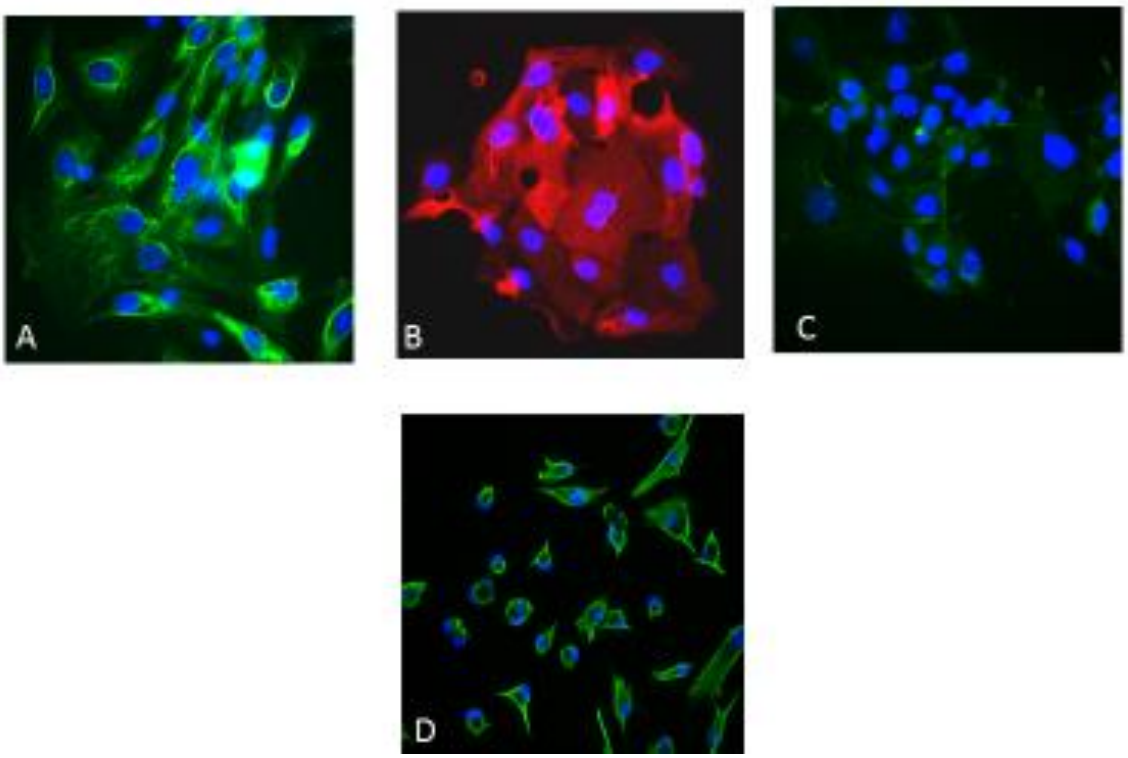


Figure 3-2: Images of primary cultures using immunofluorescence for characterisation. The technique and antibodies are described in detail in chapter 2.3. (A)- pancytokeratin stain, (B) CA125, (C) EpCAM, (D)- Vimetin, a mesenchymal marker. This primary culture was excluded from further experiments due to positive vimentin staining.

3.4 Results of AR expression

All expression data examined are summarised in *Table* **3-6** at the end of this chapter.

3.4.1 AR mRNA expression (qRT-PCR)

Primary culture cells (PCO) were seeded out in 6 well plates in full medium and incubated until they reached a confluence of 80%. RNA was then extracted and reverse transcribed and AR gene expression was examined using real time quantitative reverse transcriptase PCR (qRT-PCR) as described in chapter 2.6 and examined for AR gene expression with the Exon 3 primers (see chapter 2, *Table* **2-7**) at passage one. Gene expression was normalised to expression of the housekeeping gene HPRT1.

In order to allow comparison of expression levels, mRNA expression was also normalised to the well-studied ovarian cancer cell line OVCAR3.

3.4.1.1 AR mRNA expression in primary ovarian cultures

A total of 44 primary ovarian cultures were examined for AR gene expression and all samples showed AR gene expression, with varying levels. The majority of primary ovarian cultures showed AR gene expression at lower levels than the ovarian cancer cell line OVCAR3. PCO 170 showed higher levels of AR mRNA than OVCAR3 cells (see *Figure* **3-3**).

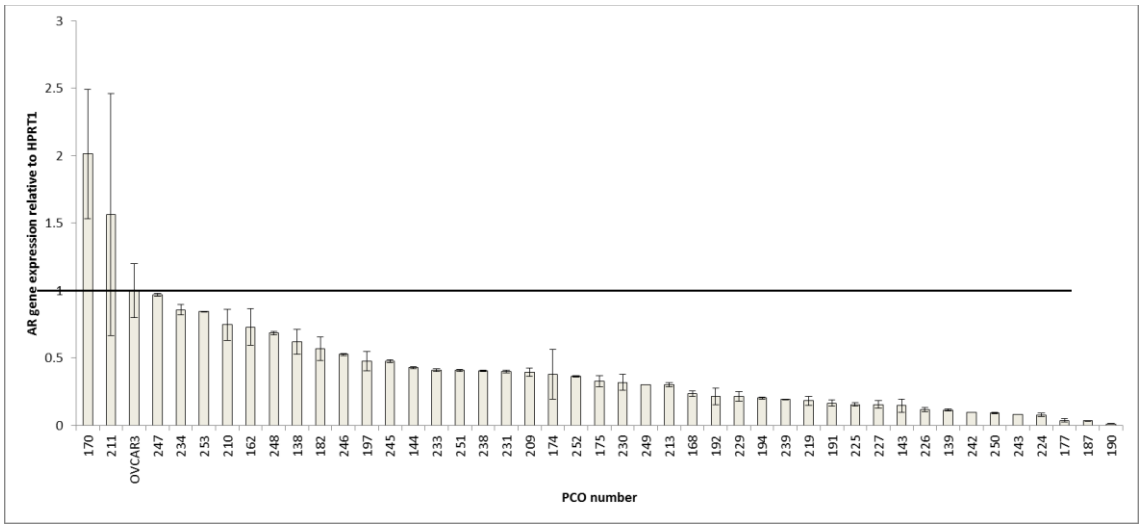


Figure 3-3: AR gene expression in 44 primary ovarian cultures at passage one relative to HPRT1 as a fold increase of AR expression of OVCAR3. Values represent the mean ±SE of the mean of three repeats with triplicates of each reaction.

3.4.1.2 *Comparison of different AR primers (Exon 3 vs 3'UTR)*

Previous studies in primary ovarian cultures measuring AR expression were conducted using Exon 3 primers (Elattar, 2010, SooHoo, 2013).

In this study a subset of 17 primary ovarian cultures AR expression was examined using the Exon 3 primers as well as 3'UTR primers in order to further validate the levels of AR expressed. The primer pairs used are documented in chapter 2, *Table* **2-7** and results for both have been normalised to HPRT1 as housekeeping gene and OVCAR3 AR mRNA expression.

AR mRNA expression measured with Exon 3 primers and 3'UTR primers was overall conforming (see *Figure* **3-4**). 4/17 of the primary ovarian cultures differed in AR expression, showing increased AR gene expression with the 3'UTR primer compared to AR expression measured with the Exon 3 primer (see *Figure* **3-4**). If these four primary ovarian cultures are excluded, the correlation was R^2=0.9202. In view of this correlation and in order to compare the obtained dataset directly with previously obtained results by Sandra SooHoo (SooHoo, 2013), all following experiments were done using Exon 3 primers.

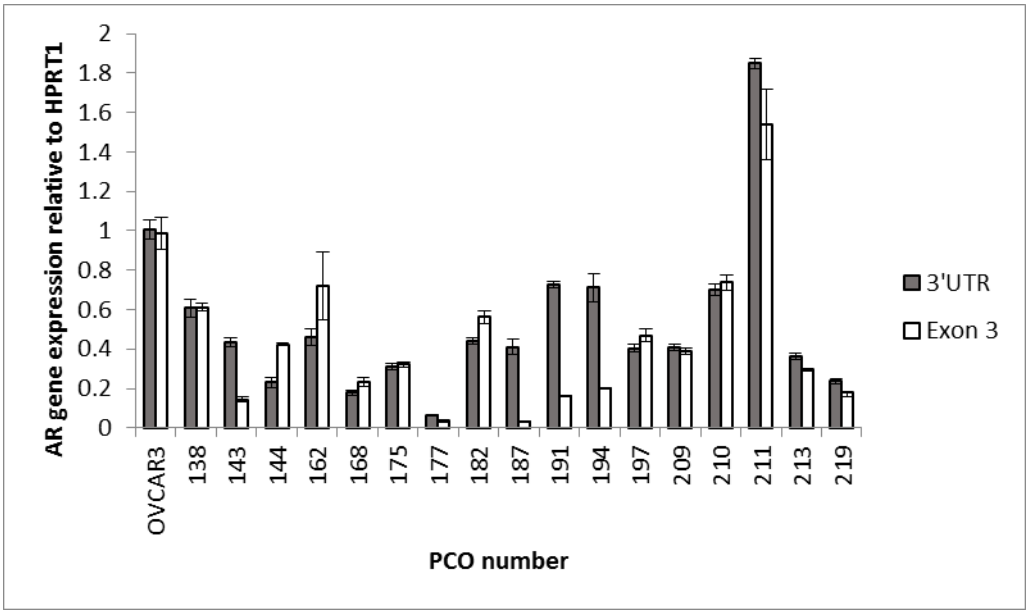


Figure 3-4: Comparison of primers for AR expression using qRT-PCR. Primers were either binding Exon 3 or the 3'UTR region. Relative gene expression was normalised to HPRT1 as housekeeping gene and OVCAR3 cells which were set to "1". Values represent the mean ±SE of the mean of three repeats with triplicates of each reaction.

3.4.2 AR Protein expression on WB

Primary cell cultures were seeded out at a density of $6x10^5$ per well in 6 well plates. After incubation for 24 hours, the cells were harvested with SDS lysis buffer and Western blot analysis was done as described in chapter 2.5. α-tubulin was used as protein loading control. Two ovarian cancer cell lines were used as controls for AR protein expression – OVCAR3 cells as positive and PEO14 cells as negative control. Densitometry was used for a semi-qualitative protein expression as described in chapter 2.5.1.

A total of 47 primary ovarian cultures were examined by Western blotting. A band of 110 kDa, corresponding to the molecular weight of AR was detectable in 16/47 (34%) samples (sample blot see *Figure* **3-5***)*. The bands were quantified with densitometry as described in chapter 2.5.1 and normalised to α-tubulin expression and OVCAR3 AR expression.

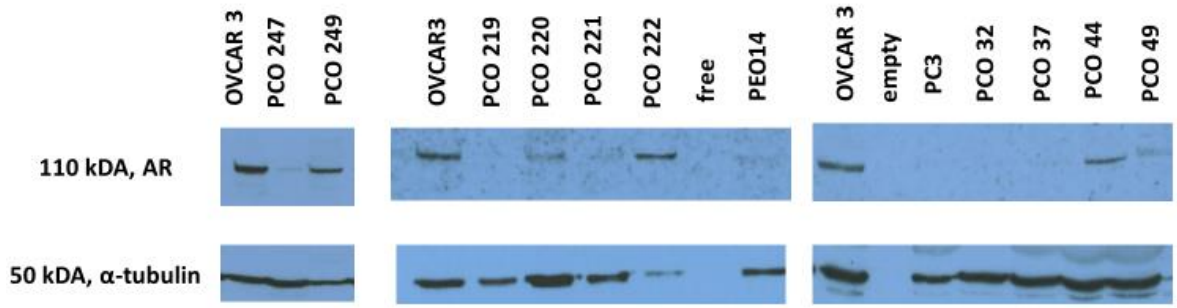


Figure 3-5: Sample Western blot of primary ovarian cultures showing bands at 110 kDA corresponding to AR expression. OVCAR3 cells were used as positive and PEO14 and PC3 cells as negative control. α-tubulin was used as loading control.

Of the 16 primary ovarian cultures which showed expression of AR protein, one showed a higher (3.5 fold) expression of AR than OVCAR3 cells. The remaining 15 primary ovarian cultures showed lower AR protein expression than OVCAR3 cells (see *Figure* **3-6**).

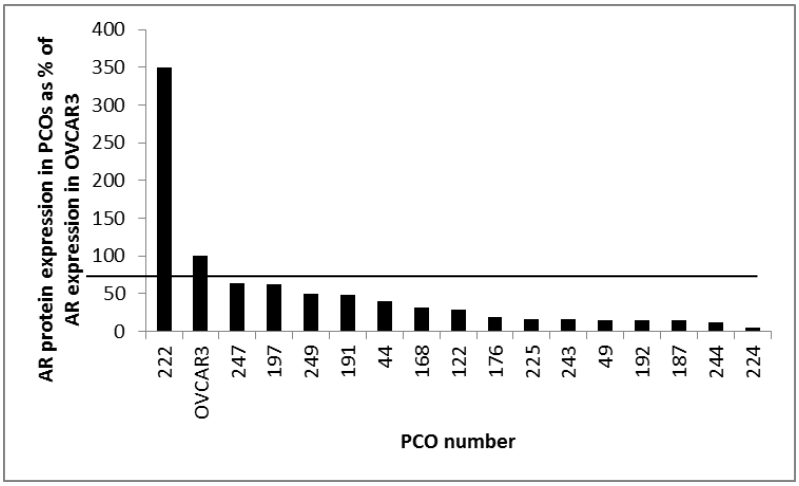


Figure 3-6: AR protein expression in 16 primary ovarian cultures measured with densitometry. The expression was normalised to the α-tubulin expression and the AR expression in OVCAR3 cells.

3.4.2.1 Examination of differences in AR expression (Isoforms)

The 47 primary ovarian cultures in chapter 3.1.3, have all been examined with an N-terminal antibody (SC441, *Table **2-5***).

Previous work on AR protein expression in primary ovarian cultures (Elattar, 2010) suggested the expression of a shorter isoform at 64kDa in addition to the normal protein expression at 110 kDa.

In view of this, a panel of 18 primary ovarian cultures were examined for potential AR isoforms with 2 N-terminal antibodies (SC441 and N20, Santa Cruz technologies) as well as a C-terminal antibody (C 19, Santa Cruz Technologies, see *Table* **2-5**). 8/18 primary ovarian cultures showed positive AR expression on WB at 110 kDa (with the SC441 antibody).

None of the AR negative and positive primary ovarian cultures incubated with either the N20 or C19 antibody showed any bands at molecular level lower than 110 kDa.

In the primary ovarian cultures examined in this study, the presence of isoforms could not be confirmed.

3.4.3 AR protein expression on immunohistochemistry (IHC)

Tissue micro arrays (TMA) for primary ovarian cultures and slides for staining were kindly prepared by Dr Peter Donoghue and stained for AR (N20 antibody, Santa Cruz, see *Table **2-11***). After the optimisations of the antibody as describe in chapter 2.7, the slides were stained with a final concentration of 1:500. The TMAs were scored independently by two different scorers (James Murray and Angelika Kaufmann). Nuclear staining was evaluated using the modified H score based on staining intensities determined for each TMA as described in chapter 2.7.1. *Figure* **3-7** shows an example of staining intensities for AR.

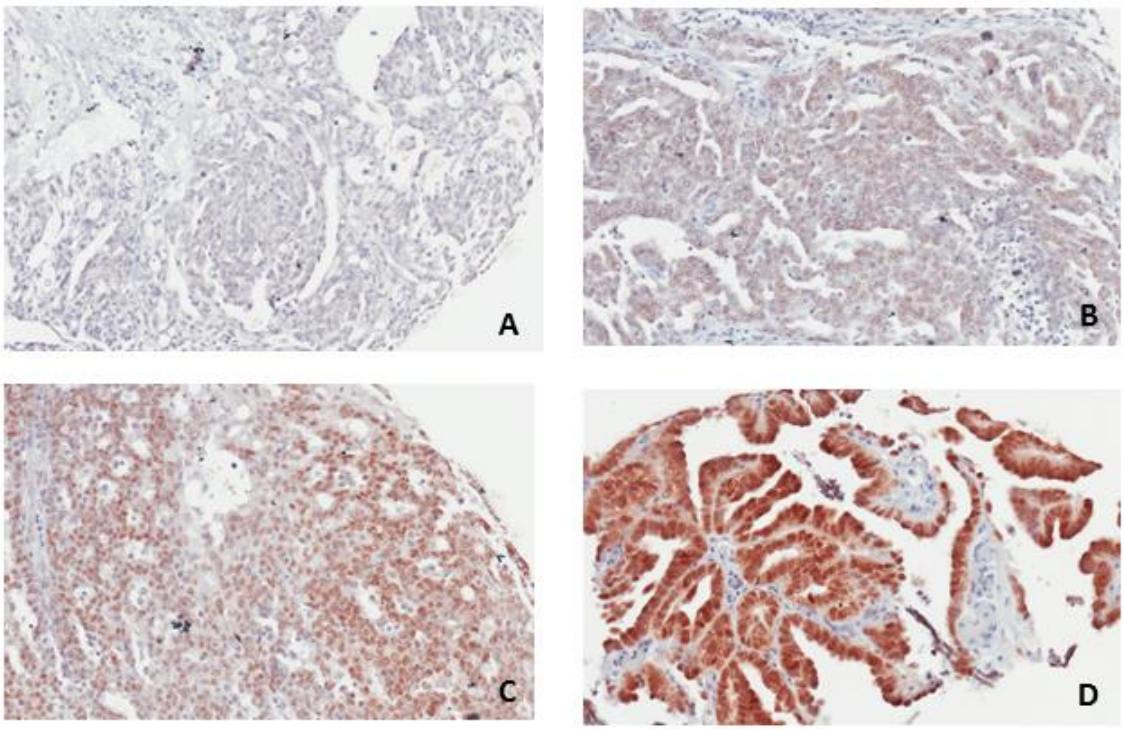


Figure 3-7: Staining intensities for the AR protein on tissue micro arrays using immunohistochemistry. Intensities shown are 0 (negative for AR) (A), intensity 1 (B), intensity 2 (C,) and intensity 3 (D). All four samples were obtained from high grade serous ovarian cancer.

The ovarian cancer tissues used for the TMAs were chosen to correspond to the primary ovarian cultures (PCO) samples derived from ascites. To outline the difference of samples obtained from ascites or tissue blocks, ascites derived samples will be referred to as PCO-A and samples derived from FFPE tissue blocks will be named PCO-T in the following paragraphs.

In total 23 PCO-T samples were examined for AR protein expression. 22/23 of these samples also had matching PCO-A samples.

20/23 (87%) of PCO-T samples expressed AR on IHC at varying levels. When comparing the AR expression on protein level on PCO-T and PCO-A samples, the AR protein expression was much lower in PCO-A samples with 34% (16/47), compared to 87% AR expression in the PCO-T samples.

3/23 (13%) of the PCO-T samples were negative for AR protein expression on IHC on examination of at least two different tumour cores- PCO 143, 211, 249. Interestingly one of these primary ovarian cultures (PCO 249) showed positive AR expression on WB. PCO 211 has shown very high AR gene expression levels. Unfortunately, no PCO-A sample was obtained for PCO 143 to measure protein expression on Western blot.

3.4.4 Correlations of AR expression levels

20/23 primary ovarian cultures were tested for AR expression with three modalities in order to allow direct correlation of AR expression levels. Protein expression was measured with Westerns blotting and IHC and qRT-PCR was used to evaluate mRNA expression and the correlations were examined using the Spearman rho test.

3.4.5 Correlation of AR protein expression measured with IHC and WB

When examining the correlation of protein expression on WB (PCO-A samples) and IHC (PCO-T samples), no correlation was found for the 22 primary cell cultures examined (see *Figure* **3-8***)*.

Statistical analysis of all 22 cultures with Spearman's correlation showed a R^2 value of 0.140 and $p<0.05$, suggesting a moderate negative correlation. As 16/22 of the samples did not express the AR on WB despite high AR protein expression on IHC (see *Figure* **3-8**), this result could be misleading and should be interpreted as no correlation between AR expression on IHC and WB instead.

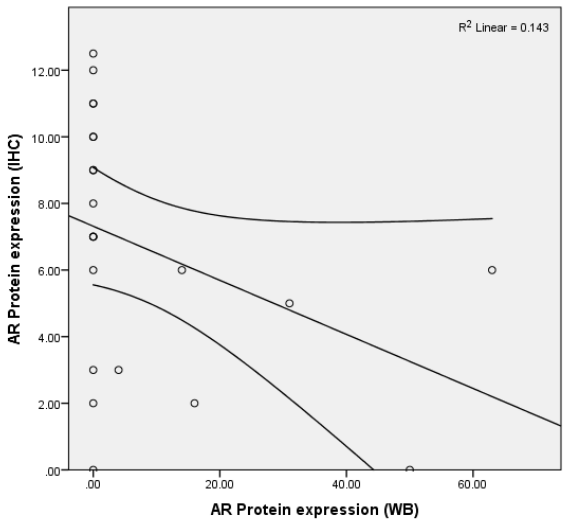


Figure 3-8: Spearman rho correlation of protein expression in 22 primary ovarian cultures on WB and corresponding cancer tissue on IHC. Statistical analysis gives a R^2 value of = 0.140 ($p< 0.05$). As 16/22 samples showed no AR expression on WB, this is best interpreted as no correlation between AR protein expression on Western blot and AR protein expression in ovarian cancer tissue.

3.4.6 Correlation of AR mRNA and protein expression

Correlations of AR protein expression and AR mRNA expression were made in two different sample groups. AR expression on mRNA level from PCO-A samples was correlated to AR

protein levels on WB for PCO-A samples (see *Figure* **3-9***)* and to AR protein expression on IHC for PCO-T samples, see *Figure* **3-11**.

In a panel of 34 PCO-A samples, the correlation of AR expression of protein measured by WB and AR mRNA measured by qRT-PCR was examined. Using the Spearman correlation test, there was no correlation found between AR expression measured by WB and qRT-PCR (see *Figure* **3-9**) with a R^2 value of 0.021 (p=0.120).

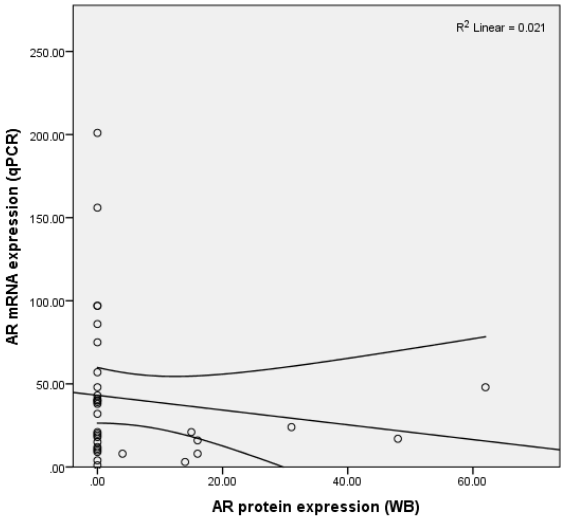


Figure 3-9: Graph showing Spearman rho correlation for AR protein expression measured by Western blotting and AR mRNA expression measured by qRT-PCR in 34 primary ovarian cultures. There is no correlation between the AR protein expression and AR gene expression with a R^2 value of 0.021 (p=0.12).

When examining a small sub-cohort of 8 PCO-A samples which excludes PCO-As with negative AR protein expression on WB, a statistically significant positive correlation for AR expression between AR protein and mRNA expression was found using the Spearman Rho test with a R^2 value of 0.655 ($p<0.05$) (see *Figure* **3-10**). However, due to the small sample size and an outlier, the result of the correlation cannot be considered as robust and should be interpreted cautiously.

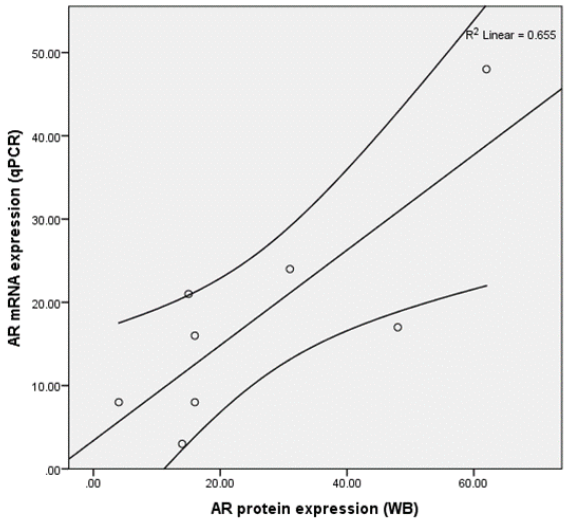


Figure 3-10: Correlation of AR protein measured by Western blotting and AR gene expression assessed by the Spearman rho correlation in 8 primary ovarian cultures with positive AR expression on Western blot. Due to the small sample size and the outlier, the calculated statistically significant positive correlation (R^2 0.655, $p < 0.05$) is not robust.

Examining AR protein expression by IHC (PCO-T) and AR mRNA expression in 20 corresponding PCO-A samples, no correlation was found with the Spearman rho correlation, R^2 value of 0.002 (p=0.062) (see *Figure* **3-11**).

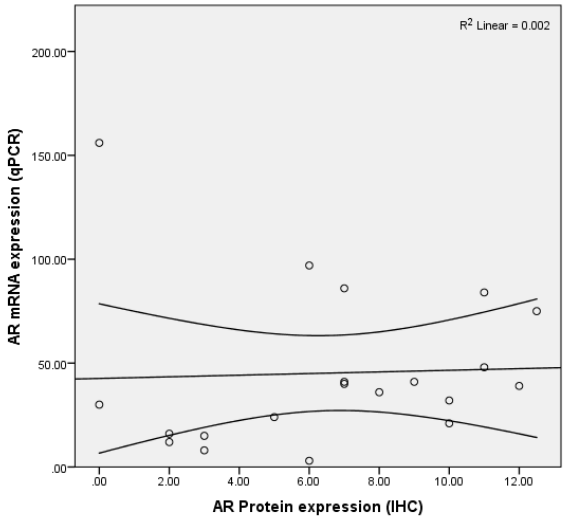


Figure 3-11: Graph showing Spearman correlation for AR protein expression measured by IHC and AR mRNA expression measured by qRT-PCR in 20 primary ovarian cultures. There is no correlation between AR protein expression on IHC and AR gene expression ($R^2 = 0.002$, $p=0.062$).

3.4.7 Clinical correlations

Survival data was calculated using the date of diagnosis which was set as the date of histological or cytological confirmation of epithelial ovarian cancer.

The progression free survival (PFS) was defined as the time interval between date of diagnosis and sign of first sign of recurrence (clinical, biochemical or radiological) and the overall survival (OS) was defined at the time interval between the date of diagnosis and the date of death (of any cause) and were obtained from medical records.

3.4.7.1 Correlations of AR expression with progression free survival and overall survival

The expression of AR with all three examined modalities was correlated to progression free survival (PFS) and overall survival (OS) with the Spearman rho test with SPSS and as shown in *Figure* **3-12**.

Correlations for survival were found for AR gene expression and AR protein expression on WB. The correlation appears more pronounced in AR gene expression when compared with protein expression on WB, but overall the correlations of mRNA AR expression to survival are weakly positive and non-significant correlation for both, PFS (R^2=0.049, p=0.168 and) and OS (R^2=0.039, p=0.222) (n=43).

The Spearman rho correlation test for AR protein expression on WB with PFS gave a R^2 of 0.013 (p=0.449) and R^2=0.020 for OS (p=0.344) (n=46).

No correlation was found for survival in the 24 women with evaluated AR protein expression on IHC, for neither PFS (R^2=3.09, p=0.979) nor OS (R^2=0.010, p=0.637).

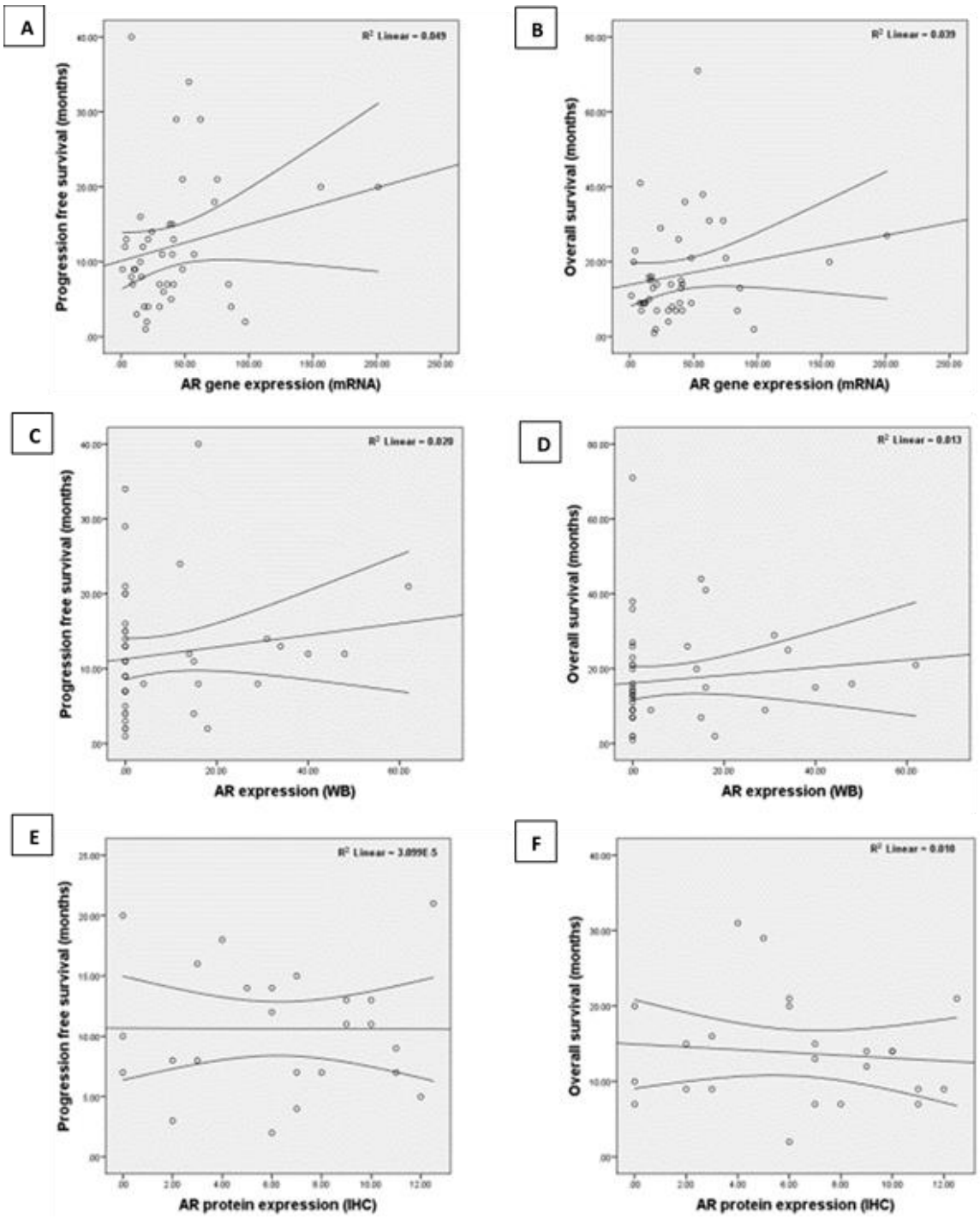


Figure 3-12: Spearman rho correlation of AR expression and progression free survival (PFS) and overall survival (OS) in months. AR mRNA gene expression shows weak non-significant correlation to PFS (A) (R^2=0.049, p=0.168) and OS (B) (R^2=0.039, p=0.222). Weak non-significant correlations can also be seen for PFS (C) (R^2=0.013, p=0.449) and OS (D) (R^2=0.020, p=0.344) and AR protein expression on WB. AR protein expression on IHC is not correlated with PFS (E) (R^2=3.09, p=0.979) or OS (F) (R^2=0.010, p=0.637).

3.4.7.2 Kaplan-Meier Curves for AR expression and PFS and OS

Univariate analyses for PFS and OS were generated by Kaplan-Meier survival curves with SPSS and differences in survival between low and high AR expression were assessed for AR gene expression and AR protein expression on IHC. The differentiation of high and low expressing group was made with the median of the AR expression as a cut-off point. For AR protein expression on WB present and absent expression of AR were compared. The log rank test was used to assess for statistical significance (for results see *Table* **3-2**).

A trend of increased OS survival with AR protein expression on WB (*Figure* **3-13**, F) and PFS and OS with high AR gene expression (*Figure* **3-13** A and B) was noticed, however this was not statistically significant *(Table* **3-2**).

A power calculation was made to further examine these observed trends. For AR mRNA expression the effect on PFS the observed power was 0.57 for a sample size of 22 in each group and a p-value of 0.09. For a calculated power of 0.8 the p-value should reach a statistical significance of 0.05 by increasing the sample size to at least 39 in each group.

For AR WB expression to reach a calculated power of 0.8 and statistical significance (p=0.05) the sample size needed to be increased to at least 126 in each group, as the effect on OS in the observed power was 0.35 for a sample size of 32 in each group and a p-value of 0.22.

In contrast, low AR protein expression on IHC suggested a potential benefit to OS (*Figure* **3-13**, D), but was non-significant on statistical analysis (*Table* **3-2**).

A power calculation to calculate for a power of 0.8 the p-value should reach a statistical significance of 0.05 with increasing the sample size to at least 57 in each group. For AR IHC expression effect on OS the observed power was 0.31 for the sample size of 22 in each group (p= 0.09).

AR protein expression on both, IHC and WB does not influence PFS (*Figure* **3-13** C and E and *Table* **3-2**).

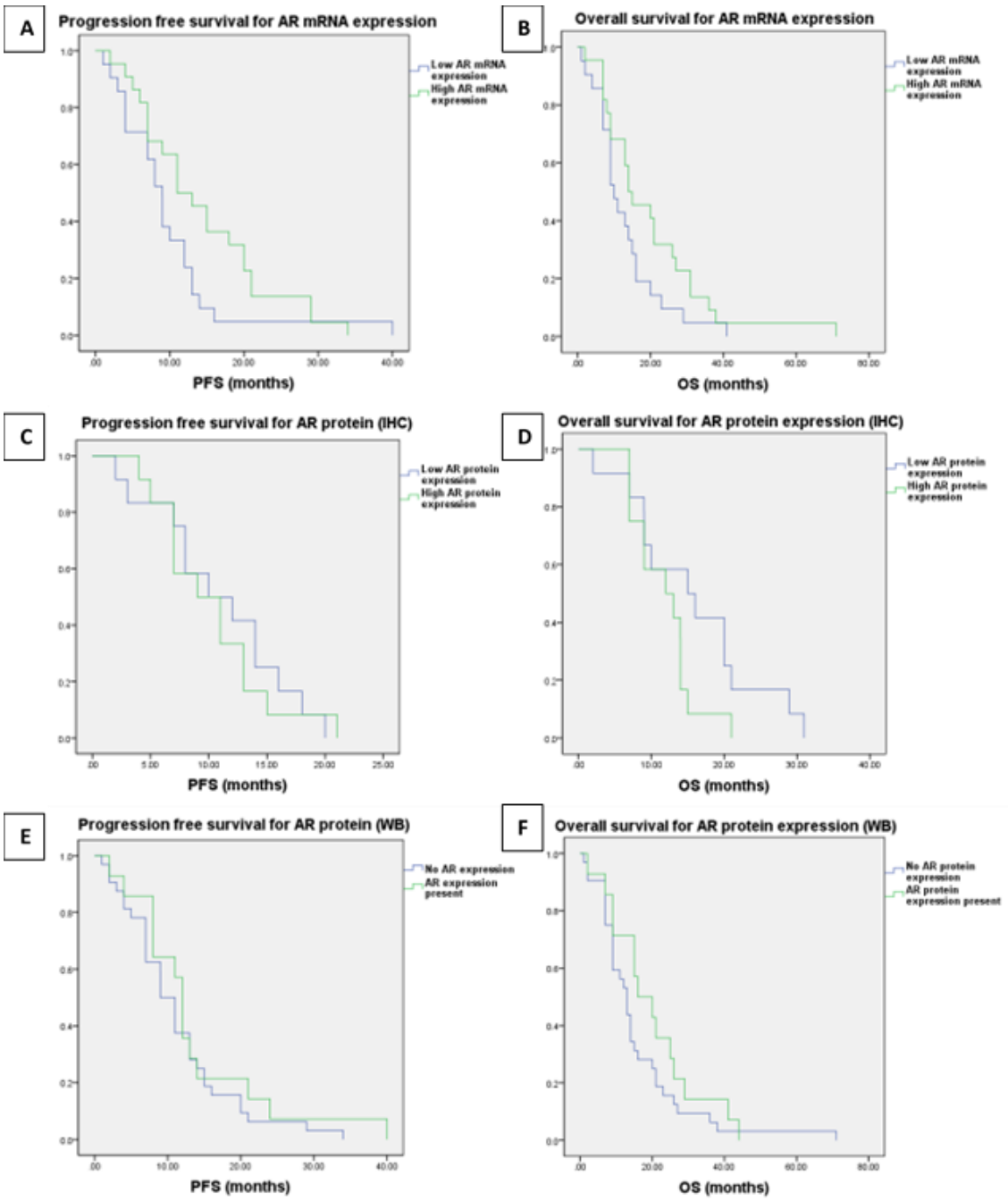


Figure 3-13: Survival in relation to AR expression visualized by Kaplan-Meier curves examining PFS and OS for AR gene expression (A and B), AR protein expression on IHC (C and D) and AR protein expression on WB (E and F).

