Investigating the Role of Cortisol and Oxytocin Signalling in Human Ovarian Cancer

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1.0 Abstract

With over 4000 deaths a year and a 10-year survival rate of just 35% it's understandable why patients with ovarian cancer report high levels of distress. Emerging data associate psychological behaviour with prevalence and stress with negative impact on prognosis. Knowing the molecular interactions in ovarian cancer cells involved with better social support could help prevent shorter survival outcomes.

Elevated levels of the stress hormone cortisol (C) have been associated with tumour cell proliferation whilst emerging studies link the social hormone oxytocin (OT) with having a moderating role on stress. We hypothesise that there is a cross-talk between these two hormones at a molecular level. Three ovarian cancer cell lines were used as *in-vitro* models; SKOV3, PEO1 and MDAH-2774, and treated with C at concentrations representative of physiological stress *in vivo* in the presence or absence of OT. In all three cell lines OT reduced cell proliferation and migration, induced apoptosis and autophagy and partially reversed the effects of C providing evidence of cross-talk *in vitro*. OT was shown to drive alternative splicing of the glucocorticoid receptor (GR) in a cell specific manner. Quantitative RT-PCR from ovarian cancer tissues revealed that the glucocorticoid receptor (splice variant GR-P) and oxytocin receptor (OTR) were significantly upregulated compared to normal ovarian tissues. Tissue microarray revealed that the expression of GRα was lower in early stage ovarian cancer tissue compared to late stage.

Data provided in this study explains why social support could be used to help distressed ovarian cancer patients and could potentially be used to produce new therapeutic interventions for socially isolated patients.

Key Words: Ovarian Cancer, Cortisol, Oxytocin, Glucocorticoid Receptor, Oxytocin Receptor

Declaration

I hereby declare that the research presented in this thesis is my own work, except where otherwise specified, and has not been submitted for any other degree.

Amanda Mankarious

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2.0 Introduction

There is growing interest concerning the relationship between psychological pressures and their impact on human health. One of the most researched, yet not fully understood, is the relationship between stress, social support and cancer. For example, Chronic stress accelerates the growth and invasion of pancreatic cancer cells *in-vivo* (Kim-Fuchs *et al.*, 2014) and increases cancer progression of squamous cell Carcinoma in mice (Dhabhar *et al.*, 2012). A study in 2009 linked the relationship between depression and cell-mediated immunity in breast cancer patients and found that women who reported more depressive symptoms showed suppressed immunity, reducing survival time in comparison to other patients (Sephton *et al.*, 2009). A survey carried out on 125 breast cancer patients concluded that a decrease in depression symptoms is associated with longer survival times (Giese-Davis *et al.*, 2011) and in a report based on the 'quality of life' inventory data reported that women who scored higher in social wellbeing had a 48% decreased risk of breast cancer recurrence (Epplein *et al.*, 2011).

It is still not possible to accurately trace stress as a causal link to cancer as there aren't any accurate and reliable methods to distinguish if a stressful event induced cancer in a patient or whether the cancer was induced long before and was only made evident due to the stressful event (Burgess, 1987). Cortisol, epinephrine and norepinephrine are used as physiological biomarkers for stress (Djuric *et al.*, 2008; Walker *et al.*, 2017) as changes in blood plasma concentrations of these hormones have been observed in patients with clinical depression, psychological stress, trauma, surgery and physical exertion (de Weerth, Zijl and Buitelaar, 2003; Lee, Kim and Choi, 2015).

Investigations into the role of social support on stress connected high levels of social support with a decreased stress response (Heinrichs *et al.*, 2003). In a systematic review on 26 published papers which have reported links between social support and cancer prognosis. Their findings suggested strong links in the relationship between breast cancer prognosis and social support however they find that for all other cancers the data is inconclusive due to methodological limitations in measuring social support (Nausheen *et al.*, 2009). The neuropeptide oxytocin is a widely accepted marker for social support (Gimpl and Fahrenholz, 2010; Cardosoa *et al.*, 2013) as it has roles in pair bonding and maternal behavioural patterns

in mothers and in social wellbeing. Baseline oxytocin levels were deemed to be inversely related to cortisol levels and distrust in a sample of 67 female volunteers assessed in the Trier Social Stress Test (TSST) and social support in the form of a close friend attenuated cortisol stress responses (McQuaid *et al.*, 2016). Also in a study with 180 women given either 24 IU of intranasal oxytocin or a placebo, those given intranasal oxytocin had reduced state anxiety levels or lower cortisol levels during the TSST (Reim *et al.*, 2019).

2.1 Ovarian Cancer

2.1.1 Structure and Function of the Human Ovaries

The ovaries are part of the female reproductive system and their primary function is for the production of female gametes known as the oocytes (Williams and Wilkins, 2006). Figure 1 shows the structure of the human ovary and the name of the cells surrounding the developing gamete. A fibrous cord known as the ovarian ligament attaches the ovaries to the wall of the uterus and another called the suspensory ligament attaches the ovaries to the wall of the abdomen (Williams and Wilkins, 2006). The outermost layer of the ovaries is the ovarian surface epithelium (OSE) (Auersperg *et al.*, 2001) and below the OSE is the ovarian cortex where the gamete develops. Flat epithelial cells from the OSE surround the oocyte and become follicular cells. As the primary oocyte develops these follicular cells change from being flat epithelium to cuboidal epithelium known as granulosa cells (Auersperg *et al.*, 2001) which produce and secrete the hormone estrogen (Garzo and Dorrington, 1984) during maturation of the oocyte. The innermost layer of the ovary is the ovarian medulla which only contain capillaries which supply blood and nutrients to the ovaries (Williams and Wilkins, 2006).

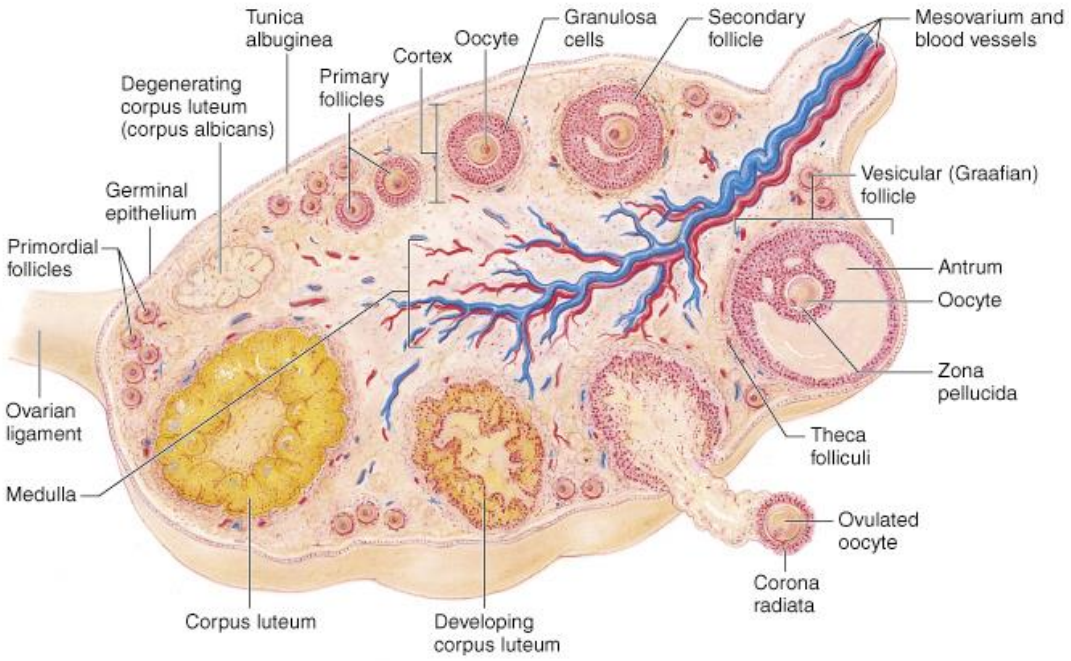

***Figure 1**. Diagrammatic representation of the Human Ovary showing the stages in follicle maturation and ovulation. At day 0 of the menstrual cycle, the primordial follicles develop and mature into one oocyte (egg cell) surrounded by several layers of granulosa cells. By day 14 of the cycle a surge in luteinizing hormone (LH) triggers ovulation where the mature oocyte leaves the raptured follicles and leaves through a hole in the ovarian surface epithelium (OSE). Image taken from [http://apbrwww5.apsu.edu/thompsonj/Anatomy%20&%20Physiology/2020/2020%20Exam%20Reviews/Exam%205/CH27%20Ovarian%20Follicles*

2.1.1.1 Structure and Function of Ovarian Surface Epithelium

The ovarian surface epithelium (OSE) is the mesothelium covering the surface of the human ovaries and has a wide range of hormone dependent functions (Auersperg *et al.*, 2001). The OSE is also referred to as the ovarian mesothelium and normal ovarian epithelium. It is composed of a single layer of cuboidal epithelial cells (figure 2) situated above the basal membrane (Auersperg *et al.*, 2001). The function of the OSE is to facilitate the transport of materials from the peritoneal cavity to the ovaries; however, the OSE also plays a significant role pre and post ovulation. The OSE need to degenerate and be removed from the site of ovulation and does this via an apoptotic mechanism (Ackerman and Murdoch, 1993). Post

ovulation the OSE is required for repair of the ovarian surface by the epithelio-mesenchymal conversion of the OSE into fibroblasts (Auersperg *et al.*, 2001). This increases motility and proliferative responses of the OSE at the site of ovulation and modifies the extra cellular matrix. These fibroblasts then become part of the stroma later in the ovarian cortex (Auersperg *et al.*, 2001). The epithelio-mesenchymal conversion has been demonstrated in culture in the presence of epidermal growth factor (EGF) and ascorbate (Siemens and Auersperg, 1988).

Corticosteroids are known to enhance OSE proliferation *in vitro* and combinations of EGF and hydrocortisone are among the most potent mitogens for cultured OSE (Siemens and Auersperg, 1988). OSE cells express the EGF receptor where EGF not only stimulates proliferation but may also alter differentiation (Siemens and Auersperg, 1988). The high concentration of EGF may be responsible for the rapid proliferation of OSE after ovulation and potentially be the reason for the epithelio-mesenchymal conversion of the OSE *in vivo*. Expression of the EGF receptor has been shown to be higher in ovarian tumours than in normal OSE (Auersperg *et al.*, 2001) but currently there is insignificant data published for the difference in expression of the corticosteroid receptors such as the glucocorticoid receptor between normal and malignant OSE.

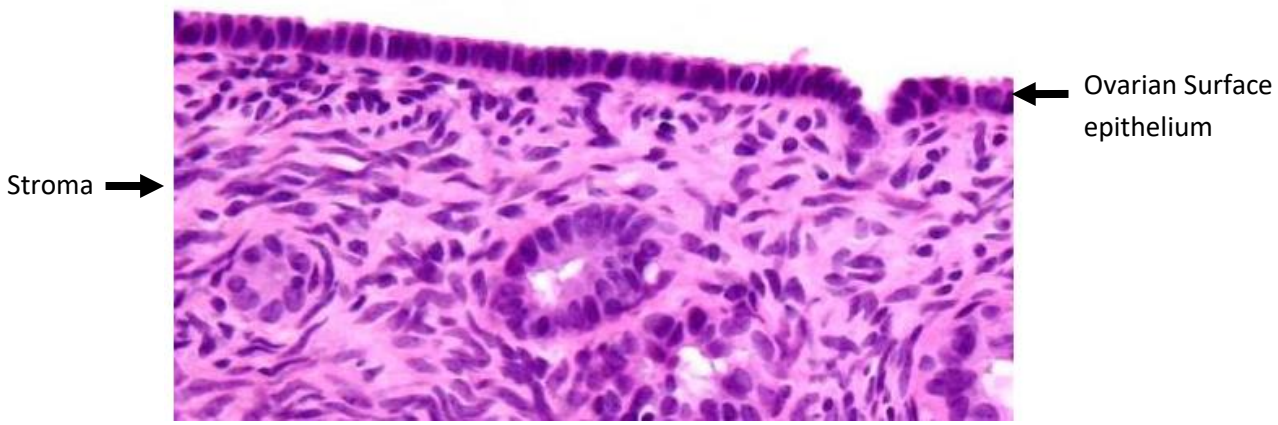

***Figure 2**. Section through a normal adult ovarian cortex, showing OSE on top as a cuboidal monolayer and the stroma underneath. Image taken from [https://secure.health.utas.edu.au/intranet/cds/pathprac/Files/Cases/Female/Case61/Case61.htm]*

Cancer cells derived from the OSE represent approximately 90% of all human ovarian malignant cancers (Auersperg *et al.*, 2001). A hypothesis put forward in 1971 by Fathalla et al suggested that frequent ovulation contributed to an increased risk of ovarian cancer because the repeated rupture and repair of the OSE at the site of ovulation made it more probable for genetic abnormalities to occur (Auersperg *et al.*, 2001)(Godwin *et al.*, 1992; Testa *et al.*, 1994).

In humans the menstrual cycle lasts 28 days with ovulation (release of the mature oocyte) happening on day 14 of the cycle. As the oocyte matures during the follicular phase of the cycle the granulosa cells undergo rapid proliferation and secret hyaluronic acid (Watson, Watson and Miller, 2010; Gupta, 2011) that is required to make up the antrum fluid surrounding the egg (seen in figure 2). Increases in the volume of antrum fluid produce a 'bulge' at the surface of the ovary known as the blister (Watson, Watson and Miller, 2010) which ruptures during ovulation. High levels of oestrogen cause the pituitary gland to release luteinizing hormone (LH) and in the presence of LH the follicular cells secrete proteolytic enzymes which degrade the tissue surrounding the blister forming a hole called the stigma (Gupta, 2011). The developed egg can now move out of the stigma towards the fallopian tubes to make its way to the uterus (Williams and Wilkins, 2006; Watson, Watson and Miller, 2010).

2.1.2 Epidemiology of Ovarian Cancer

Ovarian cancer is the 5th leading cause of cancer deaths in women (Auersperg *et al.*, 2001; Cancer Research UK, 2019), however the progression and etiology of this disease is among the least understood of the major human cancers. Only 5-10% of ovarian cancers cases are attributed to family history (Auersperg *et al.*, 2001) meaning that over 90% of cases are sporadic. This presents a challenge in terms of defining the etiology of the disease and so it remains poorly understood (Holschneider and Berek, 2000; Edmondson and Monaghan, 2001). Ovarian cancer has the greatest diversity of tumour types than any other organ of the body with currently 100 types of ovarian tumours characterized so far (Emmanuel *et al.*, 2011). There are generally three main categories; cancer derived from the surface epithelium of the human ovaries, this accounts for 85% of ovarian cancer cases, cancer derived from

germ cells or oocytes which contributes to 10-15% of cases and cancer derived from the specialized stromal tissue in the ovary which accounts for fewer than 5% of cases (Edmondson and Monaghan, 2001). The epithelial tumours are made up of an array of subtypes including serous (75%-80%), mucinous (10%), endometrioid (10%) and less common types that include; clear cell, Brenner, mixed, and undifferentiated (Holschneider and Berek, 2000; Edmondson and Monaghan, 2001).