	PFS			OS		
	HR	95% CI	p	HR	95% CI	p
AR mRNA	1.60	0.86 to 2.96	0.09	1.59	0.85 to 2.95	0.10
AR IHC	0.89	0.39 to 1.97	0.75	0.56	0.24 to 1.28	0.09
AR WB	1.29	0.70 to 2.34	0.40	1.44	0.79 to 2.61	0.22

Table 3-2: Hazard ratios and Confidence intervals for correlations between AR expression (on WB, IHC and mRNA) and PFS and OS.

3.4.8 Immunofluorescence for AR in primary ovarian cultures

WB and qRT-PCR are relatively time consuming methods to examine the expression status of a protein or mRNA. As primary ovarian cultures tend to have a short lifespan of 2-5 passages, a quicker method of establishing the AR expression would be beneficial in order to identify relevant primary cultures and allow a faster application of functional assays in these AR expressing primary ovarian cultures.

Immunofluorescence (IF) is successfully and routinely used for characterisation of primary ovarian cultures within the ovarian cancer study group for markers such as Pancytokeratin, CA125, EPCaM, and Vimentin (as listed in *Table* **2-3**).

In this study, the presence of AR was examined in five primary ovarian cultures with immunofluorescence, using the ovarian cancer cell line OVCAR 3 and the prostate cancer cell line LNCaP as reference of AR expression.

Cells were seeded out at $3x10^5$ cells/ well in full medium on sterilised coverslips in 6 well plates and fixed with ice cold Methanol when the desired cell confluence was reached. Cells were then stained with AR with the N20 antibody (see chapter 2.3).

For the IF in primary ovarian cultures, a panel of five primary ovarian cultures were selected of which three were positive and two were negative for AR expression on Western blot (see *Table* **3-3**).

Optimisation of the antibody was done in both, OVCAR3 and LNCaP cells and positive nuclear staining for AR was confirmed in both cell lines, see *Figure* **3-14**.

OVCAR3 cells were used as a positive control and slides without primary antibody for primary ovarian cultures and OVCAR3 cells were used as a negative control.

Whilst staining for AR worked well in the ovarian cancer cell line OVCAR3 cells (see *Figure* **3-14**), none of the examined primary ovarian cultures showed staining for AR on IF.

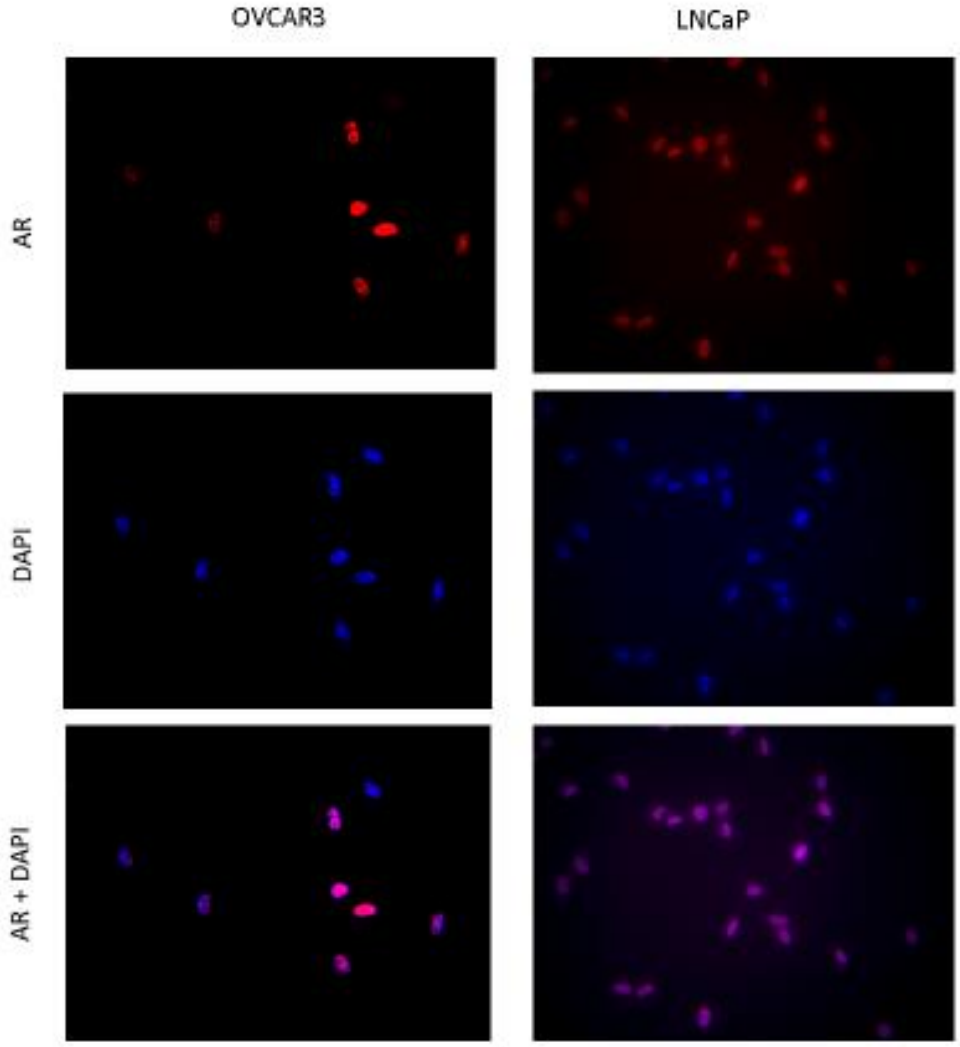


Figure 3-14: Immunofluorescence in OVCAR3 and LNCaP cells. Cells were stained for AR alone, nuclear staining (with DAPI) and a combination of the two.

PCO number	AR protein expression	AR mRNA expression
187	Positive	Positive (low)
188	Negative	Not done
190	Negative	Positive (low)
191	Positive	Positive (low)
192	Positive	Positive (low)

Table 3-3: Protein and mRNA expression status for primary ovarian cultures examined for AR on IF. IF was negative for all examined primary ovarian cultures.

3.4.9 The effect of cell passaging on AR protein and mRNA expression

Passaging of cells in cell culture has been described to change gene expression (Neumann et al., 2010). Within the ovarian group the Northern Cancer Institute in Newcastle passaging of primary ovarian cultures has suggested an altered expression in human recombination (HR) status (O'Donnell, 2016).

This study examines the effect of passaging of primary ovarian cultures on expression of AR at mRNA and protein level.

3.4.9.1 Passage effect on AR protein expression in primary ovarian cultures

The seeding out, maintenance and passaging was done as described in chapter 2.2. Cells were seeded in full medium in 6 well plates at $6x10^5$ cells/ well and were passaged when 80% confluence was reached. Passage zero refers to seeded cells of ascitic origin that were plated with full medium in 1:1 concentration. Ascending numbers were used to refer to subsequent passaging of passage zero cells. Western blotting was performed as described in chapter 2.5 and bands at 110 kDa were identified as AR with the SC441 antibody (see *Table* **2-5**). OVCAR3 cells were used as positive and PEO14 cells as negative control for AR protein expression. α-tubulin was used as loading control.

Five primary ovarian cultures were examined for AR protein expression with western blot at different passages (passages zero, one and two).

4/5 primary ovarian cultures were negative for AR on western blot at all passages (PCO 246, 251, 252 and 253). One primary ovarian culture (PCO 249) was positive for AR and showed much stronger AR expression at passage one, than in passages zero or two (see *Figure* **3-15**, *Figure* **3-3**).

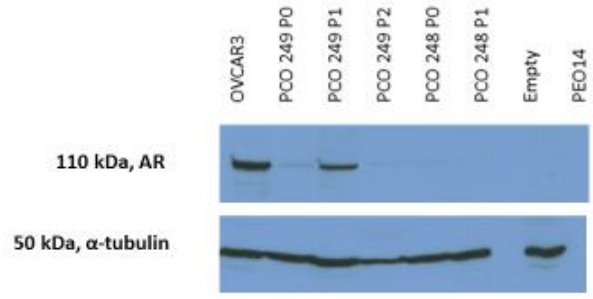


Figure 3-15: Western blot showing with AR protein expression in primary ovarian cultures at different passages. Bands at 110kDA correspond to AR expression. OVCAR3 cells were used as positive and PEO14 cells as negative control. α-tubulin was used as loading control.

3.4.9.2 Passage effect on primary ovarian cultures on AR mRNA expression

Cells were seeded in full medium at $6x10^5$cells/well in 6 well plates and RNA was extracted when confluence reached 80%. qRT-PCR was conducted as described in chapter 2.6 using the Exon 3 primer (see *Table* **2-7**). HPTR1 was used as housekeeping gene.

16 primary ovarian cultures were examined for AR expression on mRNA level at passages zero, one and two.

There was no consistent trend in AR gene expression after passaging primary ovarian cultures (*Figure* **3-16***)*. Passaging cells led to an increase in AR gene expression in six primary ovarian cultures (PCO 187, 238, 239, 247, 249 and 250), but to a decrease in AR mRNA expression levels in three primary ovarian cultures (PCO 231, 233 and 253) as shown in *Figure* **3-16**. 5/16 primary ovarian cultures showed either no or a mixed response (with increase and decrease) of AR gene expression to passaging.

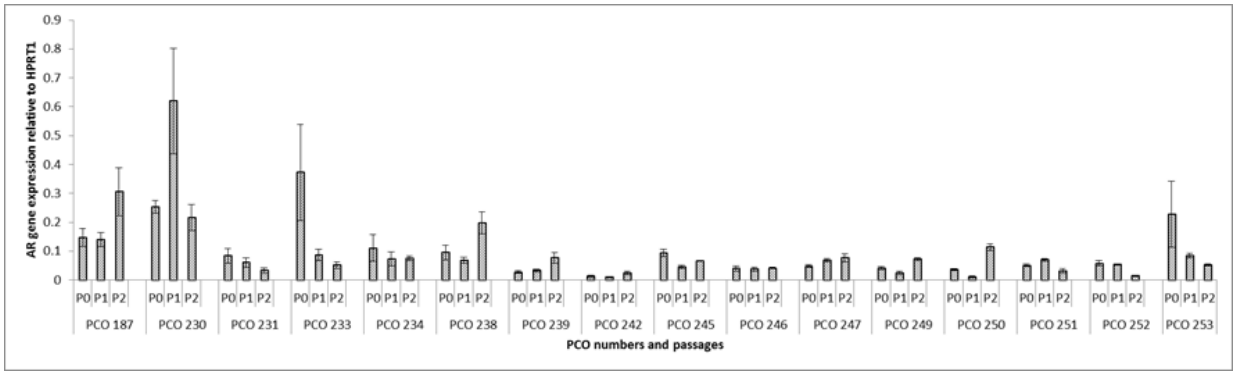


Figure 3-16: AR mRNA expression in 16 primary ovarian cultures, examined at passage zero, one and two. The relative gene expression was normalised to the housekeeping gene HPRT1. Values represent the mean ±SE of the mean of three repeats with triplicates of each reaction.

3.5 Results for Rab35 expression

3.5.1 Rab35 expression at mRNA level

A panel of 46 primary ovarian cultures was examined for Rab35 gene expression.

Primary ovarian cultures were obtained from ascites and cells seeded out as described in chapter 2.2.3. Cells at passage one were seeded out at $6x10^5$ cells/ well and RNA was extracted when cells reached 80% confluence. qRT-PCR was then performed as outlined in chapter 2.6 to assess Rab35 mRNA expression.

Rab35 was expressed in 100% of examined 46 primary ovarian cultures, and in the majority of these was present at higher levels than in OVCAR3 cells (see *Figure* **3-17**). This was in contrast to AR gene expression where most primary ovarian cultures express lower level of AR than the OVCAR3 cells (see *Figure* **3-3**).

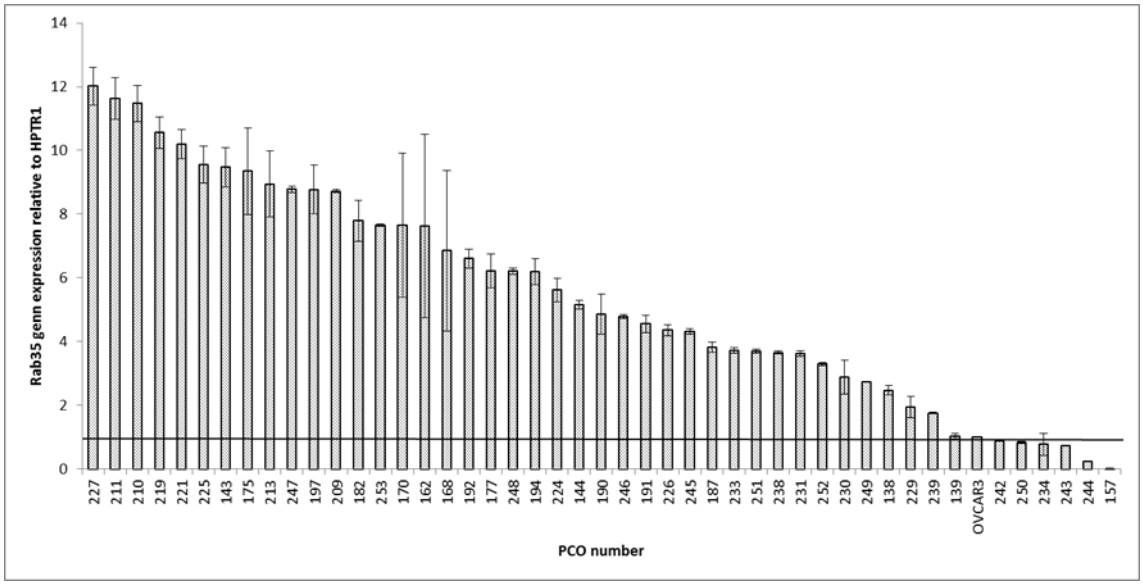


Figure 3-17: Rab35 gene expression in 46 primary ovarian cultures at passage one relative to HPRT1 as a fold increase to Rab35 expression in OVCAR3 cells. Values represent the mean ±SE of the mean of three repeats with triplicates of each reaction.

Samples	Genes- CT values		
PCO	AR	Rab35	HPRT1
139	27.720	23.790	22.419
157	33.240	26.899	21.669
190	30.865	24.817	22.160
229	29.936	28.676	25.492
230	31.511	30.656	27.584
231	30.749	29.890	23.826
233	33.473	29.698	26.491
234	32.851	30.733	26.888
238	29.260	24.662	22.392
239	30.154	25.246	22.181
242	32.326	26.697	23.289
243	32.638	25.750	23.316
244	33.504	27.059	22.533
245	30.802	29.298	24.120
246	28.491	23.961	22.034
247	28.504	25.090	22.933
248	29.034	24.622	22.941
249	28.699	23.132	21.429
250	32.974	29.722	23.759
251	32.842	30.684	25.871
252	32.177	29.859	25.069
253	28.953	27.164	23.174

Table 3-4: Sample set of raw data CT mean values from qRT-PCR for mRNA expression of AR, Rab35 and the house keeping gene HPRT1.

3.5.2 Rab35 protein expression on WB

Though Rab35 protein expression has previously been demonstrated on Western blot (SooHoo, 2013), Rab35 protein expression could not reliably be demonstrated in this study.

Western blot lysates from cell lines and primary cell cultures were obtained by lysis with SDS lysis buffer as described in chapter 2.5. HeLa cells were used as positive control.

Three antibodies were tested for Rab35 protein expression as listed in *Table **2-5***.

For optimisation purposes, all the antibodies were used in TBS with 1% and 5% milk at concentrations of 1:500, 1:1000 and 1:2000. Incubation with the primary antibody was done at 4°C overnight with incubation of the secondary antibody for one hour at room temperature. In none of the treatment conditions was it possible to obtain reliable, replicable bands at 23 kDa to demonstrate Rab35 expression.

The investigation of Rab35 protein expression on WB was hence abandoned and instead investigated with IHC and on mRNA level with qRT-PCR.

Two of the antibodies (purchased by Abcam and Proteintech) were the same as previously used by SooHoo. Their study trialled higher concentrations (1:200) of these two commercially available antibodies, but used a rabbit polyclonal antibody donated by Dr Arnaud Echard (Institute Curie, Paris) for their final experiments (SooHoo, 2013).

3.5.3 Rab35 protein expression on IHC

The same tissue microarrays used for analysis of AR were stained for Rab35 with the Proteintech antibody as listed in chapter 2.7, *Table* ***2-11***. Antigen retrieval was done using citrate buffer and the decloaker as described in chapter 2.7. Cytoplasmic staining was evaluated using the modified H score by two independent scorers (James Murray and Angelika Kaufmann) based on staining intensities determined for each TMA. *Figure* **3-18** shows an example of staining intensities for Rab35.

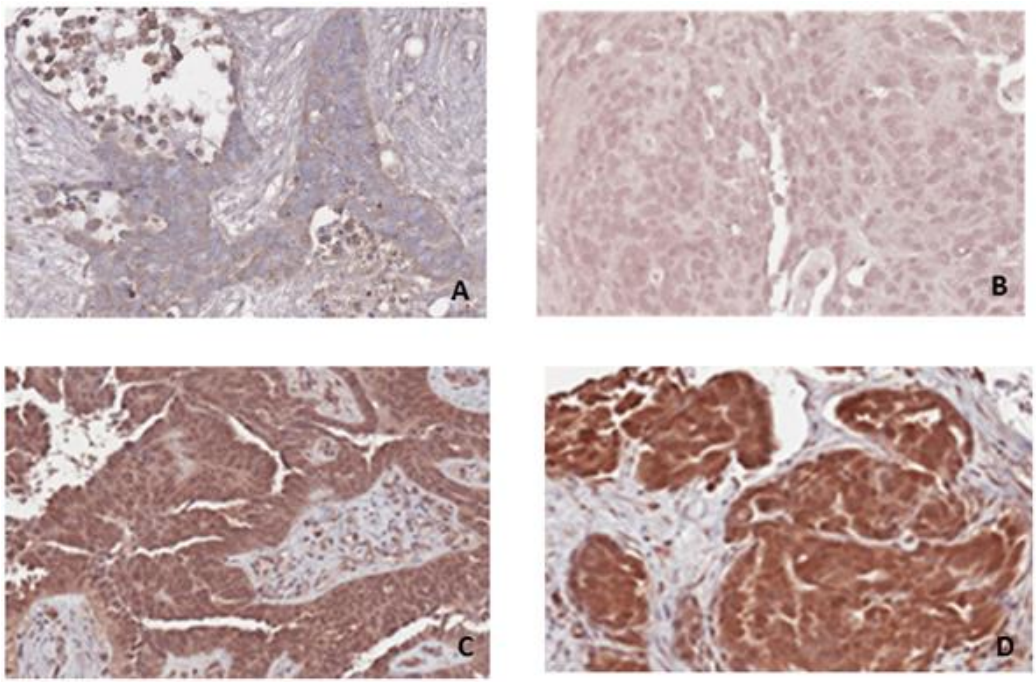


Figure 3-18: Staining intensities for the Rab35 protein expression on tissue micro arrays using immunohistochemistry. Intensities shown are 0 (negative for Rab35) (A), intensity 1 (B), intensity 2 (C) and intensity 3 (D). Samples shown are all from high grade serous ovarian cancer.

The examined tissues on the microarrays were chosen to correspond with primary cell cultures from ascites examined in this study.

A total of 27 ovarian cancers were examined for Rab35 expression with IHC, all of which expressed Rab35 at varying levels (see *Table* **3-6**).

3.5.4 Correlations for Rab35 expression

Correlations were examined with the Spearman rho test using SPSS.

3.5.4.1 Correlation of Rab35 gene expression and Rab35 protein expression on IHC

Samples from 27 patients were obtained from tumour to evaluate Rab35 protein expression by IHC and ascites was obtained from the same patient cohort to examine Rab35 gene expression by qRT-PCR.

Correlating these two expression modalities with the Spearman rho test, no correlation was found ($R^2 = 0.042$, $p>0.05$) (see *Figure* **3-19**).

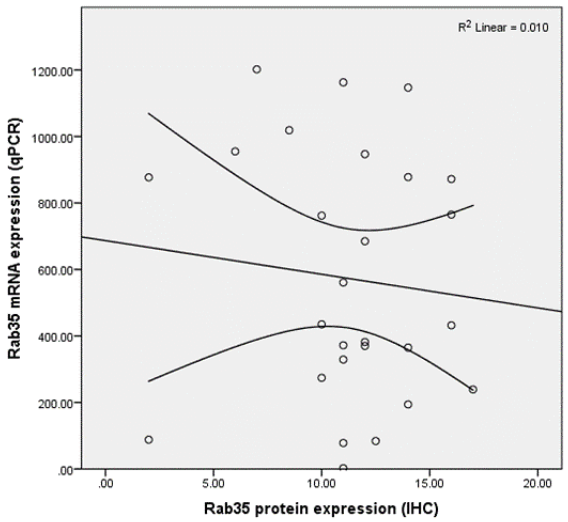


Figure 3-19: Rab35 protein expression on IHC and Rab35 mRNA expression in 27 primary ovarian cultures. There was no correlation between Rab35 protein expression on IHC and Rab35 gene expression ($R^2= 0.042$, $p> 0.05$).

3.5.4.2 Correlation of AR and Rab35 gene expression in primary cultures of ovarian cancer cells

43 primary ovarian cultures were examined for both, Rab35 gene and AR gene expression. 100% of primary ovarian cultures expressed both, the AR and Rab35 at very varying levels.

When correlating the mRNA expression of AR and Rab35 in 43 primary ovarian cultures with the Spearman rho test, a positive, non-significant correlation was found ($R^2= 0.098$, $p>0.05$) (see *Figure* **3-20**).

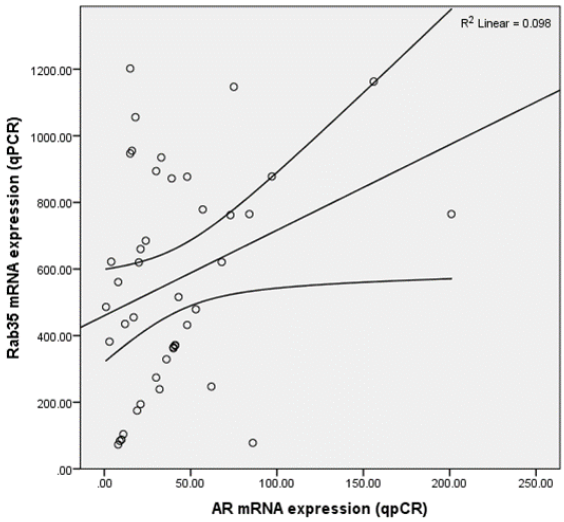


Figure 3-20: Spearman rho correlation for AR mRNA and Rab35 mRNA expression in 43 primary ovarian cultures. There is a non-significant positive correlation between AR and Rab35 gene expression with a R^2 value of 0.098 (p>0.05).

20 matching samples were examined for Rab35 protein and AR protein expression by IHC as listed in *Table* **3-6**. All these samples also had matching primary ovarian culture samples derived from ascites.

A positive, highly significant correlation was found when examining AR and Rab35 protein expression in 20 primary ovarian cultures measured with IHC (see *Figure* **3-21**) using the Spearman correlation test (R^2= 0.583, p<0.001).

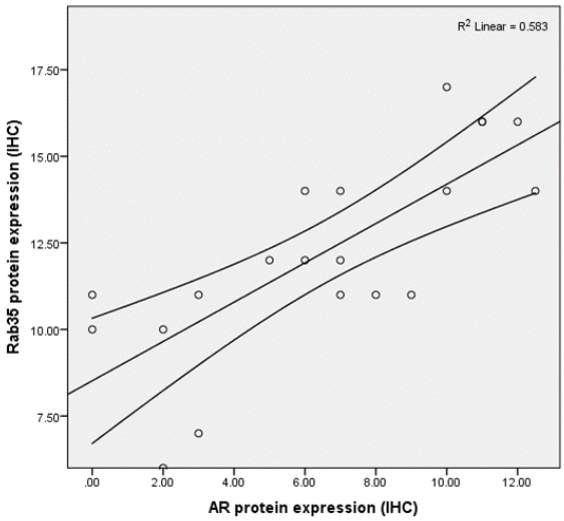


Figure 3-21: Spearman rho correlation for AR and Rab35 protein expression on IHC in 20 primary ovarian cultures. There is a significant positive correlation between the AR and Rab35 protein expression with a R^2 value of 0.583 (p<0.001).

3.5.4.3 *Correlations of Rab35 expression with progression free survival and overall survival*

PFS and OS in months were correlated with Rab35 gene and protein expression as described above in chapter 3.4.7.

There was no correlation for either PFS or OS and Rab35 gene expression for the 45 cases examined with a Spearman rho correlation, with a R^2= 0.005 (p=0.975) for PFS and R^2=2.727 (p=0.52) for OS respectively (see *Figure* **3-22**).

Rab35 protein expression showed a weak negative, non-significant correlation with survival with a Spearman rho correlation with a R^2 value of 0.046 (p=0.506) for PFS and a R^2 value of 0.034 (p=0.349) for OS (n=27) as shown in *Figure* **3-22**.

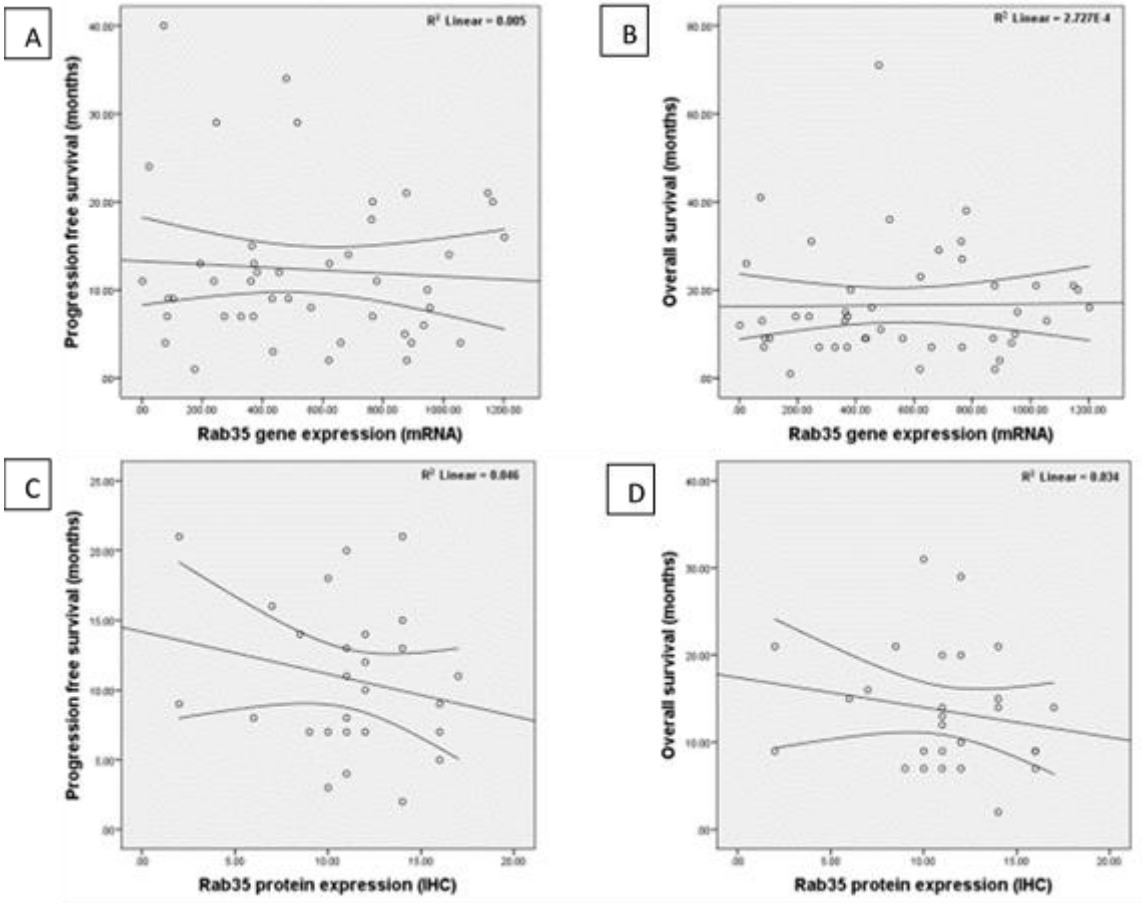


*Figure 3-22**:** Spearman rho correlation of AR expression and progression free survival (PFS) and overall survival (OS) in months. There is no correlation between Rab35 gene expression and PFS (A) (R^2=0.975, p=0.005) and OS (B) (R^2=2.772, p=0.52). A weak negative, non-significant correlation was seen for Rab35 protein expression and PFS (C) (R^2=0.046, p=0.506) and OS (D) (R^2=0.034, p=0.349).*

3.5.4.4 Survival curves for Rab35 expression and PFS and OS

Univariate analysis was used to compare the differences in PFS and OS in groups of low and high Rab35 gene and protein expression and the results demonstrated as Kaplan-Meier survival curves (*see Figure* **3-23**). The grouping into low and high expression of Rab35 was made based on the median of the expression data.

No beneficial effect on PFS of OS could be demonstrated for either Rab35 gene or protein expression (*Figure* **3-23**) which was also confirmed by statistical analysis (*Table* **3-5**).

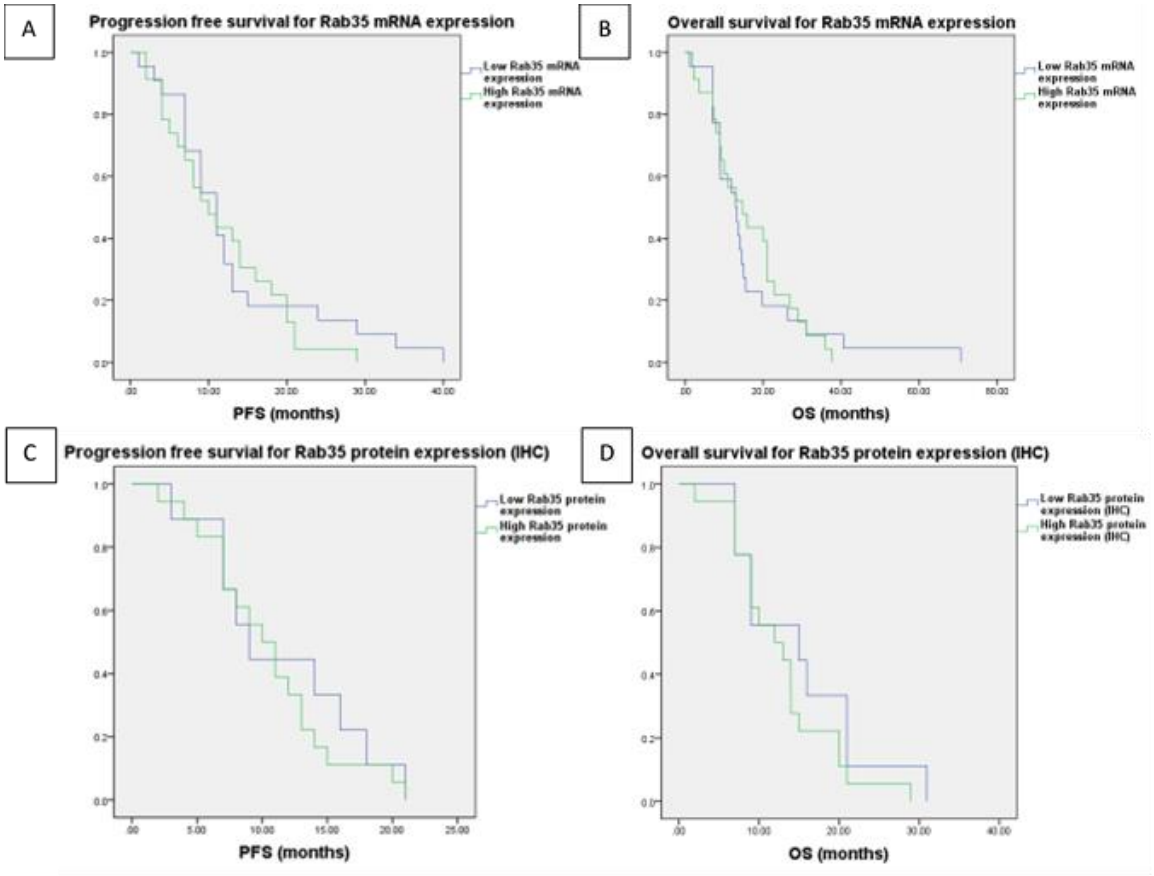


Figure 3-23: Survival in relation to AR expression visualized by Kaplan-Meier curves examining PFS and OS for Rab35 gene expression (A and B) and Rab35 protein expression on IHC (C and D).

	PFS				OS		
	HR	**95% CI**	**p**		**HR**	**95% CI**	**p**
Rab35 IHC	0.80	0.37 to 1.74	0.55		0.67	0.31 to 1.43	0.26
Rab35 mRNA	0.85	0.48to 1.53	0.56		1.06	0.59 to 1.90	0.83

Table 3-5: Hazard ratios and Confidence intervals for correlations between Rab35 expression (on WB, IHC and mRNA) and PFS and OS

3.6 Summary of result findings

The main conclusions from this chapter are as listed below:

- 100% of examined primary ovarian cultures express the AR at mRNA level (see *Figure* **3-3**)
- 87% of ovarian cancers expressed AR protein when examined with IHC (see *Table* **3-5**)
- 34% of PCOs expressed the AR on protein level when examined by Western blotting (see *Table* **3-5**)
- No isoforms could be identified with Western blotting in primary ovarian cultures
- No correlation could be found for
    - expression of AR protein measured with WB or IHC
    - expression of mRNA and protein abundance evaluated by Western blotting
    - AR gene and protein expression measured with IHC
- AR expression could not be measured with immunofluorescence
- Passaging may alter AR gene expression, however there was no consistent pattern/ effect of passaging on AR mRNA expression

- Rab35 was expressed in 100% of primary ovarian cultures at mRNA level (see *Figure* **3-17**)
- 100% of the examined tumours expressed Rab35 protein when tested with IHC (see *Table* **3-5**)
- It was not possible to use Western blotting for Rab35 protein expression despite optimization with three different antibodies
- There is no correlation between Rab35 gene and protein expression (see *Figure* **3-19**)

- ❖ There was a positive correlation of AR and Rab35 expression, at both, mRNA (see *Figure* **3-20**) and protein level (see *Figure* **3-21**)
- ❖ The correlation for AR and Rab35 at the protein level (IHC) was statistically significant (see *Figure* **3-21**)

- ▪ AR mRNA expression and AR protein expression showed a weak, non-significant positive correlation with PFS and OS (see *Figure* **3-12**)

- AR protein expression on IHC had no correlation with either PFS or OS (see *Figure* **3-13,** C and D)
- Neither AR mRNA expression, nor AR protein expression (on WB and IHC) had a statistically proven an effect on PFS or OS (see *Figure* **3-13**)
- The expression of Rab35 protein correlated weakly negative, but non-significant with PFS and OS (see *Figure* **3-22**, C and D)
- No improvement of PFS or OS was found for Rab35 expression with either Rab35 gene or protein expression (see *Figure* **3-23**)

3.7 Discussion

Translational research aims to investigate pathways, expression of substrates and pharmacological mechanisms in order to apply this knowledge to the human body with the aim to improve the course or cure a disease.

For this, several models can be used: animal models, cancer cell line models or primary cancer cell line models all of which have their own advantages and disadvantages.

Cancer cell lines are an established entity in molecular research and an easy model to use and offer the advantage to be passaged to high numbers. They allow revisiting experiments at a later date and the repeat of experiments to ensure reproducibility of data. The drawbacks of culturing live cells however are the potentials of infection and cross-contamination with other cell lines (Ye et al., 2015, Valletta et al., 2016). Furthermore, the creation of sub-populations through selection pressure (Hiorns et al., 2004) and changes in cell morphology and functionality such as altered migration and proliferation rates have also been described (Jin et al., 2017). Interestingly, Ertel *et al* compared gene expression patterns in cancer cell lines with normal and tumour tissue and found that cell lines showed unmistakably different molecular and signalling pathways compared to normal and tumour tissue (Ertel et al., 2006). In the ovarian cancers examined by this study group ovarian cancer cell lines showed a 41% and 62% upregulation of examined genes when compared to tumour or normal tissue respectively, whilst when comparing tumour to normal ovarian tissue the upregulation was only 14% (Ertel et al., 2006). Ertel *et al* suggest these observations might be due to the use of cell culture medium, further underlining the potential influence of selection pressure (Ertel et al., 2006). The use of primary cell cultures cannot exclude changes like this, but due to their short life span and shorter exposure to cell culture might be able to minimise these artificial effects and represent a model closer to *in vivo* conditions.

What's more, ovarian cancer is increasingly being recognised as a group of heterogeneous tumours sharing a tumour site, rather than representing one tumour type in a specific location (Paracchini et al., 2016). This tumour variety might account for the differences in the disease course and response to treatment of patients, even if diagnosed with the same subtype of cancer on histopathology. Protein expression and its variations, such as steroid hormone expression can easily be examined with IHC, as every patient diagnosed with ovarian cancer will have tumour tissue obtained to allow for histological confirmation and classification of the disease. However, tumour tissue can only provide information on protein expression and not be used for functional studies to help evaluate potential response to chemo- or hormonal therapy. Hence,

primary cell cultures derived from patients suffering from cancer, are a promising model to work with in molecular research and a further attempt to bridge this "translation gap".
Ovarian primary cancer cell line (PCOs) derived from malignant ascites have successfully been established and used by the ovarian group in Newcastle in several projects (O'Donnell et al., 2016).
Examining the potential involvement of AR in ovarian pathogenesis, this study uses primary ovarian cultures as a model mimicking these inter-tumour differences.

AR expression in primary cell cultures

As the AR has been associated with ovarian carcinogenesis, anti-androgens have been used in a few clinical trials, however only with little success. The trial designs suggest that the lack of response might be due to poor patient selection, as the AR status had not been determined (Papadatos-Pastos et al., 2011).
Establishing a way of quickly and reliably determining the AR status could hence be useful information in recognising patients who might respond the treatment with anti-androgens.
Primary cancer cell cultures derived from ascites can be obtained and cultured easily and have so far been examined for AR expression in two preceding projects (SooHoo, 2013, Elattar, 2010). This was however done in small numbers and with various methodologies (qRT-PCR, WB and IHC).
So far, it has not been established if any modality could be considered the most accurate and if the expression data derived from different methodologies can be equated.

Our findings show both, some agreement, but also discordance with observations from previous work within the ovarian cancer group in Newcastle.

In accordance with previous work (Elattar, 2010, SooHoo, 2013, Lau et al., 1999), all of the 44 examined PCOs in this study showed AR gene expression on q-PCR at varying levels.

In protein expression, the receptor status differed quite markedly from previous findings. Examination of protein expression in primary ovarian cancer cell lines with Western blotting has only been described in one study available and has reported a 87% expression of AR on WB in a panel of eight primary ovarian cultures with a 75% presence of a shorter AR isoform in his thesis, speculating that the presence of isoforms may confer anti-androgen resistance in EOC or determine response to anti-androgen therapy (Elattar, 2010). Our results are diverging,

as only 34% (16/47) primary ovarian cultures expressed the AR protein and no isoforms could be identified.

The contrast in the AR expression on WB between these studies might be explained in the experimental set up such as differences in antibody concentrations used. In order to objectify findings in western blotting and to ensure only true positive results were evaluated, we used a negative control as well as densitometry (Gassmann et al., 2009) in this study, which might have eliminated some results which might have otherwise been interpreted as positive. Primary ovarian cultures showed AR protein expression on WB at very varying levels- of the 16 AR expressing PCOs 15 showed an AR protein abundance much lower level than the reference cell line OVCAR3. Some bands appeared very faint and long exposure times might be required to detect expression. Without the use of a negative control an over-interpretation of AR abundance could have occurred.

Other than interpretation of protein expression, the increased number of primary ovarian cultures samples examined (n=47 in this study versus n=8 by Ahmed Elattar) might be accountable for the change in findings.

Detection of AR protein with IHC was also in contrast with previous findings in the Ovarian Cancer Research Group Newcastle, where 100% of all examined tissues (n=13) corresponding to PCOs were found to express the AR (SooHoo, 2013).

In this study, majority (87%, 20/23) though not all of the examined tumour tissues corresponding to PCOs confirmed AR protein expression.

In both studies protein abundance was assessed by two independent scorers in order to eliminate the subjective element of interpretation of expression data making bias less likely. It is hence more likely that subtle differences in the sample preparation could account for the diverging results. Though the same protocol was followed for IHC, slight differences in efficiency of the blocking step could have occurred potentially causing a difference or decrease in unspecific antibody staining in this present study and hence increased the negative receptor status.

Overall, AR protein expression on IHC shows a wide variety in literature, from 43 % (Lee et al., 2005) over 26% (van Doorn et al., 2000) to 10% (van Kruchten et al., 2015) which will mainly be contributed by technique such as the quality of microarrays examined, antibody staining and blocking steps. Some publications, such as Stringer-Reasor *et al* have even reported low to negative expression of AR on IHC without further quantification (Stringer-Reasor et al., 2015).

Immunofluorescence (IF) did not work in primary ovarian cultures for the AR receptor. This was surprising, as primary ovarian cultures were not only successfully characterised by IF for epithelial markers within the Newcastle Ovarian Cancer group, but was also described by other research groups (Bruning-Richardson et al., 2012, Strauss et al., 2011). Furthermore, the AR was detectable with IF in this study in established cancer cell lines like the ovarian cell line OVCAR3 and the prostate cancer cell line LNCaP.

Overall, whilst the AR receptor expression was detectable with three techniques- qRT-PCR, WB and IHC- methods of measuring AR expression cannot be used interchangeably. Most notably, as shown in *Table* **3-6**, no pattern emerges for the AR expression status in the same primary ovarian cultures sample when examined with different techniques.

This is mirrored in the lack of correlation between AR mRNA and protein expression, on WB and IHC, suggesting that gene expression does not necessarily lead to translation into protein (Taniguchi et al., 2010). This suggests that gene expression should not be used as surrogate marker for protein expression.

A thorough literature review failed to show any other work comparing the three examination techniques for ovarian cancer. However, the relation of protein and mRNA expression has been examined in other tumour types and predominantly suggested variable correlations. In prostate cancer poor to moderate correlations with a Pearson coefficient of 0-0.63 were described when comparing the expression of cluster designation (CD) genes on IHC and with mRNA (Pascal et al., 2008). An examination of 76 lung adenocarcinomas identified 165 protein spots with gel-electrophoresis and mass spectrometry and correlated these to mRNA identified by oligonucleotide microarrays (Chen et al., 2002) . The results were diverse, as for a subset of genes (17% of protein spots and 21% of mRNA) a significant correlation between protein and mRNA abundance was found, though this was not true for majority of the examined protein and mRNA. In a further small subset even negative correlations were seen (Chen et al., 2002).

This inconsistency in AR expression detection rates when comparing the techniques and the lack of correlation makes it impossible to determine one single examination technique as most suitable. However, in synergy, relevant information could be gained.

qRT-PCR is an easy and quick method to determine gene expression, with the drawback, as demonstrated in this chapter, that its presence might not necessarily lead to translation into protein (Taniguchi et al., 2010) as it can be influenced by post-translational modification or protein half-life.

As mRNA expression might not be representative for the protein synthetized, determination of protein expression per se might be deemed a more accurate way of assessing "true" expression status, as protein is the ultimate determinant of cell function. Whilst western blots allow quantification of a protein, IHC has the advantage to not only determine expression, but also localisation of the protein. In this study both techniques detect the AR, however, have very different detection rates. The expression also did not correlate.

The question as to which technique would be the best to reliably assess AR protein expression remains unanswered based on the data obtained.

The advantages of IHC is that because it is performed on tumour tissue, it is a direct representation of tumour material and might be a more accurate way of determining the receptor status. IHC is a technique routinely used in labs and FFPE blocks can be stored over a long time. The drawbacks are that results can be skewed if the sample cores in microarrays show poor representation of tumour as they might be too small or damaged in the preparation process. A further disadvantage is that sample collection requires invasive techniques such as operations or endoscopic biopsies and is related to morbidity of these procedures. On the other hand, tissue samples will be obtained for each patient, as they are crucial to get a histological diagnosis of cancer, so tissue samples for further investigations should be readily available.

Primary ovarian cultures, whilst having a role in representing the cancer heterogeneity, might need to be used with caution for receptor status determination. Derived from ascites, they are, compared to IHC, a more indirect measure of tumour material and bear the risk of selecting for a cell subpopulation through cell culture, which might be reflected in the finding that 14/20 primary ovarian cultures showing no AR protein expression on WB had high levels or AR protein on IHC. A possible reason for this could be i*n vitro* cultivation, which in primary breast and ovarian cancer cells has demonstrated a change in cell morphology and alterations in anticancer drug sensitivities through changes in the microenvironment (Bezdieniezhnykh et al., 2016) as well as altered gene expression status through passaging.

Furthermore, as demonstrated above, mRNA expression might not necessarily represent protein expression.

Nonetheless primary ovarian cultures do have the advantage of being the closest model representing tumour heterogeneity that allows functional assays and hence do have an important role in translational research.

Timing of sample collection

The importance to determine the AR status in order to stratify for the right patient group which could possibly benefit from anti-androgen treatment remains. A further factor, besides the presence of the AR and its functionality is the timing of AR expression evaluation.

It has been suggested that the AR expression decreases after chemotherapy exposure (Elattar et al., 2012), as well as in recurrent ovarian cancers (Feng et al., 2017), which- in the heavily pre-treated patient population in clinical trials to date- might explain the poor response rate to anti-androgens.

Feng *et al* observed a difference in AR protein expression on IHC in primary (33.6%) and recurrent ovarian cancers (17.5%) (Feng et al., 2017). In this study, the samples were obtained at primary presentation only, at either primary or interval debulking, but not after recurrence, hence no comparison could be made.

Elattar *et al* evaluated the AR protein expression on IHC on paired samples in a cohort of 29 patients with EOC (Elattar et al., 2012). Whilst we did not look at paired pre-and post-chemotherapy samples in this study, our findings suggest a different trend. We examined 24 PCO samples for AR protein expression on IHC. Of these, 19 were obtained from chemo naïve patients at primary debulking surgery and only 15% (3/19) expressed the AR, whilst all of the five samples obtained at interval debulking surgery after chemotherapy expressed the AR (100%). Whilst a direct comparison is not possible, the differences are most likely explained by differences in technique and evaluation. Elattar *et al* used a primary mouse monoclonal AR antibody at concentrations of 1:20 and evaluated nuclear as well as cytoplasmic AR expression, whilst in this study a polyclonal rabbit antibody was used at much lower concentrations (1:500) and only nuclear staining was evaluated.

Interestingly, in this study of the primary ovarian cultures examining AR protein expression on Western blot 16/47 showed AR protein expression. Majority of these (13/16) AR expressing PCOs were taken from chemo naive patients. Of the three remaining samples, one patient sample was taken a long time (20 months) after completion of chemotherapy, whereas data for the remaining two are unavailable.

On the contrary, in a paired sample (PCO 177 and 222), from the same patient, the AR protein expression on western blot increased after chemotherapy. The first ascites sample (PCO 177) was taken one year after the initial diagnosis of ovarian cancer and treatment with surgery and combined chemotherapy (Carboplatin/ Paclitaxol), and AR protein expression on WB was negative. The patient then received six further cycles on single agent chemotherapy

(Carboplatin) for progressive disease. The second sample (PCO 222) from the same patient was obtained 20 months after the initial diagnosis and 2 months after the completion of the single agent chemotherapy. PCO 222 was strongly positive for AR protein on WB expression, showing 3.5 fold AR expression when compared to OVCAR3.

This demonstrates that potential differences in expression when examining samples derived from ascites (PCOs) and tissue samples might also be accounted for by the time of collection. Quite commonly tissue samples might have been obtained at a different time point than ascites. Tissue samples could have been obtained at primary surgery of a chemo naïve patient, whilst the ascites could have been taken weeks or months later when paracentesis was performed on the ward, possibly after receiving chemotherapy. Equally paracentesis could have been performed prior to receiving chemotherapy, with tissue sample being taken later at the time of interval debulking surgery after chemotherapy.

In conclusion, using AR expression status as a criterion to stratify for patients potentially benefitting from anti-androgen treatment remains difficult and it remains undetermined which modality would be most suitable. Arguably, as tumour tissue is routinely obtained for diagnosis and at surgical treatment, IHC might be the best option, as no additional methods would need to be applied. It would we relatively easy to add AR to the routine panel of staining performed at present. Routine AR receptor expression status assessment would allow for much bigger patient cohorts and data sets to be obtained to assess (targeted) treatment response and stratification, but would at present only be done in the remits of clinical trials. Once the AR receptor status is determined, consideration should also be given the timing of potential anti-androgen treatment, ie the therapy should be administered at the time of the highest AR expression, which might be prior to chemotherapy administration.

Rab35 expression in primary cell cultures

AR expression seems to be an obvious bit of information required when determining potential targets and stratification of anti-androgen treatment and as expression might be prognostic factor regarding outcome (Nodin et al., 2010). However, hormone receptor expression alone cannot not determine functionality of a receptor.

Rab35 was identified as an androgen dependent protein in an ovarian cancer cell line and its expression was also previously examined in a small panel of primary ovarian cultures (SooHoo, 2013).

In this study, Rab35 expression was examined in a larger panel of primary ovarian cultures and as for AR, gene and protein expression were correlated.

Rab35 was expressed at mRNA level in all 43 primary ovarian cultures and on IHC in all 27 samples examined, underpinning previous results (SooHoo, 2013). In both studies, by far majority of gene expression was higher than that of the reference cell line OVCAR3.

Unfortunately, despite optimisation, detection of Rab35 protein WB was not possible in this study which could be due to the use of polyclonal antibody and the difficulty in determining single bands confirming Rab35 expression.

As seen in the AR expression, there was no correlation for Rab35 mRNA and protein expression in keeping with previous findings (SooHoo, 2013). As mentioned in the examination for the AR, qRT-PCR and IHC should be used in synergy rather than exclusively.

A promising result was the positive correlation of AR and Rab35 on mRNA as well as protein level which was noted in previous work (SooHoo, 2013) and confirmed in larger number in this study. At protein level the AR and RAb35 correlation was statistically significant. These findings support the hypothesis of Rab35 being an androgen related gene.

The functional relation of the AR and Rab35 in primary ovarian cultures are further examined in chapter 5.

AR and Rab35 expression in relation to survival

Few study groups have suggested the AR as a prognostic marker for clinical outcome.

Jonsson *et al* demonstrated an impressive statistically significant increase for both, 5-year progression-free survival (PFS) and overall survival (OS) when examining for AR protein expression with IHC in chemo naïve women with epithelial ovarian cancer (EOC) of the serous and endometrioid subtype and also saw a further improvement of prognosis in co-expression of AR and PR (Jonsson et al., 2015). Nodin *et al* who also examined the correlation of AR protein expression on IHC in 154 EOCs could however only demonstrate prolonged disease specific survival ($p=0.034$) for the serous subtype in both, univariate and multivariate analysis (adjusted for age, grade and clinical stage), but not in non-serous EOC (Nodin et al., 2010).In our study 22/24 examined ovarian cancers were of the high grade serous type, with one carcinosarcoma and one high- grade serous cancer with clear cell component. The IHC determined AR receptor expression did not correlate with either PFS or OS. The Kaplan-Meier survival shows a possible statistically non-significant trend for increased OS with low AR protein expression on IHC and no difference in PFS survival. The difference in results compared to published literature might

be partially explained due to the difference in histological subtypes but also much smaller sample size in our cohort.

Expression of the AR protein on WB has not been published by any group and in this study has not shown any statistical significance on survival.

AR gene expression in our study demonstrated a weak, non-significant positive correlation with both, PFS and OS. The Kaplan-Meier survival curves show a statistically non-significant trend of increased PFS and OS with high AR mRNA expression. This lack of difference in survival time is mirrored by Jonsson *et al's* findings who speculated that their impressive results of AR protein expression on survival could be correlated to mRNA sex steroid expression (Jonsson et al., 2015).

Their examined cohort of 285 patients however showed a wider histological distribution than the cohort examined for AR protein, including high and low grade serous epithelial cancer, endometroid cancers, borderline cancers as well as fallopian and primary peritoneal cancers. AR gene expression was, furthermore, not correlated with the histological subtypes, but with subtypes based on molecular properties classed as "immunoreactive", "proliferative", differentiated" and others. Jonsson *et al* findings found AR genes were expressed similarly in all subgroups, but also that no examined gene could predict the PFS or OS (Jonsson et al., 2015). It is hence not possible to compare our findings with findings published in literature.

List of primary ovarian cultures with expression data

PCO number	Histology	AR protein expression (WB)	AR protein expression (IHC)	AR mRNA expression	Rab35 protein expression (IHC)	Rab35 mRNA expression
44	HGS	Pos (40)	NA	NA	NA	NA
49	HGS	Pos (15)	NA	NA	NA	NA
122	HGS	Pos (29)	NA	NA	NA	2891
138	HGS	NA	NA	62	NA	247
139	Endometroid with clear cell component	0	NA	11	NA	104
143	HGS	NA	0	15	12	947
144	Endometroid with clear cell component	0	NA	43	NA	516
157	HGS	0	9	NA	11	2
162	HGS	NA	4	73	10	762
168	HGS	Pos (31)	5	24	12	685
170	HGS	0	NA	201	NA	765
174	Endometroid of the ovary	0	NA	38	NA	13190
175	Clear cell cancer	NA	NA	33	NA	935
176	HGS	Pos (18)	NA	NA	NA	NA
177 (with 222)	HGS	0	NA	4	NA	622
182	HGS	0	NA	57	NA	779
187	HGS	Pos (14)	6	3	12	382
190	Mucinous adenocarcinoma	0	NA	1	NA	486
191	HGS	Pos (48)	NA	17	NA	455
192	Clear cell/ endometrial	Pos (15)	NA	21	NA	660
194	Enteric type adenocarcinoma in mature cystic teratoma	0	NA	20	NA	620
197	HGS	Pos (62)	NA	48	2	877
209	HGS	0	12	39	16	872
210	HGS	0	12.5	75	14	1147
211	HGS	0	0	156	11	1163
213	HGS	NA	NA	30	NA	894
219 (with 234)	HGS	0	NA	18	NA	1056
221	Carcinosarcoma	0	6	NA	8.5	1019
222 (with 177)	HGS	Pos (349)	NA	NA	NA	NA

PCO number	Histology	AR protein expression (WB)	AR protein expression (IHC)	AR mRNA expression	Rab35 protein expression (IHC)	Rab35 mRNA expression
224	HGS	Pos (4)	3	8	11	561
225	HGS	Pos (16)	2	16	6	955
226	Low grade serous	0	2	12	10	435
227	HGS	0	3	15	7	1202
229	HGS	0	10	21	14	194
230	HGS and clear cell component	0	10	32	17	239
231	HGS	0	NA	40	NA	362
233	HGS	0	9	41	11	372
234 (with 219)	HGS	0	7	86	11	78
238	HGS	0	7	40	14	365
239	HGS	0	NA	19	NA	175
242	HGS	0	NA	10	2	88
243 (with 121)	HGS	Pos (16)	NA	8	NA	73
244	HGS	Pos (12)	NA	NA	NA	24
245	HGS	0	11	48	16	432
246	Endometroid adenocarcinoma	0	NA	53	NA	479
247	HGS	Pos (63)	6	97	14	878
248	HGS	0	NA	68	NA	621
249	HGS	Pos (50)	0	30	10	274
250	HGS	0	NA	9	12.5	84
251	HGS	0	7	41	12	370
252	HGS	0	8	36	11	329
253	HGS	0	11	84	16	765

Table 3-6: Primary ovarian cultures examined in chapter 3. AR expression on protein on Western blot level (WB), AR and Rab35 on mRNA level are given as percentage of AR expression in OVCAR3 cells. AR and Rab35 protein expression on immunohistochemistry (IHC) is expressed with the H score. NA: expression status has not been assessed.

4 The effect of androgen stimulation on proliferation in primary ovarian cancer cell cultures

4.1 Introduction

The expression of the AR has previously been confirmed in ovarian cancer cell lines as well as primary ovarian cultures (PCOs) (Elattar, 2010, SooHoo, 2013). As described in chapter 3, AR expression in PCOs has also been confirmed in this study albeit with some inconsistent observations. In our study AR gene expression was observed in all examined primary cell cultures, whereas AR protein expression was only seen 87% of examined samples with IHC and 34% when examined by Western blotting.

This is of interest as androgens- exerting their effect via the AR- have been implied in ovarian carcinogenesis.

Thus far, only a few publications examine the molecular effects of androgens on ovarian cancer cell lines.

An increased cell proliferative was noted in the ovarian cancer cell lines as a response to androgen exposure supporting the hypothesis of androgens being involved in tumorigenesis (Syed et al., 2001, Sheach et al., 2009, SooHoo, 2013). This proliferative androgen effect was furthermore underpinned by the abrogation of the effect with the used of anti- androgens (SooHoo, 2013, Syed et al., 2001).

In primary ovarian cell cultures the effect of androgen stimulation has only been evaluated in very small numbers by Elattar *et al* (Elattar et al., 2012), showing an increase in S-phase fraction of the cell cycle after androgen stimulation (Elattar, 2010, Sheach et al., 2009). Cell proliferation in primary cultures due to androgen stimulation in PCOs has so far not been examined.

In this study we aim to further examine the effects of androgen stimulation on proliferation in primary ovarian cultures with the hypothesis that androgen treatment would lead to an increase in cell proliferation.

Cell culture work with primary ovarian cultures differs from established cell lines, with primary ovarian cultures having a much shorter life span. In view of this and as observed proliferative androgen effects in OVCAR3 cells were subtle (Sheach et al., 2009), this study used two proliferation assays as described in chapter 2.4 to compare the potential effects of androgens on PCOs- the Sulforhodamine B (SRB) assay and the Bromodeoxyuridine enzyme linked immunosorbent assay (BrdU ELISA).

Both assays have previously been used in ovarian cancer cell lines- the SRB to examine proliferative and anti-proliferative effects of agents (Jia et al., 2013, Yan et al., 2013, Mao et al., 2016, Sheach et al., 2009) and the BrdU ELISA to demonstrate the effect of agents on proliferation (Al-Alem et al., 2011, Wan et al., 2015).

The difference between these two assays lies in the measured entity. The SRB assay determines cell density by measuring the cellular protein content where dye is bound to the basic amino acids of cellular proteins and the spectrometric quantification by colour change. The BrdU ELISA was used as described in chapter 2.4.2 and evaluated as an alternative, as due to measuring cell proliferation through assessment of DNA synthesis rather than protein content it was hypothesised to potentially be more accurate. In short, Bromodeoxyuridine (BrdU) is incorporated in the DNA of proliferating cells instead of the pyrimidine deoxynucleoside thymidine. Cells are then fixed and the DNA is denatured in order to allow the antibody binding of the incorporated DNA. An anti-BrdU probe binds to the newly synthesised cellular DNA. Formed immune complexes undergo a colorimetric reaction and their absorbance is measured with a spectrometer. The amount of colouric reaction and absorbance values correlate directly to the DNA synthesis and therefore with the number of proliferating cells.

Optimisation experiments in this chapter for the proliferation assays and cell viability were done in OVCAR3 cells.

The non-aromatisable dihydrotestosterone (DHT) was used in the following experiments to ensure that observed effects were due to androgen exposure alone. DHT was used at a dose of 10nM, to mimic a near normal physiological environment, based on experiments by previous study groups (Syed et al., 2001, Sheach et al., 2009, SooHoo, 2013).

The cut off for proliferation was set arbitrarily at a 10% increase of cells.

4.2 Aims

- To identify a suitable assay for evaluation of the proliferative response of androgens in primary ovarian cultures using optimisation with established cell lines
- To examine inter assay variability between androgen proliferation experiments using basal or steroid depleted media
- To measure the effects of androgen treatment in primary ovarian cultures on proliferation
- To evaluate if passaging of primary ovarian cultures might have an effect on the proliferative effect of androgen
- To determine if freeze- thawing would influence the androgen effect on primary ovarian cultures

4.3 Results of proliferative effects of androgens on primary ovarian cancer cell lines

4.3.1 SRB and BrdU assays used for the evaluation of proliferative effects of androgen on ovarian cancer cell lines

In order to evaluate a suitable proliferation assay for primary cancer cell lines, the SRB and BrdU assay were optimised in the AR expressing and androgen responsive ovarian cancer cell line OVCAR3.

Effects of androgen exposure measured by the SRB was also evaluated using LNCaP cells, a prostate cancer cell line expressing the AR and known to proliferate in response to androgen exposure.

Dihydrotestosterone (DHT) was used for androgen stimulation in experiments at 10nM DHT, as previously optimised by Sandra SooHoo from the ovarian cancer group in Newcastle (SooHoo, 2013). The optimal time point determined by SooHoo to observe proliferation after androgen exposure in OVCAR3 cells was previously seen at 96 hours, compared to 48 hours in LNCaP cells (SooHoo, 2013).

4.3.1.1 Effects of androgen stimulation on a prostate cancer cell line (LNCaP) and on ovarian cancer cell line (OVCAR3) using the SRB assay

Optimisation of exposure times

Firstly, the doubling times for LNCaP cells and OVCAR3 cells were evaluated with the SRB assay as described in chapter 2.4.1. The mean doubling time for LNCaP cells was 30.5 hours and for OVCAR3 cells 48 hours. The media used are listed in *Table* **2-2**.

To optimise cell densities used for the proliferation assay, LNCaP and OVCAR3 cells were seeded out in 96 well plates in concentrations of 10^3, $2x10^3$ or $5x10^3$ cell per well in sextuplicate. The cells were allowed to adhere for 24 hours, then washed with PBS twice and incubated in one of the following treatment conditions: basal (=serum free) medium, basal medium with 10 nM DHT and full medium. In view of the doubling times as stated above, LNCaP cells were fixed at 24, 48 and 72 hours and OVAR3 cells were fixed at 48, 72 or 96 hours and the proliferation was measured with the SRB assay as described in chapter 2.4.1. All experiments were done in triplicate.

The proliferative effect of LNCaP cells was best demonstrated at a cell concentration of $2x10^3$ and for OVCAR3 cells at cell densities of $5x10^3$ cells/ well. These cell densities were used for seeding out LNCaP and OVCAR3 cells in all further proliferation experiments.

As shown in *Figure* **4-1**, LNCaP cells showed an increase in proliferation of 18% and 40% when treated with 10 nm DHT after 48 and 72 hours exposure as opposed to treatment in basal medium only.

OVCAR3 cells grew well in full medium. Compared to growth in basal medium, treatment with 10 nM DHT did not lead to proliferation after 48 and 72 hours, but a 25% increase in proliferation was seen after 96 hours (see *Figure* **4-2**).

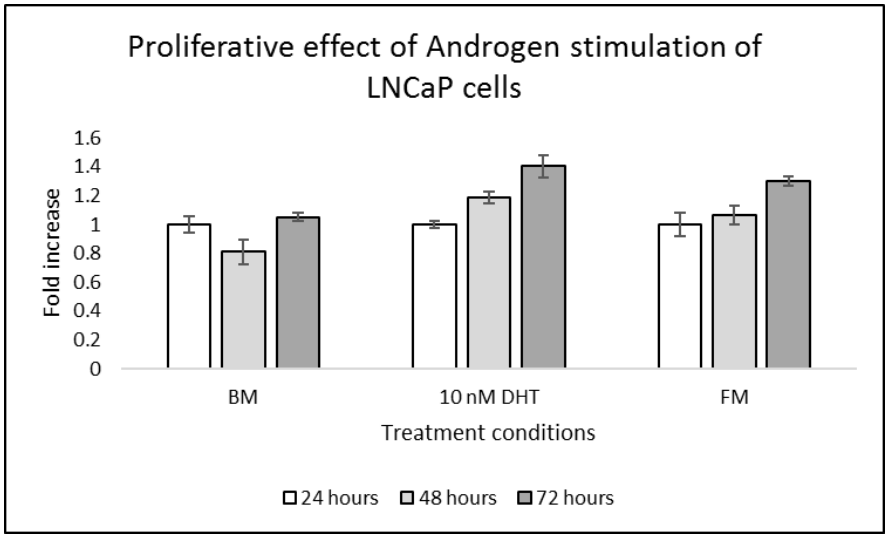


Figure 4-1: LNCaP cells were seeded out as 2x10^3 cells/ well, stimulated with 10 nM DHT in basal medium (BM) and proliferation was measured with the SRB assay. Cells grown in basal medium (BM) only have been used as negative control, cells grown in full medium (FM) as positive control. Values represent the mean ±SE of the mean of three repeats with sextuplicate in each experiment.

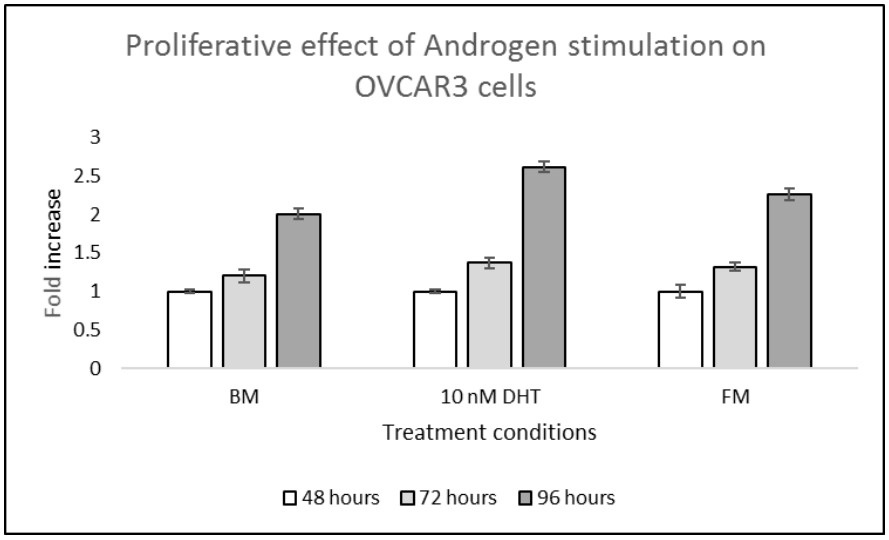


Figure 4-2: Proliferative effect of androgen stimulation on OVCAR3 cells using the SRB assay. Cells were seeded out at 5x10^3 cells/well and stimulated with 10nM DHT in basal medium (BM). Cells treated with basal medium only (BM) were used as negative control and cells treated with full medium (FM)

were used as positive control. Values represent the mean ±SE of the mean of three repeats with sextuplicate in each experiment.

4.3.1.2 Comparison of proliferative effects and growth of OVCAR3 cells in two different types of media using the SRB assay

Rationale

Previous evaluation of a cohort of 30 primary ovarian cultures has shown a wide range of doubling times from 79-195 hours, with a mean of 100 hours (RL et al., 2014). In general, these doubling times are much longer than those of established ovarian cancer cell lines. Hence, in order to see proliferative effects, potentially long exposure of primary ovarian cultures would be required.

The majority of assays evaluating hormone or growth factor response involve quiescing the cells into a growth arrested phase prior to introduction of the growth factor of interest. In a long assay, therefore cells are exposed to quiescent medium for long periods of time, which could potentially mimic abrogating proliferative effects due to starvation of cells.

To optimise conditions for later use with primary ovarian cultures different media were therefore investigated, specifically basal medium was compared to steroid depleted medium (SDM) (see *Table* **2-2**). OVCAR3 cells were used for these assays in preference to primary ovarian cultures to provide a uniform model.

Aims

- To evaluate viability of OVCAR3 cells over a period of 10 days in basal medium and SDM
- To examine inter assay variability between androgen proliferation experiments using basal medium or SDM

Method

For the evaluation of viability, OVCAR3 cells were seeded out at $2x10^3$ cells/ well in 96 well plates in full medium in six wells and incubated for 24 hours. After two washes with PBS they were quiesced for 24 hours in basal (=serum free) medium. After a further two washes with PBS cells were incubated either in basal or SDM medium. Plates were fixed every 24 hours until day ten and stored at -4°C, until at day ten all plates were washed, treated with SRB and read with the spectrometer as described in chapter 2.4.1.

For androgen proliferation experiments, cells were seeded and quiesced as described above. After quiescence, cells were treated with basal medium (negative control), full medium (positive control) and 10 nM DHT in basal medium. As ethanol was used as DHT solvent, cells not treated with DHT were treated with corresponding doses of ethanol alone to exclude any observed effects were due to ethanol.

To examine androgen effects in SDM, cells were seeded and quiesced as above and treated with 10nM DHT in SDM, using SDM only as negative and full medium as positive control.

For the proliferation experiments, plates were also fixed in 24 hour intervals with trichloracetic acid (TCA) for ten days and stored at -4°C before being washed and treated with SRB as described in chapter 2.4.1.

Results

Evaluation of viability of OVCAR3 cells over a period of 10 days in basal medium and steroid depleted medium

Treatment with full medium led to a continuous cell growth over all ten days. As demonstrated in *Figure* **4-3** (A), exposure of cells to basal medium (BM) only showed maintenance of cell viability, but no growth over the same time period. Treatment with steroid depleted medium (SDM) showed continuous proliferation for 10 days, with a growth pattern very similar to cells grown in full medium (see *Figure* **4-3**, B).

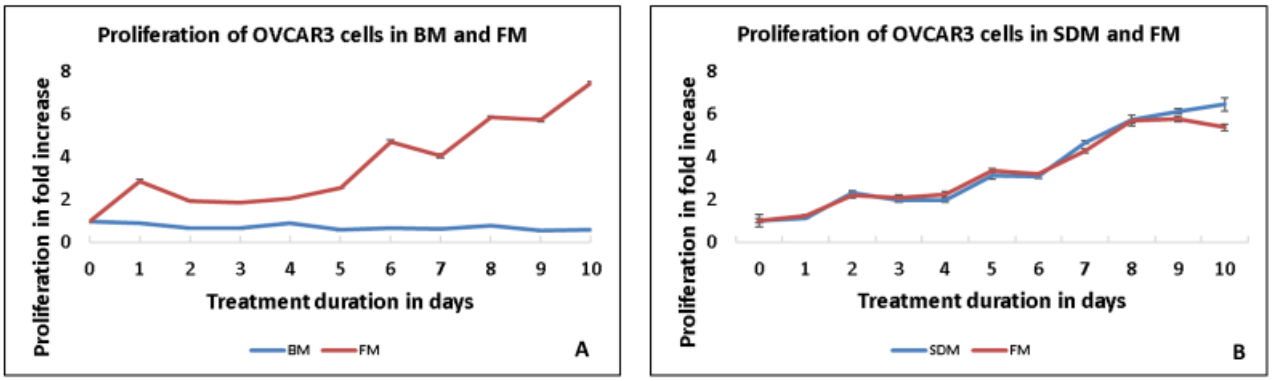


Figure 4-3: Comparison of proliferation of OVCAR3 cells in basal medium (BM), steroid depleted medium (SDM) and full medium (FM) using the SRB assay. Values represent the mean ±SE of the mean of three repeats with sextuplicate in each experiment.