2.1.2.1 Ovarian Cancer Statistics

In 2016 the number of women diagnosed with ovarian cancer was over 7500 in the UK alone. In that same year 4227 deaths were as a direct result of having ovarian cancer. Age is a major risk factor of ovarian cancer with women above the age of 50 more likely to develop the disease (Cancer Research UK, 2019) although the incidence rates after the year 2000 are decreasing due to raised awareness and earlier screening.

There is also a significant difference in the incidence rates of ovarian cancer cases in women from economically developed countries compared to the number of cases in economically developing countries. North, Central and Eastern Europe have the highest incidence rate whereas Africa and many countries in Asia have the lowest incidence rates (Cancer Research UK, 2019). It is estimated that in economically developed regions the incidence rate for ovarian cancer is 9 per 100,000 of the population compared to 5 per 100,000 of the population of economically developing regions of the world (Ferlay *et al.*, 2008).

	INCIDENCE (ASR) PER 100,000	MORTALITY (ASR) PER 100,000
LESS DEVELOPED	5.0	3.1
MORE DEVELOPED	9.2	5.0
WORLD AVERAGE	6.1	3.7

***Table 1**. Age standardised incidence and mortality rates (ASR) from 2012 in more and less developed regions of the world against the world average. Table taken from (Reid, 2018)*

Table 1 presents data collected by the World Ovarian Cancer Coalition on the incidence rate of ovarian cancer as an average of economically developed countries to less developed countries. Rates in developed areas exceed 9 per 100,000, and were lowest in SubSaharan Africa with rates below 5 per 100,000 (Reid, 2018). The increase in incidence rate might be due to economically developed countries being more affluent and affluent populations having lower parity (NCIN, 2008). As early as 1997 high parity was established to be a protective factor against ovarian cancer (Banks, Beral and Reeves, 1997). Another contributing factor could be greater social support networks in economically developing countries, however there is currently no quantifiable data to support this.

2.1.2.2 The Cell Cycle

The cell cycle is a highly regulated cycle of events where all the genetic information in a cell is replicated and the cell divides to produce two new genetically identical daughter cells from the one parent cell. The cycle is split into two major stages; interphase and mitosis (Figure 3) which are then further subdivided into several phases. To ensure that cell division is carried out correctly and only takes place when the conditions are suitable, there are several

checkpoints as well as regulators within the cycle that are able to initiate or inhibit division and correct damage to prevent uncontrollable cell growth (Elledge, 1996).

Interphase is made up of the phases; Gap 1 (G_1), DNA Synthesis (S) and Gap 2 (G_2), although cells no longer undergoing cell division enter another gap phase known as Gap 0 (G_0) (McLennan *et al.*, 2005). During G_1 all the biochemical processes and protein synthesis required by the cell takes place at a very fast rate so that all enzymes needed for the S phase are made (Elledge, 1996). DNA replicates during the S phase using semi-conservative replication to form two genetically identical daughter strands from one parent strand. Each new daughter strand remains attached to the other by the centromere ready for cell division. After replication the cell enters another gap phase G_2 where it continues to grow and recheck the replicated strands until mitosis begins (Elledge, 1996; McLennan *et al.*, 2005).

Mitosis is nuclear division where the sister chromatids (the newly made daughter strands) are separated from each other at the centromere. The first phase of mitosis is prophase, where the nuclear envelope disappears and the centromeres make their way round to either end of the cell (McLennan *et al.*, 2005). This is then followed by metaphase where all the chromosomes line up along the equator of the cell and spindle fibres produced from the centrioles attach to the centromere of each chromosome. The spindle fibres then contract during anaphase and pull the sister chromatids towards opposite poles of the cells before the nuclear envelope reappears in telophase around each set of chromosomes. Cytokineses happens immediately after where the cell membrane pinches in to create two new daughter cells (Elledge, 1996; McLennan *et al.*, 2005).

2.1.2.3 Regulation and check points

There are principle check points that occur at the end of G_1 and G_2 essential for regulating the cell cycle. There is a point during G_1 known as the restriction point (R point) (Elledge, 1996; McLennan *et al.*, 2005) where the cell is able to determine if it should enter another cycle or enter G_0 depending on the presence of mitogens (Elledge, 1996). After the R point there is also an intra-S-phase check point, a G_2 phase check point and a metaphase check point. In order for the cycle to progress to the S phase from the G_1 phase, activation of the factor E2F is required (McLennan *et al.*, 2005). E2F is inhibited by the retinoblastoma tumour suppressor

protein (Rb) which upon phosphorylation by cyclin dependant kinases (CDK's) moves away from E2F enabling the E2F protein to initiate transcription of the genes required for DNA synthesis (Elledge, 1996). It additionally initiates transcription of CDK's needed for the next phases of the cell cycle (Elledge, 1996). Inhibitor proteins called CDK inhibitor proteins (CIP) also exist to repress the activity of CDK's causing the cell to enter the G_0 phase (McLennan *et al.*, 2005). Tumor suppressor genes such as p53 induce transcription of CIP's to prevent cell cycle progression (Elledge, 1996; McLennan *et al.*, 2005).

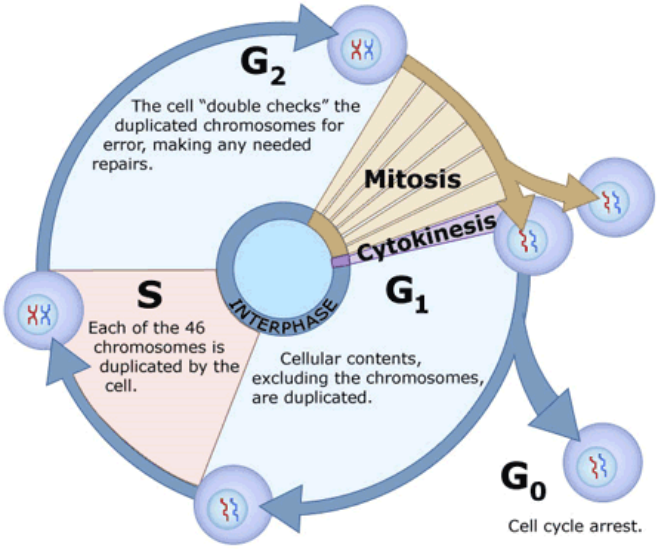

***Figure 3**. A diagrammatic representation of the stages of the cell cycle showing. Interphase consists of Growth 1 (G1) stage, where all cellular contents are duplicated, the Synthesis (S) stage where semi-conservative replication of all the chromosomes takes place, and Growth 2 (G2) where the cell proof reads the copied chromosomes and repairs any errors, before undergoing nuclear division by mitosis and followed by cytokinesis to create two genetically identical daughter cells. Cell leaving the cycle enter cell cycle arrest (G0) (McLennan, et al., 2005)*

2.1.2.4 The cell cycle and ovarian cancer

Oncogenes are mutated forms of proto-oncogenes, which have regulatory functions within the cell cycle. Alterations can be point mutations which alter protein function, or over expression which means the cell is exposed to mitogens constantly and cell division is constantly switched on.

Disruption of oncogenes and tumour suppressor genes can also be caused by epigenetic mechanisms (Chen, Hardy and Tollefsbol, 2011) such as hypermethylation and histone modifications. The biochemical process of DNA methylation involves the addition of a methyl group to the C^5 of the cytosine nucleotides of a gene which stops the transcription and reduces expression of the gene. Hypermethylation is associated with gene silencing and the hypermethylation of tumour suppressor genes is implicated in the progression of cancer. It has been found that the promoter hypermethylation of the breast cancer type 1 (*BRCA1*) gene leads to the silencing of this gene and is the cause for sporadic cases of ovarian cancer (Baldwin *et al.*, 2000). Modifications on the N-terminal tails of core histones include acetylation, methylation and phosphorylation (Chen, Hardy and Tollefsbol, 2011). Histone acetylation makes the chromatin easily accessible to transcription, whereas histone deacetylation prevents transcription (Turner, 2000). Studies have shown that hypoacetlylation of core histones 3 and 4 (H3, H4) causes the gene silencing of transcription factors GATA4 and GATA6, subsequently leading to the loss of tumour suppressor Disabled-2 gene. This is the proposed mechanism for dedifferentiation in ovarian carcinogenesis (Caslini *et al.*, 2006) .

2.1.3 Stages of Ovarian Cancer

The International Federation of Gynaecology and Obstetrics (FIGO) set out four stages of ovarian cancer diagnosis depending on how far the cancer has progressed in a patient when they were first diagnosed. Ovarian Cancer has four stages, the first three stages are subdivided into three categories, a, b and c (Helm, 2011). Both stage I and II are considered early stages in the disease prognosis and patients have a higher survival rate (93% and 70% respectively) if the cancer is diagnosed early on. As stage III and IV are considered the later stages of the disease, diagnosis at this stage has a significantly lower survival rate (37% and 25% respectively) than stage I and II (Holschneider and Berek, 2000). Table 2 is a summary of

the spread of the cancer in each stage and the course of treatment set out by FIGO (Katopodis *et al.*, 2019).

2.1.4 Diagnosis of Ovarian Cancer

The majority of patients diagnosed with ovarian cancer are at stages III or IV (Holschneider and Berek, 2000) of the disease and this is largely due to the fact that ovarian cancer is asymptomatic. As the diseases progresses and cancerous growth metastasises outside the ovaries patients may report general/non-specific symptoms such as constipation, bloating, abdominal pain, irregular periods and pain during sex; all of which are similar to symptoms presented to women with polycystic ovaries, or pre and post menopause or women suffering from other illnesses associated with the abdomen (Cancer Research UK, 2019). By stages III and IV the symptoms may include sickness, tiredness, constipation, a significant swelling of the abdomen and shortness in breath, all of which lack specificity (Edmondson and Monaghan, 2001). It has therefore become essential that efficient screening programmes which are specific to ovarian cancer be developed as the late diagnosis dramatically reduces the 5 year survival rates from 67% at stage I to 12% at stage IV (Holschneider and Berek, 2000). Currently tests include checking for abdominal swelling, a transvaginal ultrasound to check for the presence of cysts on the surface of the ovaries, and a blood test to detect the elevated levels of protein CA-125 (Holschneider and Berek, 2000; Edmondson and Monaghan, 2001).

Stage	Sub stage	Cancer spread and description	Treatment
I	Ia	Tumour present on one of the ovaries	Surgery to remove the tumour
	Ib	Tumour present on both the ovaries	
	Ic	Presence of a small tumour focused on the ovarian epithelial surface and the possible presence of cancer cells in ascites	Surgery to remove the tumour followed by chemotherapy
II III	IIa	Metastasis outside the ovaries on the fallopian tubes or in the uterus	Surgery to remove the tumour as much as possible followed by chemotherapy Surgery as extensive as necessarily possible, followed by Chemotherapy
	IIb	Metastasis to pelvic regions such as the bladder	
	IIIa	Metastasis to retroperitoneal lymph nodes, malignancy found in the peritoneum and/or the omentum	
III	IIIb	Tumors or implants on the ovaries that are around 2 cm wide	Surgery as extensive as necessarily possible, followed by Chemotherapy Surgery as extensive as necessarily possible then chemotherapy and radiotherapy to follow
	IIIc	Tumors of implants that have a diameter greater then 2cm wide found in the pelvic region and on the lymph nodes	
IV	IVa	Cancer has metastasized and there is growth in lung tissue and in the liver	
	IVb	Cancer has metastasized to distant sites including parenchymal liver and spleen	

Table 2. *Description of each stage in OvC diagnosis and the course of treatment recommended, summarised from The International Federation of Gynaecology and Obstetrics (FIG0) guidelines for Ovarian Cancer Stages. Adapted from (Katopodis et al., 2019)*

Cancer antigen-125 (CA-125) is a glycoprotein encoded for by the gene *mucin16* (Osman *et al.*, 2008). The role of this protein is to create a hydrophilic environment on the membrane of epithelial cells which acts as a lubricating barrier against foreign particles and infections (Göcze and Vahrson, 1993). Approximately 80% of ovarian cancer sufferers have elevated levels of CA-125 in their blood which is why it is currently the most frequent biomarker used for the detection of ovarian cancer (Auersperg *et al.*, 2001; Edmondson and Monaghan, 2001). CA-125 is also used to monitor how well a patient is responding to treatment, with lowering levels of the protein correlating to successful treatments (Göcze and Vahrson, 1993). However, there are limitations to the use of CA-125 in ovarian cancer diagnosis. CA-125 levels in pre-menopausal women can fluctuate during their normal menstrual cycles leading to false-positive results (Göcze and Vahrson, 1993) or in early stage ovarian cancer the test can give false-negative results meaning patients are not diagnosed until too late (Göcze and Vahrson, 1993; Osman *et al.*, 2008). Another major limitation is that the use of elevated CA-125 as a biomarker is not specific to only ovarian cancer, but to endometrial cancer, fallopian tube cancer, breast cancer and lung cancer as well as in other gynaecological diseases such as endometriosis (Bast *et al.*, 1998). If 80% of ovarian cancer patients show elevated levels of CA-125, then there remains a population of 20% who have ovarian cancer and do not express elevated levels, highlighting the importance of developing new, sensitive and specific screening for ovarian cancer since the biomarker CA-125 is not sensitive enough (Osman *et al.*, 2008).

2.1.5 Causes and Risks of Ovarian Cancer

Family history of ovarian cancer is the most significant risk factor (Holschneider and Berek, 2000). The majority of cases of inherited ovarian cancer are due to mutations on the breast cancer type 1 susceptibility protein coded for by the *BRCA1* gene (around 70% of inherited cases) and the breast cancer type 2 susceptibility protein coded for by the *BRCA2* gene. The risk of developing the disease however can differ depending on the age of the patient at diagnosis and if they have a first or second degree relative with ovarian cancer (Holschneider and Berek, 2000). Reproductive factors, pharmacological agents, environmental and dietary factors have all been associated with an increase in the risk of developing ovarian cancer (Emmanuel *et al.*, 2011). Pooled literature on all potential risk factors of ovarian cancer

concluded that increasing age, having a family history of ovarian cancer and mutations on the BRCA1 and BRCA2 genes were all established risk conferring factors. Established protective factors from ovarian cancer include oral contraceptive usage, hysterectomy, increasing parity and having an oophorectomy. Little to no evidence has yet been established that links controversial theories such as menopausal age, hormonal replacement therapy (HRT), fertility drugs, breast feeding, diet, childhood viruses, talc powder usage and socio-economic status as risk factors. (Banks, Beral and Reeves, 1997; Reid, Permuth and Sellers, 2017).

2.2 The Physiology of Stress

2.2.1 Biological Stress

The term stress is used to describe an emotional and biological response to a threatening situation that enables an organism to adapt (Riley, 1981; McEwen, 2007). Stressors can be any physical, chemical, psychological and physiological stimuli which induce a physiological change in the organism (Everly and Lating, 2013). The stress response is controlled and regulated by both the nervous and the endocrine systems. In acute or short term stress response the hypothalamus stimulates the adrenal medulla to secret the hormones epinephrine and norepinephrine which bring about physiological changes such as increasing blood pressure, breathing rate, metabolic rate and ensuring more blood is supplied to the heart and brain in preparation for 'fight or flight' (Belk and Borden, 2009; Everly and Lating, 2013).

When the body is constantly exposed to stressful events such as disease (chronic stress) the hypothalamus responds by stimulating the adrenal cortex to secrete mineralocorticoids and glucocorticoids which have long term effects on the body such as suppressing the immune system, breaking down fats and proteins for energy and increasing blood pressure and volume (Belk and Borden, 2009). Figure 4 summarises the effects of both short and long term exposure to stress.

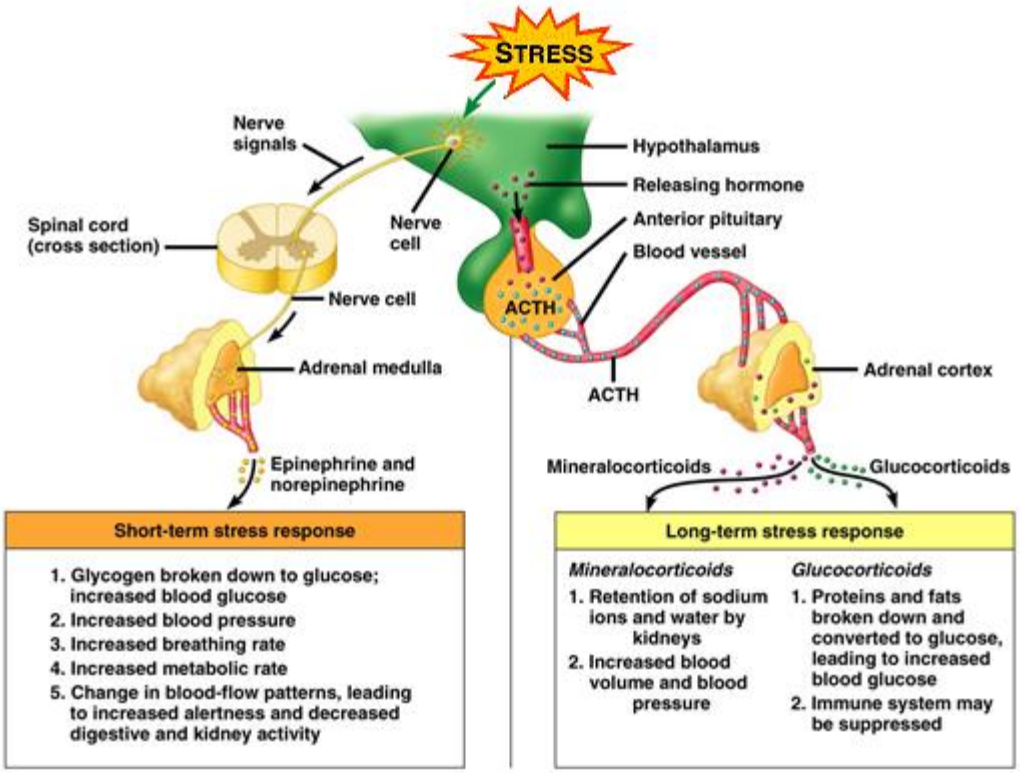

Figure 4. *Diagram illustrating how the adrenal glands respond to stress. Stressors effecting the Adrenal medulla via the nervous system, produce short term response to stress by breaking down glycogen to glucose and increasing metabolic rate, breathing rate and blood pressure to prepare the body for fight or flight. (Barot et al., 2005).*

2.2.2 Glucocorticoids and the Glucocorticoid Receptor

2.2.2.1 Steroid Hormones

Steroids are a group of organic compounds which contain 17 carbon atoms which are arranged in four rings (King and Mainwaring, 1974). Hormones which have this arrangement are called steroid hormones and are all derived from the molecule cholesterol (Evans, 1988). Only three glands in the body produce steroid homones; the adrenal cortex, the testes and the ovaries, their synthetic pathways are represented in figure 6 (Payne and Hales, 2004). The adrenal cortex produces glucocorticoids (such as cortisol) for regulation in metabolic pathways, and mineralocorticoids for water and mineral retention (figure 4) (Evans, 1988; Belk and Borden, 2009).

2.2.2.2 Cortisol Production

Cortisol, a glucocorticoid, is a steroid hormone that binds to the glucocorticoid receptor (GR) in cells. Cortisol has several functions including the release of glucose by breaking down proteins to increase blood sugar levels and fat metabolism. The release of cortisol is achieved via activation of the hypothalamic-pituitary-adrenal axis (HPA axis) (Everly and Lating, 2013). In response to stress the hypothalamus releases corticotrophin releasing hormone (CRH). CRH binds to receptors on the anterior pituitary gland causing the synthesis and secretion of adenocorticotrophic hormone (ACTH). ACTH is transported via the blood and binds to GPCR on the adrenal cortex where it increases the availability of cholesterol substrate via activation of StAR for the production of cortisol. The process is regulated by a negative feedback mechanism, where cortisol inhibits the production of ACTH and CRH from the pituitary gland and the hypothalamus respectively as summarised in figure 5 (Belk and Borden, 2009; Everly and Lating, 2013).

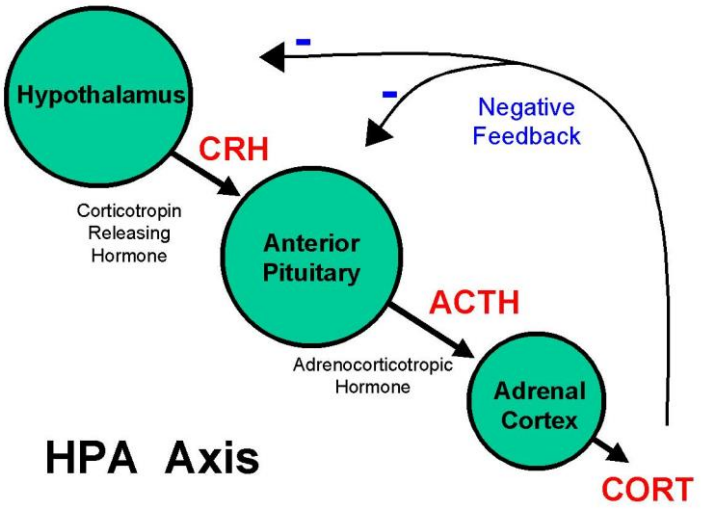

Figure 5*. A diagrammatic representation of the HPA axis showing negative feedback control by cortisol on the hypothalamus and the pituitary gland. Cortisol has inhibitory effects on the production of both adenocorticotropic hormone (ACTH) and corticotrophin releasing hormone (CRH), preventing excess cortisol production in the response to stress (Everly & Lating, 2013).*

ACTH binds to receptors present on the cell membrane of cells in the adrenal cortex and upon binding they activate low-density lipoprotein receptors to take up cholesterol (Margioris and Tsatsanis, 2011). The cholesterol is cleaved and converted into pregnenolone by the enzyme cytochrome P450scc. Pregnenolone is subsequently converted to 17-OH-pregnenolone which makes its way to the endoplasmic reticulum where it is further converted to 11-deoxycortisol. Finally 11-deoxycortisol is taken up by the mitochondria where it is converted to cortisol and immediately secreted out of the cell (Margioris and Tsatsanis, 2011).

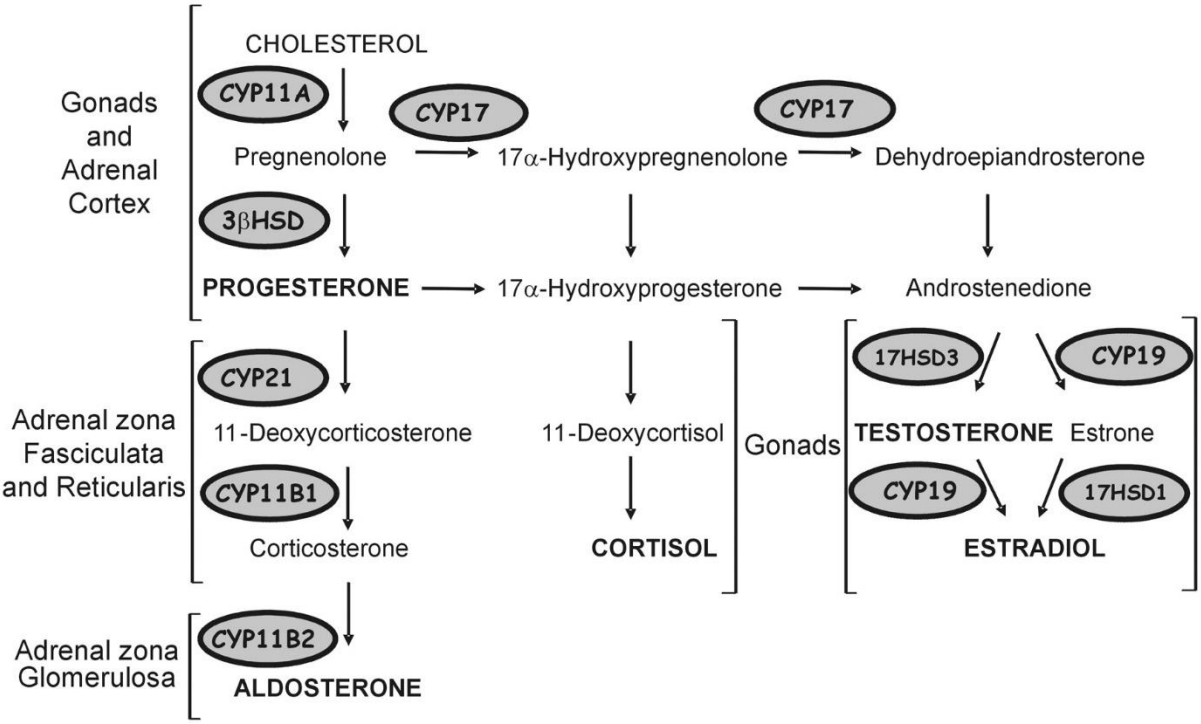

***Figure 6**. Chemical pathways in the biosynthesis of steroid hormones from cholesterol. Image taken from (Payne and Hales, 2004). Final steroid hormone is written in lock capital letters with intermediate molecules in the path way in lower case. Enzymes involved are shown in the grey ovals. Cholesterol in the adrenal cortex is converted to pregnenolone in the presence of the enzyme CYP11A (aka cytochrome P450scc) Pregnenolone is subsequently converted to 17α-Hydroxypregnenolone (17-OH-pregnenolone), then into 11-deoxycortisol in the Adrenal Zona Fasciculata and Zona Reticularis before making cortisol. The image also shows production of progesterone, aldosterone and estrasdiol from cholesterol and the tissues in which they are made.*

2.2.2.3 Steroid Hormone Receptors

Steroid receptors are all ligand-inducible transcription factors; upon binding of a ligand they can control specific target gene transcription (Heitzer *et al.*, 2007). Steroid receptors share a common molecular structure which consists of three functional domains. The first is a variable amino terminal domain (NTD). This domain is also known as activation-factor-1 (AF-1) and it can function as a transcription regulator independent of ligand binding. The second domain is located at the centre of the protein and consists of 8 cysteine residues located around two zinc atoms known as the zinc fingers which make up the DNA binding domain (DBD) (Yudt and Cidlowski, 2002). The 3D configuration of the zinc fingers allows the receptor to bind to the DNA molecule. The third domain is the ligand-binding domain (LBD) at the carboxylic terminal of the protein and is the site where the hormone binds to the receptor. In the absence of a ligand, the LBD can function in other protein binding interactions or in the folding of the receptor, preventing it from binding to DNA (Yudt and Cidlowski, 2002; Heitzer *et al.*, 2007).

2.2.2.4 The Structure of the Glucocorticoid Receptor

The glucocorticoid receptor (GR) is coded for by a single gene; the *NR3C1* gene located on chromosome 5q31-q32 (Yudt and Cidlowski, 2002). The GR gene consists of 8 protein coding exons (numbered 2-9) and a first exon which remains untranslated (Turner *et al.*, 2010). The *N3C1* gene has three different promoter regions (1A, 1B and 1C) and the human GR gene has been found to have at least five, potentially more, different exon 1 sequences. These multiple promoter regions and multiple exon 1 sequences give rise to several mRNA scripts from the one gene (Yudt and Cidlowski, 2002). There are four protein splice variants of the GR; GR-α, GR-β, GR-γ and GR-P. GR-α is the conventional, ligand binding receptor whereas GR-β has a dominant negative action on GR-α (McMaster and Ray, 2007). The α and β variants differ in the C-terminal exon splicing with the translation of exon 9α for GR-α and exon 9β for GR-β as shown in figure 7. GR-γ has the same structure as GR-α except for the presence of an extra amino acid; arginine, in the DBD (McMaster and Ray, 2007). The splice variant GR-P lacks exons 8 and 9, and mRNA levels of this splice variant is abundant in all cells (Yudt and Cidlowski, 2002; Turner *et al.*, 2010).

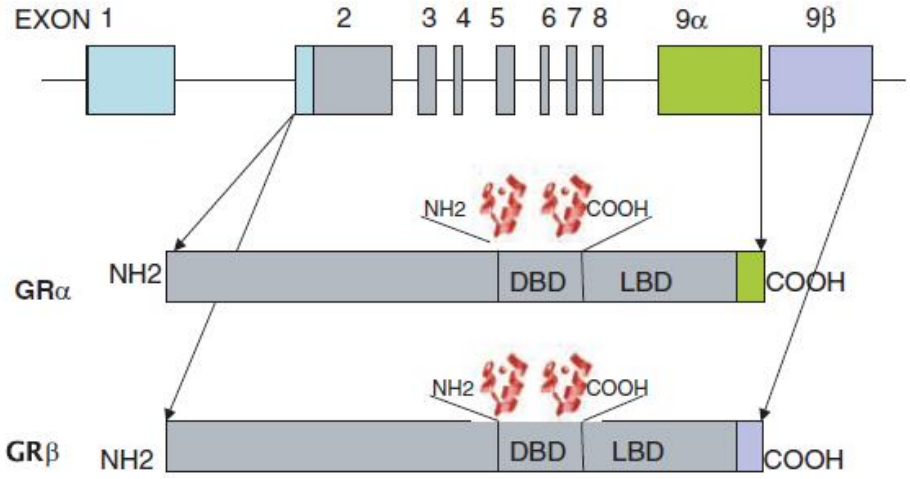

***Figure 7**. Structure of the two main splice variants; GR α and β. Exon 2-8 are translated in both variants and they both have an identical ligand binding domains (LBD) and DNA binding domain (DBD), however they have different isoforms of exon 9. (McMaster and Ray, 2007)*

2.2.2.5 GR splice variants in disease

Lange (2010) studied the expression of the GR splice variants (α, β and P) in a number of haematological malignancies. In all tumours studied, mRNA expression of the GR-β variant was extremely low in comparison to GR-α, however in all tumours, there was a considerable amount of GR-P mRNA being expressed. They also found that in several types of myeloma cell lines GR-P increased the activity of GR-α suggesting that the ratio of GR-P:GR-α and the ratio of GR-P:GR-β affects the GR responsiveness to glucocorticoids (Lange, Segeren and Koper, 2010).