Examination of inter-assay variability between androgen proliferation experiments using basal or steroid depleted media

OVCAR3 cells treated with 10 nM DHT show a different proliferation pattern if grown in basal medium or steroid depleted medium (see *Figure* **4-4** and *Figure* **4-5**).
Cells stimulated with 10 nM DHT in basal medium showed more growth than treatment with basal medium only, though the differences were subtle (see *Figure* **4-4**). The proliferative effect was significant on day three and four ($p < 0.05$) and on day 10 ($p=0.02$).

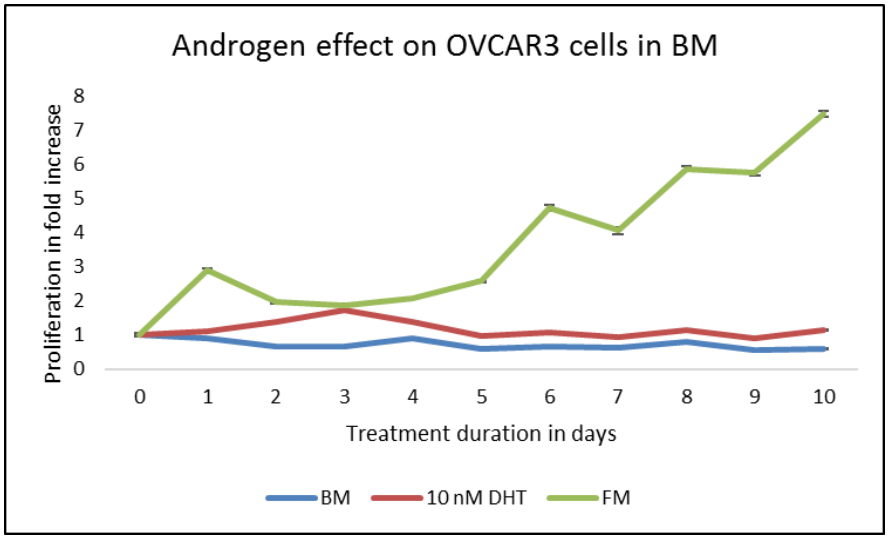


Figure 4-4: Androgen effect on OVCAR3 cells in basal medium. Cells were treated with 10 nM DHT in basal medium (BM) and fixed in 24 hour intervals for 10 days. Cells grown in full medium (FM) were used as positive control, cells in basal medium (BM) only were used as negative control. Values represent the mean ±SE of the mean of three repeats with sextuplicate in each experiment.

In contrast, treatment of OVCAR3 cells with SDM showed cell growth throughout all ten days of treatment. Proliferation for all three treatment conditions – FM, SDM alone and SDM with 10nM DHT- was very similar, with no distinguishing or statistically significant differences (when calculated with the t-test) in proliferation due to androgen exposure (as seen in *Figure* **4-5**).

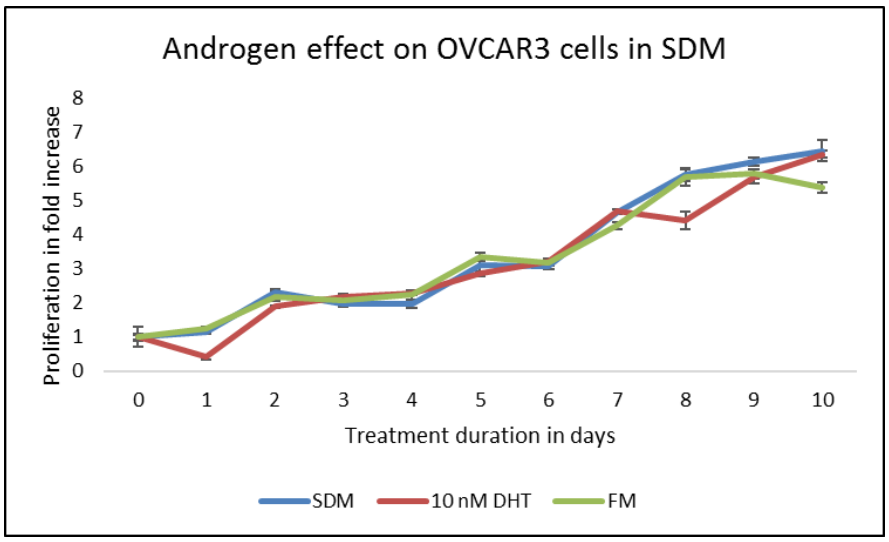


Figure 4-5:Androgen effect on OVCAR3 cells in steroid depleted medium (SDM). Cells were grown in full medium (FM) as positive control, SDM only (negative control) and 10 nM DHT in SDM over ten days. Values represent the mean ±SE of the mean of three repeats with sextuplicate each experiment.

4.3.1.3 *Effects of androgen stimulation on an ovarian cancer cell line (OVCAR3) using the BrdU assay*

Rationale

Proliferative effects of androgens on OVCAR3 cells measured with the SRB assay have been seen, though they were subtle.

As the SRB assay measures total protein content and might not be able to evaluate subtle changes in proliferation, it was postulated, that the BrdU assay, which measures DNA synthesis and might be a more accurate method to detect these small effects.

As reagents are available in limited amount and costly and daily fixation for ten days is not feasible for the BrdU assay, three time points for fixation were set- 24 and 96 hours and 10 days.

The 96 hour time point was based on the doubling time of OVCAR3 cells of 30.8 hours and the observation that proliferation was measurable after four days with SRB assays. The 24 hour time and 10 days points were chosen to establish a baseline, in order to compare findings with BrdU assays obtained on primary ovarian cultures cells.

Aim

- To evaluate the proliferative effect of androgen stimulation in OVCAR3 cells at different time points using the BrdU assay

Methods

The optimisation for cell density was done in preliminary experiments. OVCAR3 cells were seeded out in triplicate at 10^3, $2x10^3$, $5x10^3$ cells/ well in 96 well plates in full medium and allowed to adhere for 24 hours. On day two, the cells were washed with PBS twice and quiesced with serum free medium for 24 hours. After two further washes on day three, cells were treated with the desired treatment medium (full medium, 10 nM DHT in basal medium, 100 nM DHT in basal medium and basal medium only as negative control). Time points of fixation and measuring the absorbance were either at 24 or 96 hours or 10 days after the treatment.

Cells were then treated with BrdU labelling solution 24 hours prior to fixation. An anti-BrdU labelling solution and a substrate solution to allow colorimetric absorbance readings were added as described in chapter 2.4.2.

The cell density of $5x10^3$ cells/ well showed the best proliferation and was used for further experiments. All experiments were done in triplicates.

Results

Androgen treatment of OVCAR3 cells showed no proliferation after 24 hours (see *Figure* **4-6**). OVCAR3 cells treated with 10 nM DHT for 96 hours showed an increase in proliferation by 18% which was statically significant (p=0.02) as previously observed with the SRB assay (*Figure* **4-2**) whilst treatment with 100 nM DHT showed 6% increase in proliferation, which was statistically not significant (p=0.45) (see *Figure* **4-6**).

After ten days, the increase in proliferation observed was smaller- 11% after 48 hours and 4% after 96 hours (see *Figure* **4-6**). Neither of these increases were statistically significant.

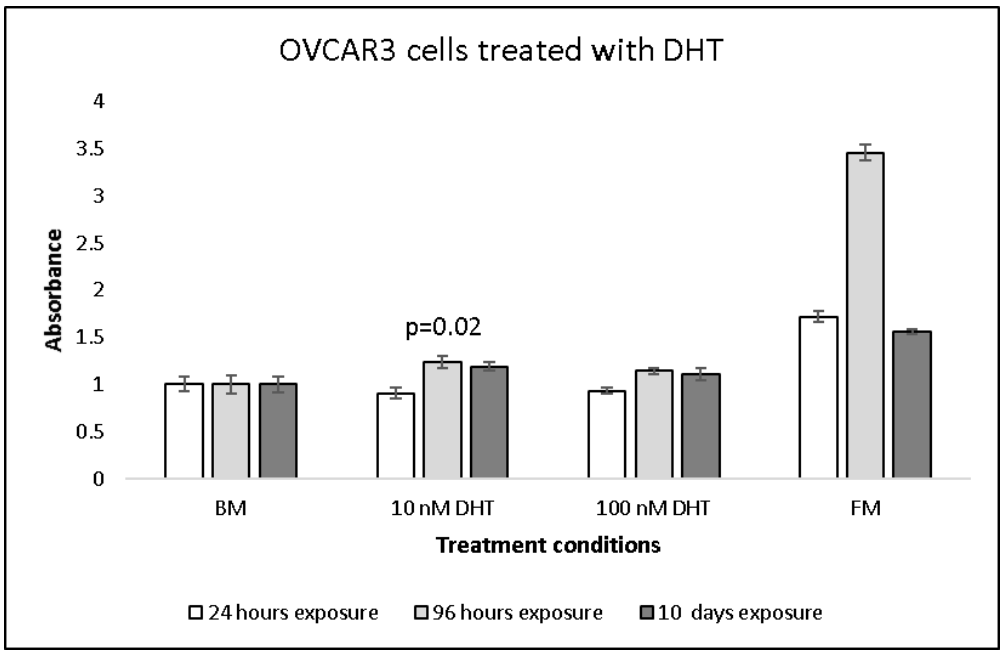


Figure 4-6: Proliferation of OVCAR3 cells measured with the BrDU assay after treatment with 10 or 100 nM DHT for 24 or 96 hours or 10 days. Basal medium (BM) was used as negative and full medium (FM) as positive control. Values represent the mean ±SE of the mean of three repeats with triplicate in each experiment. Values obtained were normalised to cells treated with BM only and set as "1".

4.3.2 SRB and BrdU assays for the evaluation of proliferative effects in primary ovarian cultures

Rationale

Established cancer cell lines offer the opportunity to repeat and plan experiments in sequential order due to the continued supply of cells.

Primary cancer cell lines on the contrary tend to be viable for a short life span of two to three passage numbers and lack the opportunity to repeat experiments. Information gathering, such as establishing doubling times and running experiments often need to be done in parallel with data evaluation in retrospect, as opposed to determining the treatment time required to observe proliferative effects in advance.

As demonstrated in chapter 4.3.1.2 and 4.3.1.3, both- SRB and BrdU assays- can be used to examine proliferative effects of androgen in cell lines with AR expression.

Aims

- To evaluate the viability of primary ovarian cultures over a period of ten days in basal medium and SDM
- To assess the inter-assay variability between androgen proliferation experiments using basal and SDM using the SRB assay
- To evaluate androgen effects in primary ovarian cultures with the BrdU assay

Methods

The optimisation of cell densities was done as in chapter 4.1.1.4 and cells were seeded out in $5x10^3$ cells/ well.

In both experiments, primary ovarian cultures cells were seeded out at $5x10^3$ cells/well in sextuplicate 96 well plates in full medium for 12-24 hours and allowed to adhere. After two washes with PBS, cells were quiesced for 24 hours in basal medium. After a further two PBS washes, primary ovarian cultures were incubated with either full medium (positive control), basal medium (negative control) or 10 nM DHT in basal medium or full medium (positive control), SDM (negative control) or 10 nM DHT in SDM. Ethanol corrections were done for all experiments.

Cells were then fixed every 24 hours and stored at 4°C until the course of the experiment was completed. The SRB assay was then completed as described in chapter 2.4.1.

4.3.2.1 *Examination of androgen stimulation on primary culture cells using the SRB assay*

4.3.2.1.1 *The SRB assay in primary ovarian cultures using basal medium*

Seven primary cultures (PCO 229, 230, 231, 233, 234, 251 and 252) were treated with 10 nM DHT in basal medium using the SRB assay.

In all seven primary cultures only the ones treated with full medium grew continuously over ten days as previously observed in OVCAR3 cells. Primary cultures treated with basal medium or 10nM DHT in basal medium maintained viability, but showed minimal, statistically non-significant proliferation only, with no marked difference of exposure to basal medium only or stimulation with androgens. *Figure* **4-7** depicts the results for 4/7 examined primary cultures.

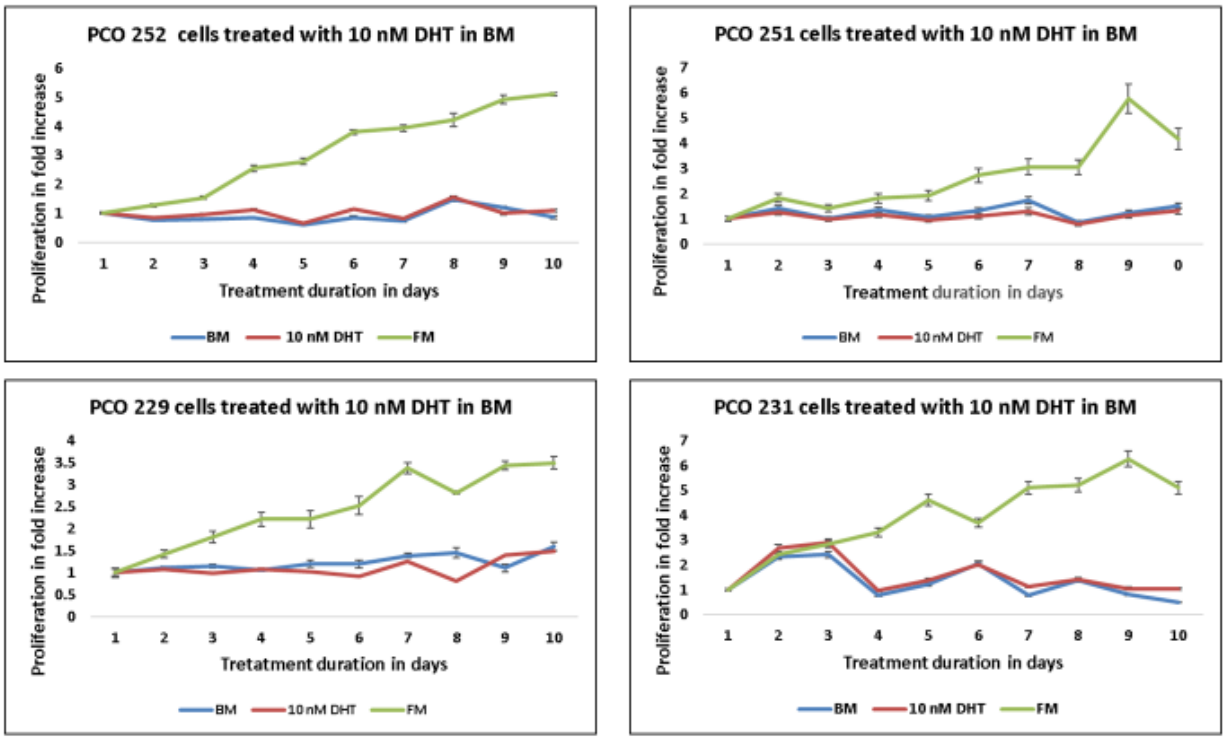


Figure 4-7: Primary cultures cells (PCO 229, 231, 251 and 252) were treated with full medium (FM) (positive control), basal medium (BM) (negative control) and 10nM DHT in basal medium. Values represent the mean ±SE of six replicates in each experiment.

4.3.2.1.2 *The SRB assay in primary ovarian cultures using steroid depleted medium (SDM)*

Six primary cultures (PCO 230, 231, 233, 234, 251, 252), which were also examined for response to androgen treatment in basal medium with the SRB assay, were furthermore investigated for response to androgen exposure with the SRB assay using steroid depleted medium. The seeding out and treatment was done as described above.

The response to androgen exposure using SDM as medium differed markedly within these six primary cultures as described in detail below and are shown in *Figure* **4-8**.

In summary, one primary cultures (PCO 251) showed a proliferative effect when treated with androgens *Figure* **4-8** (A), whilst one primary culture (PCO 234) suggested an increase in proliferation at one time point only (day eight) (see *Figure* **4-8**, B). In another primary cell culture (PCO 252) cells showed a similar growth pattern with all three treatment conditions, without a significant proliferation through DHT exposure *(Figure* **4-8**, C). In three primary cultures (PCO 230, 231 and 233) androgen exposure did not increase proliferation (*Figure* **4-8**, D- depicting PCO 231 as representative).

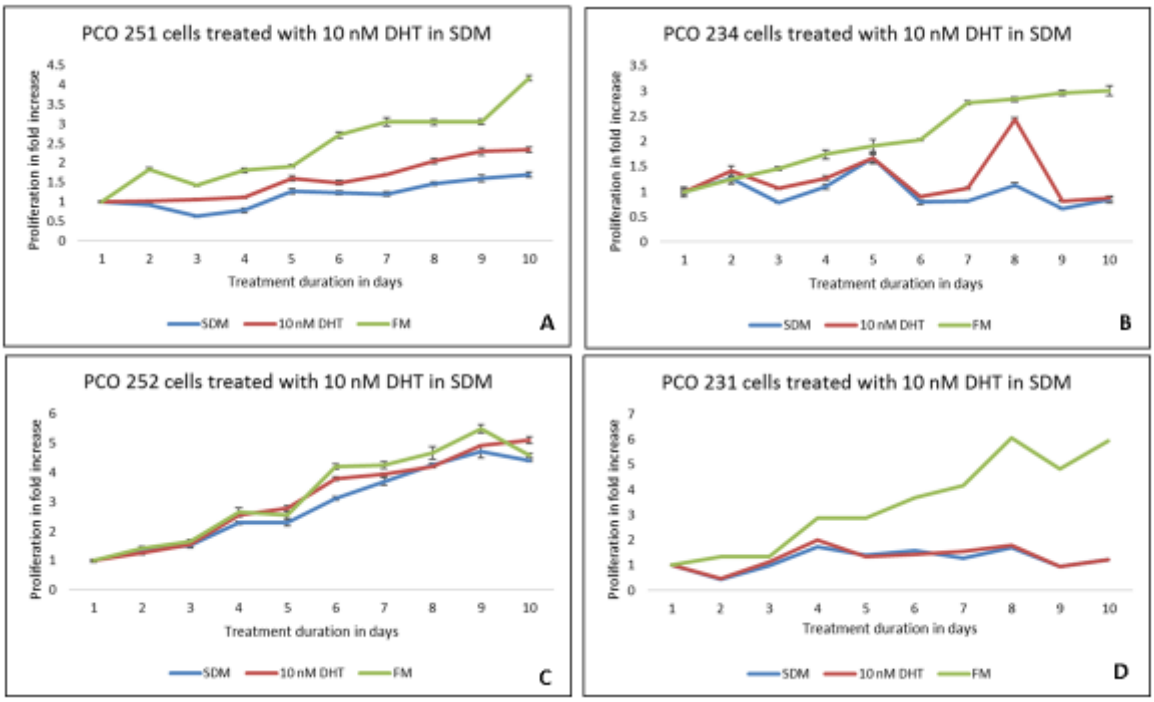


Figure 4-8: PCO cells 251, 252, 231 and 234 were treated with 10nM DHT in SDM. Cells cultures in full medium (FM) were used as positive control and in SDM alone as negative control. The effects of androgens seen in PCO 231 (D) were also observed in two further primary cultures (PCO 230 and 233).

PCO 251 cells (see *Figure* **4-8**, *A*) showed a proliferative response to DHT stimulation. Proliferative effects were seen on all ten days of treatment which were higher than in cells treated with SDM only. Overall, the growth of PCO 251 cells was slow with the most effect in full medium, with an increase of proliferation after day 6. On statistical analysis (t-test), the proliferation was significantly higher after treatment with DHT compared to treatment with SDM alone on day 2 and 6 with p-values of 0.003 and 0.08 respectively.

One primary culture (PCO 252) followed the growth pattern observed when OVCAR3 cells were treated with androgens in steroid depleted medium (*Figure* **4-5***)*, showing similar growth

when treated with all three treatment condition without any distinguishable or statistically significant differences in proliferation (see *Figure* **4-8**, *C)*. Whilst cells grew over all ten days, no marked, statistically significant androgen related proliferation was observed.

In 3/6 primary cultures (PCO 230, 231, 233) treatment with FM, SDM alone and 10nM DHT in SDM resembled findings observed in OVCAR3 cells treated with basal medium (see *Figure* **4-4**), showing good growth when treated with full medium. Treatment with SDM medium in these cells confirmed cell viability throughout the ten days, with some proliferative effects until day five. Androgen stimulation did not result in an increase in proliferation. *Figure* **4-8** *(C)* shows PCO 231 as a representative example for these three primary ovarian cultures.

PCO 234 showed slow growth when treated with full medium, doubling proliferation only on day 6 and showing a three-fold increase on day 10. Treatment with DHT suggested an increase in cell growth on day eight when treated with androgen when compared to treatment with SDM only, however, this is not statistically significant (see *Figure* **4-8**, *D*).

4.3.2.2 *Examination of effects of androgen stimulation on primary culture cells using the BrdU assay*

Rationale

As demonstrated on OVCAR3 cells (chapter 4.3.1.3) BrdU might be a more sensitive assay to evaluate potentially subtle proliferative changes and the effect might depend on media used.

Aims

- To compare the androgen effect on primary ovarian cultures in basal medium and SDM medium (at time points 24 and 96 hours and ten days)
- To examine androgen effects on proliferation on primary ovarian cultures in basal medium at 24 and 96 hours and after ten days.
- To examine the effects of androgen stimulation on primary ovarian cultures thawed from frozen (resurrected PCOs) measured with BrdU assays.

Methods

Primary ovarian culture cells were seeded at concentrations of $5x10^3$ cells/ wells in triplicate in 96 well plates and treated as described in chapter 4.3.1.3 and chapter 2.4.4. Values obtained were normalised to untreated cells which were set "1". An increase of 10% was set as arbitrary cut off to determine proliferation.

4.3.2.2.1 *Comparison of androgen effects in primary ovarian cultures in basal medium and steroid depleted medium*

Examination of growth using the SRB assay showed different growth curves when cells were treated in basal (BM) or steroid depleted medium (SDM) medium (see chapter 4.3.1.2 and 4.3.1.3).

In view of this, the BrdU assay was done in five primary cultures (PCO 230, 231, 233, 234, 247) in both treatment conditions at set times points: 24 hours, 96 hours and 10 days.

Cells were treated with 10 nM DHT in either medium (BM or SDM). In both settings (BM and SDM) cells used as negative controls were treated with the corresponding amount of ethanol to rule out proliferative effects due to the solvent.

Overall, the same trend was observed in both treatment conditions. When proliferation (defined as 10% increase from the baseline) occurred, it was observed in both treatment conditions (see *Table* **4-1**). Equally, the absence of proliferation or even anti-proliferative effects were mirrored

in both treatment regimens, though the statistical significance (evaluated with the t-test) differed (*Table* **4-1**).

For comparability basal medium was used for all future experiments.

PCO number	DHT exposure time					
	24 hours		96 hours		10 days	
	BM	SDM	BM	SDM	BM	SDM
230	1.23 (*)	1.17	1.28	1.14	1.27	1.12
231	0.84	0.96	0.95	1.02	0.93	1.02
233	1.18	1.33	1.48 (*)	1.26	1.14	1.98
234	1.68(*)	1.27 (**)	1.17	1.22	1.42	1.44
247	0.87	0.9	0.83	0.78	0.81	1.03

Table 4-1: Comparison of proliferative effect of androgens on primary ovarian cultures (PCO) in different media (basal medium=BM, steroid depleted medium= SDM) using the BrdU assay at different time points. Shown values are the means of three measurements, with cells treated with DHT normalised to non-treated cells at each time point. Primary ovarian cultures were either treated with 10 nM DHT in basal medium or basal medium only (negative control). An increase in growth of 10% was classed as proliferation. Some primary ovarian cultures show significant proliferation with p< 0.05 (*) or highly significant proliferation with a p< 0.005 (**). Overall, a similar trend is observed in cells treated with either BM or SDM.

4.3.2.2.2 Proliferative response of primary ovarian cultures cells to androgen stimulation in basal medium at different time points in the BrdU assay

As shown in *Table* **4-2**, 31 primary cultures were examined for androgen induced growth effects and all of these were exposed to androgen for 96 hours.

For 2/31 primary cultures (PCO 209 and 210) this is the only time point available, as they were used for the preliminary optimisation experiments of the BrdU assay in primary ovarian cultures. 9/31 primary cultures were then treated with androgens at two time points (24 and 96 hours) to evaluate the effect of androgen on proliferation (PCO 211, 213, 219, 224, 225, 226, 227, res 191 and res 194). 2/31 further primary cultures (PCO 242 and 243) senesced prior to 10 days DHT exposure.

Following the results of the preliminary panel and the observation of some proliferative effects, 16 more primary cultures were treated with androgens at three time points (24 hours, 96 hours and ten days)- PCO 229, 230, 231, 233, 234, 238, 239, 245, 246, 247, 251, 252, 143res, 174res, 187res, 177res as shown in *Figure* **4-9** and *Table* **4-2**.

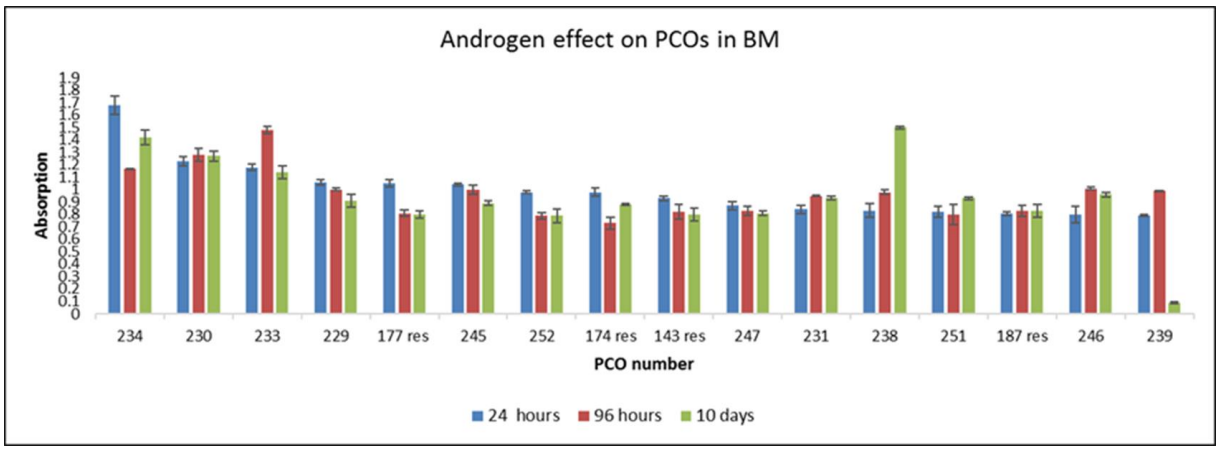


Figure 4-9: Effect of androgen on 16 primary cultures measured with the BrdU assay at all three time points. Cells were treated with 10 nM DHT for 24 and 96 hours and ten days. Values shown were normalised to untreated cells. An increase of 10% was considered as proliferative effect.

PCO	Proliferation status after exposure time			AR expression status			Treatment
	24 hours	96 hrs	10 days	WB	IHC	qPCR	
229	1.06	1	0.91	0	10	21	Primary
230	**1.23 (*)**	**1.28**	**1.27**	0	10	32	Primary
231	0.84	0.95	0.93	0	NA	40	no treatment
233	**1.18**	**1.48 (*)**	**1.14**	0	9	41	Chemo only
234	**1.68(*)**	**1.17**	**1.42**	0	7	86	IDS
238	0.83	0.98	**1.5 (**)**	0	7	40	IDS
239	0.79	0.99	0.09	0	NE	19	No treatment
245	1.04	1	0.89	0	11	48	Primary
246	0.8	1.01	0.96	0	NE	53	Primary
247	0.87	0.83	0.81	0	6	97	No treatment
251	0.82	0.8	0.93	0	7	41	IDS
252	0.98	0.79	0.79	0	8	36	Primary
143 res	0.93	0.82	0.8	NE	NE	NE	Primary
174 res	0.98	0.73	0.88	NE	NE	NE	Primary
187 res	0.81	0.83	0.83	NE	NE	NE	Primary
177 res	1.05	0.81	0.8	NE	NE	NE	IDS
211	**1.2**	0.81	NE	0	0	156	Primary
213	**1.2**	**1.13**	NE	NE	NE	30	Chemo only
219	0.93	**1.4**	NE	0	NE	18	IDS
224	0.82	0.86	NE	4	3	8	Primary
225	0.88	1.02	NE	16	2	16	Primary
226	0.92	**1.2 (*)**	NE	0	2	12	Primary
227	0.99	**1.1**	NE	0	3	15	Primary
242	0.95	0.98	NE	0	NE	10	Primary
243	0.95	0.91	NE	16	NE	8	Primary
191 res	0.78	0.85	NE	NE	NE	NE	Primary
194 res	0.93	0.67	NE	NE	NE	NE	Primary
194	NE	0.78	0.94	0	NE	20	Primary
197	NE	1.07	1.08	62	NE	48	Primary
209	NE	**1.14**	NE	0	12	39	IDS
210	NE	**1.14 (*)**	NE	0	12.5	75	Primary

Table 4-2: Summary of proliferation of primary ovarian cultures (PCO) measured with the BrdU assay. The numbers show the means of three measurements, with cells treated with DHT normalised to non-treated cells at each time point. Primary ovarian cultures were either treated with 10 nM DHT in basal medium or BM with ethanol (negative control) to allow for solvent correction. An increase in growth of 10% was classed as proliferation. Res= resurrected cells. These primary ovarian cultures were stored in liquid nitrogen for several months at passage one, prior to resurrection and use in this experiment. Some primary ovarian cultures show significant proliferation with p< 0.05 (*) or highly significant proliferation with a p< 0.005 (**).NE= not examined. Expression data of the AR are as per chapter 3. AR expression on protein on Western blot level (WB) and AR on mRNA level (qRT-PCR) are given as percentage of AR expression in OVCAR3 cells. AR protein expression on immunohistochemistry (IHC) is expressed with the H score. Primary= primary debulking, IDS= interval debulking surgery.

Statistics

Proliferation was measured with the BrdU ELISA assay as described in chapter 2.4.4. for primary cultures treated with 10 nM DHT and untreated primary cultures. For each treatment condition the measurements were done in triplicate and their means used for further evaluation. Means of primary cultures treated with 10 nM DHT were normalised to untreated primary ovarian cultures, as shown in *Table* **4-2**.

As the data were not normally distributed, the Wilcoxon Sign rank test was used to compare the proliferation of treated with untreated primary cultures for each time point separately at 24 hours, 96 hours and 10 days. At neither time point did the Wilcoxon Sign rank test indicate a significant proliferation of primary cultures after treatment with 10 nM DHT, with Z= -1.604 and p= 0.109 at 24 hours, Z= -1.391 and p=0.164 after 96h hours and Z= -1.221 and p= 0.222 after 10 days treatment.

For comparison of proliferation of treated and untreated conditions in individual primary cultures the paired-tests was used and the findings described below.

Overall, a statistically significant increase in proliferation was observed in 6/31 primary cultures, though at different time points.

After 24 hours of DHT treatment, 5/27 primary cultures showed increased growth, with two primary ovarian cultures having statistically significant growth (PCO 230 p=0.03, PCO 234 p=0.04).

9/31 primary cell cultures showed proliferation after 96 hours of androgen stimulation, with statistically significant growth for three primary ovarian cultures (PCO 233 p=0.03, PCO 226 p=0.02, PCO 210 p=0.02).

Of the 16 primary cultures treated with androgen for 10 days, four (PCO 230, 233, 234 and 238) showed increased proliferation, with a significant proliferation of one primary ovarian cultures (PCO 238, p=0.002), see *Table* **4-2**.

For the primary cultures showing no proliferation, but a potential anti-proliferative trend, no statistical significance could be found.

Comparing the expression data of the 11 primary ovarian cultures that responded with proliferation to the DHT treatment, no clear trend arises. None of these primary cultures expressed the AR on protein level when evaluated with the Western Blot. The H scores for AR protein expression on IHC in the same primary cultures are very varied, showing high scores (10>=) in 3/11, moderate expression (H score 5-9) in 3/11 and low scores (0-5) in 3/11 samples.

In two primary cultures, the IHC had not been done. Unfortunately, most samples of the primary cultures showing no response to DHT stimulation were not evaluated by IHC, hence the AR protein expression of primary ovarian cultures showing and lacking proliferation cannot be compared. No clear distinguishing trend between proliferating and non-proliferating primary ovarian cultures after DHT stimulation could be seen in either AR mRNA expression, nor in the distribution of histology.

4.3.2.2.3 Comparing the response of primary ovarian cultures to androgens with the SRB and BrdU assay

Six primary cultures (PCO 230, 231, 233, 234, 251, 252) have been examined for androgen related effects with both assays- the BrdU and the SRB assay using basal medium (in chapter 4.3.2.1.2.).

Comparing the effect of androgens on primary cultures measured with the two different proliferation assays showed different results (see *Table* **4-3**). With the SRB assay, none of the primary cultures responded with proliferation to androgen exposure. However, with the BrdU assay, three primary cultures (PCO 230, 233 and 234) expressed statistically significant increase in proliferation at one time point each (see *Table* **4-3***).*

All the PCOs have the same histological diagnosis of high grade serous cancer and do not show any particular distinguishing features regarding the AR expression, as evaluated in chapter 3.

PCO	Response to androgen treatment		AR expression		
	SRB assay	BrdU assay	Western blot	IHC	qPCR
230	no proliferation	proliferation (at 1 time point)	negative	10	32
231	no proliferation	no proliferation	negative	NE	40
233	no proliferation	proliferation (at 1 time point)	negative	9	41
234	no proliferation	proliferation (at 1 time point)	negative	7	86
251	no proliferation	no proliferation	negative	7	41
252	no proliferation	no proliferation	negative	11	86

Table 4-3: Summary of response to androgen stimulation in six primary ovarian cultures evaluated with the SRB and BrdU assay. AR expression data on protein level (western blot and IHC) as well as RNA level (qRT-PCR) are included as discussed in chapter 3. IHC= immunohistochemistry.

4.3.3 Effect of passaging on androgen effects in primary ovarian cultures

Rationale

Passaging of cells has been shown to cause changes due to spreading, migration and cell-surface ultra-structures (Liao et al., 2014). This phenomenon was mainly observed with high passage numbers of 30 or above. Though primary cell cultures have a much shorter lifespan (2-3 passages), there might be potential for change in AR expression, functionality and androgen response with passaging.

Aim

- To evaluate if passaging of primary cultures might change the effect of androgen stimulation at set time points.

All results above examining the proliferative effect of DHT on primary cultures measured with the BrdU assay were evaluated at passage one.

Methods

Three primary cultures (PCO 246, 247, 249) were examined for proliferative effects of androgen at different times point at passages one, two and three (see *Table* **4-4**).

Cells were seeded out at $5x10^3$ cells/well in 96 well plates in triplicate and allowed to adhere for 24 hours. They were then washed with PBS twice and treated with 10 nM DHT in basal medium, or in basal medium with the corresponding ethanol concentration (negative control) for 24 hours, 96 hours and ten days at different passages. Cells were passaged as described in chapter 2.2.2. The BrdU assay was done as described in chapter 2.4.4.

Results

For two primary cell cultures (246 and 247), neither the treatment time, nor passaging made a difference to proliferation (*Table* **4-4**).