There was also an increase in GR-β mRNA expression in patients with Cushing's syndrome (hypercortisolism) suggesting that GR-β is produced to compensate the over exposure of the cells to cortisol. Conversely patients with hypocortisolism had much greater GR-α mRNA expression and an increase in the number of receptors within the mononuclear leukocytes (Hagendorf *et al.*, 2005).

2.2.3 GR signalling

Before hormone binding, the GR is kept in the cytoplasm by the binding of a heat shock protein (HSP90) at the LBD, a molecule of p23 bound to the HSP90 to stabilize the complex, and a variety of different tetratricopeptide repeat (TPR) proteins that bind to the HSP90. however their function in GR regulation is still unknown (Heitzer *et al.*, 2007). Binding of cortisol to the GR activates the receptor and causes the complex (containing the HSP90, TPR and p23) to dissociate (figure 8). The GR-α is now able to form a dimmer by binding with another GR-α receptor-ligand complex. There are two nuclear import signals within the DBD and LBD of the GR which are the recognition sites for the nuclear import receptors, allowing the GR dimmer to translocate into the nucleus (Heitzer *et al.*, 2007; Turner *et al.*, 2010). Inside the nucleus the activated GR-α molecules bind to glucocorticoid response elements (GRE's) on the promoter regions of target genes and recruit coactivators to the DNA chromatin. The assembly of the coactivator macromolecules cause the target chromatin to become re-modelled, changing its shape to allow the binding of other activators and proteins. This leads to the recruitment of RNA polymerase II to begin gene transcription (Yudt and Cidlowski, 2002; Heitzer *et al.*, 2007; Turner *et al.*, 2010). There is also present negative GRE's (nGRE's) which function to repress transcription (Turner *et al.*, 2010). Interaction of the activated GR-α homodimer with nuclear transcription factors such as activator protein 1 (AP1) and nuclear factor *k* B (NF *k*B) can also repress transcription by stopping histone acetylation activity and so stopping transcription in the absence of the nGRE's (Turner *et al.*, 2010).

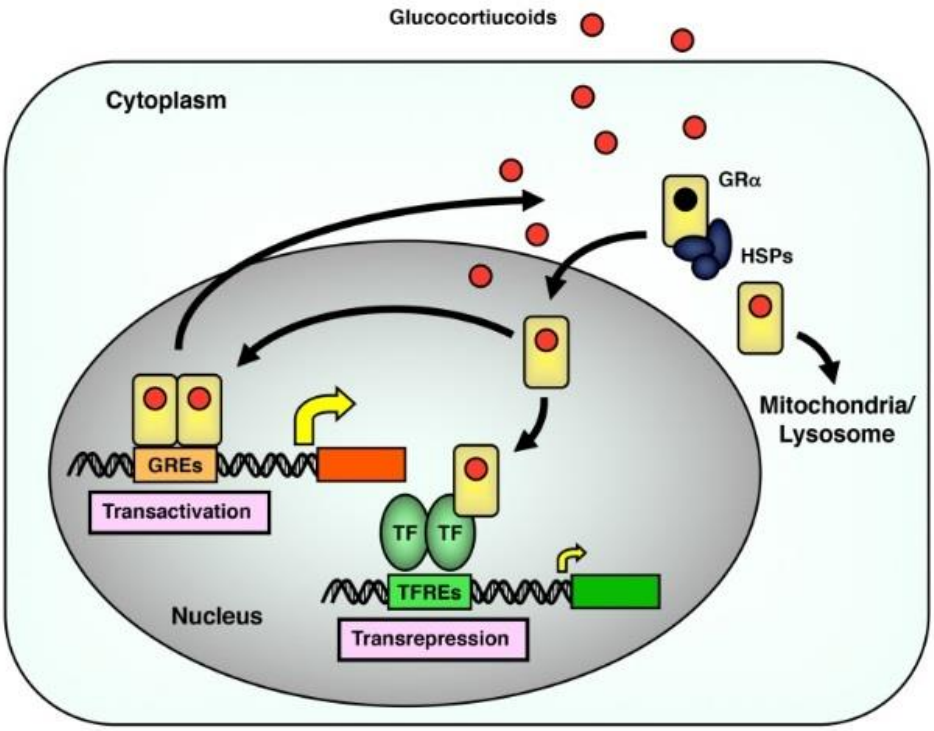

***Figure 8**. The binding of cortisol to a GR causes cytoplasmic activation of the glucocorticoid receptor (GR) and it drops the protein complexes. The active GR monomer joins with another GR monomer and the resulting dimmer moves into the nucleus and binds to the glucocorticoid response element (GRE) where it regulates transcription by allowing the binding of RNA polymerase II (Kino, 2017)*

2.2.4 Stress and Cancer

Riley (1981) carried out a seminal study on the effect of stress and the role of stress hormones on the growth and prognosis of cancers. Lymphosarcoma was transplanted into two groups of female mice and one group was subjected to stress whilst the other was the control. The volume of the tumour was measured and compared to tumour volume of control mice, the data collected is shown in figure 8. The mice exposed to stress have a rapid increase in tumour volume compared to control mice (Riley, 1981).

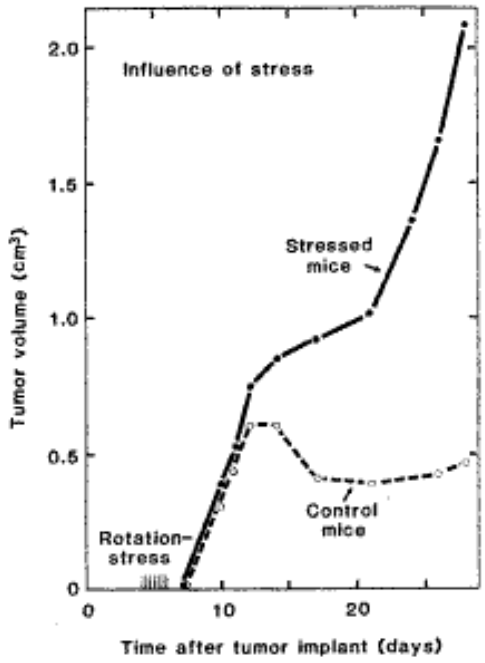

Figure 9*. The influence of Stress on Lymphosarcoma volume in female mice. Stress was induced by rotation at 45 rev/min for 10 minutes of every hour on days 4, 5 and 6 after tumour implantation. Tumours in mice exposed to stress significantly increases in volume compared to tumours in mice not exposed to the stress (Riley, 1981).*

To ensure that the rapid tumour growth in stressed mice was due to corticoids, Riley (1981) inoculated mice with the Moloney Sarcoma Virus (MSV) and implanted a pellet into their hip that released corticosterone slowly into the blood plasma. As before, the size of the tumor was measured every day and compared to tumour growth in control mice who were also inoculated with MSV but without the corticosterone pellet (Riley, 1981). The results showed a dramatic increase in tumour growth for mice with the corticosterone pellet. Another study by Arranz (2010) looked at the impact of CRH on breast cancer cell lines *in vivo*. CRH treated 4T1 breast cancer cells increased expression of the proliferative genes SMAD2 and β-catenin. The tumour grew in mice subjected to chronic stress, whereas mice given a CRH antagonist suppressed tumour growth (Arranz *et al.*, 2010).

2.2.4.1 Stress and Ovarian Cancer

In 2003 Lutgendorf, observed that there were lower levels of vascular endothelial growth factor (VEGF) in ovarian cancer patients with greater social support and that VEGF levels were higher in patients that were distressed. VEGF promotes the production of new blood vessels around a cancer to supply the tumour with oxygen (Lutgendorf *et al.*, 2003a). In the study, they also studied the effects of the stress hormones norepinephrine, epinephrine and cortisol on the VEGF in ovarian cancer cell lines. Their findings indicated that Cortisol was stimulated VEGF production in some ovarian cancer cell lines. This study was one of the first to suggest stress hormones are a contributing factor in ovarian cancer prognosis (Lutgendorf *et al.*, 2003a). Thaker and Sood (2008) demonstrated that chronic stress elevated norepinephrine and cortisol levels which enhanced the pathogenesis of ovarian cancer *in vivo*, increased tumour weight and increased invasiveness of the cancer (Thaker and Sood, 2008).

2.3 Structure and Function of the Oxytocin Receptor System

2.3.1 The structure of Oxytocin

Oxytocin (OT) is a small protein consisting of nine amino acids and a molecular mass of 1007 daltons (Gimpl and Fahrenholz, 2010). It is a highly abundant neuropeptide expressed in almost every cell of the body. OT is coded for by the human gene *OT-neurophysin I* which is found on chromosome 20 p13. When translated, the gene makes the OT-Neurophysin protein dimmer which is the initial molecule in the OT synthesis pathway (Gimpl and Fahrenholz, 2010). The molecule is made and stored in the paraventricular nucleus (PVN) of the hypothalamus and is then transported to the neurosecretory granules in the posterior pituitary gland. Electrostatic interactions and hydrogen bonding between the neurophysin and the OT enable the two to exist as a dimmer in the neurosecretory granules in the pituitary gland which is essential for the packaging and the storage of the OT. The dimmer is sensitive to pH changes so it remains stable in the neurosecretory granules which has a pH of 5.5 but will readily dissociated once released into blood plasma as the pH is less acidic (Gimpl and Fahrenholz, 2010).

2.3.2 The Function of Oxytocin

When OT is released from the neurosecretory granules in the hypothalamus it binds to the Oxytocin Receptor (OTR); a G-protein coupled receptor (GPCR). Extensive research on the role and function of OT in mammals has revealed that OT has a role in sexual arousal, pair bonding in mammals and maternal behavioural patterns in mothers towards offspring (Kosfeld *et al.*, 2005; Gimpl and Fahrenholz, 2010). The hormone is also vital for muscle contraction of the uterus during labour and for breastfeeding (Kosfeld *et al.*, 2005; Lowrie and Goodger, 2009). During labour, estrogen makes the myometrium more sensitive to OT. OT released from the pituitary gland causes myometrial contractions, causing the fetus to apply pressure onto the cervix. As the cervix stretches, impulses are sent to the hypothalamus which in turn causes the pituitary gland to produce more OT. This process is known as positive feedback (Kosfeld *et al.*, 2005; Lowrie and Goodger, 2009; Gimpl and Fahrenholz, 2010). During breastfeeding the suckling action of the baby on the tissue around the nipples stimulates receptors to send nerve impulses to the hypothalamus to enable the release of OT. OT causes the muscle wall around the milk ducts to contract releasing milk into the baby's mouth (Lowrie and Goodger, 2009).

2.3.2.1 The role of Oxytocin on the Stress Response

It has been documented in several reports that OT has the ability to decrease stress response in mammals (Nishioka *et al.*, 1998). Nishoika et al (1998) exposed male rats to stress using a shaker and measured the OT levels in the PVN and in blood plasma. They found there was a significant increase in OT levels both in the blood and in the PVN in response to stress. In 2003, Heinrichs carried out a double-blind experiment to study the effect of OT on cortisol levels in participants exposed to stress. Healthy males were given intranasal OT or a placebo before being exposed to the Trier Social Stress Test and salivary levels of cortisol was measured. They found that there was a significant decrease in cortisol levels compared to the group taking the placebo (Heinrichs *et al.*, 2003). Both studies indicate that OT may play a role in stress response.

2.3.2.2 GPCR signalling

The GPCRs are from a family of proteins that all share four common elements. The receptor has seven transmembrane domains, and near it on the cytoplasmic side there is a hetro-trimeric G protein complex that acts as a switch upon activation by the GPCR. Near the receptor there is a membrane bound effector protein and there is a mechanism in place for feedback regulation of the signalling pathway (Dorsam and Gutkind, 2007). The signal pathway is initiated by the binding of the primary messenger to the membrane bound receptor causing a conformational change in the structure of the receptor. There is a G-protein complex that is made up of three subunits; α, β and γ. The α subunit has a GDP molecule bound on its surface. Conformational changes in the receptor cause the G-protein complex to bind to the C-terminal of the receptor and in doing so the α subunit lets go of the GDP molecule exposing a site for GTP to bind instead. The binding of the GTP causes the α-GTP complex to dissociate from the still bound β and γ counterparts. The α-GTP complex then binds to the nearby effector protein (usually an enzyme) and the now activated enzyme catalyses intermediates found in the cytoplasm into secondary messengers that will be used in the signalling pathways (Dorsam and Gutkind, 2007; Cotton and Claing, 2009; Gimpl and Fahrenholz, 2010). The GTP is hydrolysed back into GDP and the α-GDP complex leaves the effector protein to re-join the βγ G-protein complex back in the resting position. (Cotton and Claing, 2009; Gimpl and Fahrenholz, 2010). Figure 10 is a diagrammatic representation of this mechanism.

There are 4 major families of G proteins; G_s, $G_{i/10}$, $G_{q/11}$, and $G_{12/13}$. The OTR mainly couples to G_q however it has also been found to couple to G_i (Cotton & Claing, 2009). Upon activation of the receptor, the G_q-α subunit of the trimeric G_q protein attaches to a membrane bound enzyme called Phospholipase C (PLC). The enzyme hydrolyses the membrane bound molecule phosphoinositol 4, 5 bisphosphate (PIP2) into two intracellular messengers; 1, 2-diacylglycerol (DAG) and inositol 1, 4, 5 triphosphate (IP_3) (Dorsam and Gutkind, 2007; Cotton and Claing, 2009). IP_3 production results in Ca^{2+} ions moving out of the sarcoplasm and into the cytosol and binding to calmodulin to form calcium-calmodulin complexes which activate the enzyme myosin light chain kinase (MLCK) needed for muscle contraction. DAG remains membrane bound but it promotes the translocation of protein kinase C (PKC) from the

cytoplasm to the membrane where it is activated. Once activated the PKC phosphorylates intermediate proteins belonging to the MAPK signalling (Dorsam and Gutkind, 2007). This activates various gene transcription factors (TF's) that are involved in promoting cell growth (Gimpl and Fahrenholz, 2010).

If the G_i trimeric protein is activated it inhibits the activity of the enzyme adenylyl cyclase, preventing the production of cyclic AMP. This decreases the activity of the cyclic dependant protein kinases which in turn reduces glucose production from the breakdown of glycogen. It is thought that in the presence of OT, if G_i couples with the OTR instead of G_q, cell growth is inhibited and cell migration of cancerous cells are significantly reduced (Dorsam and Gutkind, 2007).

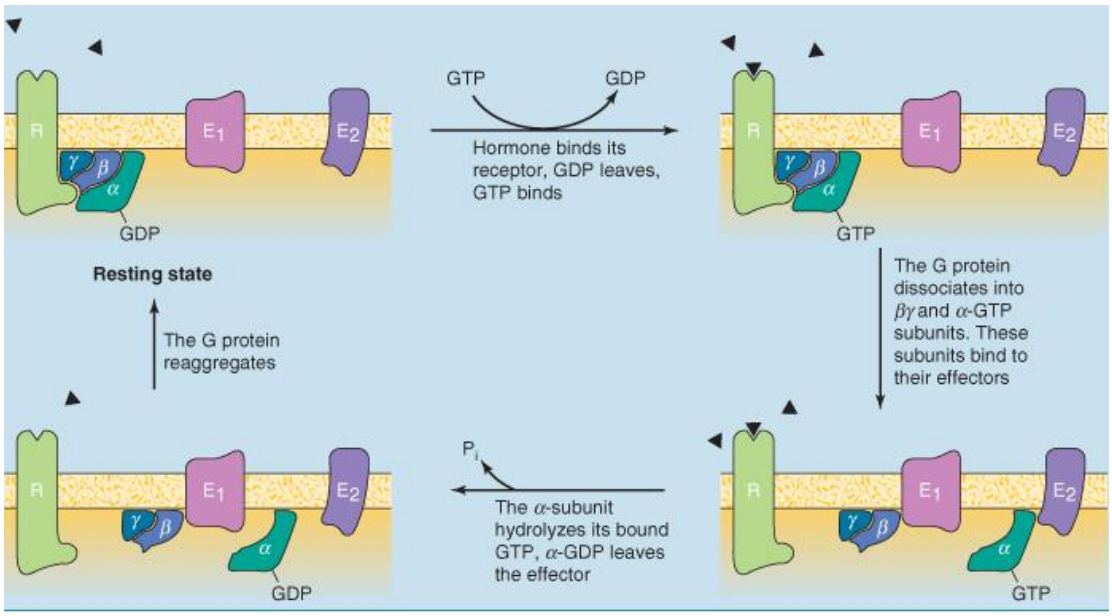

***Figure 10**. Coupling of a hormone receptor (R) to effector proteins (E1, E2) in the plasma membrane through a G protein. By an allosteric mechanism, the activation of the receptor causes GDP-GTP exchange and the dissociation of the heterotrimeric G protein into βγ and α-GTP subunits. These subunits act allosterically on the effectors. The action on the effector is terminated when the α subunit hydrolyzes its bound GTP. The most important effectors of hormone-regulated G proteins are second messenger-synthesizing enzymes such as adenylate cyclase and phospholipase C, but some calcium and potassium channels also are regulated by this mechanism. (Meisenberg and Simmons, 2012)*

2.3.2.3 Oxytocin and Cancer

Reversi (2005) found that in human myometrial cells, OTR's coupled to G_q stimulated cell proliferation, whereas OTR's coupled to G_i inhibited cell proliferation (Reversi *et al.*, 2005). Using a drug called atosiban (which acts as an antagonist to the OT) they were able to inhibit cell growth in kidney cells that were transfected with the OTR (Reversi *et al.*, 2005; Reversi, Cassoni and Chini, 2006). Past studies have tried to outline the effects of OT and the OTR on the proliferation of cancer cells. In 2006 Reversi compiled together all studies associated with OTR signalling and breast cancer cell growth. The report highlighted the diverse and quite contradictive roles of OT as studies by Bussolati and Cassoni in 1996 found that high expression of the OTR stimulated breast cancer cell proliferation; however Cassoni's work in 1994 concluded that OT inhibited cell proliferation in three different breast cancer cell lines (Cassoni, Sapino and Negro, 1994; Bussolati *et al.*, 1996; Reversi, Cassoni and Chini, 2006). Although the difference in previous studies could be due to the concentration of OT used and the duration of the treatment; the promiscuous coupling of G_i or G_q to the OTR generates different secondary messengers which leads to different responses (Reversi *et al.*, 2005; Reversi, Cassoni and Chini, 2006).

2.4 Project Aims

To this date, little is known about potential cross-talk between cortisol and oxytocin in the context of cancer in general and in ovarian cancer specifically. Because of the often grave prognosis of ovarian cancer due to it being diagnosed frequently at advanced stages (Longuespée *et al.*, 2012), and since it is easy to administer OT, it seemed important to examine the effects of OT and cortisol on ovarian cancer cells first *in vitro*.

The aims of this project are;

1. To investigate if OT is able to reverse proliferative effects of cortisol in ovarian cancer cells *in vitro*
2. To investigate if OT can affect GR splicing *in vitro*
3. To investigate if clinical tissue from ovarian cancer patients show differential expression of OTR and GRs

Based on their opposing effects, we hypothesised that the activity of cortisol in ovarian cancer cells might be compromised if OTR signalling is activated.

Solution/ Buffer Used	**Constitution**
10% TBS	24.2g Tris Base, 80g NaCl, 1L dH_2O
1M Tris HCl, pH 8	121.1 g Tris base, 1L dH_2O adjusted to pH 8
1.5 M Tris, pH 8.8	181.65 g Tris base, 1L dH_2O adjusted to pH 8.8
1.5 M Tris, pH 6.8	181.65 g Tris base, 1L dH_2O adjusted to pH 6.8
0.5M EDTA	93.05 g $Na_2EDTA \bullet 2H_2O$, 9g NaOH, 500ml dH_2O adjusted to pH 8 using NaOH
10% SDS	10g SDS in 100ml dH_2O
DNA lysis buffer	2ml of 0.5M EDTA, 1ml of 1M Tris-Hcl at pH 8, 200µl of 100% triton X-100, 97ml of TE buffer
10xTBE buffer	108g Tris Base, 55g boric acid, 7.5g EDTA, 1L dH_2O
TE buffer	10 mM Tris-Cl, pH 7.5,1 mM EDTA
Laemmli buffer	2ml glycerol, 1ml 1M Tris HCl, 0.5 ml mercaptoethanol, 4ml 10% SDS and 2.5ml dH_2O
4x Separating Gel Buffer	1.5 M Tris, pH 8.8, 0.4% SDS
4x Stacking Gel Buffer	0.5 M Tris, pH 6.8, 0.4%
Running Buffer	30g Tris Base, 44g Glycine, 10g SDS, 1L dH_2O
Transfer Buffer	2.41g Tris Base, 11.25g Glycine, 200ml Methanol, make up to 1L with dH_2O
5% Blocking Buffer	5g Dried milk powder in 100ml 1% TBS tween
1% TBS tween	1ml Tween20 to 1L 1% TBS
RNA lysis Buffer	2.5µl 2-Mercaptoethanol in 250µl of RNA lysis solution from GenElute™ Kit

***Table 3**. Table detailing how all solutions and buffers were made up.*

3.1 Tissue Culture

3.1.1 Cell Lines

SKOV3, PEO1 and MDAH-2774 cell lines were selected for this project based on previous studies that have shown them to be suitable candidates for an *in-vitro* model for ovarian cancer (Buick, et al., 1985) (Wang, et al., 2006). The SKOV3 cell line was derived from ascites around the ovaries of a patient suffering from epithelial ovarian adenocarcinoma. Mutations present on the p53 gene in the SKOV3 cell line means they do not express endogenous levels of the p53 protein (an important regulator for the cell cycle control). These cells are also resistant to tumour necrosis factor and some cytotoxic drugs making them an appropriate model for late stage diagnosis of ovarian cancer and a good model for patients who are non-responsive to chemotherapeutic treatments. The PEO1 cell line was taken from the peritoneal ascites of a patient with a poorly differentiated serous adenocarcinoma. Ovarian cancers that are diagnosed at the later stages tend to be very poorly differentiated which makes the PEO1 cells a good *invitro* representation of the majority of ovarian cancers since it also demonstrates an epithelial phenotype. The cells are not p53 null and there aren't any reports of cytotoxic resistance. MDAH-2774 cell line was developed from cells in the ascitic fluid from a patient with endometrioid ovarian cancer and is used to represent ovarian cancer which has not originated from the ovaries. Similarly, this cell line is not p53 null and there is currently no report of cytotoxic resistance (Wang *et al.*, 2006).

3.1.2 Cell Culture

SKOV3, PEO1 and MDAH-2774 ovarian cancer cell lines were cultured in Gibco RPMI phenol red-free complete media containing 10% foetal bovine serum (FBS), 5% penicillin/streptomycin (P/S) solution (5000 µg/ml) and 5% Gibco 100x non-essential amino acids (NEAA) at 37^{o}C and 5% CO_2. For cell treatments, cells were seeded overnight into 6-well plates with 2ml complete media before media was aspirated and wells washed with Phospho Buffered saline (PBS) solution. The cells were then incubated for 3hours in serum free media (phenol red-free media with 5% NEAA and 5% P/S but lacking FBS). The cells are re-fed with 2ml complete media with the addition of dH_2O, oxytocin (OT) and/or cortisol (C), and staurosporine (ST) to make up the final concentrations as seen in table 4.

Treatment	Volume added to 2ml Media	Final treatment concentration
NS	2µl of dH_2O	Control
OT	2µl of 10µM OT	100nM OT
OTC	2µl of 10µM OT and 2µl of 10µM of C	100nM OT with 100nM C
C	2µl of 10µM of C	100nM C
ST	2µl of 10µM of ST	100nM ST

Table 4. *The volume and concentrations of each solution added to 2ml complete media to make up a final concentration of 100nM for cell treatments.*

3.1.3 Cell Fixing

Sterilized glass cover slips were placed at the bottom of 6-well plates before cells were cultured as described above. Media was aspirated and 150µl of 4% paraformaldehyde solution (PFA) was added for 20 minutes followed by three 5 minute washes in PBS. Cover slips were stored at 4° C.

3.1.3.1 DAB staining of fixed cells

Fixed cells were blocked using 200µl 1% donkey serum for one hour at room temperature before washing. Primary antibody (GRα and GRβ) were left on overnight at 4°C and washed off the following day using PBS. The coverslips were then incubated at room temperature for an hour in HRP conjugated secondary antibody followed by another three washes. Cover slips were then subjected to DAB staining, counterstained with haematoxylin and washed with 0.1% sodium bicarbonate.

3.1.4 Cell Viability Assay

After treatment, media is removed and cells are incubated for 2min in 0.25% Trypsin-EDTA solution to lift cells from the wells before re-suspending the cells in 1ml complete media. 10µl of treated cells was then taken and mixed with 10µl of trypan blue solution in a new tube. Viability values were determined using Countess™ Automated Cell Counter (Invitrogen, Paisley, Renfrewshire, UK).

3.1.5 Wound Healing Assay

Wound Healing Assay: A solid line spanning the diameter of each well on a 6-well plate was drawn on the reverse side before cells were seeded at equal density and treated as stated above. The 'wound' was created using a 200-µl yellow pipette tip (Fisher) and scratching a line through the cells which was perpendicular to the line drawn along the well. Images of each wound at 0 hours (h), 6, 12 and 18 h after treatment were inspected by the Olympus IX71 Microscope and the images captured using the Photometrics Cool Snap™ CF camera. Percentage migration of cells into the wound after 18 h was calculated using the following formula: 1 − average width of wound at 18 h/average width of wound at 0 h*100.

3.2 RNA Extraction

RNA extraction was carried out using the GenElute™ Mammalian total RNA miniprep kit from Sigma (catalogue no. RTN70). Treated cells were lysed in 250µl RNA lysis buffer and stored at -80°C before extraction. The lysed cells were placed into a GenElute™ filtration column and centrifuged at maximum speed for 2min. 250µl of 70% ethanol (essential for RNA binding) was added to the flow through liquid and pipette into a GenElute™ binding column and centrifuged again for 15 seconds. The binding column was then placed into a new collection tube and 500µl of Wash Solution 1 was added to the column and the samples were centrifuged again for 15 seconds. Flow through liquid was removed and 500µl of Wash Solution 2 was added before the samples were spun down again, this time for two minutes. This step is repeated before the binding column is moved to a fresh collection tube and 50µl of Elution solution is added to remove RNA from binding column. Measurement of RNA quality was carried out using the NanoDrop 200c (Thermo Scientific) by using 1 µl Elution solution as a blank reading. For each sample the concentration of RNA in ng/µl and the protein contamination ratio (260/280) were recorded. For clinical samples, RNA extraction (As describes above) was preformed from ready lysed tissue samples.

3.3 Complementary DNA (cDNA) synthesis

cDNA synthesis was carried out using the Precision NanoScript Reverse Transcription Kit from Primer Design (catalogue no. RT-NanoScript). All RNA extracts were standardised to a final concentration of 25ng/µl and 1µl of Random Nanomer primer was added to 10µl of RNA and left for 5 minutes at 65^{o}C before immediately being placed on ice. For each RNA sample; 2µl NanoScript 10x Buffer, 1µl 10mM dNTP mix, 2µl 10OnM DTT, 4µl RNase/DNase free H_2O and 1µl nanoScript Reverse Transcriptase Enzyme were added and allowed to incubate at room temperature for 5minutes. The samples were heated at 55^{o}C for 20 min and 75^{o}C for another 15 minutes before storage at -20^{o}C.