In PCO 249, some changes were seen with androgen exposure time, showing almost anti-proliferative effects with increased treatment duration at passage zero and two (*Table* **4-4**). Proliferation, though not statistically significant, was only seen at two time points- after 24 hours at passage two and after 96 hours at passage one (see *Table* **4-4**).

Overall, the numbers of the primary cell cultures examined for different passages are too small to draw solid conclusions and would have to be repeated in a bigger cohort.

PCO number	Passage	Duration of DHT exposure		
		24 hrs	96 hrs	10 days
246	1	0.80	1.01	0.96
	2	0.96	0.96	0.86
	3	1.01	0.96	0.94
247	1	0.87	0.84	0.81
	2	1.05	0.92	0.87
	3	1.01	0.93	1.09
249	1	0.99	0.92	0.94
	2	0.90	1.13	1.01
	3	**1.15**	**1.08**	**0.89**

Table 4-4: Effects on proliferation after androgen stimulation of primary ovarian cell cultures using the BrdU assay. Primary ovarian cultures were either treated with 10 nM DHT in basal medium or BM with ethanol (negative control) to allow for solvent correction. The values shown are the means of three measurements, with cells treated with DHT normalised to untreated cells at each time point and passage.

4.3.4 Effects of androgen stimulation on primary ovarian cell cultures thawed from frozen (resurrected PCOs)

Rationale

As primary cultures have a short life span and some experiments might want to be conducted after preliminary results, it would be useful to freeze them at passage zero or one in order to use them for experiments further on.

Aim

- To determine if freeze-thawing would influence the androgen effect on primary ovarian cell cultures

Methods

A majority of the primary cultures were examined "fresh". As described in chapter 2.2.3, ascites would have been obtained from consented patient and seeded out in a 1:1 mix of medium and

let to seed down. This was defined as passage zero. Further passages would have been used for experiments.

At the time of seeding and passaging primary cultures, excess cells not used for experiments would be frozen (as described in chapter 2.2.4) in the -80° freezer and stored in liquid nitrogen. Cells with the suffix "res" describe primary culture cells (see *Table* **4-3***)*, were derived from ascites as described in chapter 2.2.3, but were frozen in liquid nitrogen for several months prior to resurrection (chapter 2.2.5) and utilisation in this experiment.

Results

The four primary cultures used in this experiment (PCO 143, 174, 177, 187) were stored for 16, seven, six and two months respectively prior to use for the proliferation BrdU assay.

None of the resurrected primary cultures showed proliferation after treatment with DHT. Instead, an anti-proliferative effect was observed, though it was not statistically significant (see *Table* **4-2**).

This anti-proliferative effect can also be seen in some "fresh" primary ovarian cultures, though so a small extent (*see Table* **4-2***).*

4.4 Conclusions

- Androgen effects on proliferation show different results when compared with different growth assays (see *Table* **4-3**).
- The BrdU ELISA might be the preferred assay when examining androgen related changed in primary ovarian cell cultures as it detects subtler changes compared to the sulforhodamine B assay (see *Table* **4-3**)
- Incubation with basal medium is suitable to examine androgen related effects in primary ovarian cultures.
- The effect of androgens on primary ovarian cell cultures is varied (see *Table* **4-2**) Using the BrdU assay
    - Androgen exposure led to statistically significant proliferation in 6/31 (19%) primary cultures
    - No change or anti-proliferative trends without statistical significance were observed in 25/31 primary cultures
- Treatment of primary culture cells used after thaw- freezing showed an anti-proliferative effect (see *Table* **4-2**)
- Passaging of primary ovarian cancer cells did not demonstrate an effect on the androgen response on proliferation (see *Table* **4-4**)

4.5 Discussion

Evaluation of a suitable assay to examine the response of androgen treatment in primary ovarian cultures

In this chapter the androgen effect on established and primary ovarian cancer cells was evaluated using two different proliferation assays (the SRB and BrdU assays).

For the established ovarian cancer cell line OVCAR3, the cell line examined in this study, a small, but statistically significant increase in proliferation after androgen treatment was seen using both assays.

While in the primary cell cultures, the effects of androgen exposure on proliferation was different in the same PCO and in fact showed opposing trends when examined with two separate growth assays. Of the six examined PCOs, the primary cancer cultures showing increased proliferation when examined with the SRB assay did not show any increase in proliferation when examined with the BrdU assay and vice versa. Whilst the detection of increased proliferation with the BrdU assay in its absence in the SRB assay (in PCO 230, 233 and 233) could support the hypothesis that the BrdU is more sensitive and can detect more subtle changes, it does not explain the reverse effect observed in three other PCOs.

One explanation for the observed inter-assay variability could be assay specific as the mechanisms in how proliferation is assessed in each assay. Whilst in the SRB assay proliferation is estimated through the change in total protein mass, it is the de-novo incorporation of BrdU into DNA during the S-phase of the cell cycle which is used for assessment of proliferation in the BrdU assay.

Both examined assays, the SRB (Jia et al., 2013, Yan et al., 2013, Mao et al., 2016, Sheach et al., 2009, SooHoo, 2013) and BrdU assay (Wan et al., 2015, Al-Alem et al., 2011) have been described for evaluation of proliferation in ovarian cancer cell lines, though a direct comparison of the two assays could not be found in literature search.

The SRB assay has previously been used to specifically evaluate the proliferative effects of androgen on EOC cell lines (SooHoo, 2013, Sheach et al., 2009). Researchers have also described the use of this technique to evaluate potential proliferative or anti-proliferative effects of substrates such as the flavone baicalin on ovarian cancer cell lines (Gao et al., 2017), corilagin (an active component of a medicinal herb) (Jia et al., 2013) or bile acid diamides (Mao et al., 2016). The BrdU assay has reportedly successfully investigated the proliferation in EOC

cell lines to assess the effect of the eukaryotic initiation factor 4E (Wan et al., 2015) or Peroxisome Proliferator Activated Receptor gamma (PPARc) agonists (Al-Alem et al., 2011). In contrary, primary ovarian cell cultures have been less investigated and literature search confirmed examination of primary ovarian cell cultures mainly by one group (Elattar et al., 2012, SooHoo, 2013). Assessment of proliferation with the BrdU assay in primary ovarian cultures has to date not been reported in the literature, hence no comparisons can be made.

Further contributing factors to explain the differences observed between the two assays could be the handling per se. The SRB assay is technically easy and allows the examination of proliferation over a long period of time with multiple time points, i.e. daily fixation of samples over several days. Samples can also be stored at 4°C until the treatment course is finished and then all processed together. The assay relies on the binding of SRB to proteins under mild acidic conditions and its extraction under basic conditions (Orellana and Kasinski, 2016). Changes in storage temperatures or in the pH of the fixing solution used in the SRB assay could hence influence the result. Other steps, such as the washing of the SRB dye are also crucial and might influence the result as incomplete washes would lead to a false increased reading (Orellana and Kasinski, 2016).

The BrdU assay unlike the SRB assay measures the incorporated BrdU into newly synthesized DNA as opposed to protein quantity. Its main disadvantage compared to the SRB assay other than higher cost due to the limited provision of substrates. Time points hence need be to be carefully chosen, which in the work with primary cell cultures proved to be difficult. PCOs are used as a cell model for inter-tumour heterogeneity. Due to their short life span, the individual primary culture is barely characterised at the time of the proliferation assessment and experiments need to be conducted in parallel rather than in a staggered timeline. Setting time points for examination of proliferation is hence arbitrary and might not be set optimally. This might have led to the loss of information, as changes occurring between the chosen time point will not be assessed.

A further potential pitfall to consider is the possible interaction of substrates with the cells examined. Due to its mechanism of incorporation as a label during DNA synthesis, adult neurogenesis research has used BrdU extensively in animal models. Taupin has described BrdU as a toxic and mutagenic substance with potential effect on the cell cycle, transcription and translation on properties in adult neuronal cells (Taupin, 2007). Breunig *et al*. considered that due to these effects, conclusions based on results from the BrdU assay should be considered

with caution without further cross-examination (Breunig et al., 2007). It should be taken into consideration that some changes or interaction could also occur at the cellular level.
A further point to consider is that BrDU incorporation will not only measure DNA synthesis de novo, but also unscheduled DNA repair synthesis even if the culture is not proliferating. The lack of proliferation measured with the SRB assay in PCO samples showing a statistically significant proliferation of the same PCO with the BrDU assay (*Table* **4-3**) might hence be a false positive result and should be considered cautiously.

One of the weaknesses of this study is the limited number of samples. It might have been more conclusive to examine several androgen expressing and responsive ovarian cancer cell line with the two proliferation assays to confirm the reliability of the assays.

In conclusion, we have found that the BrdU assay is suitable to assess the effect of substrates in primary ovarian cultures. A bigger sample size of ovarian cultures with more time points in each set experiment would provide more clarity of potential trends.

The effects of androgens on primary ovarian cancer cells

Examining the effect of androgen exposure on primary ovarian cultures using the BrdU assay we have found small statistically significant increase in proliferation in 19% of the cultured primary cell cultures. In the remaining primary ovarian cultures either no effect was detected or a potential anti-proliferative trend, though not statistically significant, was observed.
In the responsive primary cell cultures following DHT treatment, the increase in proliferation was observed at different time points in the different primary cultures. Three primary ovarian cultures (PCO 230, 233 and PCO 234) that showed an increase in proliferation at each of the three chosen time points, already had an increase in proliferation after 24 hours. This suggests that these primary cultures might be particularly susceptible to androgen exposure. The observed differences are not unexpected and would most likely be accounted for the heterogeneity of the disease, as each primary culture represents a separate EOC from a different patient.

To date only few other authors have reported the androgen effect in primary ovarian cultures. The findings, however, are difficult to compare due to variation in factors such as generation of cultures, examination techniques used or androgen doses used.

Elattar *et al.'s* generation of primary cultures is the most comparable to this study as all the primary cultures were also obtained from fresh ascitic fluid (Elattar et al., 2012). In his study 75% (6/8) primary ovarian cultures showed a significant increase in the cells in the S-phase in response to androgen treatment (Elattar et al., 2012). A further group has observed an increase in proliferation due to androgen exposure, though in a small number of cultures (Syed et al., 2001). Four of their cell cultures were obtained from women with late-stage serous adenocarcinomas, but these cultures were obtained with different methods, such as culturing ascites and tumour transplants (Syed et al., 2001). 3/4 of their malignant cultures showed an increase in proliferation following androgen exposure (Syed et al., 2001). Despite the fact that these results are comparable to Elattar *et al's*, the influence on generation of cultures cannot be excluded.

The choice of the examining technique could potentially influence the results and prohibits direct comparability. As demonstrated in chapter 4.3.2.2.3, direct comparison of proliferation with different tests is not necessarily possible. Whilst Syed *et al* chose to examine proliferation with the MTT assay (Syed et al., 2001), Elattar *et al* did not assess proliferation, but used flow cytometry in order to assess androgen response via cell cycle analysis (Elattar et al., 2012).

Furthermore, the choice of treatment dosage has been shown to potentially influence the observed effects. A dose-dependent effect resulting in increased proliferation with higher doses has been previously described (Sheach et al., 2009, Syed et al., 2001). Most authors treating malignant ovarian cell cultures chose a dose of 10 nM, as it is deemed the closest to the normal physiological environment (SooHoo, 2013, Sheach et al., 2009, Syed et al., 2001, Evangelou et al., 2003), though Elattar et al. used relatively high doses of androgen, stimulating their primary cell cultures with 20 nM and 100 nM of DHT (Elattar et al., 2012).

Interestingly, the choice of androgen type has been described to have a potential effect on investigated outcomes (Syed et al., 2001). When comparing the stimulation effect of both testosterone and DHT on ovarian cells, the cells obtained from non-malignant ovaries showed higher proliferation when treated with DHT compared to testosterone incubation. Malignant

ovarian cells on the other hand responded in the same way to both agents, with a two- to four-fold increase in proliferation (Syed et al., 2001). A further variable in Syed *et al*'s study which was not accounted for, was that women whose cell cultures derived from normal ovaries, had also concomitant diseases such as cervical or breast cancer. It is, however, impossible to conclude whether this would impact on androgen response.

However, the difficulty in stratification of which primary culture would be androgen responsive, remains to be overcome. In this study, for the 6/31 primary ovarian cultures showing proliferation after androgen treatment, no obvious common factors could be identified. No uniform pattern in AR expression could be seen in these primary cultures. Some of the primary cultures showing proliferation had high AR protein expression, while some had low AR protein expression on IHC. Contrary to these findings, some of the primary cell cultures showing high AR protein expression on IHC did not display increased proliferation after androgen treatment. None of the androgen responsive primary ovarian cultures expressed AR on Western blotting, which is in stark contrast to Elattar's findings, where 8/11examined primary cultures were expressing the androgen receptor at the protein level.

One of the hurdles in this study was to define the cut-off for proliferation. Due to the potential small effect observed, it was set rather low at 10% increase. Would it have been set higher at 40-50% increase, none of the primary cultures would classify as androgen responsive.
Regarding the clinical data, all the androgen responsive cultures in this study were of the same histological subtype (high grade serous cancers), however this is also the most common EOC type. Of these six primary ovarian cultures three were chemo-naiive and three were pre-treated with chemotherapy. Taking into consideration that the AR expression decreases after chemotherapy, this would suggest that even those women who had received chemotherapy would potentially benefit from anti-androgen therapy, if they could be identified.

In order to further strengthen the results of androgen-response of some primary ovarian cultures, it would have been useful to treat these with anti-androgens such as casodex or flutamide at the same time. An abrogation of the proliferative effect would have substantiated the assumption that the observed effect is due to androgen treatment. This has previously been successfully shown in ovarian cancer cell lines (SooHoo, 2013, Sheach et al., 2009)as well as in primary ovarian cultures (Elattar et al., 2012, Syed et al., 2001).

Another weak point of this study is that only the direct effect of androgens on proliferation in primary ovarian cultures was examined. The steroid hormone receptor signalling is controlled by proteins such as the transforming growth factor β (TGFβ). TGFβ inhibits the growth of benign and malignant ovarian cells and has been shown to be influenced by DHT. Treatment with DHT has shown to reverse this inhibitory effect in one cancer cell line and one primary ovarian culture (Evangelou et al., 2000).

Proliferation is a hallmark of carcinogenesis and was the only factor examined in this study. Further aspects, such as cell migration (SooHoo, 2013), invasion (Chan et al., 2017) and apoptosis (Chan et al., 2017) have been examined in ovarian cancer cell lines and could be an adjunct to examine primary cultures.

5 The effect of androgen stimulation of Rab35 expression

5.1 Introduction

In molecular cancer research, other than examining expression, functionality of the examined protein or gene of interest is crucial.

Androgens have been implied in ovarian tumorigenesis and AR expression has been confirmed in ovarian cancer.

We were looking for a marker of androgen stimulation that can be used principally in experimental systems, but which could also have some clinical utility as a predictor of response to anti androgen therapy.

Rab35 has been proposed as such a marker of androgen stimulation, as it was the most upregulated gene in a cDNA microarray following acute exposure of OVCAR3 cells to androgen stimulation (Sheach et al., 2009). This finding has been confirmed in independent qRT-PCR experiments (Sheach et al., 2009). In this study we aimed to see if Rab35 could be confirmed as an androgen-responsive marker in primary ovarian cultures.

Furthermore, functional effects of Rab35 have previously been reported (SooHoo, 2013). Silencing of the Rab35 gene was shown to decrease in the proportion of cells in S-Phase of the cell cycle and cell proliferation as well as an increase of cell apoptosis (SooHoo, 2013). The hypothesis has been postulated that Rab35 overexpression could increase cell proliferation and decrease cell apoptosis and hence mimic oncogenic effects (SooHoo, 2013).

Androgen sensitivity of Rab35 was confirmed by a two-fold increase of Rab35 gene expression two hours after 10 nM DHT stimulation in the ovarian cancer cell line OVCAR3 (SooHoo, 2013). Moreover, this effect was abrogated when the AR was silenced, underpinning the androgen-dependence of the Rab35 gene expression (SooHoo, 2013).

These promising findings led to further examination of Rab35 gene expression after androgen stimulation in primary cultures of the ovary in this thesis.

5.2 Aim

- To investigate Rab35 gene expression after androgen stimulation of primary cell cultures.

5.3 Methods

Rab35 mRNA expression was measured by qRT-PCR after the stimulation of 30 primary ovarian cell cultures (PCO) with 10 nM DHT for two, four, eight and twelve hours. Unstimulated cells at time point zero were used as reference and set at the level "zero".

Primary ovarian culture cells were seeded at passage one at $6x10^5$ cells/ well in 6 well plates in full medium. They were left to adhere for 24 hours before two washes with PBS. They were then stimulated with 10 nM DHT in basal medium for two, four, eight and twelve hours and thereafter examined for Rab35 gene expression with qRT-PCR, as described in chapter 2.6. Cells were harvested at time point zero (at the time of androgen stimulation) without being stimulated with DHT for mRNA extraction and used as reference point. Rab35 mRNA expression at all time points was normalised to the housekeeping gene HPRT1.

The same panel of primary ovarian cultures examined for Rab35 gene expression response following DHT stimulation has also been examined for AR mRNA expression with qRT-PCR as described in chapter 3, *Figure* **3-3**. AR gene expression was normalised to HPRT1 as housekeeping gene and furthermore to AR mRNA expression measured in OVCAR3 cells to have a reference point for expected AR gene levels.

5.4 Results

The response of Rab35 gene expression after androgen stimulation in the 30 primary ovarian cultures examined was variable, showing either an increase, a decrease or mixed response with increased and decrease in Rab35 mRNA expression compared to untreated cells (see *Table* **5-1** and *Figure* **5-2**).

Overall 10/30 primary ovarian cultures responded to androgen stimulation with an increase of Rab35 gene expression at all time points, 10/30 showed a mixed response with an increase and decrease in Rab35 mRNA expression compared to untreated cells and 10/30 primary cultures responded with persistently lower Rab35 gene expression than untreated cells.

Most of the gene expression changes are subtle. If an increase of 50% of Rab35 gene expression after androgen stimulation would be set as arbitrary cut-off, then 12/30 primary cultures would be considered as responsive to androgen.

In the previous examination of OVCAR3 cells (SooHoo, 2013), a two-fold increase in Rab35 gene expression has been observed after androgen stimulation. If this increase in Rab35 gene expression is applied to primary cell cultures, only 3/30 primary cultures (PCO 231, 247 and

230) would have shown an increase in Rab35 RNA expression after DHT exposure (*Table* **5-1** *and Figure* **5-1**).

The time points with the highest increase or most decrease in Rab35 gene expression varied in different primary cell cultures, as shown in (see *Table* **5-1**).

PCO number	Rab35 gene expression normalised to untreated cells (in %)				AR gene expression (in % of OVCAR3 AR gene expression)	AR protein expression (on WB)
	Hours after stimulation					
	2	4	8	12		
231	**1340**	320	30	1081	40	Negative
247	84	0	**583**	125	97	Negative
230	166	176	**241**	99	32	Negative
191	98	176	**193**	110	17	Positive
250	5	15	**138**	60	9	Negative
211	31	54	38	**64**	156	Negative
238	**63**	3	7	37	40	Negative
243	**63**	15	38	13	8	Positive
187	10	**52**	6	6	3	Positive
225	6	**48**	8	32	16	Positive
246	4	14	-16	49	53	Negative
229	-39	-49	**53**	-25	21	Negative
227	58	**66**	-17	5	15	Negative
233	40	-46	**83**	-27	41	Negative
219	-40	17	13	5	18	Negative
252	44	43	57	**-67**	36	Negative
210	36	36	**-53**	37	75	Negative
174	19	4	-39	-24	38	Negative
209	-38	**-53**	32	47	39	Negative
177	**-79**	28	-31	33	4	Negative
194	**-35**	-17	-8	-22	20	Negative
224	**-38**	-30	-33	-8	8	Positive
213	-32	**-38**	-30	-23	30	NA
245	-34	**-50**	0	35	48	Negative
249	-6	-29	-7	**-52**	30	Negative
251	-30	**-71**	-25	-39	41	Negative
239	**-76**	-46	-4	-12	19	Negative
143	-7	**-92**	-90	-89	15	NA
234	-89	-90	-93	**-97**	86	Negative
253	**-97**	-97	-79	-5	84	Negative

Table 5-1: Rab35 mRNA expression in primary ovarian cultures after stimulation with 10 nM DHT. Exposure of primary ovarian cultures to DHT was done for 2, 4, 8 or 12 hours and the values were normalised to the housekeeping gene HPRT1 and untreated cell at time point zero. Positive values indicate an increase and negative values a decrease in Rab35 gene expression compared to untreated cells (in %). Figures in bold show the most increase or decrease of Rab35 gene expression. AR gene expression for the same primary ovarian cultures was measured with qRT-PCR and normalised to

HPRT1 and AR mRNA expression measured in OVCAR3 cells. AR protein expression was evaluated with Western blots as described in chapter 3.1.3. NA= not assessed.

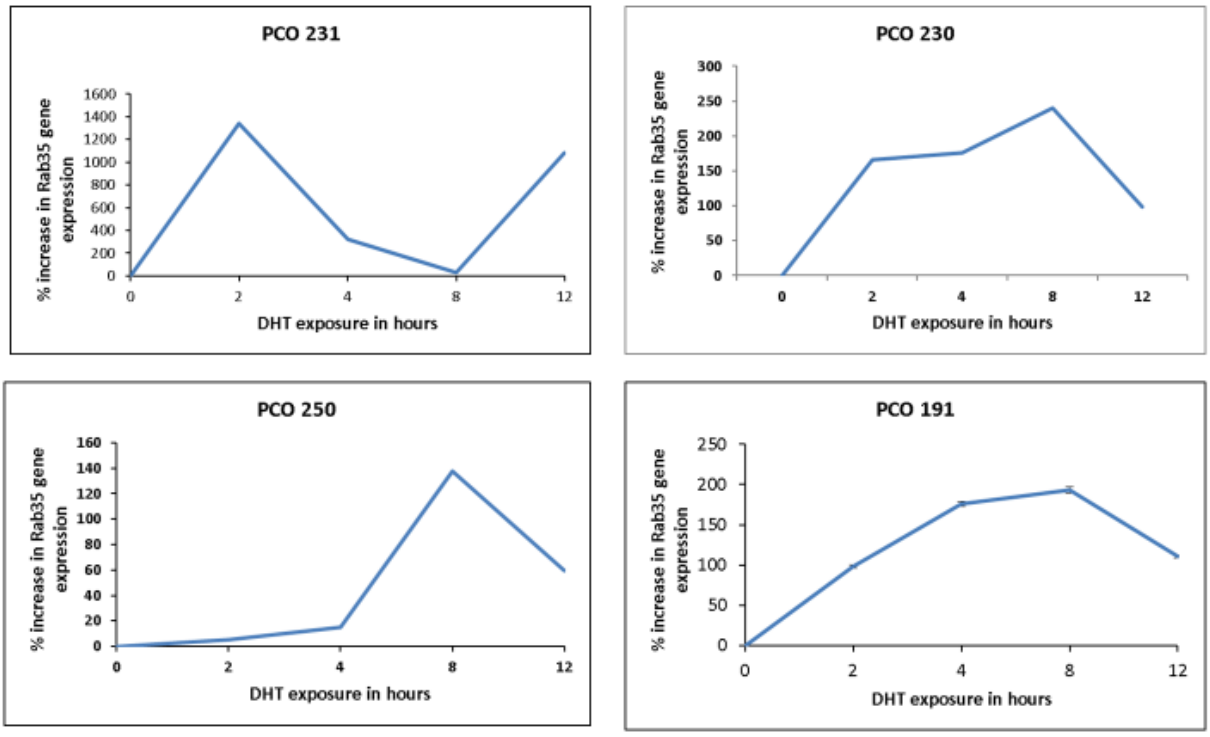


Figure 5-1: Rab35 gene expression in primary ovarian cultures after stimulation with 10 nM DHT at different time points. Compared to untreated cells, all these primary ovarian cultures showed an increase in Rab35 gene expression of at least 190%. Only PCO 191 expressed AR on Western blotting.

PCO number	DHT exposure	CT values for genes		PCO number	DHT exposure	CT values for genes	
	(in hours)	Rab35	HPRT1		(in hours)	Rab35	HPRT1
143	0	27.574	22.931	234	0	24.469	24.916
	2	27.090	23.138		2	24.897	28.592
	4	24.149	23.995		4	24.880	28.734
	8	24.236	23.886		8	25.170	29.129
	12	24.119	23.549		12	24.938	30.779
174	0	25.759	24.501	238	0	30.155	25.177
	2	25.573	24.113		2	32.117	29.132
	4	24.647	23.357		4	30.608	27.143
	8	25.570	25.025		8	29.944	28.212
	12	25.943	25.091		12	29.730	27.738
187	0	24.321	23.883	239	0	25.762	24.078
	2	26.192	25.993		2	26.767	27.341
	4	24.384	23.513		4	26.767	27.341
	8	24.388	24.236		8	25.163	24.587
	12	24.018	23.871		12	25.281	23.861
191	0	26.611	27.377	247	0	33.576	28.340
	2	27.088	26.811		2	26.548	25.454
	4	25.679	24.992		4	27.281	25.412
	8	26.019	25.280		8	27.845	25.550
	12	27.265	26.901		12	33.609	25.838
191	0	25.000	22.782	248	0	28.113	26.064
	2	24.646	23.104		2	25.846	24.763
	4	25.143	23.300		4	25.947	24.985
	8	26.160	24.040		8	26.188	24.877
	12	25.801	23.992		12	24.019	24.264
231	0	24.440	25.628	249	0	25.358	27.527
	2	24.050	24.764		2	27.527	25.358
	4	24.366	26.455		4	27.416	25.129
	8	24.133	24.458		8	27.794	25.119
	12	24.420	26.066		12	26.643	24.401

Table 5-2: Raw data CT mean values from qRT-PCR for Rab35 gene expression in primary ovarian cultures (PCO) after stimulation with 10nM DHT at different time points. The results of all examined PCOs are summarised in Table **5-1** *with some sample figures shown in Figure* **5-1**.

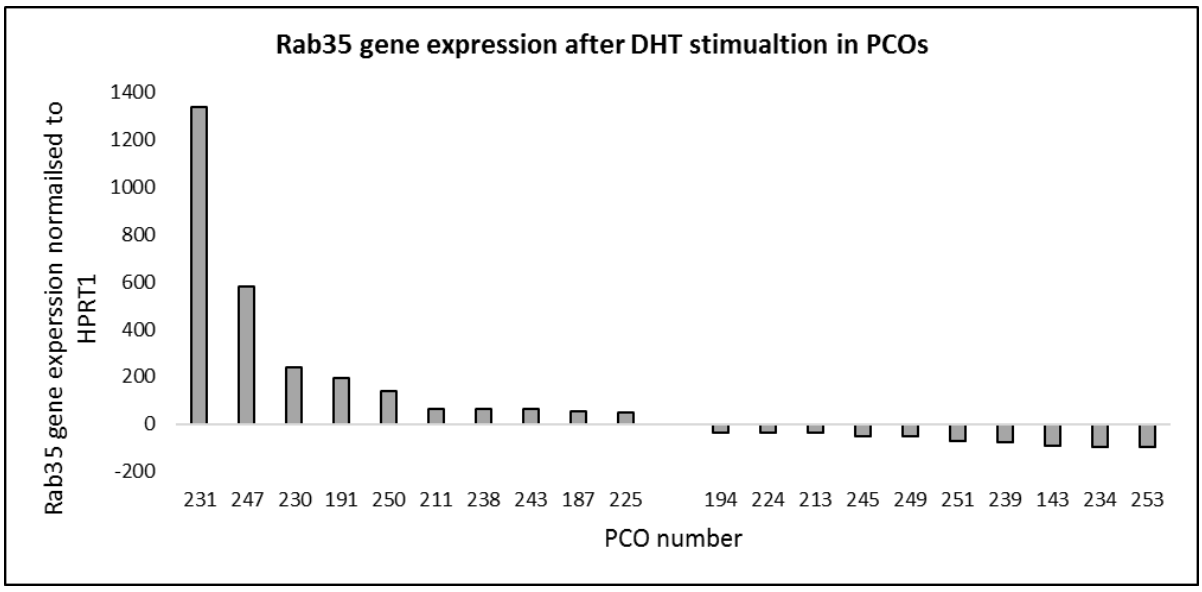
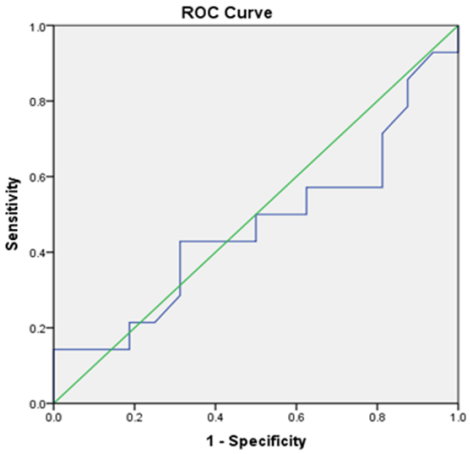


Figure 5-2: Rab35 gene expression in primary ovarian cultures after androgen stimulation. Primary ovarian cultures samples were stimulated with 10 nM DHT for 0, 2, 4, 8 and 12 hours and Rab35 mRNA expression was measured by qRT-PCR. Rab35mRNA expression was normalised to HPRT1 as housekeeping gene and Rab35 gene expression of unstimulated cells at time point zero. Positive values show the maximum increase in mRNA expression post androgen stimulation in the primary ovarian cultures and negative values represent the highest decrease of Rab35 gene expression following androgen exposure.

Combining the AR expression status, of those three primary ovarian cultures with Rab35 mRNA expression of more than 200% after androgen exposure, none showed AR protein expression on Western blot (PCO 191), but low levels of AR mRNA expression (*Table* **5-1**). 5/30 primary ovarian cultures examined were positive for AR protein expression on Western blot, but only one (PCO 191) showed an increase of Rab35 gene expression after DHT stimulation (*Table* **5-1** and *Figure* **5-1**). 3/5 primary ovarian cultures (PCO 243, 187 and 225) showed a small increase in Rab35 mRNA expression after androgen stimulation with a maximum increase of 48-63% increased gene expression compared to untreated cells. PCO 224 was positive for AR on Western blot, but showed decreased Rab35 gene expression levels after androgen treatment at all examined time points (see *Table* **5-1**).

To analyse if AR expression could predict the Rab35 gene expression after androgen exposure, a ROC curve was generated with SPSS (see *Figure* **5-3**). With an area under the curve on 0.467 and a p-value of 0.755, AR gene expression cannot predict the increase in Rab35 gene expression after DHT stimulation.



AUC	0.467
95% CI	0.257-0.682
p	0.755

Figure 5-3: ROC curve for AR mRNA expression and increase of Rab35 gene expression after androgen stimulation.

5.5 Discussion

Rab35 is a small GTPase and member of the RAS oncogene family. It is located in endosomes and plasma membranes and controls many essential cell functions such as cytokinesis, phagocytosis, exosome release, cell migration, neurite outgrowth and pathogen hijacking (Klinkert and Echard, 2016). The underlying mechanism is the activation of the inactive GDP-bound form through guanine nucleotide exchange factors (GEFs) to an activated GTP form, which can in reverse be de-activated by GTPase activating proteins (GAPs) (Allaire et al., 2013). Whilst three effector proteins have been discovered for Rab35 located in endosomes, GEFs activating Rab35 at the plasma membrane are yet to be determined (Klinkert and Echard, 2016). Overall, the influence on Rab35 on cell functions named above has been observed and attributed to its location in the plasma membranes. These effects are well documented, however poorly understood and signalling pathways are yet to be further determined. The same is true for potential posttranslational modifications of Rab35 which could potentially modulate Rab35 function in normal cells (Klinkert and Echard, 2016).

The relation of androgen and Rab35 gene expression have so far only been published by the ovarian cancer group Newcastle (Sheach et al., 2009) (SooHoo, 2013).

A literature search showed no published work examining Rab35 expression in relation to androgen exposure, not even in known androgen dependent or androgen sensitive cancers such as prostate cancer or breast cancer.

In this study Rab35 was examined as a potential androgen dependent downstream product of the AR. This relation was identified in an ovarian cancer cell line by Sheach *et al* through cDNA microarray where OVCAR3 cells were stimulated with 10nM DHT for 2 and 8 hours. In their study Rab35 was one of the most upregulated genes and further validated by qRT-PCR (Sheach et al., 2009).

Microarrays offer the advantage of the examination of hundreds of genes at the same time rather than just one. In microarrays, mRNA is converted to cDNA, labelled with a fluorescent marker and changes in colour intensity indicate gene up- or down regulation. Microarrays are an established technique in cancer research and have been used in ovarian cancer to identify potential proliferation factors (Yin et al., 2016), biomarkers (Siamakpour-Reihani et al., 2015), tumour suppressive factors (Bernaudo et al., 2016), prognostic markers to predict chemotherapy and drug resistance (Zou et al., 2015) and cancer progression (Liu et al., 2015).
The difficulty with using microarrays lies in the overwhelming amount of data collected and the challenge to identify critical genes and distinguish these from genes with no effect, thus further validation is required.
Following the ovarian cancer cell microarray (Sheach et al., 2009) Sandra SooHoo has confirmed an increase in Rab35 gene expression in OVCAR3 cells after DHT stimulation and underpinned this relation by demonstrating an abrogation of increase of Rab35 gene expression after AR silencing (SooHoo, 2013).

In this study, we aimed to examine the correlation of Rab35 and its potential role as androgen related downstream product.
Rab35 gene expression was examined in primary ovarian cultures after androgen stimulation. Only 3/30 cultures showed a two-fold or more increase of Rab35 gene expression, comparable to findings in OVCAR3 cells after androgen stimulation (SooHoo, 2013). A further 9/30 showed an increase of 50% or more at one time point, but three of these nine primary ovarian cultures also showed a decrease in Rab35 gene expression after androgen treatment.

Furthermore, statistical analysis does not confirm a correlation between AR mRNA expression and response of Rab35 gene expression to androgen treatment.
Overall, findings in this study suggest that Rab35 expression is not androgen regulated in PCO's and that gene expression cannot predict which primary culture is androgen sensitive in the Rab35 mRNA expression.

The only other downstream effects of Rab35 described in the literature is placing Rab35 action downstream of epidermal growth factor (EGF) receptors in the renal monkey cell line COS-7 (Allaire et al., 2013). In COS-7 cells, the knockdown of Rab35 led to enhanced recycling and re-routing of the EGF away from lysosomal degradation with the consequence of increased EGF receptor expression. As EGF is required for cell migration and proliferation, the knockdown of Rab35 resulted in significantly increased proliferation rates (Allaire et al., 2013).

The main limiting factor of examining Rab35 expression of this study is the small amount of data, as only one experiment was used to examine functional effects of Rab35 gene expression. To further examine if the expression of Rab35 is androgen related, a larger panel of experiments should be conducted in primary ovarian cultures. This would require optimisation and successful knockdown of Rab35 in primary ovarian cultures. The knockdown technique was attempted for three primary ovarian cultures, all of which died, and hence the technique was abandoned.
A Rab35 knockdown model would allow to examine mechanisms defining carcinogenesis, such as cell cycle analysis and apoptosis.
Interestingly, Rab35 silencing in OVCAR3 cells led to a significant reduction of S-Phase and an increase in apoptosis (SooHoo, 2013). However, only the increase in apoptosis was demonstrated to be an androgen dependent effect. The decreases of S-Phase after Rab35 gene silencing was not confirmed to be androgen dependent, as they were the same in serum-free and androgen rich medium (SooHoo, 2013).
The knockdown of the AR in primary cell cultures with high Rab35 gene expression after androgen stimulation would allow the examination of whether the effect could be abrogated and if the increase in Rab35 gene expression could be contributed to by androgen action.
Furthermore, could flow cytometer be used to examine the Rab35 expression after androgen exposure in primary cultures? These data could then be correlated to mRNA expression data gathered in this study.

6 The effects of abiraterone on ovarian cancer

6.1 Introduction

Androgens have been implicated in the pathogenesis of ovarian cancer based on epidemiological data and signalling pathways.

Treatments targeting the hormonal (androgen) axis seem appealing, as they are generally well tolerated with a low side effect profile.

In ovarian cancer, compounds suppressing the androgen axis such as GnRH analogues as well as blocking the AR with antagonists such as bicalutamide and flutamide have been evaluated in clinical trials, however with little success (chapter 1, *Table **1-4***).

A further potential approach targeting the androgen axis is enzymatic blockade of androgen synthesis (see *Figure* **6-1**).

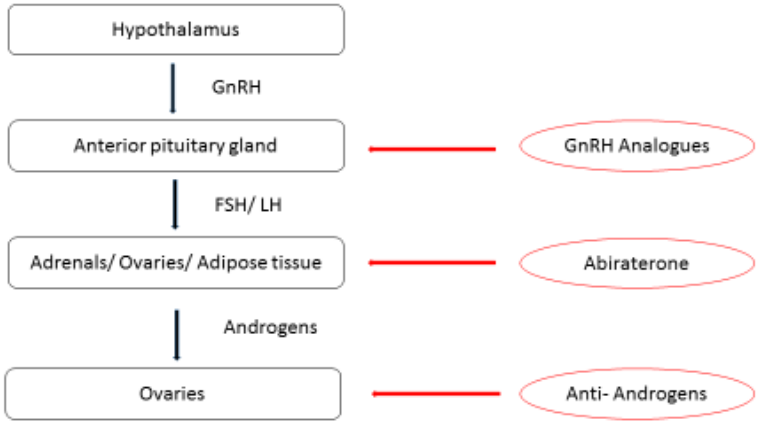


Figure 6-1: Androgen axis in the female and potential treatment strategies.

Cytochrome P450 17α (CYP17) is a key enzyme in the generation of oestrogens and androgens, such as testosterone and DHT via two steroid reactions in the androgen biosynthesis pathway, as described in chapter 1 (see *Figure **1-2***). In the female, CYP17 is expressed in the adrenal glands and the theca interna of the ovaries (Ang et al., 2009).

Abiraterone, a compound which selectively and irreversibly inhibits CYP17 seems a promising treatment option for androgen dependent cancers and is an established treatment for castration-refractory prostate cancer (CRPC) (Smith et al., 2015, Ryan et al., 2015, Fizazi et al., 2012). It has also been trialled in breast cancer (Bonnefoi et al., 2016, O'Shaughnessy et al., 2016) and suggested for treatment in ovarian cancer (Papadatos-Pastos et al., 2011).

To date one phase II clinical trial (CORAL- Cancer of the OvaRy Abiraterone trial) has examined the effect of CYP17 inhibition on AR positive recurrent ovarian, primary peritoneal and tubal cancer in women with previous chemotherapy. The response, measured by evaluation of radiological criteria (RECIST) and biomarker levels (CA 125), was very limited. Only one of 42 recruited patients responded to the abiraterone treatment with a disease- free interval of 47 weeks. However, in a subgroup of women disease stabilisation was achieved for 12 weeks and six months in 26% and 14% of women respectively (Banerjee et al., 2016). This study also aimed to examine the molecular effect of abiraterone on epithelial ovarian cancers, but these results have not been published.

Despite promising results in clinical response of CYP17 inhibition in CRPC, the molecular mechanisms are poorly understood and have only been studied by a small number of research groups.

Direct anti-androgenic effects such as decreased AR protein and mRNA expression after treatment with Abiraterone were demonstrated in the androgen dependent and responsive prostate cancer cell line LNCaP (Soifer et al., 2012) as well as dose-dependent inhibition of proliferation (Richards et al., 2012). Abiraterone was furthermore suggested to interfere with the AR signalling pathway through inhibition of androgen-induced AR translocation by 58% in the human liver cancer cell line Hep3B. This compared to a complete inhibition with the nonsteroidal anti-androgen enzalutamide (Soest et al., 2013).

No studies have been published examining CYP17 expression in either established ovarian cancer cell lines or primary ovarian cancer cell lines to date.

Rab35 has been proposed as an androgen dependent protein and potential biomarker in ovarian cancer (SooHoo, 2013, Sheach et al., 2009). In this study the relationship between Rab35 mRNA expression and CYP17 inhibition was investigated.

6.2 Aims

- To confirm the expression of CYP17 in a panel of established ovarian cancer cell lines at the protein and mRNA level
- To examine the expression of the CYP17 enzyme at the protein and mRNA level in a panel of primary ovarian cell cultures
- To investigate the effect of Abiraterone in two ovarian cancer cell lines (OVCAR3 and PEO4) with respect to
    - Proliferation
    - CYP17 and AR expression at the protein and mRNA level
    - Rab35 gene expression

6.3 Results

6.3.1 CYP17 expression at the protein level in ovarian cancer cell lines

A panel of seven ovarian cancer cell lines alongside the prostate cancer cell line LNCaP and the cervical cancer cell line HeLa (see *Table* **6-1**) were examined. Cells were cultured in full medium and protein lysates were subsequently extracted and analysed with Western blotting as described in chapter 2.5. A goat polyclonal antibody (Santa Cruz) was used at the concentration 1:100 to detect CYP17 protein expression at the molecular weight of 55kDa and GAPDH (Santa Cruz) was used a loading control detecting a protein at 37 kDa. All examined cell lines showed expression of CYP17 protein (see *Figure* **6-2**) (n=2).

Cell line	Derivatives
OVCAR 3	Human ovarian adenocarcinoma, established from malignant ascites after combination chemotherapy with cyclophosphamide, adriamycin, and cisplatin.
LNCaP	Human prostate adenocarcinoma, established from a lymphnode metastasis.
HeLa	Human adenocarcinoma of the cervix.
PEO1	Human poorly differentiated serous adenocarcinoma, derived from ascites, after previous treatment with cisplatin, 5-fluorouracil and chlorambucil. Derived from the same patient as PEO4.
PEO4	Human serous adenocarcinoma, derived from the same patient as PEO1. Derived from ascites collected after the development of resistance to chemotherapy. Oestrogen receptor positive.
PEO14	Human well differentiated serous adenocarcinoma, established from ascites, collected prior to treatment. Oestrogen receptor negative.
IGROV1	Human ovarian adenocarcinoma.
A2780	Human ovarian carcinoma, cisplatin sensitive.
CP70	Human ovarian carcinoma, derived from the A2780 cell line, cisplatin resistant.

Table 6-1:Cancer cell lines used for CYP 17 expression

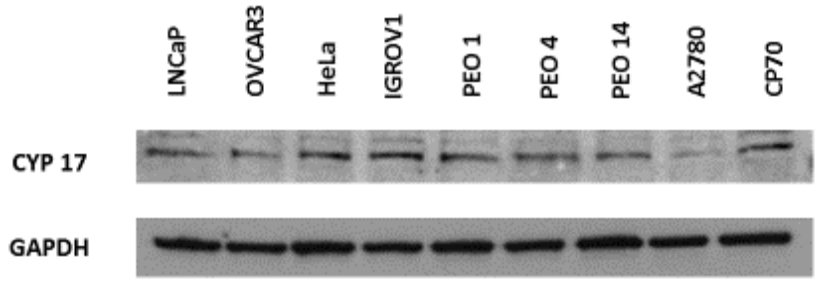


Figure 6-2: CYP17 protein expression in cell lines examined using Western Blotting. GAPDH was used as loading control.

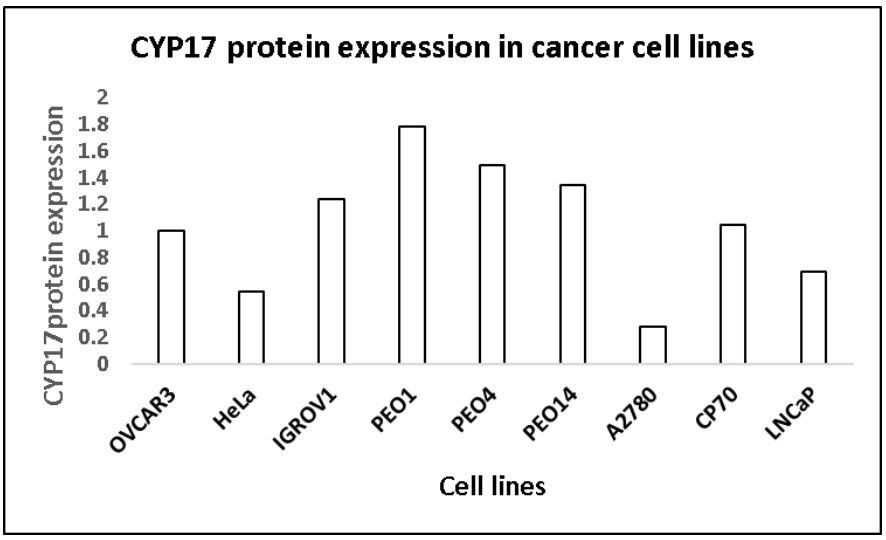


Figure 6-3: Densitometry measurements for CYP17 protein expression for the Western blot in Figure 6-2.

6.3.2 CYP17 mRNA expression in ovarian cancer cell lines

mRNA encoding the CYP17 gene was then examined in the same panel of cell lines.

Cells were seeded in 6 well plates at concentrations of $5x10^6$ cells/ well in full medium for 24-48 hours. RNA was extracted and reverse transcribed as described in chapter 2.6. Four primer sets were used for preliminary experiments- one previously described in a publication examining LNCaP cells (Cai et al., 2011) and three further exon spanning primers were designed for CYP17 and used for qRT-PCR as described in chapter 2.6. Three of the primer sets were not suitable for use due to either their poor binding or the formation of primer dimers. The primer set chosen for final CYP17 mRNA evaluation was fwd 5'-CCGTAAGGGTATCGCCTTCG-3' and rev 5'-CCATCCTTGAACAGGGCAAAG-3'. The relative gene expression of CYP17 was measured against the relative expression of the

housekeeping gene HPRT1. The experiment was done in triplicate and the means of the results are shown in *Figure* **6-4**.

All of the cell lines were found to express CYP17 at the mRNA level at varying levels (*Figure* **6-4**).

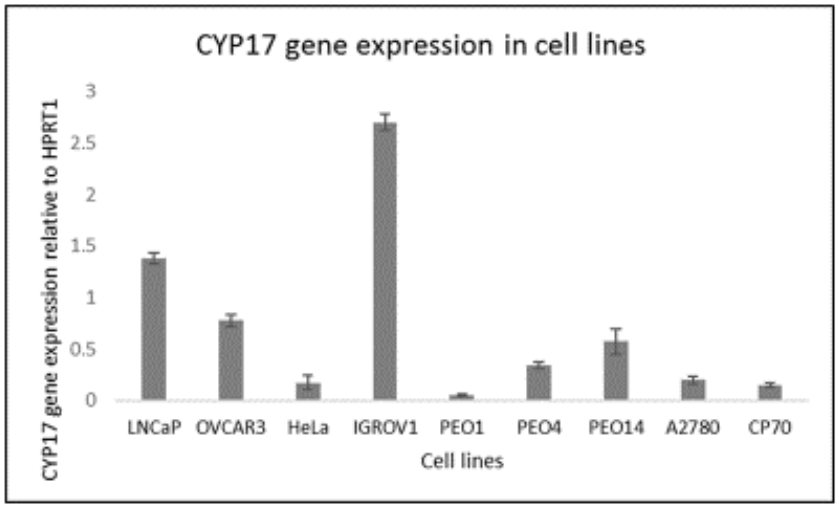


Figure 6-4: CYP17 gene expression in cell lines, normalised to HPRT1 mRNA expression. Values represent the mean +/- SE of the mean of three repeats with triplicates of each reaction.

For further evaluation related to the effects of abiraterone treatment on ovarian cancer cell lines, the two AR expressing cell lines OVCAR3 and PEO4 were chosen. As OVCAR3 has extensively been used in this study it was chosen for further characterisation. PEO4 was chosen as it had high protein CYP17 levels and also expressed CYP17 on mRNA level.

6.3.3 Effects of abiraterone on proliferation in ovarian cancer cell lines

CYP17 inhibition with abiraterone in the prostate cancer cell line LNCaP previously demonstrated promising direct inhibitory effects on proliferation whilst maintaining cell viability (Richards et al., 2012).

In this study, OVCAR3 and PEO4 cells were chosen to examine whether abiraterone treatment would have a direct inhibitory effect on proliferation. The effect was studied in two different treatment conditions- steroid depleted medium (SDM) to minimise steroid hormone effects and full medium (FM) as a model closer to real life.

Cells were seeded out in 96 well plates at concentrations of $5x10^3$ cells/ well in sextuplicates in either full medium or steroid depleted medium and left to adhere for 24 hours. They were then treated with 0.1, 0.5, 1, 5 or 10 µM abiraterone before being fixed with TCA at 24 hour intervals for nine days. Plates were stored at 4°C until the completion of the treatment course and upon

completion of the treatment stained and read with the SRB assay as described in chapter 2.4.2 and 2.4.3, respectively. Values shown are the means of the six readings. The t-test was used to evaluate statistical significance of the results, comparing each time points to no-treated cells.
The results are shown in *Figure* **6-5** and *Table* **6-2**.

In both cell lines, cell viability was maintained over the treatment course of all nine days (*Figure* **6-5*).***

An overall decrease in proliferation after abiraterone exposure was noted in both cell lines and both culture media. This anti-proliferative effect was more pronounced with longer treatment time. Both examined cell lines showed a statistically significant decrease of proliferation when treated with lower doses of abiraterone (0.1 µM and 0.5 µM) from day six to ten irrespective of the media used *Table* **6-2**.

OVCAR3 cells cultured in either treatment condition (FM or SDM) exposed to abiraterone showed less proliferation than untreated cells when treated with doses of 0.1, 0.5, 1 or 5 µM. This effect was more marked and statistically significant with longer exposure (six or more days). However, treatment with higher doses of abiraterone (10 µM) caused a different response depending of the culture environment. OVCAR3 cells grown in steroid depleted medium showed a highly statistically significant decrease in proliferation when treated with 10 µM abiraterone, whilst in cells cultured in full medium exposed to the same dose showed an initial increase in proliferation until day six, followed with a decrease in proliferation which was statistically significant (see *Table* **6-2**).

In contrast, whilst PEO4 cells cultured in full medium also showed a statistically significant decrease proliferation with doses of 0.5 µM and 1 µM of abiraterone (*Table* **6-2**.), PEO4 cells in steroid depleted medium showed a highly significant increase in proliferation at high doses abiraterone treatment (5 µM and 10 µM) from as early as three days exposure.

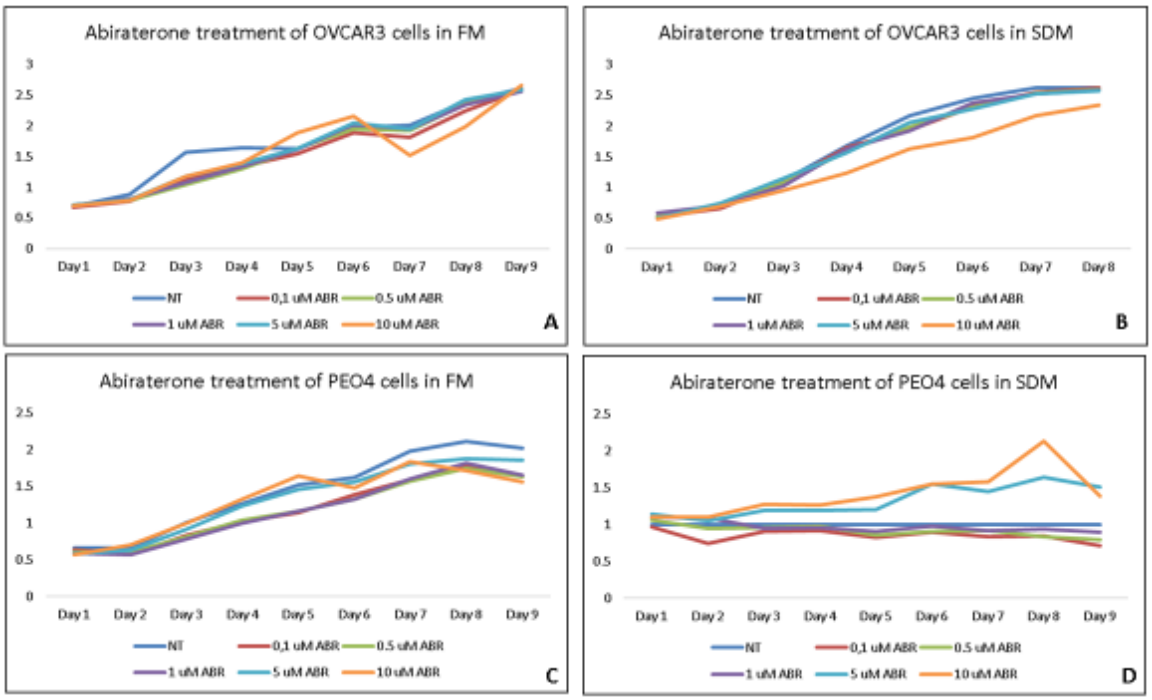


Figure 6-5: Effects of abiraterone on proliferation examined in OVCAR3 and PEO4 cells. Cells were treated with abiraterone at concentrations of 0.1, 0.5, 1, 5 or 10 µM in full medium (FM) or steroid depleted medium (SDM). Cells not treated (NT) with abiraterone were used as negative control.

	OVCAR3 FM					OVCAR3 SDM				
	Treatment dose of abiraterone					Treatment dose of abiraterone				
	0.1 µM	0.5 µM	1 µM	5 µM	10 µM	0.1 µM	0.5 µM	1 µM	5 µM	10 µM
Day 1	0.807	0.227	0.445	0.351	0.706	0.888	0.895	< 0.05	0.290	0.140
Day 2	< 0.05	< 0.05	0.057	< 0.05	< 0.05	< 0.05	0.686	0.378	0.747	0.256
Day 3	0.154	0.092	0.118	0.197	0.195	0.517	0.476	0.108	0.408	< 0.05
Day 4	*< 0.001*	*< 0.001*	*< 0.001*	*< 0.001*	*< 0.001*	0.536	0.304	0.235	0.236	< 0.05
Day 5	< 0.05	< 0.05	< 0.05	< 0.05	0.059	0.580	0.056	0.364	< 0.05	*< 0.001*
Day 6	0.000	0.002	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	*< 0.001*
Day 7	0.128	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	0.160	< 0.05	*< 0.001*
Day 8	0.094	*< 0.001*	*< 0.001*	*< 0.001*	*< 0.001*	< 0.05	< 0.05	< 0.05	< 0.05	*< 0.001*
Day 9	*< 0.001*	*< 0.001*	*< 0.001*	*< 0.001*	*< 0.001*	0.911	0.224	< 0.05	0.158	*< 0.001*

	PEO 4 FM					PEO4 SDM				
	Treatment dose of abiraterone					Treatment dose of abiraterone				
	0.1 µM	0.5 µM	1 µM	5 µM	10 µM	0.1 µM	0.5 µM	1 µM	5 µM	10 µM
Day 1	0.504	0.496	0.417	0.281	0.234	0.521	0.218	0.160	< 0.05	0.189
Day 2	< 0.05	< 0.05	0.131	0.343	0.194	0.617	0.996	0.561	0.293	0.085
Day 3	< 0.05	< 0.05	< 0.05	< 0.05	0.754	< 0.05	0.258	0.488	< 0.05	*< 0.001*
Day 4	< 0.05	< 0.05	*< 0.001*	0.595	0.261	< 0.05	0.424	0.391	*< 0.001*	*< 0.001*
Day 5	*< 0.001*	*< 0.001*	*< 0.001*	0.169	0.169	< 0.05	0.008	0.063	< 0.05	*< 0.001*
Day 6	< 0.05	< 0.05	< 0.05	0.536	0.151	0.112	0.204	0.611	*< 0.001*	*< 0.001*
Day 7	*< 0.001*	*< 0.001*	*< 0.001*	< 0.05	*< 0.001*	< 0.001	< 0.05	0.084	*< 0.001*	*< 0.001*
Day 8	*< 0.001*	*< 0.001*	*< 0.001*	< 0.05	*< 0.001*	< 0.05	< 0.05	0.348	*< 0.001*	*< 0.001*
Day 9	*< 0.001*	*< 0.001*	*< 0.001*	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	*< 0.001*	*< 0.001*

Table 6-2: p-values for the effect of abiraterone on proliferation in OVCAR3 and PEO4 cells were evaluated with the t-test. Cells were seeded out in sextuplicate in either full medium (FM) or steroid depleted medium (SDM) and treated with abiraterone at concentrations of 0.1, 0.5, 1, 5 or 10 µM. Untreated cells (NT) in either FM or SDM were used as negative control. Values in red indicate a decrease in proliferation after abiraterone treatment, values in black show an increase in proliferation after abiraterone exposure.

6.3.4 Effects of abiraterone on CYP17 and AR protein and mRNA expression in ovarian cancer cell lines

6.3.4.1 Protein expression of CYP17 and AR after abiraterone treatment in ovarian cancer cell lines

The molecular effect of abiraterone has so far only been investigated by very few studies on prostate cancer cell lines (Soifer et al., 2012, Richards et al., 2012). One such investigation observed a dose dependent decrease of AR protein expression after CYP17 blockade treatment (Soifer et al., 2012), suggesting direct anti-androgenic properties of abiraterone.

We aimed to examine if this effect could be reproduced in ovarian cancer cell lines. Two treatment conditions were chosen- cells were either grown in steroid depleted medium to exclude proliferative effects due to exogenous steroid presence or in full medium.

OVCAR3 and PEO4 cells were seeded at $6x10^5$ cells/ well in 6 well plates in either full or steroid depleted medium and left to adhere for 24 hours. They were then treated with abiraterone at different concentrations (0.1, 0.5, 1, 5 or 10 µM) for 48, 72 or 96 hours. Cells were then lysed and processed with Western blotting as described in chapter 2.5. Protein bands at 110 kDa were identified as AR with the SC441 antibody. CYP17 expression was measured with the antibody as listed in *Table* **2-5** at the molecular weight of 55kDa and GAPDH (Santa Cruz) was used a loading control identifying detecting a protein at 37 kDa. The experiments were done in triplicate and the Western blots in *Figure* **6-6** are representative for the findings.

The AR expression response to abiraterone was different between the two examined cell lines. Whilst AR protein expression was unchanged in OVCAR3 cells, it appeared to have a dose dependent effect in the PEO4 cells, with an increase in AR expression at higher doses (5 and 10 µM) which was also more pronounced with longer exposure duration (see *Figure* **6-6**).

CYP17 protein expression did not appear to alter in either cell line following abiraterone treatment for 48 or 72 hours. After 96 hours of exposure to abiraterone, a small increase in CYP17 expression was noted in both cell lines at high concentrations (5 and 10 µM) as shown in *Figure* **6-6**.

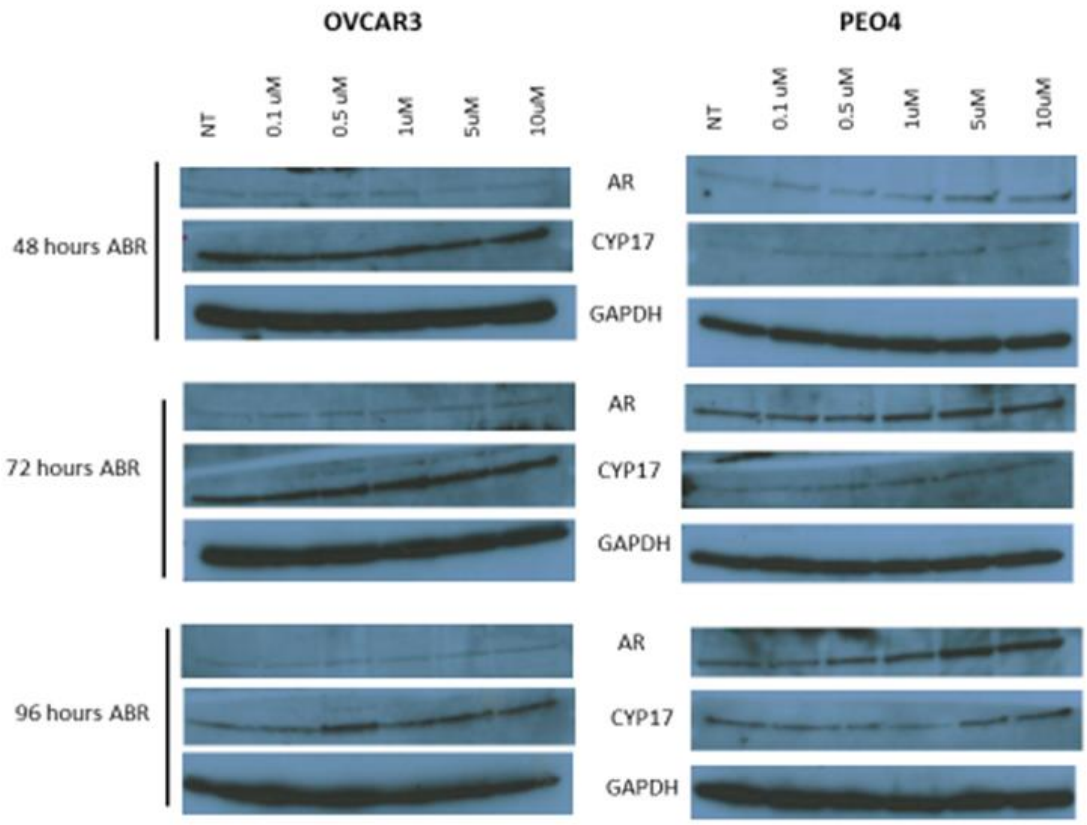


Figure 6-6: Western blots showing protein expression of CYP17 and AR in OVCAR3 and PEO4 cells after treatment with abiraterone (ABR) in the given concentrations (0.1-10 µM) after 48, 72 and 96 hours. GAPDH was used as loading control.

Cell line	Protein	Treatment duration	Treatment dose				
			NT	0.1 umM ABR	0.5 uM ABR	1 uM ABR	5 uM ABR	10 uM ABR
OVCAR 3	AR	48 hours	1	0.51	0.35	0.82	1.24	0.73
		72 hours	1	1.12	1.13	1.10	1.26	1.59
		96 hours	1	1.34	1.82	1.49	1.19	0.93
			NT	0.1 umM ABR	0.5 uM ABR	1 uM ABR	5 uM ABR	10 uM ABR
OVCAR 3	CYP17	48 hours	1	0.62	0.92	1.14	0.90	1.46
		72 hours	1	1.25	1.00	1.40	1.49	1.52
		96 hours	1	1.03	2.10	0.76	1.33	1.67
			NT	0.1 umM ABR	0.5 uM ABR	1 uM ABR	5 uM ABR	10 uM ABR
PEO4	AR	48 hours	1	1.02	0.44	1.06	2.59	2.42
		72 hours	1	1.10	0.86	1.40	1.46	1.27
		96 hours	1	0.83	0.98	1.05	1.32	1.32
			NT	0.1 umM ABR	0.5 uM ABR	1 uM ABR	5 uM ABR	10 uM ABR
PEO4	CYP17	48 hours	1	0.63	1.32	1.58	1.37	1.48
		72 hours	1	0.66	0.62	0.42	0.72	0.94
		96 hours	1	2.56	1.84	0.92	3.87	3.28

Table 6-3: Densitometry values for protein expression of AR and CYP17 in OVCAR3 and PEO4 cells after Abiraterone (ABR) treatment with given concentrations (0.1-10 µM) after 48, 72 and 96 hours. The Western blot is shown in Figure **6-6**. *Values were normalised to the loading control GAPDH and non-treated cells (NT).*

6.3.4.2 The effect of abiraterone treatment on mRNA expression of CYP17 and AR in ovarian cancer cell lines

Abiraterone treatment has demonstrated the inhibition of AR-regulated gene expression in the prostate cancer cell line LNCaP (Richards et al., 2012).

The aim in this study was to determine whether abiraterone treatment would affect the AR and CYP17 gene expression in the ovarian cancer cell lines OVCAR3 and PEO4.

OVCAR3 cells were seeded out at $6x10^5$ cells/ well in 6 well plates in full medium and treated with 0.1, 0.5, 1, 5 or 10 µM abiraterone in DMSO as solvent for 8 hours. Untreated cells with dose adjusted solvent only were used as negative control. RNA was then extracted and used for qRT-PCR as described in chapter 2.6. All experiments were done in three repeats and the results are expressed as the means of values observed. Expression values were normalised to the house keeping gene HPTR1 and to untreated cells.

6.3.4.2.1 Effects of abiraterone treatment on CYP17 and AR mRNA expression in OVCAR3 cells

OVCAR3 cells responded to abiraterone treatment with decreased CYP17 gene expression, which was most pronounced with smaller doses, showing a decrease of CYP17 gene expression of more than 50% at doses of 0.1 and 0.5 µM abiraterone (*Figure* **6-7**). The reduction in gene expression was evaluated with the t-test and was statistically significant for all treatment doses: 0.1 µM p= 0.006, 0.5 µM p=0.007, 1 µM p= 0.011, 5 µM p= 0.009 and 10 µM p=0.029.

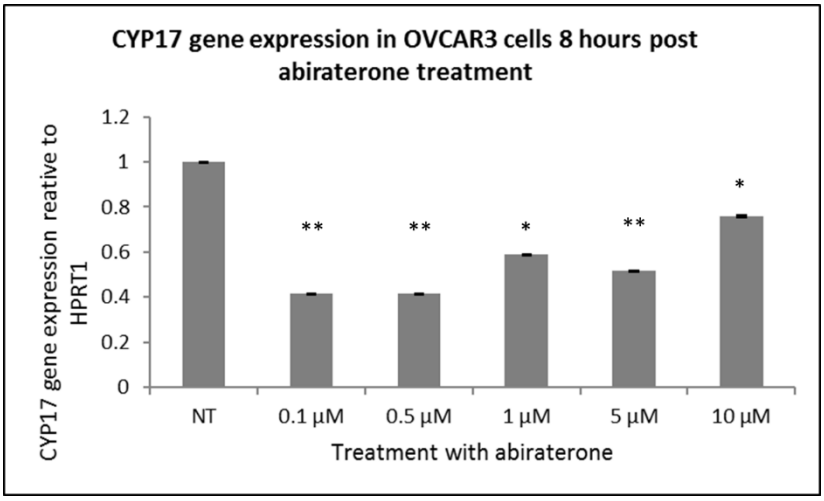


Figure 6-7: CYP17 gene expression in OVCAR3 cells after treatment with abiraterone in different concentrations (0.1- 10 µM) after 8 hours. Untreated cells (NT) were used as negative control. Relative gene expression was normalised to the housekeeping gene HPRT1 and untreated control. Values represent the mean ±SE of the mean of three repeats with triplicates of each reaction. Statistical evaluation was done by t-test.= p <0.05, **= p<0.01.*

AR gene expression also decreased after 8 hours abiraterone treatment in OVCAR 3 cells. The effect is not clearly dose dependent, but statistically significant at doses of 0.5 µM (p= 0.004), 5 µM (p= 0.021) and 10 µM (p=0.004) as shown in *Figure* **6-8**.

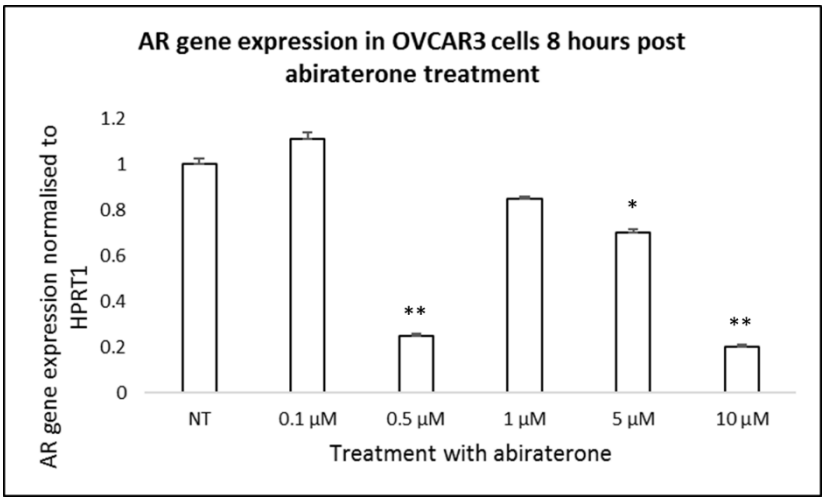


Figure 6-8: AR gene expression post abiraterone treatment for 8 hours in OVCAR3 cells in the given concentrations (0.1- 10 µM). Untreated cells (NT) were used as negative control. Relative gene expression was normalised HPRT1 as housekeeping gene and the untreated control. Values represent the mean ±SE of the mean of three repeats with triplicates of each reaction. Statistical evaluation was done by t-test.= p <0.05, **= p<0.01.*

6.3.4.2.2 Effects of abiraterone on CYP17 and AR expression in PEO4

In contrast to OVCAR3 cells, in PEO4 cells CYP17 gene expression showed an increase in CYP17 mRNA expression after 8 hours of treatment. Treatment with 1 µM abiraterone led to a 2-fold increase and exposure to 10 µM abiraterone to a 4-fold increase of CYP17 gene expression as shown in see *Figure* **6-9**. Statistical significance was evaluated by t-test and was highly statistically significant for the increase of CYP17 gene expression after 10 µM treatment with a p value of 0.0003. p values for treatment doses of 0.1, 0.5, 1 and 5 µM are 0.007, 0.152, 0.002 and 0.044 respectively.

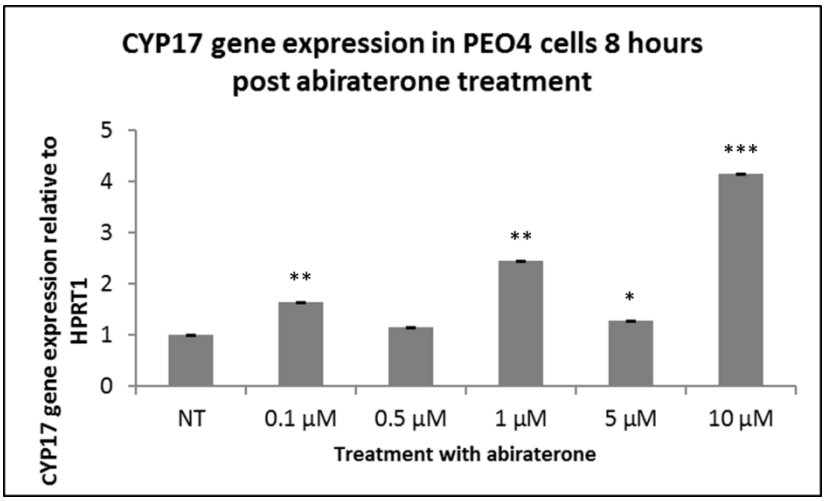


Figure 6-9: CYP17 gene expression in PEO4 cells after treatment with abiraterone in different concentrations (0.1- 10 µM) after 8 hours. Untreated cells (NT) were used as negative control. Relative gene expression was normalised to the housekeeping gene HPRT1 and untreated control. Values represent the mean ±SE of the mean of three repeats with triplicates of each reaction. Statistical evaluation was done by t-test.= p <0.05, **= p<0.01 *** p<0.001.*

In contrast, AR mRNA expression was found to decrease with all concentrations following CYP17 inhibition (*Figure* **6-10**). The decrease of CYP17 gene expression was evaluated with the t-test and was statistically significant for all doses of treatment (0.1 µM p= 0.004, 0.5 µM p=0.003, 1 µM p=0.003, 5 µM p=0.002, 10 µM p=0.005).