3.4 Quantitative Polymerase Chain Reaction (qPCR)

Precision MasterMix with ROX and with SYBRgreen from Primer Design (Catalogue no: Precision-R-SY) was used to quantify RNA expression in treated cells. For each sample 10µl of the master mix (which contains a thermo-stable TAQ Polymerase as well as buffer and $MgCl_2$ at concentrations optimised for the enzyme) was mixed with forward and reverse primers (Table 5) and RNAse free water. 19µl of the mixture was added to each well on a MicroAmp™ Fast Optical 96-Wells Reaction Plate from Applied Biosystems (Catalogue No. 4314320) and 1µl of cDNA was carefully added to each well to make a final volume of 20µl. As a negative control for all the reactions, distilled water was used in place of the cDNA. The plate is sealed using MicroAmp® Optical Adhesive Film from Applied Biosystems (Category No. 4314320) and centrifuged for 1 minute before being placed into the ABI 7400 instrument (Applied Biosystems) and a thermal cycle programme (50^{o}C for 2 min; 95^{o}C for 10 min; 95^{o}C for 15 sec; 50^{o}C for 1 min; 72^{o}C for 1min) was set for 40 cycles. Once completed, the instrument was reprogrammed to carry out a dissociation stage with a thermal cycle programme (95^{o}C for 15 sec; 60^{o}C for 15 sec; 95^{o}C for 15 sec). RNA levels were expressed as a relative quantification (RQ) using the house keeping gene GAPDH using the ΔCt method:

- For Expression in clinical samples and in untreated cells;

  ΔCt = Ct (gene of interest) – Ct (housekeeping gene)

  Relative Quantity (RQ) = $2^{-\Delta Ct}$

- For Expression in treated cells;

  ΔCt = Ct (gene of interest) – Ct (housekeeping gene)

  $\Delta\Delta Ct$ = ΔCt (treated) – ΔCt (untreated)

  Relative Quantity (RQ) = $2^{-\Delta\Delta Ct}$

Primer		Sequence	Company
GRα	Forward	5'-CTATGCATGAAGTGGTTGAAAA-3'	Sigma
	Reverse	5'-TTTCAGCTAACATCTCGGG-3'	
GRβ	Forward	5'-GAAGGAAACTCCAGCCAGAA-3'	Sigma
	Reverse	5'-CCACATAACATTTTCATGCATAGA-3'	
GRγ	Forward	5'-TTCAAAAGAGCAGTGGAAGGTA-3'	Sigma
	Reverse	5'-GGTAGGGGTGAGTTGTGGTAACG-3'	
GRp	Forward	5'-GCTGTGTTTTGCTCCTGATCTGA-3'	Sigma
	Reverse	5'-TGACATAAGGTGAAAAGGTGTTCTACC-3'	
GAS5	Forward	5'-CAGTGTGGCTCTGGATAGCA-3'	Sigma
	Reverse	5'-TTAAGCTGGTCCAGGCAAGT-3'	
OTR	Forward	5'-TTACAATCACTAGGATGGCTACAA-3'	Primer Design
	Reverse	5'-CATTTACATTCCCACCAACAATTTAA-3'	
GAPDH	Forward	5'-TGATTCTACCCACGGCAAGTT-3'	Primer Design
	Reverse	5'-TGATGGGTTTCCCATTGATGA-3	

***Table 5**. Sequence of forward and reverse primers used for qPRC and the company where it was purchased*

3.5 Western blotting

Treated cells were lysed in 250 µl Laemmli buffer and denatured at 100°C for 10 min. A 12.5% resolving gel was made (2.37ml dH_2O, 3.13ml Acylamide/Bisacrylamide 30%, 1.9ml 4x Separating Gel Buffer, 112µl 10% Ammonium per Sulphate, 5µl TEMED) and set between

1mm glass plates. Stacking gel (1ml dH_2O, 300µl Acylamide/Bisacrylamide 30%, 444µl 4x Stacking Gel buffer, 28µl 10% Ammonium per Sulphate, 5µl TEMED) was poured on top of the resolving gel and set before the samples were loaded. Running buffer covered the cell, and a current of 80mA was applied for one hour. To transfer the proteins from the gel to a nitrocellulose membrane the gel was removed from the tank and laid between two sheets of filter paper and a sponge pad then soaked transfer buffer. The proteins were transferred to a nitrocellulose membrane by electrophoresis at 100 volts for 1h. The nitrocellulose membrane was left overnight to incubate in 5% blocking buffer and washed twice for 10 minutes in 1% TBS tween. Primary antibodies (see table 6) were prepared and added to the nitrocellulose membrane to incubate overnight at 4^{o}C before washing the membrane three times for 15 minutes in 1% TBS tween. The membrane was incubated at room temp for 1h with a horseradish peroxidise (HRP) conjugated secondary antibody and washed three times for 15 minutes with 1% TBS tween. After washing the membrane Coumaric acid and luminol solution (10ml Tris pH8, 22µl Coumaric acid, 50µl luminol and 3µl 30% Hydrogen peroxide) were added to the nitrocellulose membrane for 5 minutes in the dark room and the membrane was exposed on an Amersham Hyperfilm® ECL film. The film is developed and fixed before being washed and then analysis of bands is carried out using the AlphaEaseFC software.

Primary Antibody	Dilution	Company	Species
Caspase-3	1:1000	Cell Signaling Technology	Rabbit
Beclin-1	1:1000	Cell Signaling Technology	Rabbit
GAPDH	1:2000	Cell Signaling Technology	Rabbit

Secondary Antibody	Dilution	Company	Species
Anti-Rabbit	1:2000	Cell Signaling Technology	Goat

***Table 6**. Primary and secondary antibodies used in Western Blotting.*

3.6 Clinical Samples

Clinical samples were of ovarian origin (n=12) and were taken from patients admitted to the First Department of Obstetrics and Gynecology, Papageorgiou General Hospital, Medical School, Aristotle University, Thessaloniki, Greece. Ethical approval was obtained by the local authority and Brunel University. The majority of ovarian cancers were deemed to be grade 3 stage III (poorly differentiated and involving the whole peritoneal cavity, not just confined to ovaries/tubes or pelvis) (10/12). Control samples (n=10) were also used in this study, obtained from patients undergoing total hysterectomy and bilateral salpingo-oopherectomy for benign reasons. None of the two groups (ovarian cancer and control) received hormone replacement therapy, and ovarian cancer patients were all post-menopausal. Table 7 provides further information on the stage, grade, patient age and CA125 status of ovarian cancer patients.

Histology	Grade	Stage	Age (years)
Serous	3	IIIC	64
Serous	3	IIIC	48
Serous	3	IIIC	61
Serous	2	IIIC	54
Serous	3	IIIC	69
Serous	3	IV	65
Serous	3	IIIC	75
Serous	3	IIIC	65
Serous	3	IIIC	56
Serous	3	IIIC	64
Serous	3	IIIC	64
Serous	2	IIIC	56

Table 7. *Patient details showing Histology, grade, stage and age of clinical samples taken from Ovarian Cancer patient (n=12)*

3.7 Ovarian Tissue Microarray

Unstained paraffin tissue micro-array slides containing multiple ovarian carcinoma and normal tissue micro-array (70 cases of ovarian carcinoma, 5 cases of tumour adjacent normal ovary and 5 normal ovarian tissue from different biopsies; Biomax USA, see table 8 for tissue detail) were used for this study. The paraffin-embedded slides were deparaffinised and rehydrated by a series of washes in reducing concentrations of ethanol (100, 95, 70 and 50%) followed by rinsing in tap water for 10 min. Antigen retrieval was accomplished by incubating the slide in sodium citrate (pH 6.0) for 20 min in a microwave. Slides were washed in 0.4% of PBS-T for 5 min and then incubated for 15 min in the PBS containing 0.3% H_2O_2 to stop the interference of the endogenous peroxidase activity. Blocking was carried out with 5% goat serum, followed by overnight incubation with primary GRα, GRβ (did not work), antibodies. The following day, after several washes with PBS, slides were incubated with HRP conjugate-secondary antibody for 60 min. Further washing in PBS-T was carried out for 20 min before performing staining. Slides were then subjected to DAB staining, counterstained with haematoxylin and washed with 0.1% sodium bicarbonate.

Position	**Age**	**Pathology Diagnosis**	**Stage**
A1	40	Clear cell carcinoma	I
A2	57	Serous papillary carcinoma	Ic
A3	48	Clear cell carcinoma	II
A4	57	Serous papillary carcinoma	IIIc
A5	43	Serous papillary carcinoma	IIIc
A6	54	Serous papillary adenocarcinoma	Ic
A7	63	Serous papillary adenocarcinoma	IV
A8	46	Serous papillary adenocarcinoma	IIIc
A9	54	Serous papillary adenocarcinoma	IIIc
A10	56	Hyperplastic fibrous tissue	-
B1	44	Granular cell tumor	-
B2	49	Serous papillary adenocarcinoma	II
B3	18	Immature teratoma	-
B4	15	Endodermal sinus carcinoma	Iia
B5	38	Metastatic adenocarcinoma	-
B6	39	Serous papillary adenocarcinoma	IV
B7	24	Endodermal sinus carcinoma	II
B8	42	Serous papillary adenocarcinoma	II

B9	50	Serous papillary adenocarcinoma	I
B10	49	Serous papillary adenocarcinoma	IIIc
C1	62	Serous papillary adenocarcinoma	II
C2	53	Mucinous papillary carcinoma	IV
C3	38	Metastatic adenocarcinoma	-
C4	43	Clear cell carcinoma	Ia
C5	26	Serous papillary adenocarcinoma	Ic
C6	47	Serous papillary adenocarcinoma	I
C7	62	Squamous cell carcinoma	I
C8	35	Dysgerminoma	Ia
C9	41	Dysgerminoma	I
C10	47	Serous papillary adenocarcinoma	I
D1	42	Clear cell carcinoma	Ic
D2	39	Metastatic adenocarcinoma	-
D3	66	Metastatic adenocarcinoma	-
D4	48	Malignant theca cell tumor	III
D5	51	Serous papillary adenocarcinoma	IIIc
D6	33	Metastatic signet-ring cell carcinoma	-
D7	18	Mixed germ cell tumor	Ib
D8	40	Metastatic signet-ring cell carcinoma	-
D9	43	Granular cell tumor	-
D10	55	Serous papillary adenocarcinoma	II
E1	46	Serous papillary adenocarcinoma	IIIc
E2	57	Serous papillary adenocarcinoma	IIIc
E3	75	Serous papillary adenocarcinoma	IIIc
E4	69	Serous papillary adenocarcinoma (Sparse)	Ia
E5	30	Serous papillary adenocarcinoma	I
E6	42	Serous papillary adenocarcinoma	IIIc
E7	48	Clear cell carcinoma	I
E8	22	Serous papillary adenocarcinoma	Iib
E9	50	Clear cell carcinoma	I
E10	32	Serous papillary adenocarcinoma	I
F1	48	Serous papillary adenocarcinoma	I
F2	50	Serous papillary adenocarcinoma	II
F3	65	Serous papillary adenocarcinoma	IIIc
F4	38	Serous papillary adenocarcinoma	IIIc
F5	31	Metastatic adenocarcinoma	-
F6	55	Metastatic adenocarcinoma	-
F7	51	Serous papillary adenocarcinoma	II
F8	65	Serous papillary adenocarcinoma	I
F9	26	Serous papillary adenocarcinoma	IIIc
F10	55	Serous papillary adenocarcinoma	I
G1	49	Serous papillary adenocarcinoma	II

G2	48	Metastatic signet-ring cell carcinoma	-
G3	46	Serous papillary adenocarcinoma	IIIc
G4	63	Serous papillary adenocarcinoma	II
G5	37	Serous papillary adenocarcinoma	IV
G6	35	Malignant tumor (sparse)	IIIc
G7	12	Dysgerminoma	Ib
G8	55	Serous papillary adenocarcinoma	I
G9	20	Malignant tumor (sparse)	I
G10	55	Serous papillary adenocarcinoma	I
H1	39	Cancer adjacent normal ovary tissue	-
H2	53	Cancer adjacent normal ovary tissue	-
H3	48	Cancer adjacent normal ovary tissue	-
H4	39	Cancer adjacent normal ovary tissue	-
H5	17	Cancer adjacent normal ovary tissue	-
H6	42	Normal ovary tissue	-
H7	41	Normal ovary tissue	-
H8	18	Normal ovary tissue	-
H9	19	Normal ovary tissue	-
H10	27	Normal ovary tissue	-

***Table 8**. Details for each core embedded in the 80 core slide provided by Biomax USA*

3.7.1 Scoring

10 Random field of view were selected per tissue sample and a percentage of brown staining was determined by three individuals. The average percentage cover of positive staining was assigned a score.

Percentage of Tissue showing positive staining	Score
< 5%	0
5% - 25%	1
26%-50%	2
51%-75%	3
>75%	4

***Table 9**. Scoring system based on percentage cover of positive staining*

3.8 Statistical Analysis

All Statistical analysis was carried out using GraphPad Prism® Software. A value of $P<0.05$ was regarded as statistically significant. For all results plotted and analysed the paired t-test was selected as a mean of assessing if two means were statistically significant. For all data presented from clinical samples, the unpaired t-test was used as samples have all come from different test subjects. All data is presented ± standard deviation and statistical significance is indicated using * for $p< 0.05$ and ** for $p<0.01$.

3.9 In-silico analysis

Oncomine is a cancer microarray database which houses online genome expression analysis from published data made available to the public. The data is accessed from www.oncomine.org. *In silico* datasets for expression of GR, GAS5 and OTR in both normal and ovarian cancer tissue was used in this study.

Kaplan-Meier Plots are survival probabilities constructed using very large patient participation data and non-parametric statistical analysis to estimate the probable survival time for patients after treatment. The plots in this study are used to measure differences in probability of survival depending on high and low expressions of GR, the OTR and GAS5. Plots are accessed from www.kmplots.com/analysis.

4.0 All three OvC cell lines express the four GR splice variants, with different ratios of GR α:P

4.1 Introduction

As previous studies have presented SKOV3 and PEO1 and MDAH-2774 cell lines as suitable candidates for an *in-vitro* model of ovarian cancer (Bukovský *et al.*, 1995; Lutgendorf *et al.*, 2003a; Wang *et al.*, 2006; Karabulut *et al.*, 2010) they were selected for this study. These three cell lines vary in origin, differentiation and cytotoxic resistance (see materials and methods for cell line information) which allowed for more inclusive representation of ovarian cancer in patients.

In order to fully dissect any crosstalk between cortisol and oxytocin in ovarian cancer cells the presence and expression of the different Glucocorticoid receptors must first be established at an mRNA and protein level. The GR's are Steroid receptors that control specific target gene transcription (Hagendorf *et al.*, 2005; Heitzer *et al.*, 2007) and the four known spice variants are GR-α, GR-β, GR-γ and GR-P. Upon binding with cortisol, the GR-α splice variant binds with another GR-α receptor to form a dimmer and translocate into the nucleus where the dimer binds to glucocorticoid response elements (GRE's) and subsequently begins gene transcription in target cells (Yudt and Cidlowski, 2002; Turner *et al.*, 2010; Lutgendorf *et al.*, 2012). Of the four known splice variants, GR-β is the only one that does not bind to cortisol and has the lowest levels of mRNA expression of the four in majority of tumours studied. GR-P has the highest expression levels (Hagendorf *et al.*, 2005; McMaster and Ray, 2007). The ratios of mRNA levels of the splice variant GR-P to GR-α and GR-β affects the GR responsiveness to cortisol (Lange, Segeren and Koper, 2010).