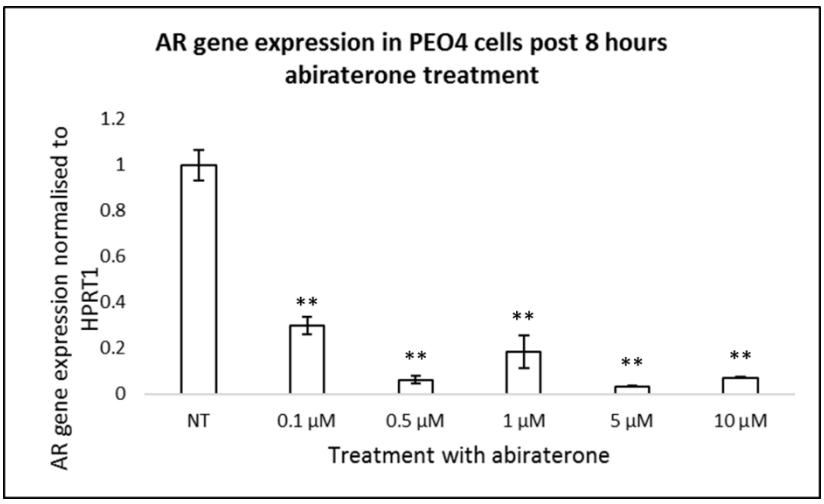


Figure 6-10: AR gene expression in PEO4 cells after 8 hours treatment with abiraterone in different concentrations (0.1- 10 µM). Untreated cells (NT) were used as negative control. Relative gene expression was normalised to the housekeeping gene HPRT1 and untreated control. Values represent the mean ±SE of the mean of three repeats with triplicates of each reaction. Statistical evaluation was done by t-test.= p <0.05, **= p<0.01 *** p<0.001.*

In summary, PEO4 cells responded to abiraterone treatment with an increase in CYP17 gene expression and a decrease in AR gene expression.

6.3.5 CYP17 expression in primary ovarian cancer cell lines (PCO)

6.3.5.1 CYP 17 gene expression in primary ovarian cultures

As described in chapter 3, primary cancer cell lines express the AR in all of the examined cultures at the mRNA level (with qRT-PCR).

For the examination of CYP17 gene expression, the same PCO RNA samples which had been investigated for AR gene expression were used. All examined primary ovarian cultures were at passage one. Each PCR reaction was done in triplicate and primer set 4 (see *Table* **2-7**) was used to detect CYP17 mRNA. Values were normalised to the housekeeping gene HPRT1 and the cell line OVCAR3 and represent the mean ±SE of triplicates of each reaction.

In total, a panel of 27 primary ovarian cultures were examined for CYP17 expression (see *Figure* **6-11**). All of these cultures were found to express CYP17, but at very variable levels. When compared to CYP17 gene expression in OVCAR3 cells, some primary ovarian cultures expressed more than 1000 times more CYP17 mRNA than OVCAR3 cells (PCO 138, 143, 144) as shown in *Figure* **6-11**.

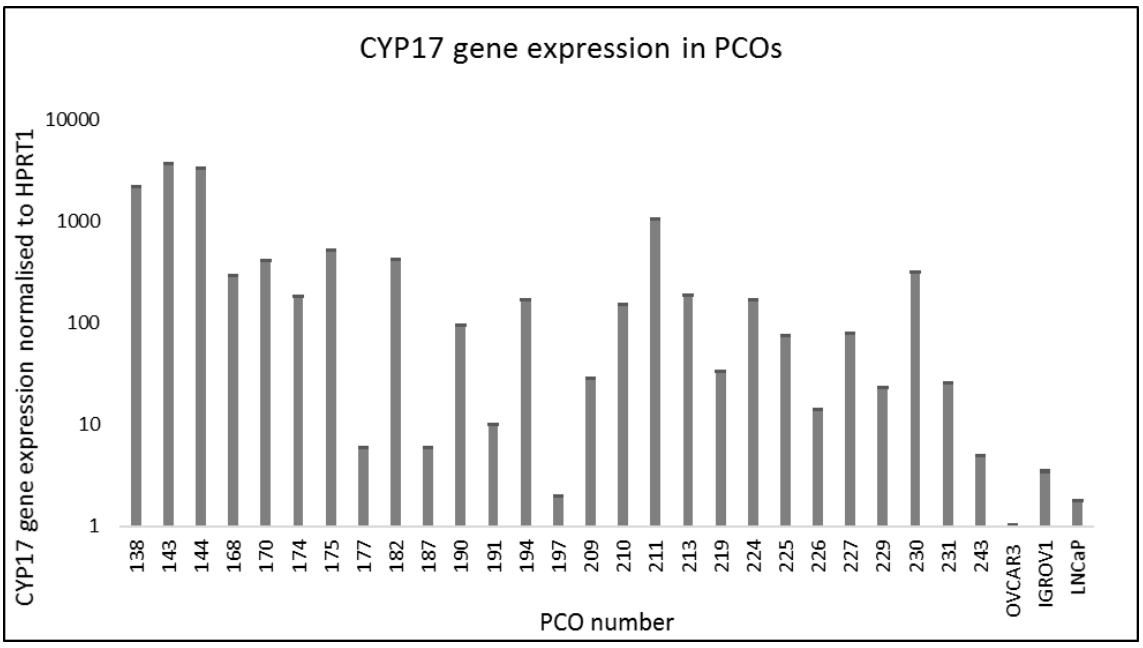


Figure 6-11: CYP17 gene expression in 27 primary ovarian cultures. mRNA expression was normalised to the house keeping gene HPRT1 and expression was standardised to OVCAR3 cells which was set at a value of 1. Values represent the mean ±SE of triplicates of each reaction.

In contrast, AR gene expression in the same panel of primary cell cultures showed much lower expression levels (see *Figure* **6-12**) with only two primary cultures expressing AR mRNA at higher levels than OVCAR3 cells. PCO 170 and PCO 211 showed a 1.5 and 2fold increase of AR relative gene expression than OVCAR3 AR gene expression (see *Figure* **6-12**).

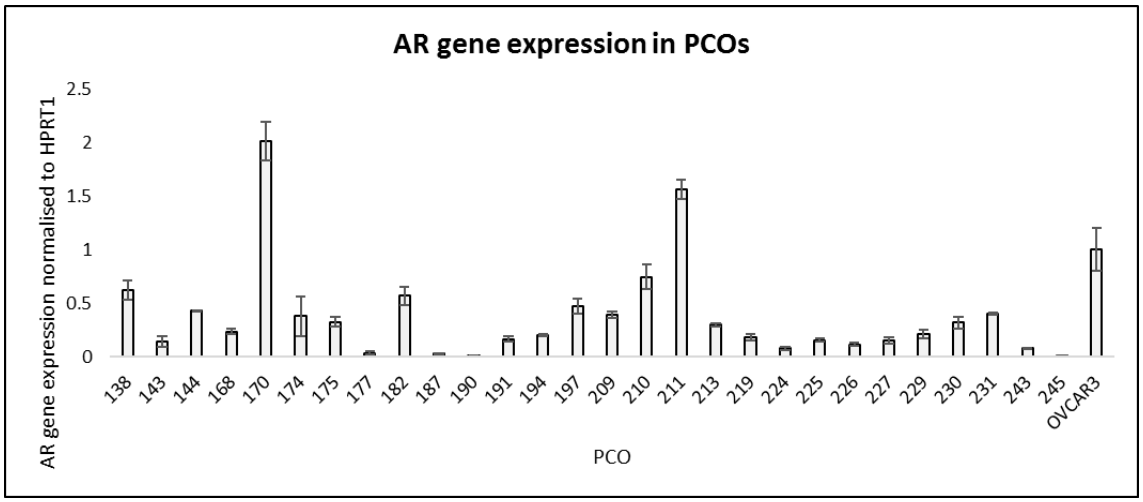


Figure 6-12: AR gene expression in 27 primary ovarian cultures. mRNA expression was normalised to the house keeping gene HPRT1 and expression was standardised to OVCAR3 cells which was set at a value of 1. Values represent the mean ±SE of triplicates of each reaction.

CYP17 and AR gene expression of the 27 examined primary ovarian cultures were evaluated with the Spearman rho correlation coefficient using SPSS (see *Figure* **6-13**). The Spearman correlation test with a R^2 of 0.019 and a p-value of 0.011 suggested a moderate positive correlation between AR and CY17 gene expression. However, the result is best interpreted with caution in view of the outliers and small sample size.

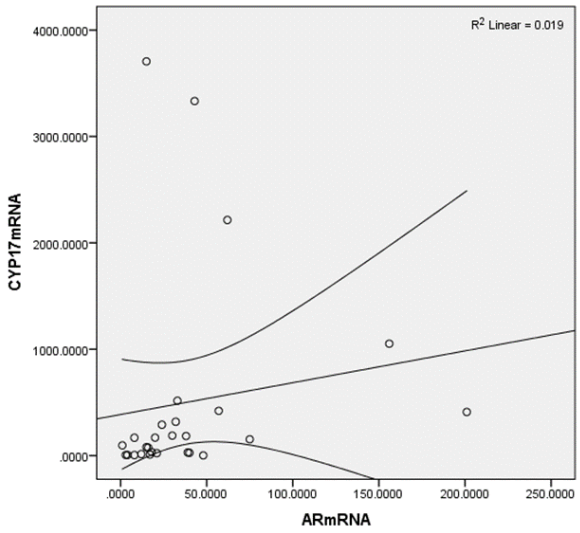


Figure 6-13: Spearman rho correlation of AR and CYP17 mRNA expression in 27 primary ovarian cultures A moderate positive correlation was calculated by the Spearman correlation test (R^2= 0.019, p=0.011), but should be interpreted as no correlation due to the outliers.

6.3.5.2 CYP17 protein expression in primary ovarian cultures

12 primary ovarian cultures were examined for CYP17 protein expression with Western blotting.

Cells were seeded at $6x10^5$ cells/ wells in 6 well plates in full medium, and lysed when 80% confluent. The protein content was quantified, and Western blotting performed to detect CYP17 and AR as described in chapter 2.5. GAPDH protein level was used as a loading control.

8/12 primary ovarian cultures expressed CYP17 as shown in *Figure* **6-14**. Some primary ovarian cultures showed a strong CYP17 expression (PCO 219, 222 and 224). Two of the primary ovarian cultures (PCO 197 and 222) expressed both CYP17 and AR (at low levels) protein (*Figure* **6-14**).

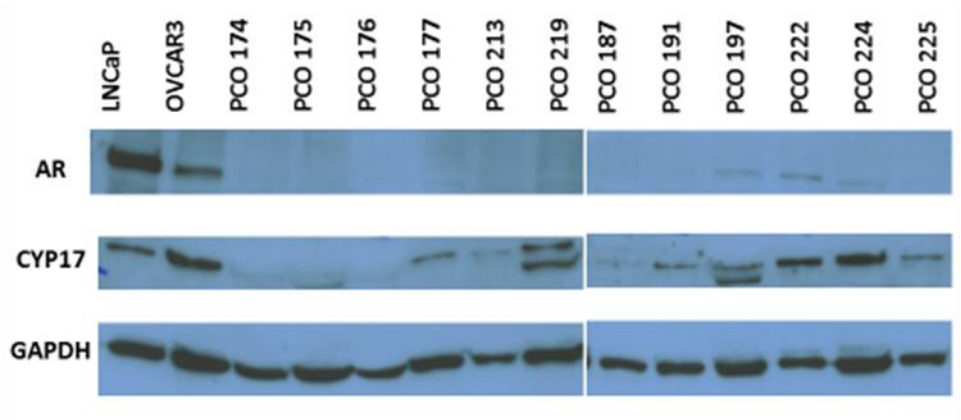


Figure 6-14: CYP17 and AR protein expression in a panel of primary ovarian cancer cell lines. LNCaP and OVCAR 3 cells were used as positive control. Protein expression was normalised to the house keeping gene GAPDH which acted as a loading control.

For 10/12 primary ovarian cultures which have been examined for CYP17 protein expression, CYP17 gene expression was tested (see *Figure* **6-15**). Two primary ovarian cultures (176 and 222) could not be tested as no RNA sample had been obtained at the time of collection.

The protein and the gene expression appeared to have an inverse correlation, as it was found that primary ovarian cultures with high CYP17 mRNA expression displayed a low protein expression (PCO 174, 175, 213), whilst primary ovarian cultures with pronounced CYP17 protein expression had a low mRNA expression (PCO 197 and 219). Only one PCO (224) demonstrated both a strong protein and gene expression for CYP17 (see *Figure* **6-14** *and Figure* **6-15**).

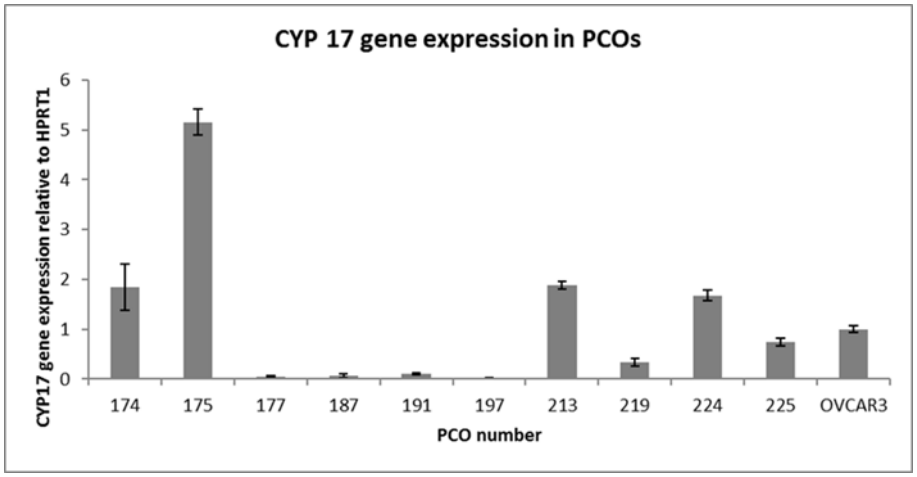


Figure 6-15: CYP 17 gene expression in the same panel of 10 primary ovarian cultures which was examined for protein expression of CYP17. Values represent the mean ±SE of triplicates of each reaction and were normalised to the housekeeping gene HPRT. Values were standardised to relative levels of CYP17 gene expression in OVCAR3 cells that were set at '1'. PCO 176 and 222 were not examined, as no mRNA sample was available.

Statistical evaluation with the Spearman rho test was done with SPSS. A non-significant, weak negative correlation between CYP17 protein expression on Western blot and CYP17 mRNA expression was found (see *Figure* **6-16**) with a R^2 value of 0.122 (p=0.174). This should however be interpreted with caution due to the outliers.

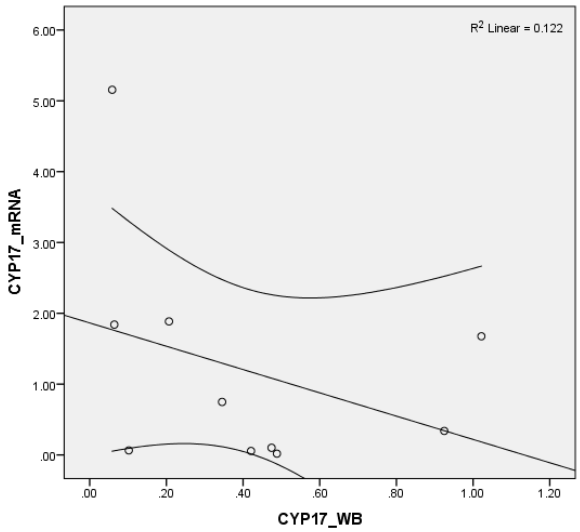


Figure 6-16: Spearman rho correlation of CYP17 protein and CYP17 mRNA expression in 10 primary ovarian cultures A weak, negative correlation was calculated by the Spearman correlation test (R^2=0.122, p= 0.174), but should best be regarded as negative due to the outliers and small sample size.

6.3.5.3 CYP17 gene expression in primary ovarian cultures at different passage numbers

Altered gene expression after passaging of cells has been described (Neumann et al., 2010). The expression data detailed above were made on cells at passage one. These experiments investigated whether cell passage numbering has an effect on CYP17 gene expression.

The collection of primary cancer cells was described in chapter 2.2.3. Ascites obtained from patients was seeded 1:1 in full medium and labelled as passage zero. Passage one would be obtained when cells were first passaged. RNA was extracted and reverse transcribed and evaluated for CYP17 gene expression with qRT-PCR as described in chapter 2.5

The effect of passaging of primary ovarian cultures cells on CYP17 gene expression was examined in five primary ovarian cultures (see *Figure* **6-17**). All of the examined primary ovarian cultures show decreased CYP17 gene expression with increased passaging.

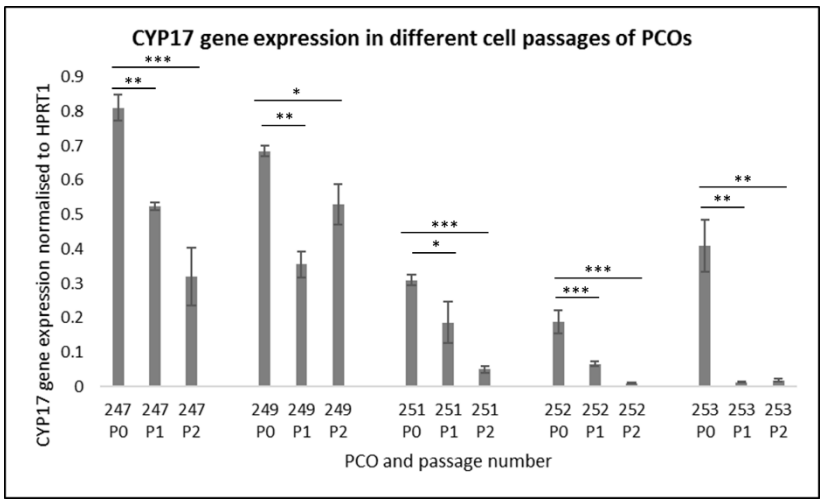


Figure 6-17: CYP17 gene expression in five primary ovarian cultures at passages zero, one and two. Values represent the mean ±SE of triplicates of each reaction and were normalised to the housekeeping gene HPRT. Statistical evaluation was done by t-test.= p <0.05, **= p<0.01 *** p<0.001.*

In contrast and as previously described in chapter 3, AR gene expression was not uniformly affected by passaging of cells, as passaging cells only showed a decrease in AR mRNA expression in 2/16 primary ovarian cultures (see chapter 3, *Figure **3-12***).

Although CYP17 gene expression decreased in all five primary ovarian cultures examined with passaging, it was observed that the AR gene expression shows the same effect only in one primary ovarian culture (PCO 253) (see *Figure* **6-18**).

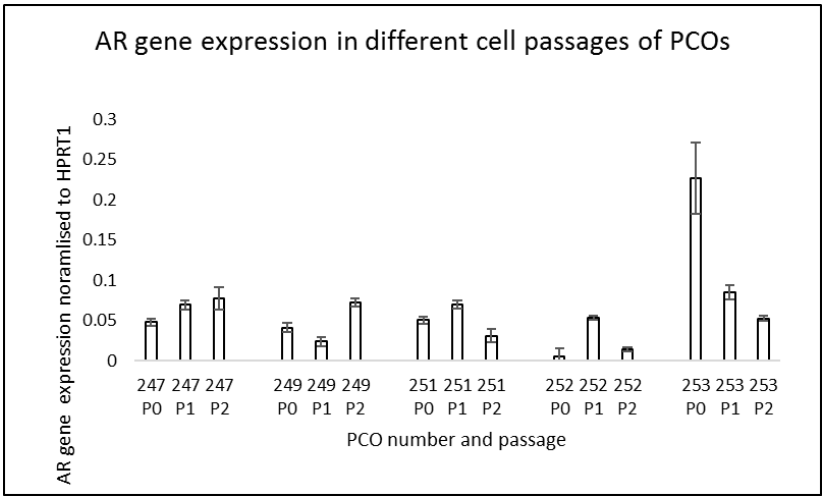


Figure 6-18: AR gene expression in five primary ovarian cultures at passages zero, one and two. Values represent the mean ±SE of triplicates of each reaction and were normalised to the housekeeping gene HPRT.

6.3.5.4 CYP17 protein expression in primary ovarian cultures at different passages

Three primary ovarian cell cultures were examined for CYP17 protein expression with Western blotting.

Primary ovarian cultures were obtained and passaged as described in chapter 2.2.3. Cells were seeded at $6x10^5$ cells/well in 6 well plates in full medium and lysates obtained when cells had reached 80% confluence. Western blotting was performed as outlined in chapter 2.5.

For all three examined primary ovarian cultures, CYP17 protein expression varied with different passages (see *Figure* **6-19**). Unlike the CYP17 gene expression however, the expression was not necessarily decreased with increased passage numbering but was observed to increase in some cases with increasing passage number.

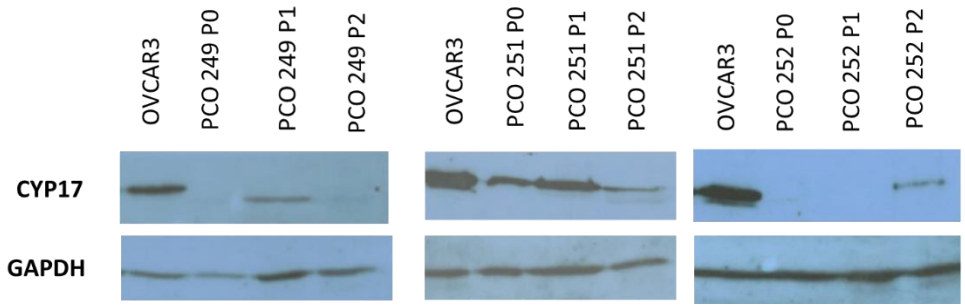


Figure 6-19: CYP17 protein expression on Western blots in primary ovarian cultures at passages zero one and two. GAPDH was used as loading control.

6.3.6 Effects of abiraterone on Rab35 gene expression

In prostate cancer, an androgen dependent cancer, disease progression is routinely monitored with serum levels of the prostate-specific antigen (PSA) (Cuzick et al., 2014, Schroder et al., 2009). At the molecular level, PSA is known as a downstream target for androgen function used for evaluation of the AR function.

A decrease in PSA gene expression after abiraterone treatment of LNCaP cells was demonstrated by Richards *et al*. The inhibitory effect of treatment with abiraterone was comparable to treatment with established anti-androgens such as bicalutamide and MDV3100 (Richards et al., 2012).

In ovarian cancer, though subject to extensive research, the only established biomarker in clinical practice is CA125 (Bottoni and Scatena, 2015), which can also be expressed in benign conditions and is not specific for any cancer subtypes.

Literature search has not shown any publications linking androgen function or AR receptor expression to CA125. As shown in chapter 3, expression of AR mRNA using qRT-PCR and protein using IHC in primary ovarian cultures did not show any correlation.

Rab35 has been proposed as an androgen dependent protein and potential biomarker in ovarian cancer in an ovarian cancer cell line (Sheach et al., 2009, SooHoo, 2013) .

Based on this, we hypothesized that if abiraterone has an inhibitory effect on the AR, treatment with abiraterone may decrease Rab35 expression as a measurement of androgen function

In this chapter we examined the effect of abiraterone on Rab35 gene expression in two ovarian cancer cell lines (OVCAR3 and PEO4).

Cells were seeded out in full medium in 6 well plates at concentrations of $6x10^5$ cells/ well. They were treated with abiraterone for 8 hours at concentrations of 0.1, 0.5, 1, 5 or 10 µM. Untreated cells were used as control. RNA extraction and qRT-PCR were performed as described in chapter 2.6.

The effect of abiraterone on Rab35 gene expression was found to differ in the two examined cells lines.

In PEO4 cells, a dose-dependent decrease in Rab35 gene expression was seen after eight hours of abiraterone treatment (*Figure* **6-20**). The decrease of Rab35 gene expression was compared to untreated cells and evaluated with the t-test. The decrease of Rab35 gene expression after abiraterone treatment was statistically significant for all treatment doses with a p-value of 0.005, 0.004, 0.003, 0.002 and 0.002 for treatments with 0.1, 0.5, 1, 5 and 10 µM abiraterone respectively.

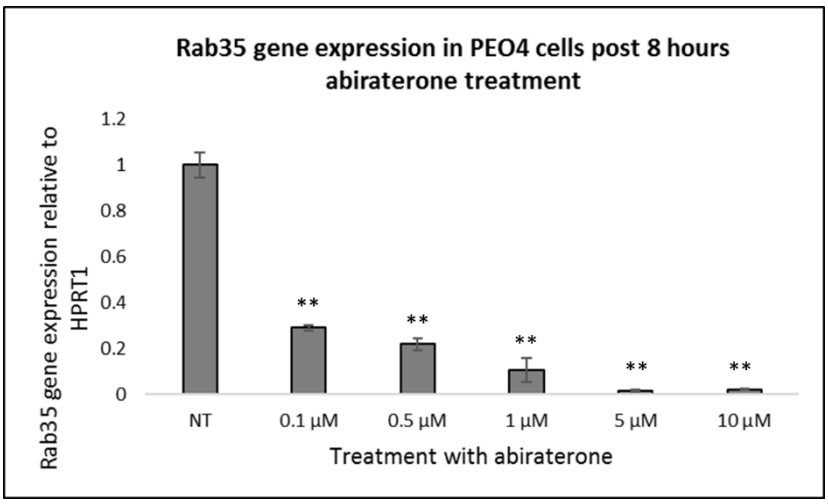


Figure 6-20: Rab35 gene expression after abiraterone treatment in PEO4 cells after 8 hours at different concentrations (0.1 – 10 µM). Untreated cells (NT) were used as negative control. Relative gene expression was normalised to the housekeeping gene HPRT1 and untreated control. Values represent the mean ±SE of the mean of three repeats with triplicates of each reaction. Statistical evaluation was done by t-test.= p <0.05, **= p<0.01.*

In OVCAR3 cells, abiraterone exposure showed a rather mixed response, with an increase in Rab35 gene expression at 0.1 µM, 1 µM and 5 µM, but a statistically significant decrease in Rab35 gene expression at doses of 0.5 µM and 10 µM with p-values of 0.003 and 0.004 respectively (see *Figure* **6-21**).

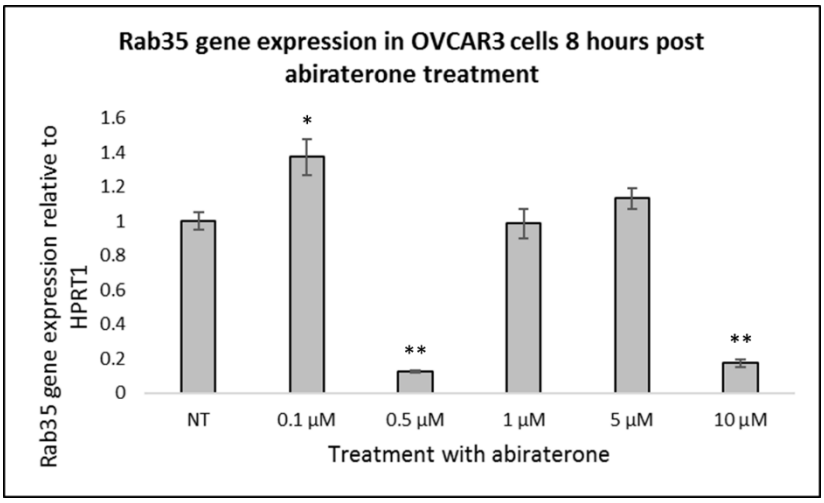


Figure 6-21: Rab35 gene expression in OVCAR3 cells after abiraterone treatment for 8 hours at different concentrations (0.1 – 10 µM). Untreated cells (NT) were used as negative control. Relative gene expression was normalised to the housekeeping gene HPRT1 and untreated control. Values represent the mean ±SE of the mean of three repeats with triplicates of each reaction. Statistical evaluation was done by t-test.= p <0.05, **= p<0.01.*

Interestingly however, the gene expression change was almost identical with the AR gene expression after CYP17 inhibition (see *Figure* **6-8** and *Figure* **6-10**, *Figure* **6-20** and *Figure* **6-21**).

Abiraterone treatment dose (in uM)	CT values for genes	
	Rab35	HPRT1
NT	25.228	21.751
0.1	24.771	21.745
0.5	28.420	21.916
1	25.261	21.746
5	25.152	21.854
10	28.24974	22.219275

Table 6-4: Raw data CT mean values for Rab35 gene expression in OVCAR3 cells after abiraterone treatment for 8 hours at different concentrations (0.1 – 10 µM). Untreated cells (NT) were used as negative control. The results are shown in Figure 6-21.

PCO number	Histology	CYP17 mRNA expression	CYP17 protein expression	AR mRNA expression	AR protein expression	Rab35 mRNA expression
138	HGS	2215	NA	62	NA	247
143	HGS	3706	NA	15	NA	947
144	Endometroid with clear cell component	3333	NA	43	(-)	516
168	HGS	290	NA	24	31	685
170	HGS	410	NA	201	(-)	765
174	Endometroid of the ovary	184	NA	38	(-)	13190
175	Clear cell cancer	516	NA	33	NA	935
177	HGS	6	40	4	(-)	622
182	HGS	421	NA	57	(-)	779
187	HGS	6	NA	3	14	382
190	Mucinous adenocarcinoma	96	NA	1	(-)	486
191	HGS	10	53	17	48	455
194	Enteric type adenocarcinoma in mature cystic teratoma	169	NA	20	(-)	620
197	HGS	2	52	48	62	877
209	HGS	29	NA	39	(-)	872
210	HGS	153	NA	75	(-)	1147
211	HGS	1052	NA	156	(-)	1163
213	HGS	188	24	30	NA	894
219	HGS	34	84	18	(-)	1056
224	HGS	168	102	8	4	561
225	HGS	75	40	16	16	955
226	Low grade serous	14	NA	12	(-)	435
227	HGS	79	NA	15	(-)	1202
229	HGS	23	NA	21	(-)	194
230	HGS and clear cell component	318	NA	32	(-)	239
231	HGS	26	NA	40	(-)	362
243	HGS	5	NA	8	16	73

Table 6-5: Summary of primary ovarian cultures examined in this chapter. CYP17, AR and Rab35 mRNA expression are given as percentage of respective relative gene expression in OVCAR3 cells. CYP17 and AR protein expression were measured with Western blotting and values normalised to the CYP17 and AR protein expression in OVCAR3 cells.

6.4 Conclusions

The main conclusions from this chapter are listed below:

Ovarian cancer cell lines

- Ovarian cancer cell lines have varying expressions of CYP17 at the protein and mRNA level
- Effects observed following abiraterone treatment of OVCAR3 and PEO4 cell lines:
    - Low dose treatment led to a statistically significant decrease of cell proliferation in OVCAR3 cells cultured in FM at 0.5- 10µM for eight days, in OVCAR3 cells in SDM at 10µM after six days and in PEO4 cells in FM at 0.1-10 µM after seven days
    - No effect on protein expression of CYP17
    - Increased AR protein expression with high treatment doses and long treatment duration (in PEO4 cells)

The effects of abiraterone treatment on gene expression of CYP17 and the AR differ in the OVCAR3 and PEO4 cell line.

- In the PEO4 cell line model, abiraterone exposure
    - increased the CYP17 gene expression
    - decreased the AR gene expression
    - led to changes in Rab35 gene expression mirroring the AR gene expression after abiraterone treatment.
- In the OVCAR3 cell line model, abiraterone treatment
    - decreased the CYP17 gene expression.
    - led to mixed response in AR gene expression with mainly a decrease of AR gene expression.
    - led to changes in Rab35 gene expression mirroring the AR gene expression after abiraterone treatment.

Primary cancer cell lines (PCO)

- All examined samples expressed CYP17 at mRNA level (100%)
- 66% of examined primary ovarian cultures expressed CYP17 at the protein level

- CYP17 mRNA and AR mRNA expression showed a weak positive correlation
- Cell passaging led to a decrease in CYP17 gene expression after passage zero.
- Cell passaging altered the protein expression of CYP17. The expression showed a mixed response of increased or decreased CYP17 protein expression.

6.5 Discussion

Anti-androgens for treatment in ovarian cancer have shown promising treatment result *in vitro* in ovarian cancer cell lines (SooHoo, 2013) and on a small panel of primary ovarian cultures (Elattar, 2010), though no good response could be achieved in patients (Kosaka et al., 2014) (Vassilomanolakis et al., 1997, van der Vange et al., 1995, Tumolo et al., 1994, Thompson et al., 1991).

Abiraterone, a CYP17 inhibitor is successfully used in prostate cancer, as CYP17 inhibition prevents steroidogenesis. One study has described direct anti-androgenic effect of abiraterone (Soifer et al., 2012). This could suggest a potential enhanced effect of abiraterone via dual action- the suppression of hormone ligand synthesis as well targeting the end organ by reduction of receptor expression.

Expression

Confirmation of CYP17 expression at the protein and at mRNA level in a panel of seven ovarian cancer cell lines was demonstrated (see *Figure* **6-2** and *Figure* **6-3**) as well as CYP17 protein in 66% of examined primary cell cultures *Table* **6-3** and could hence provide a promising prospect as a potential target for abiraterone treatment in ovarian cancer.

Furthermore, relative CYP17 gene expression was confirmed in 100% of 27 examined primary ovarian cell cultures at varying levels and in 100-1.000fold higher expression than in the reference cancer cell line OVCAR3 (see *Figure* **6-11**).

Interestingly, for some primary cultures (PCO 175, 197 and 219) a second band was detected on Westerns blots, opening the suggestion for a spliced variant of the protein.

Proliferation

In this study, two ovarian cancer cell lines (OVCAR3 and PEO4) expressing AR and CYP17 were treated with the CYP17 inhibitor abiraterone. Abiraterone has been shown to inhibit the synthesis of steroid hormones as well as the AR itself, as Richards *et al* have demonstrated a dose dependent reduction in proliferation in the prostate cancer cell lines LNCaP and VCaP (Richards et al., 2012)

In keeping with this we have found that treatment courses of six days or more with low dose of abiraterone led to an inhibition of proliferation in both examined cell lines (see *Table* **6-2**).

Interestingly, when cultured in steroid depleted medium, the response to high doses of abiraterone differed between cell lines. Whilst in OVCAR3 cells a significant decrease of

proliferation was seem following high dose abiraterone, a significant increase in proliferation due to abiraterone exposure was noted.

The proliferative effect of abiraterone has been described in breast cancer cell lines. Capper *et al* have confirmed a dose dependent proliferative effect when treating the oestrogen dependent breast cancer cell lines MCF7 and T47D with abiraterone in oestrogen-free conditions and attributed this effect to the structural similarity of abiraterone to sex steroid hormones (Capper et al., 2016). The same study group further validated these results by demonstrating the inhibition of abiraterone induced proliferation by the oestrogen receptor (ER) antagonist ICI 182,780 and showing that abiraterone antagonizes oestrogen-induced growth competitively in MCF-7 cells (Capper et al., 2016).

In contrast, a further study group found no anti-proliferative effects following abiraterone treatment, though abiraterone did inhibit the CYP17A1 expression in their examination of the castrate resistant cancer cell lines C4-2 and C4-2AT6- (Kosaka et al., 2014) This study group concluded that in view of the lack of anti-proliferative effect of abiraterone, its efficacy in vivo might be limited (Kosaka et al., 2014).

Abiraterone and protein expression of AR and CYP17

A dose-correlated decrease of AR protein expression following abiraterone treatment demonstrating the direct anti-androgenic effect of abiraterone has been described in the prostate cancer cell lines LNCaP and LAPC-4 by Soifer *et al* (Soifer et al., 2012).

In this study we found that the effect of abiraterone exposure on AR protein expression differed-whilst in OVCAR3 cells CYP17 inhibition did not alter the AR protein expression, abiraterone treatment of PEO4 cells increased the AR protein expression at higher treatment doses and with increased treatment duration (see *Figure* **6-6**).

One possible explanation of difference in findings could be the medium used for cell culture. Whilst Soifer *et al* cultured their cells in steroid depleted medium, full medium was used in this project. It would be possible that subtle anti-androgenic effects could be counteracted by steroids present in full medium.

Another possible explanation for this observed lack of inhibition and even proliferation could be explained by findings of one study group who demonstrated that abiraterone treatment drives androgen synthesis within the tumour (Cai et al., 2011). Cai *et al* also described an increase in

selection pressure for CYP17 expression in tumours treated with abiraterone (Cai et al., 2011), explaining possible mechanisms for resistance in tumours to CYP17 inhibition.
CYP 17 protein expression after abiraterone treatment has interestingly not been affected in either of the two examined ovarian cancer cell lines (see *Figure* **6-6**).

Abiraterone and mRNA expression of CYP17 + AR

To further evaluate potential direct anti-androgenic properties of abiraterone, we examined AR mRNA and CYP17 mRNA expression after abiraterone treatment.
The statistically moderate positive correlation between AR mRNA and CYP17 mRNA expression was calculated with the Spearman Rho test (see *Figure* **6-13**). This could lead to the hypothesis that CYP17 inhibition would decrease AR expression, however the result should best be interpreted as no correlation, due to the outliers and small sample size.
The effects of abiraterone exposure led to varied results in the two cell lines.
Whilst abiraterone treatment caused the expected decrease in CYP17 gene expression in OVCAR3 cells and was statistically significant (*Figure* **6-17**), the CYP17 gene expression in PEO4 cells was in contrast increased (*Figure* **6-9**).
The AR gene expression was overall decreased in both cell lines- with a more dose dependent effect in PEO4 cells (*Figure* **6-8** and *Figure* **6-10**). In OVCAR3 cells, though most treatment concentrations caused a decrease in AR expression, low dose treatment caused an increase in AR expression. A similar phenomenon was observed in the oestrogen positive breast cancer cell line MCF7, where abiraterone induced the expression of the oestrogen receptor responsive gene GREB1 in ER-positive cell lines and this induction was blocked by the oestrogen receptor antagonist, ICI 182,780 *(Capper et al., 2016).* Though some promising effects were seen in this study, the differences in CYP17 gene expression in response to abiraterone treatment pose more questions and could be interpreted in view of tumour heterogeneity.
A potential shortcoming of this study might be the time course chosen, as in LNCaP cells, which are the best characterised cell line for AR, a treatment duration of 8-16 hours is required to see detectable changes in the downstream gene PSA.
The two chosen cell lines (OVCAR3 and PEO4) only expressed low levels of CYP17. Further work with ovarian IGROV1 cells which express CYP17 mRNA at high levels *Figure* **6-3** should be considered.

Passaging effect

Working with cell line models is an established approach in translational research, but has pitfalls, such as selecting for a fast-growing sub-population of cells with cell culturing and change in gene expression through passaging.

In this study, a passaging effect on CYP17 expression was demonstrated in primary ovarian cultures, both at the mRNA and protein levels. Whilst CYP17 gene expression showed a clear statistically significant decrease with advanced passaging (*Figure* **6-17**), protein levels showed a varied response with either increased or decreased CYP17 expression after passaging (*Figure* **6-19**). This demonstrates one of the potential challenges in working with primary cultures, as it might be difficult to determine which cell passage should be examined for reliable results.

Primary cancer cells

Primary cancer cells are nevertheless a desirable model in translational research, as they more accurately reflect the heterogeneity of cancer to a greater extent than established cell lines.

Hence, CYP17 expression was investigated in primary ovarian cancer cell lines in this study. CYP17 gene expression was confirmed in all examined primary ovarian cultures (*Figure* **6-15**) and CYP17 protein expression was seen in 66% of primary ovarian cultures (*Figure* **6-14**).

A trend of inverse expression of protein and mRNA levels of CYP17 was noticed for the cancer cell lines as well as most of the primary ovarian cultures (*Figure* **6-16**). This should however be interpreted cautiously in view of the outlier as well as the small sample size.

Whilst the assumption seems logical that mRNA expression could be equalised to protein expression levels, as protein is translated from mRNA, this does not seem to be necessarily true. Protein synthesis is influenced by factors such as post-translational modification or the protein half-life. Errors and noise in experiments examining both, protein and RNA expression could be further factors potentially decreasing the correlation (Baldi and Long, 2001). Overall, the correlation between protein and mRNA expression levels has been reported with conflicting results. Whilst some studies suggest poor correlation between protein and mRNA expression (Taniguchi et al., 2010), others could demonstrate good correlations (Koussounadis et al., 2015, Orntoft et al., 2002).

In view of the decrease in proliferation seen after abiraterone treatment in the cancer cell lines in this study, and the confirmation of CYP17 protein and mRNA expression in the primary cancer cell model functional studies in primary cultures would help gain better understanding.

Effects such as cell viability, proliferation and the exploration of AR signalling pathways might be more representative to gauge treatment effects.

Considering that inhibition of steroidogenesis is the main mode of action of abiraterone, molecular research examining receptor expression or cell proliferation might only be of limited use. Even investigations evaluating the effects of abiraterone on steroidogenesis *in vitro* might not add knowledge required to gauge treatment success.

Clinical correlation has so far only been examined in one clinical trial. The CORAL trial, a multi-centre phase II study, has only shown little response in women pre-treated with chemotherapy with recurrent ovarian cancer. The one woman of the 42 recruits with measurable response to abiraterone treatment had low grade serous cancer, a disease known to respond poorly to chemotherapeutic agents. This led to the conclusion that abiraterone should be further explored in this subgroup of women with epithelial ovarian cancer. However, in view of the low response rate and despite the observation that 14-26% of women experienced disease stabilisation, the study did not proceed to the next phase (Banerjee et al., 2016). Tissues and blood samples were obtained from probands in the CORAL trial to allow further investigation into the AR signalling pathway in EOC. Which elements of the pathway or which tumour markers would be evaluated has not been stated and to date no results have been published (Banerjee et al., 2016).

The difficulty of stratification to select the patient population most susceptible for the treatment remains. In the absence of reliable biomarkers, AR status might be considered.

7 Overall discussion

Targeted therapies have begun to emerge as additions to current standard treatment of ovarian cancer treatment (cytoreductive surgery and chemotherapy) and include therapies such as angiogenesis inhibitors (Burger et al., 2011, Della Pepa and Banerjee, 2014) and PARP inhibitors (Fong et al., 2010, Lokadasan et al., 2016).

Hormonal therapies, specifically anti-androgen therapies, have rather been passed over, probably largely because trials have shown little success (Vassilomanolakis et al., 1997, Tumolo et al., 1994, Thompson et al., 1991, van der Vange et al., 1995). This might be due to a heavily pre-treated patient population, but also due to the lack of stratification of potentially susceptible individuals.

Primary ovarian cancer cell lines (PCOs), as a model as close to "real life" as possible, have been chosen for this study in order to evaluate the role of AR expression and effects of androgen stimulation. Furthermore, Rab35 as a potential androgen dependent biomarker, as previously established in ovarian cancer cell lines, was explored in the primary ovarian cultures and molecular effects of CYP17 inhibition have been examined in primary cell cultures.

7.1 AR Expression data

Previously, AR expression had been confirmed in small cohorts of primary ovarian cultures applying different techniques (Elattar et al., 2012, SooHoo, 2013). In this study we evaluated the AR expression with quantitative PCR (qRT-PCR), Western blotting (WB) and immunohistochemistry (IHC) and aimed to assess the correlation between the different modalities (chapter 3.4). Our findings demonstrate that the AR expression differs widely with these three techniques, showing 100% AR mRNA expression by qRT-PCR, 87% protein expression with IHC and 34% protein expression on WB. As none of the modalities measuring the expression status correlate, the question if any of these examination techniques is superior or more accurate remains unanswered.

Studies and clinical trials tend to examine AR on IHC to classify AR expression (Feng et al., 2017, Elattar et al., 2012, ClinicalTrials.Gov, 2018, Banerjee et al., 2016). However, no set criteria are agreed upon which cut off defines AR positivity. The trial examining the effects of enzalutamide in pre-treated women with ovarian, primary peritoneal and fallopian tube cancer uses AR expression as an inclusion criterion and defines AR positivity as AR expression of 5% or more in one or more IHC stained slices (ClinicalTrials.Gov, 2018). The CORAL trial on the

contrary uses a cut off of 10% AR expression on IHC to define AR receptor positivity and identified that 69% of their probands had AR positive ovarian tumours (Banerjee et al., 2016). The definition of the target population is crucial, as a low cut off for AR expression might lead potential treatment failure. The inclusion of women with tumours of low AR receptor expression might not express the therapeutic target to levels where treatment could exert its effect. On the other hand, a too high cut off could prevent treatment of women potentially susceptible to the drug.

Other than patient stratification of women potentially responding to anti-androgen treatment, the AR receptor expression might help to identify the right window of opportunity to exert maximal treatment effect. Elattar *et al* have seen a significant decrease in the AR expression post chemotherapy (Elattar et al., 2012) which might partially explain the poor response seen in trials, but also opens the possibility for a better treatment response if the timing of anti-androgen treatment would be adjusted and be made available to women prior or in conjunction with chemotherapy. Even the newer trials, like CORAL or the enzalutamide trial include heavily pre-treated patients with up to three courses of chemotherapy. Tying in with Elattar *et al's* observation, Feng *et al* have demonstrated in a panel of 18 matched high-grade serous ovarian cancers, the AR protein expression on IHC decreases by 41% (Feng et al., 2017).

Further consideration should be given to the current perception that ovarian cancer is in fact a cancer not only from the ovary, but likely arising from the fallopian tube and the peritoneal surface. The sole examination of AR expression of the ovary might hence not be a reliable predictor, and primary ovarian cultures generated from ascites (a fluid in close contact with all the pelvic and abdominal surfaces) seems a useful model. As demonstrated above, the difficulty in finding the most accurate and predictive method to examine AR expression remains.

7.2 Androgen effects on primary ovarian cultures

Androgen- responsiveness has been previously demonstrated in a small number of primary ovarian cultures by increase of percentage of cells in S-phase following stimulation with DHT (Elattar, 2010), but proliferation, as a measure of potential stimulatory effects, has so far not been examined in primary ovarian cultures and was therefore investigated in this study (see chapter 4.3.2). We also aimed to identify the most suitable assay to evaluate potential androgen induced changes.

Overall, the effect of androgens on primary ovarian cultures was found to be varied (see *Table* **4-2**). In 37% of primary ovarian cultures some proliferative effects were observed, whilst some others showed no or even anti-proliferative effects. This varied response is not unexpected in view of the known heterogeneity of behaviour seen in ovarian cancer. The difficulty of predicting which primary ovarian cultures (or primary cancer) will show proliferative response remains, as neither AR gene nor protein expression was found to be associated with the proliferative response, ie AR protein on Western blot did not correlate with proliferative response to DHT (*Table* **4-2**).

Regarding the choice of the proliferation assay, the BrdU ELISA assay, measuring DNA synthesis rather than protein content, might be a more preferred option, as changes in proliferation after androgen treatment were observed to be subtle. This was demonstrated in three primary ovarian cultures which appeared to be androgen insensitive when examined with the SRB assay but showed proliferation at all three measured time points with the BrdU assay (see *Table* **4-3**). The drawback of the BrdU ELISA however, compared to the SRB assay, is that timings for examination need to be carefully chosen and a continuous assessment for a large number of cultures over a long period of time is less feasible due to the expensive nature of the substrate.

Another point to consider when working with primary ovarian cultures, is the careful timing of processing samples as well as measurement of expression. Freeze-thawing of primary ovarian cultures was examined in a small number of samples and therefore does not allow a definite conclusion, but for the primary ovarian cultures examined it might suggest a potential tendency to exhibit anti-proliferative effects to androgen stimulation.

7.3 Rab35 as a biomarker

Though subject to many studies, so far only one biomarker (CA 125) has been established for common clinical use in epithelial ovarian cancer.

Biomarkers need to be both specific and sensitive in order to avoid over-diagnosis and over-treatment. Rab35 has been suggested as a specific, androgen dependent downstream target of the androgen receptor activation and possible biomarker for androgen responsive ovarian cancers based on cell line models (SooHoo, 2013).

A previous small study on primary ovarian cultures confirmed Rab35 expression in all primary cultures examined (SooHoo, 2013). In this study (see chapter 3.5) a larger cohort of primary

cell cultures was examined and observed Rab35 mRNA expression in 100% of examined samples (n=46) and Rab35 protein expression in all samples examined by IHC (n=27).
The expression, as the heterogeneity of ovarian cancer would suggest, was mirrored in the widely varying levels of Rab35 expression, for both, protein and mRNA levels between primary ovarian cultures. However, the expression levels for protein and Rab35 expression in individual POC samples did not correlate either, suggesting that these techniques cannot be used ubiquitously.

A more promising result though, was the positive correlation for AR and Rab35, suggesting that Rab35 might indeed be an androgen related protein. These correlations were positive for gene *Figure* **3-20**) and protein expression (*Figure* **3-21**) and underpin findings from S. SooHoo's work (SooHoo, 2013).
Following this, based on cancer cell line experiments and the fact that 100% of primary ovarian cultures expressed the AR at the mRNA level, we hypothesised, that stimulation of primary cultures with androgen should lead to an increase in Rab35 gene expression. However, primary cell lines treated with androgen, showed a variable response (see *Table* **5-1**). 47% showed an increase of more than 50% in Rab35 gene expression following androgen stimulation, 16% showed an increase of <50% and 37% showed no increase. Limitations of this experiment were the inability to validate the results with protein expression after androgen stimulation, as Rab35 expression on Western blot was not possible. Based on the changes observed in gene expression, Rab35 could not be verified as androgen dependent in primary cell cultures. It does however seem that some primary ovarian cultures are androgen sensitive rather than androgen regulated.

7.4 Passaging effect

As passaging can potentially involve a selection pressure and alter gene expression, the effect of expression data with passaging primary ovarian cultures was examined.
Whilst CYP17 mRNA expression generally decreased after passaging (*Figure* **6-17**), there was no consistent trend in the AR gene expression (*Figure* **6-18**). AR mRNA was found either to be increased, decreased or to have a mixed response to passaging.
Protein expression was altered through cell culture maintenance for both, the AR and CYP17 expression. For AR protein expression no conclusion could be made, as only 1/5 primary

ovarian cultures examined was positive for AR. In this single primary ovarian culture, the expression was much more pronounced at passage one, compared to passage zero and two.

For CYP17, protein expression was either increased or decreased after passaging cells (*Figure* **6-19**).

These variations in expression demonstrate one of the difficulties in working with primary cell cultures and the caution required in data interpretation, considering the potential passage number effects.

7.5 CYP17 inhibition

An alternative approach to treat cancers related to the hormone axis, other than using receptor blockade (ie AR-antagonists) or inhibiting the negative feedback mechanism (ie GnRH analogues) is the blockade of hormone synthesis. Abiraterone is an inhibitor of enzymes responsible for androgen synthesis. One study has reported a direct anti-androgenic effect *in vitro* on LNCaP cells of the compound (Soifer et al., 2012).

In this study some of the molecular effects of abiraterone on ovarian cancer cell lines were examined.

We have demonstrated in chapter 6.3 that ovarian cancer cell lines as well as primary cell cultures all expressed CYP17 at the mRNA level (100%) and at the protein level (66%) when examined by qRT-PCR and WB, respectively.

Treatment of ovarian cancer cell lines however showed effects contrary to those expected. Treatment with abiraterone did not lead to inhibition of cell proliferation and the cell viability was maintained (see *Figure* **6-5**).

Though treatment with abiraterone showed some reduction in CYP17 gene expression, the effect was not dose related and it occurred at different treatment times in either cell line.

More importantly, exposure to abiraterone did not lead to a decrease in protein expression, for either CYP17 or AR. In contrast, a proliferative effect was observed. One explanation for this might be that some ovarian cancers could have existing mutations of the AR, causing a selective growth advantage in a low androgen environment by utilization of low doses of systemic androgen or available intra-tumoural/ ovarian androgen, similar to effects seen in castrate resistant prostate cancer cell lines as described by Cai *et al* (Cai et al., 2011).

The lack of inhibition on cell proliferation or the reduction of the protein AR level after abiraterone treatment however does not equal unsuccessful treatment in a clinical context, as its main mode of action is the repression of steroidogenesis.

7.6 Limitations of the project

Primary cancer cell lines are a useful model to examine the concept of cancer heterogeneity, but can pose difficulties in practical terms, the main hurdles being their short life span and limited time available to gather information from experiments.

Cells used for the generation of primary ovarian cultures are derived from ascites, an accessible source as many patients with high stage ovarian cancer develop ascites. Ascites can be easily obtained with a minimally invasive abdominal puncture which can be performed in an outpatient setting.

Ascites, however, is multicellular fluid, containing not only cells from epithelial ovarian cancer, but also components such as mesenchymal cells and lymphocytes. Characterisation of the primary ovarian cultures with IF was hence used in this study (in combination with cross reference to formal histopathology) to ensure only epithelial ovarian cancer cells were investigated.

These cultures are derived from patients with advanced disease and might be obtained at different time points in the disease and treatment stage. Ascitic drainage could occur not only at presentation in a chemo-naïve state, but also at interval debulking surgery (after chemotherapy) or whilst receiving palliative chemotherapy. Chemotherapy given to women with EOC might have led to mutations and potential acquisition of resistance to certain therapies. The very subtle response of androgen stimulation in some of the primary ovarian cultures, may be attributed to this effect.

Furthermore, culturing of primary ovarian cultures itself could select for clones with favourable growth under certain experimental conditions and might hence not represent the original tumour directly.

Expression data are important and interesting, but by themselves might be a too simplistic approach. Functional assays to supplement the information are hence crucial, however they can sometimes be difficult to perform. In this study it was for example not possible to silence the

AR in primary ovarian cultures or to measure Rab35 protein expression using WB, which could be a key investigation to establish whether Rab35 is an androgen dependent marker.

With regards to examining the AR function, results should be seen in the context that the AR is not only activated by androgens as ligands, but can underlie mechanisms, such as potential cross talk between steroid hormone receptors, ie oestrogen can stimulate the androgen receptor.

AR signalling, which can be influenced by other pathways and AR co-activators has not been examined in this study.

A potential target to be explored could be TGF-beta. Androgens have been shown to decrease the TGF-beta receptor level, and therefore counteracted the TGF-beta growth inhibition with the consequence of promoting ovarian cancer progression (Evangelou et al., 2000).

Other pathways examined in prostate cancer interfering with the AR signalling, such as loss of PTEN or constitutive activation of the PI3K pathway, might need consideration (Jefferies et al., 2017).

Co-Activators, such as p44 have been found to influence the AR. The subunit p44/Mep50/WDR77 has been characterised as a coactivator for steroid receptors, enhancing transcriptional activity for both the AR and ER. Whilst cytoplasmic p44 is found in the normal ovary and fallopian tube, nuclear p44 is observed in invasive ovarian carcinoma. Overexpression of nuclear-localized p44 was described in stimulation of proliferation and invasion in ovarian cancer cells in the presence of oestrogen or androgen (Ligr et al., 2011).

Also, the role of the AR might have further implications other than being a target for anti-androgen therapy which should be considered. The AR is not only a transcription factor but has also been implied as a prognostic marker (Nodin et al., 2010).

A further interesting aspect is that the AR might impact on already established chemotherapy used for the treatment of ovarian cancer. The presence of the AR was associated with increased sensitivity to platinum-based chemotherapy (Feng et al., 2016). It was also suggested that silencing of the protein FKBP5, which form a protein complex with the AR, might regulate the expression of taxol resistance and hence affect cancer cell sensitivity to taxol (Sun et al., 2014). More recently, silencing of the AR has confirmed the sensitisation of taxol resistant cells to taxol in ovarian carcinoma (Sun et al., 2015). Correlation with data on chemo sensitization

which have been obtained by other members of the ovarian cancer study group Newcastle (Rachel O'Donnell and Aiste McCormick), could be correlated to gain further information.

In conclusion, the fact that androgen exposure caused proliferation in some primary ovarian cultures suggests confirmation of androgens and the AR playing a role in ovarian cancer, albeit the proliferative effects are subtle. The search for a reliable biomarker for androgen effects remains, as Rab35 remains to be confirmed as androgen dependent.
CYP17 inhibition, demonstrating some inhibitory effects in ovarian cancer cell lines, might be a potential treatment strategy in view of its dual action, on the molecular level and its effect on steroid synthesis.

7.7 Future research

- Expansion of functional assays in primary ovarian cultures, such as establishment and use of knockdown models could be useful to further explore the correlation of Rab35 and AR in primary cell cultures.
- The expansion of examination of the molecular effects of abiraterone on proliferation should include treatment of primary ovarian cancer cells with the CYP17 inhibitor.
- RNA Sequencing of primary ovarian cultures for certain mutations could expand the understanding of the primary ovarian cultures and the heterogeneity of ovarian cancer as a whole.
- The creation of a stable cell line from primary ovarian cultures would allow further in-depth examination of functional effects of androgens, anti-androgens and CYP17 inhibition.
- The reproduction of the tumour microenvironment *in vitro* could help explore possible paracrine interactions and help understand the disease process.

Appendices

Appendix 1: Overview of clinical and demographic detail of examined primary ovarian cultures

PCO number	Date of collection	Age (years)	FIGO stage	Histological subtype	CA125 (U/l)	Surgery	Cytoreduction	NACT	Adjuvant Chemo	PFS (months)	OS (months)
32	22-09-08	68	3C	HGSC	NA	Primary	Optimal	NA	6x carbo/ taxol	NA	NA
37	05-11-08	65	4	HGSC	324	Primary	Suboptimal	NA	6x carbo/ taxol	9	13
44	19-01-09	61	4	HGSC	731	Primary	Optimal	NA	6x carbo/ taxol	12	15
49	13-03-09	53	3	HGSC	2421	Primary	Complete	NA	6x carbo/ taxol	11	44
67	02-09-09	68	3C	HGSC	3845	Primary	Optimal	NA	6x carbo/ taxol	17	30
68	07-09-09	67	4A	HGSC	6024	No surgery	NA	NA	3x carbo/ taxol	3	3
121	21-09-53	57	3C	HGSC	10477	Primary	Optimal	NA	6x carbo/ taxol	40	41
122	12-11-10	71	4A	HGSC	520	Delayed	Optimal	4x carbo/taxol	2x carbo/ taxol	8	9
124	17-11-10	74	3C	HGSC	2757	Primary	Optimal	Na	6x carbo/ taxol	22	43
138	12-10-11	70	3C	HGSC	1412	Primary	Suboptimal	NA	6x carbo/ taxol	29	31
139	21-10-11	77	4	Endometrioid / Clear cell	1445	Primary	Optimal	NA	NA	9	9
143	21-11-11	46	3C	HGSC	1465	Primary	Optimal	NA	6x carbo/ taxol	10	10
144	02-12-11	60	1A	Endometrioid / Clear cell	1317	Primary	Optimal	NA	6x carbo/ taxol	29	36
157	02-03-12	58	3C	HGSC	500	Primary	Suboptimal	NA	6 carbo/taxol + tamoxifen	11	12
162	23-04-12	68	3C	HGSC	186	Primary	Optimal	NA	6x carbo/ taxol	18	31
168	19-06-12	66	3C	HGSC	1500	Primary	Complete	NA	6x carbo/ taxol	14	29
170	03-08-12	73	3C	HGSC	10000	IDS	Suboptimal	4 x carbo/taxol	2 x carbo/taxol + bevacizumab	20	27

PCO number	Date of collection	Age (years)	FIGO stage	Histological subtype	CA125 (U/l)	Surgery	Cytoreduction	NACT	Adjuvant Chemo	PFS (months)	OS (months)
174	12-09-12	45	3A	Endometrioid	552	Primary	Complete	NA	6x carbo, 2x taxol, 4x docetaxol	15	26
175	12-09-12	66	3B	Clear	250	Primary	Optimal	NA	5x carbo/taxol	6	8
176	12-10-12	60	3C	HGSC	6000	NA	no surgery	Nil	NA	2	2
177	16-10-12	46	3C	HGSC	6	IDS	Optimal	4 x carbo/taxol	2 x carbo/taxol	13	23
182	23-11-12	61	3C	HGSC	286	IDS	Complete	3 x carbo/taxol	3 x carbo/taxol; tamoxifen; 5 x rucaparib	11	38
187	10-01-13	71	3C	HGSC	2013	Primary	Optimal	NA	6x carbo/ taxol	12	20
190	28-01-13	75	3C	mucinous	135	Primary	Complete	NA	6x carbo/ taxol	9	11
191	31-01-13	75	3C	HGSC	346	Primary	Optimal	NA	6x carbo/ taxol	12	16
192	06-02-13	66	3C	Clear cell / Endometrioid	2425	Primary	Optimal	NA	3x carbo/taxol	4	7
194	25-02-13	59	NA	Enteric type adeno- carcinoma in mature cystic teratoma	1000	Primary	Suboptimal	NA	NA	2	2
197	06-03-13	63	3C	HGSC	2000	Primary	Optimal	NA	6x carboplatin	21	21
209	26-04-13	52	3C	HGSC	419	IDS	Optimal	4x carbo/ taxol	2x carbo/ taxol	5	9
210	04-05-13	63	3C	HGSC	420	Primary	Optimal	NA	6x carbo/ taxol	21	21
211	04-05-13	53	3C	HGSC	869	Primary	Optimal	NA	6x carbo/ taxol	20	20
213	10-05-13	85	4	HGSC	1295	NA	NA	NA	NA	4	4
219	03-06-13	60	3C	HGSC	1445	IDS	Optimal	4x carboplatin	2x carboplatin	4	13

PCO number	Date of collection	Age (years)	FIGO stage	Histological subtype	CA125 (U/l)	Surgery	Cytoreduction	NACT	Adjuvant Chemo	PFS (months)	OS (months)
221	05-06-13	72	2C	Carcinosarcoma	1000	IDS	Optimal	4x carbo/ taxol	2x carbo/ taxol	14	21
222	07-06-13	47	3C	HGSC	6	IDS	NA	4x carbo/ taxol	2x carbo/ taxol	13	25
224	12-07-13	70	3C	HGSC	325	Primary	Optimal	NA	5x carbo/ taxol	8	9
225	15-07-13	48	3C	HGSC	2852	Primary	Optimal	NA	6x carbo/taxol; bevacizumab	8	15
226	24-07-13	83	3C	Low grade serous	1964	Primary	Optimal	NA	Tamoxifen	3	9
227	26-07-13	63	3C	HGSC	8430	Primary	Suboptimal	NA	6x carbo/ taxol	16	16
229	11-09-13	65	4	HGSC	6521	Primary	Optimal	NA	2x carbo/taxol, 4 carboplatin	13	14
230	11-09-13	41	3C	HGSC / Clear cell	1743	Primary	Optimal	NA	6x carbo/ taxol	11	14
231	01-10-13	69	3C	HGSC	388	NA	NA	6x carbo/ taxol	NA	11	13
233	02-10-13	69	4	HGSC	8407	NA	NA	6x carbo/ taxol	NA	13	14
234	02-10-13	60	3C	HGSC	1252	IDS	Optimal	4x carbo/ taxol	2x carboplatin	4	13
238	25-10-13	76	3C	HGSC	1317	IDS	Suboptimal	4x carbo/ taxol	2x carbo/ taxol	15	15
239	25-10-13	77	3C	HGSC	4361	NA	NA	NA	NA	1	1
242	13-01-14	65	3C	HGSC	1053	Primary	Open + close	6x carbo/ taxol	NA	9	9
243	03-02-14	57	3C	HGSC	10477	Primary	Optimal	NA	6x carbo/ taxol	40	41
244	04-02-14	62	3C	HGSC	NA	IDS	Optimal	NA	3x carbo/ taxol; 3x carboplatin	24	26
245	10-02-14	69	3C	HGSC	6196	Primary	Suboptimal	NA	6x carboplatin	9	9
246	07-03-14	70	3C	Endometroid adenocarcinoma	62	Primary	Optimal	NA	6x carbo/ taxol	34	71
247	10-03-14	70	3C	HGSC	5936	NA	NA	NA	NA	2	2
249	02-04-14	75	3C	HGSC	702	Primary	Optimal	NA	6x carbo/ taxol	7	7

PCO number	Date of collection	Age (years)	FIGO stage	Histological subtype	CA125 (U/l)	Surgery	Cytoreduction	NACT	Adjuvant Chemo	PFS (months)	OS (months)
250	12-04-14	55	4	HGSC	447	IDS	Complete	4x carbo/ taxol	2x carbo/ taxol	7	7
251	15-05-14	73	4	HGSC	6679	IDS	suboptimal	4x carbo/ taxol	2x carbo/ taxol	7	7
252	19-05-14	46	3C	HGSC	251	Primary	Complete	NA	6x carbo/ taxol	7	7
253	30-05-14	61	3C	HGSC	1878	Primary	Optimal	NA	6x carbo/ taxol	7	7

HGSC: high grade serous cancer, IDS= interval debulking surgery, NACT= neoadjuvant chemotherapy, NA- not applicable, PFS- progression free survival, OS= overall survival.

Appendix 2: List of expression data for examined primary ovarian cultures

PCO number	Histology	AR protein expression (WB)	AR protein expression (IHC)	AR mRNA expression	Rab35 protein expression (ICH)	Rab35 mRNA expression	Increase in Rab35 expression in response to DHT	CYP17 mRNA expression	CYP 17 expression (WB)
32	HGS	(-)	NE	NE	NE	NE	NE	NE	NE
37	HGS	(-)	NE	NE	NE	NE	NE	NE	NE
44	HGS	40	NE	NE	NE	NE	NE	NE	NE
49	HGS	15	NE	NE	NE	NE	NE	NE	NE
67	HGS	(-)	NE	NE	NE	NE	NE	NE	NE
68	HGS	(-)	NE	NE	NE	NE	NE	NE	NE
121 (paired with 243)	HGS	NE	NE	NE	NE	3394	NE	NE	NE
122	HGS	29	NE	NE	NE	2891	NE	23	NE
124	HGS	(-)	NE	NE	NE	NE	NE	NE	NE
138	HGS	NE	NE	62	NE	247	NE	2215	NE
139	Endometroid with clear cell component	(-)	NE	11	NE	104	NE	NE	NE
143	HGS	NE	0	15	12	947	(-)	NE	NE
144	Endometroid with clear cell component	(-)	NE	43	NE	516		3333	NE
157	HGS	(-)	9	NE	11	2	NE	2	NE

PCO number	Histology	AR protein expression (WB)	AR protein expression (IHC)	AR mRNA expression	Rab35 protein expression (ICH)	Rab35 mRNA expression	Increase in Rab35 expression in response to DHT	CYP17 mRNA expression	CYP 17 expression (WB)
162	HGS	NE	4	73	10	762	NE	NE	NE
168	HGS	31	5	24	12	685	NE	NE	NE
170	HGS	(-)	NE	201	NE	765	NE	410	NE
174	Endometroid of the ovary	(-)	NE	38	NE	13190	20%	184	NE
175	Clear cell cancer	NE	NE	33	NE	935	NE	516	NE
176	HGS	18	NE	NE	NE	NE	NE	NE	NE
177 (paired with 222)	HGS	(-)	NE	4	NE	622	negative	6	40
182	HGS	(-)	NE	57	NE	779	NE	421	NE
187	HGS	14	6	3	12	382	50%	6	NE
190	Mucinous adenocarcinoma	(-)	NE	1	NE	486	NE	96	NE
191	HGS	48	NE	17	NE	455	150%	10	53
192	Clear cell/ endometrial	15	NE	21	NE	660	NE	NE	NE
194	Enteric type adenocarcinoma in mature cystic teratoma	(-)	NE	20	NE	620	Negative	169	NE

PCO number	Histology	AR protein expression (WB)	AR protein expression (IHC)	AR mRNA expression	Rab35 protein expression (ICH)	Rab35 mRNA expression	Increase in Rab35 expression in response to DHT	CYP17 mRNA expression	CYP 17 expression (WB)
197	HGS	62	NE	48	NE	877	NE	2	52
209	HGS	(-)	12	39	16	872	20%	29	NE
210	HGS	(-)	12.5	75	14	1147	30%	153	NE
211	HGS	(-)	0	156	11	1163	50%	1052	NE
213	HGS	NE	NE	30	NE	894	(-)	188	24
219 (paired with 234)	HGS	(-)	NE	18	Ne	1056	20%	34	84
221	Carcinosarcoma	(-)	6	NE	8.5	1019	NA	10	NE
222 (paired with 177)	HGS	349	NE	NE	NE	NA	NA	NE	79
224	HGS	4	3	8	11	561	(-)	168	102
225	HGS	16	2	16	6	955	50%	75	40
226	Low grade serous	(-)	2	12	10	435	NA	14	NE
227	HGS	(-)	3	15	7	1202	60%	79	NE
229	HGS	(-)	10	21	14	194	50%	23	NE
230	HGS and clear cell component	(-)	10	32	17	239	150%	318	NE
231	HGS	(-)	NE	40	NE	362	1000%	26	NE
233	HGS	(-)	9	41	11	372	80%	NE	NE

PCO number	Histology	AR protein expression (WB)	AR protein expression (IHC)	AR mRNA expression	Rab35 protein expression (ICH)	Rab35 mRNA expression	Increase in Rab35 expression in response to DHT	CYP17 mRNA expression	CYP 17 expression (WB)
234 (paired with 219)	HGS	(-)	7	86	11	78	(-)	NE	NE
238	HGS	(-)	7	40	14	365	80%	NE	NE
239	HGS	(-)	NE	19	NE	175	(-)	NE	NE
242	HGS	(-)	NE	10	NE	88		NE	NE
243 (paired with 121)	HGS	16	NE	8	NE	73	50%	5	NE
244	HGS	12	NE	NE	NE	24	NA	25	NE
245	HGS	(-)	11	48	16	432	(-)	NE	NE
246	Endometroid adenocarcinoma	P0/1/2 (neg)	NE	53	NE	479	50%	NE	NE
247	HGS	Pos (+)- P2	6	97	14	878	500%	NE	NE
249	HGS	P0/1/2 (1 pos)	0	30	10	274	(-)	NE	NE
250	HGS	(-)	NE	9	NE	84	130%	NE	NE
250	HGS	(-)	NE	9	NE`	84	130%	NE	NE
251	HGS	P0/1/2 (-)	7	41	12	370	(-)	NE	NE
253	HGS	P0/1/2 (-)	11	84	16	765	(-)	NE	NE

Overview of primary cell cultures (PCOs) examined in this study. Histology are listed as determined by the pathologist at the Queen Elizabeth Hospital. HGS= high grade serous cancer. (-)- no expression. WB= western blot, IHC= immunohistochemistry, DHT= dihydrotestosterone. NE- not examined. Values given for protein expression examined by Western blot are given as percentage of protein expression in OVCAR3 cells for both, AR and CYP17 expression. Protein expression values for immunohistochemistry are given as H-score. mRNA expression data are normalised to OVCAR3 gene expression. Increase in DHT expression is given as

Appendix 3: Consent form for patients

NGOC
Northern Gynaecological Oncology Centre

Gateshead Health NHS
NHS Foundation Trust

Cancer Research Involving Tumour Samples
Blood and Patient Information

CONSENT FORM

	Please initial box
1. I have read the attached information sheet on the above project and have been given a copy to keep. I have had the opportunity to ask questions and understand why tumour samples, blood and patient information is being collected.	☐
2. I agree to donate a sample of tumour tissue and blood for research. I understand how the sample will be collected, that giving the sample for this research is voluntary and that I am free to withdraw my approval at any time without giving a reason and without my medical treatment or legal rights being affected.	☐
3. I give permission for certain anonymised medical details, as have been explained to me, to be looked at and information taken from them to be analysed in strict confidence by staff at the Northern Gynaecological Oncology Centre or from organisations supervising the research.	☐
4. I understand that future research using the sample I have given may include genetic research aimed at understanding the genetic influences relating to cancer, but that the results of these investigations are unlikely to have any implications for me personally.	☐
5. I agree that the samples I have given and the information gathered about me can be looked after and stored in the Northern Gynaecological Oncology Centre for use in future projects, as described in the attached information sheet. I understand that some of these projects may be carried out by researchers other than from the NGOC.	☐
6. I understand that all studies will be anonymised, coded and unlinked. This means that no results will be given to me directly of any research carried out on tissue, blood or data that I donate.	☐
7. I understand some research may take part in conjunction with commercial organisations. I will not benefit financially if this research leads to the development of a new treatment or medical test.	☐
8. I know how to contact the research team if I need to.	☐

Name of patient: ..... Signature: ..... Date: .....

Name of doctor: ..... Signature: ..... Date: .....

Thank you for agreeing to participate

White Copy – Patient Copy

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