Activated GR's are blocked in the presence of a non-protein coding RNA called Growth Arrest-specific 5 (GAS5). It is generally found in abundance in cells that exit the cell cycle and acts as a decoy for GRE's. GR-α dimers instead bind to GAS5 not the GRE's therefore subsequently preventing transcription (Smith and Steitz, 1998).

Binding of oxytocin (OT) to the oxytocin receptor (OTR) causes conformational changes depending on which G-protein α subunit the OTR couples to. Coupling of OTR to G_q brings about phosphorylation of intermediate proteins and subsequently promotes cell growth

(Gimpl and Fahrenholz, 2010). Coupling of OTR to G_i instead prevents production cyclic AMP, inhibiting cell growth and reducing migration of cancerous cells (Dorsam and Gutkind, 2007).

4.2 Objectives

1. To validate gene expression of the four GR splice variants; α, β, γ and P, as well as the expression for the non-coding RNA GAS5 and the OTR in SKOV3, PEO1 and MDAH-2774 ovarian cancer cell lines.
2. To validate protein expression of the two GR splice variants, GRα and GRβ in SKOV3, PEO1 and MDAH-2774 cells.

4.3 Results

4.3.1 Baseline Expression of four GR splice variants in SKOV3, PEO1 and MDAH-2774 cells.

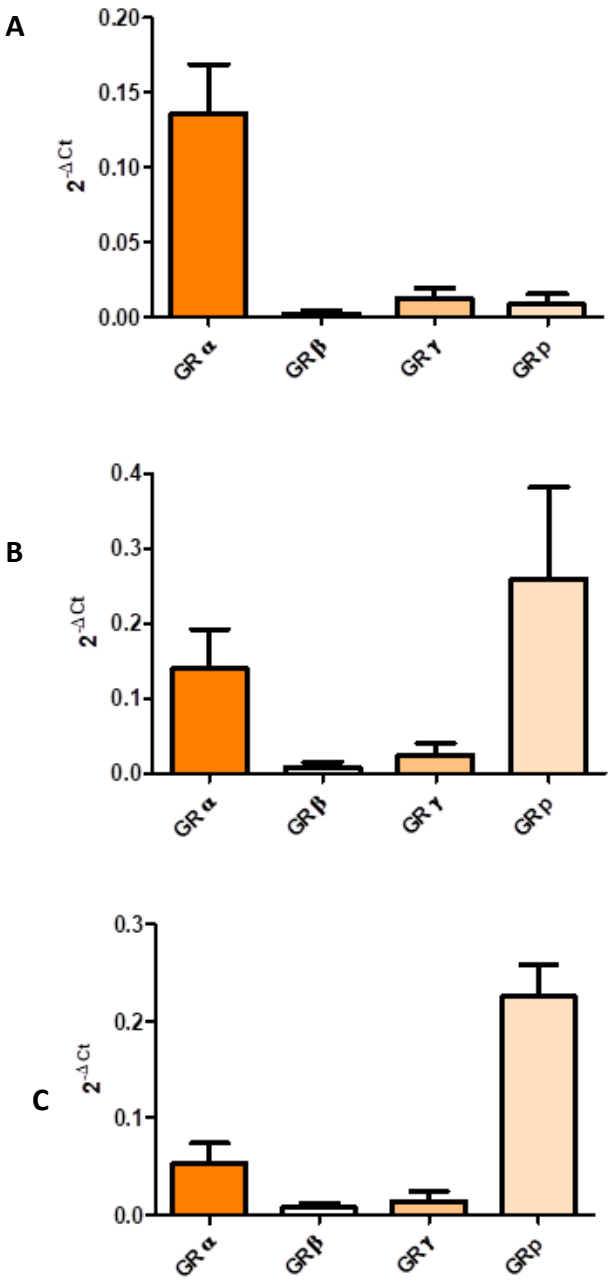

***Figure 11**. Gene expression measured in fold change ($2^{-\Delta Ct}$) of GR splice variants GRα, GRβ, GRγ, and GR-P in SKOV3 (A), PEOI (B) and MDAH-2774 (C) cells (n=3). Data are expressed as the mean ± SD. PEO1 and MDAH-2774 cell showed elevated GR-P expression. SKOV3 cells has overall lower expression of all four splice variants compared to the other two cells.*

In all three cell lines, SKOV3, PEO1 and MDAH-2774, gene expression of the four splice variants was confirmed. Consistently in all three cell lines GRα levels were significantly ($p<0.0001$) greater than GRβ expression. All three lines also express low levels for the GRγ variant. For both PEO1 and MDAH-2774 cells there is greater expression of GR-P to GRα. In SKOV3 cells this is reversed, with GRα expression being significantly greater than GR-P.

4.3.2 Baseline Expression of GAS5 and the OTR in SKOV3, PEO1 and MDAH-2774 cells.

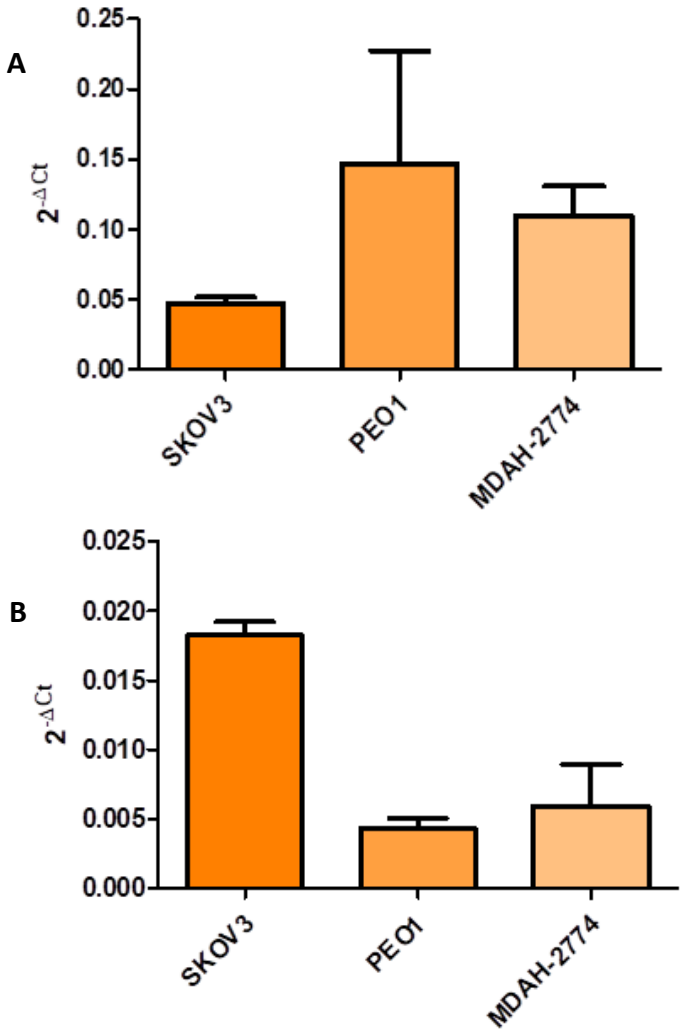

***Figure 12**. Gene expression measured in fold change ($2^{-\Delta Ct}$) of GAS5 (A) and OTR (B) in SKOV3, PEO1 and MDAH-2774 cells (n=3). Data are expressed as the mean ± SD. SKOV3 cells have lower levels of GAS5 compared to PEO1 and MDAH-2774 cells, and greater expression of OTR than PEO1 and MDAH-2774 cells.*

Expression of GAS5 and the OTR is confirmed for all three cell lines. There is lower expression of GAS5 in SKOV3 cells than in PEO1 cells (not significant) and in MDAH-2774 cells this difference is significant (p=0.007). Interestingly for OTR expression, SKOV3 cells had greater relative quantity of the receptor when to PEO1 cells and MDAH-2774 cells. OTR expression in SKOV3 was greater than in PEO1 (P<0.0001) and in MDAH-2774 (p=0.0026).

4.3.3 DAB immunohistochemistry for Protein expression of GRα and GRβ in SKOV3, PEO1 and MDAH-2774 Cells

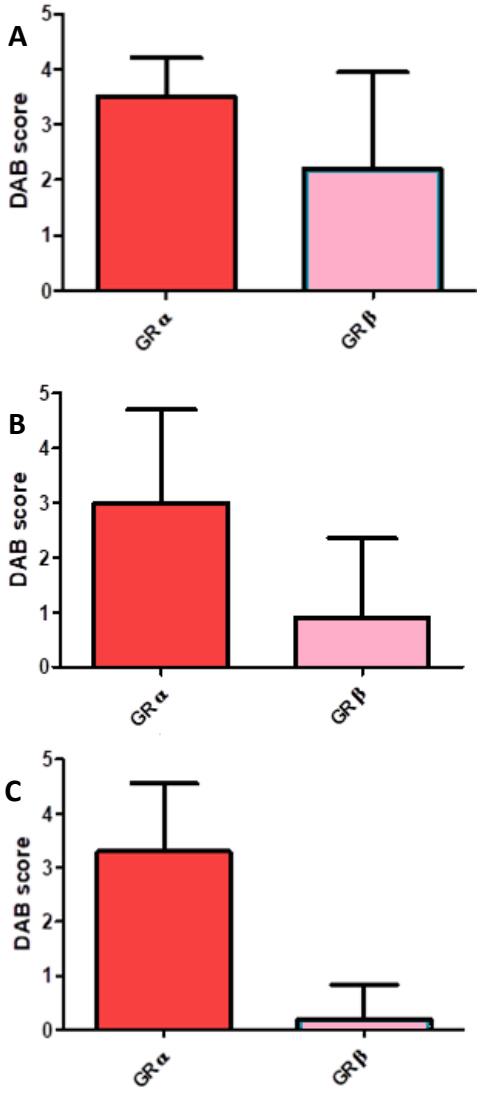

***Figure 13**. Protein Expression measured as a score (1-4) of GR splice variants GRα and GRβ in SKOV3 (A), PEOI (B) and MDAH-2774 (C) cells (n=3). Data are expressed as the mean ± SD. GRβ is less expressed than GRα in all cell lines.*

Protein expression for both GRα and GRβ was confirmed in all three cell lines. MDAH-2774 cells results mirrored qPCR data where GRβ levels were significantly lower than expression of GRα ($p=0.0147$), and although not to a statistically significant level, both SKOV3 and PEO1 cells had lower levels of GRβ protein expression compared to GRα.

4.4 Discussion

SKOV3, PEO1 and MDAH-2774 cells were selected for this study as their variations in origins, cytotoxicity and differentiation enabled better representation of ovarian cancer *in-vitro*. The above data interestingly highlighted that both PEO1 and MDAH-2774 cell lines displayed similar expression patterns for the four GR splice variants, for GAS5 expression and for expression of the OTR. Interestingly SKOV3 had the reverse pattern in comparison. Where PEO1 and MDAH-2774 cells expressed higher GR-P and GAS5 mRNA levels, and expressed lower GRα and OTR levels, SKOV3 had the opposite; lower GR-P and GAS5 expression and higher GRα and OTR expression.

Cortisol binds to GRα in target cells. A cell/tissues responsiveness to cortisol has always been correlated with the abundance of GRα present (Schrepf *et al.*, 2013) and oppression of this pathway is associated with increasing expression of GRβ (Turner *et al.*, 2010). Not much is known about the GR-P slice variant or its role in stress response, however, in haematological malignancies, increasing expression of GR-P can increase the activity of GRα (Lange, Segeren and Koper, 2010). To date not much is known about GR-P role in ovarian cancer, however, looking at the above data there are differences in GRα and GR-P expression in all three cell lines.

Data from the baseline expression presented above was taken to calculate the ratio of GR-P to GRα in SKOV3, PEO1 and MDAH-2774 (Table10).

	GRα Expression	*GR-P Expression*	*Ratio of GR-P:GRα*
SKOV3	0.122	0.0019	0.0155
PEO1	0.1387	0.25817	1.86
MDAH-2774	0.0525	0.2253	4.29

***Table 10**. mRNA expression of GRα and GR-P presented as a ratio of GR-P:GRα*

As seen in the above table, PEO1 and MDAH-2774 had a greater ratio of GR-P:GRα (1.86:1 and 4.29: 1 respectively) whereas in SKOV3 cells the ratio was 0.0155:1, potentially making PEO1 and MDAH-2774 cells more responsive to cortisol. Interestingly GAS5 data seemed to complement this finding.

GAS5 is responsible for the suppression of activated GRα as it acts as a decoy for the GREs. If GR-P does indeed activate and increase activity of GRα and increases cells responsiveness to cortisol, than higher levels of GAS5 would be required to control this increase. This is reflected in the above data, PEO1 and MDAH-2774 cells both have higher expression of GAS5 whereas SKOV3, with its low ratio of GR-P:GRα also has lower expression of GAS5.

High expression of GAS5 in ovarian cancer cells suppresses cell proliferation and migration. In ovarian tumour tissue, lower expression levels of GAS5 was associated with poor prognosis, deeper invasive depth and higher tumour stage (Li *et al.*, 2016). SKOV3 cells had lower levels of GAS5. Of all three cell lines, it has the greatest cytotoxicity resistance and does not express endogenous levels of the p53 protein. SKOV3 are also resistant to tumour necrosis factor so they are an appropriate model for late stage diagnosis of ovarian cancer and a good model for patients who are non-responsive to chemotherapeutic treatments.

Relative quantification of the OTR revealed that in all the cell lines expression of OTR was low (RQ values all being lower than 0.02). SKOV3 again differed from the other two cell lines as expression of OTR was significantly greater than expression of OTR in PEO1 and MDAH-2774

cells. Present data has proved that OT in SKOV3 cells inhibits proliferation by repression of VEGF (Ji *et al.*, 2018). This inhibition is further seen in other ovarian cancer cell lines; HEYA8, OVCAR8 and OV432 (Schachner, 2017) but no data is published on the effects of OT on PEO1 and MDAH-2774 cells. It would be interesting to see if the differences in the mRNA expression of the OTR in SKOV3, PEO1 and MDAH-2774 cause different response to stress treatments in the next chapter.

Protein expression of the GRα and GRβ in SKOV3, PEO1 and MDAH-2774 cells was established by DAB immunochemistry. As expected for all three cell lines, there seemed greater expression of GRα than GRβ which correlates with the mRNA data for GRα and GRβ expression. Only GRα and GRβ primary antibodies are commercially available therefore protein expression of GR-γ and GR-P were not included in this study. Attempts at using the OTR primary antibody to measure OTR expression in ovarian cancer cells were unsuccessful, as was the use of the antibody on our positive control. Re-ordering the antibody did not change the outcome and attempts were later abandoned.

Protein expression was measured using DAB staining as chromogenic stains are longer lived and tend to be more resistant to photobleaching when compared to using fluorescent dyes. Preliminary experiments using immunofluorescence for the expression of GRα, GRβ and OTR in SKOV3 and PEO1 cells did produce protein signals, however with very high background staining that prevented a reliable quantitative reading for protein expression, it was therefore not used in this study.

5.0 OT is able to lower Viable Cell Count, increase activity of caspase-3 and Beclin-1 whilst reducing cell migration. OT and C alter expression of the different splice variants in a cell specific manner

5.1 Introduction

Now that expression of all four GR splice variants, GAS5 and the OTR has been confirmed in all three selected cell lines, potential cross-talk between cortisol and oxytocin was assessed by measuring the effect treatment with these hormones have on proliferation and cell migration of ovarian cancer cells *in-vitro*. Cells are treated with the hormone oxytocin (OT), the hormone cortisol (C), and a combination of the two (OT+C) to study changes in cell viability, cell migration, expression levels of GR's and in expression of apoptotic cell markers.

Cell viability assays are an effective method to measure the success of cancer cell proliferation after treatment. To measure whether OT potentially has a role in pro-apoptotic mechanisms, protein expression of activated caspase-3 and beclin-1 was assessed in the presence and absence of C. The role of caspase-3 in cells is to execute cell apoptosis after cell death initiation by caspase-9 (Galluzzi *et al.*, 2016). The protein exists in cells in an inactive state known as the procaspase (also known as total) which is 32 kDa in size. Upon activation by caspase 9, caspase-3 is cleaved into two subunits; 17 kDa and 12kDa, which begin the process of DNA fragmentation for apoptosis (Riedl and Shi, 2004). Beclin-1 is a protein involved in autophagy essential for the protection of organisms by initiating cell death in damaged cells (Kumar, Shankar and Srivastava, 2013). Lower levels of autophagy promotes cancer progression as ovarian tumours with high beclin-1 expression were less aggressive and more responsive to chemotherapy than ovarian tumour expressing significantly low levels of beclin-1 (Valente *et al.*, 2014). If treatment with OT upregulates expression of beclin-1 in ovarian cancer cells, the proliferative effects of cortisol should be reduced, indicating potential cross talk between the two hormones.

Assessing the effect of OT treatment on GR's expression will highlight if OT is able to drive differential splicing of the GR thus altering, or even reducing, the cells responsiveness to cortisol and stress.

5.2 Objectives

1. To investigate if Cortisol and Oxytocin have opposing effects on one another on pathways regulating cell survival
2. To measure if and how oxytocin, cortisol, and a combination of both hormones influence the differential expression of the four GR splice variants, GAS5 and the OTR

5.3 Results

5.3.1 Effects of OT and C treatment on Cell Viability of SKOV3, PEO1 and MDAH-2774 Cells

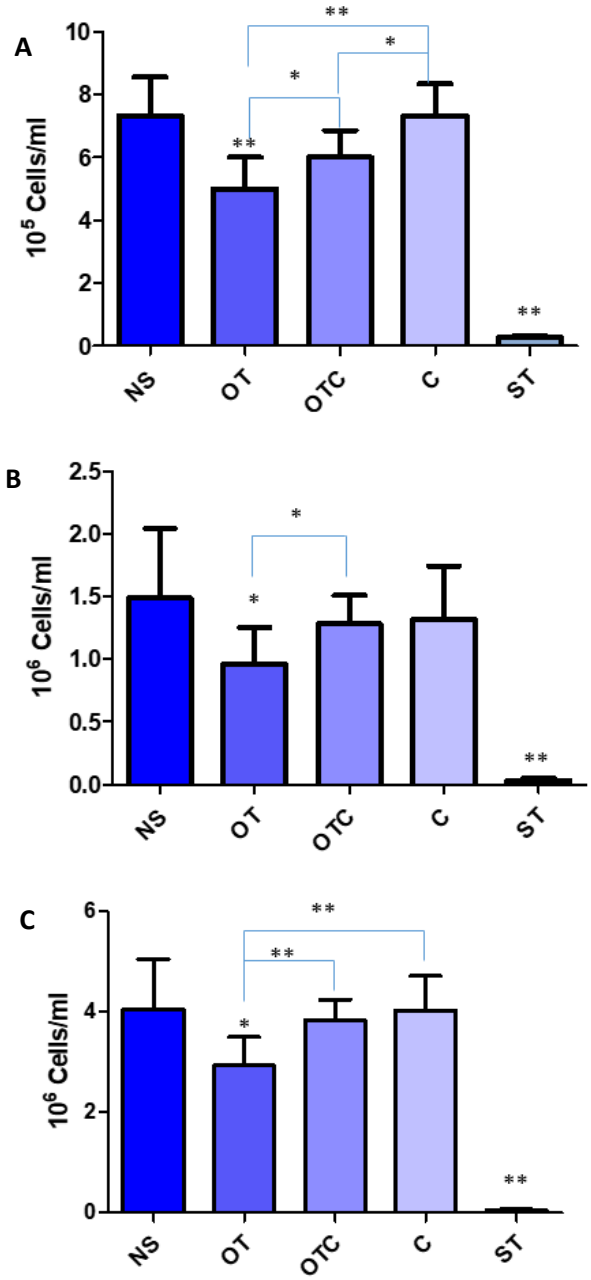

***Figure 14**. Cell viability assay measured as cell concentration per ml for control cells (NS), cells treated with 100nM oxytocin (OT), 100nM oxytocin and 100nM cortisol (OTC), 100nM cortisol (C) and 1µM staurosporine (ST) in SKOV3 (A), PEO1 (B) and in MDAH-2774 (C) cells. All results are present ± SD and statistical significance was calculated using the paired t-test; *p<0.05, **p<0.01. OT was able to reduce significantly cell viability in all three cell lines.*

It can be seen for all three cell lines that treatment with OT significantly produced lower viable cell count compared to untreated cells. Treatment with OT was able to arrest/reduce cell proliferation compared to untreated cells in SKOV3 ($p=0.0047$), PEO1 ($p=0.01$) and MDAH-2774 ($p=0.02$), whereas treatment with C alone did not drive cell proliferation. For both SKOV3 and MDAH-2774 cells, C treated cells had a significantly higher viable cell count in comparison with OT treated cells ($p=0.007$ and $p=0.0035$ respectively). This reduction in proliferation seemed to be counteracted by the addition of C to OT as OTC treatment had significantly greater cell count than OT treated cells ($p=0.04$, $p=0.032$ and $p=0.007$) in all three cell lines. What was interesting is that for all three cell lines; SKOV3, PEO1 and MDAH-2774, OT treated cells had a 33%, 31% and 34% less viable cell count than the C treated cells respectively.

5.3.2 Effects of OT and C on Caspase 3 Cleavage in SKOV3, PEO1 and MDAH-2774 Cells

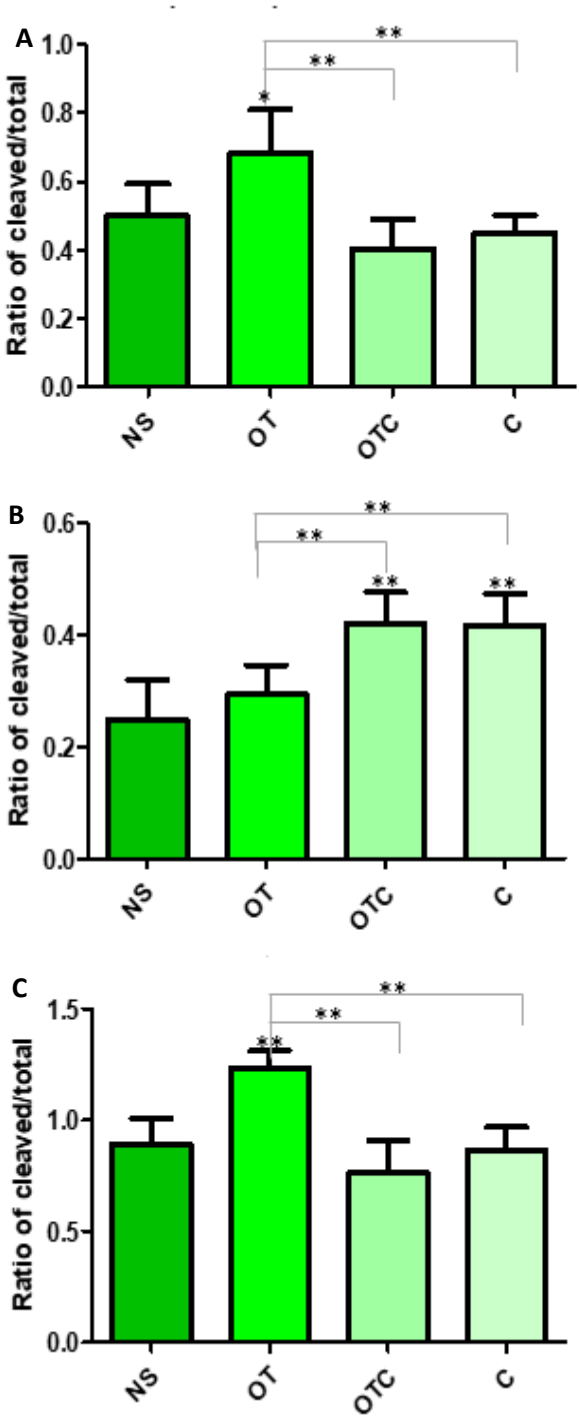

Figure 15*. Expression of cleaved caspase3 measured as a ratio of band intensity over total caspase 3 band intensity in SKOV3 (A), PEO1 (B) and MDAH-2774 (C) cell lines treated for 48 hours in NS,100nM OT, 100nM OTC and 100nM C. All results are present ± SD and statistical significance was calculated using the paired t-test; *p<0.05, **p<0.01. OT drives the cleavage of caspase3 in both SKOV3 and MDAH-2774 cells.*

The results for SKOV3 and MDAH-2774 cells presented in figure 15 are the same. OT treatment significantly increased cleavage of caspase-3 ($p<0.05$ in SKOV3 and $p<0.01$ in MDAH-2774) whereas treatment with C had no effect when compared to NS. For both these cell lines, the greater expression of cleaved caspase-3 in the presence of OT is overturned in the presence of C, as treatment with OT+C produced exactly the same results as treatment with C alone. Expression of cleaved caspase-3 is significantly lower in OTC and C treated cells compared to OT treated cells ($p<0.01$) for both cell lines. PEO1 was the only cell line where C treatment increased expression of cleaved caspase. Unlike SKOV3 and MDAH-2774, OT did not increase expression of cleaved caspase-3, although, just like in SKOV3 and MDAH-2774 cells, treatment with OT+C produced exactly the same result as treating with C alone.

5.3.3 Effects of OT and C on Beclin-1 expression in SKOV3, PEO1 and MDAH-2774 Cells

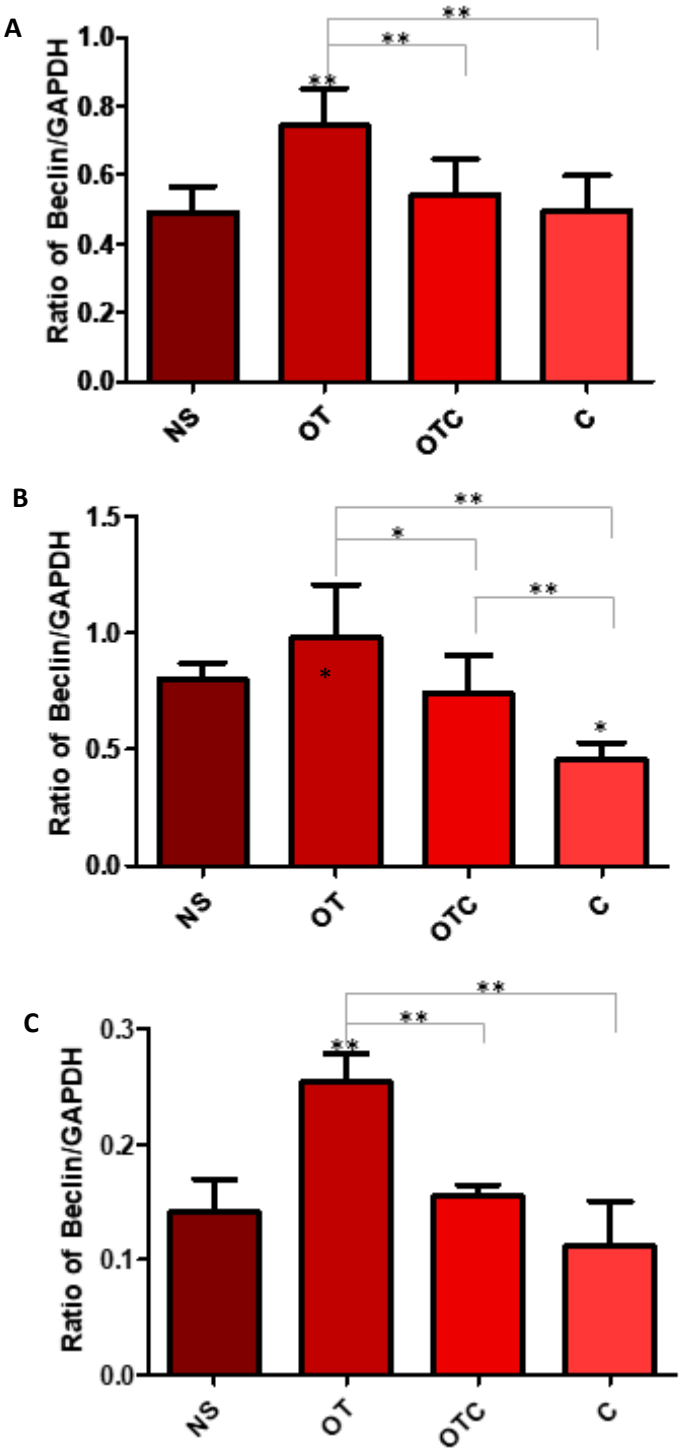

***Figure 16**. Expression of Beclin-1/GAPDH measured by ratio of band intensity in SKOV3 (A), PEO1 (B) and MDAH-2774 (C) cell lines treated for 48 hours in NS, 100nM OT, 100nM OT+C and 100nMC. All results are present ± SD and statistical significance was calculated using the paired t-test; *p<0.05, **p<0.01. Cortisol is able to reverse the increase in Beclin-1 expression brought on by OT treatment.*

As seen in figure 16 with caspase-3 expression, SKOV3 and MDAH-2774 cells responded the same way during treatments. OT treatment significantly increases expression of beclin-1 ($p<0.01$) for both SKOV3 and MDAH-2774 but not in PEO1 cells. C treatment does not reduce expression of beclin-1 in cells, however the increase caused by OT treatment alone is reversed as OTC and C treatments had significantly lower expression of beclin-1 to OT treated cells ($p<0.01$ for both SKOV3 and MDAH-2774 cells). Although PEO1 cells did not express higher beclin-1 levels in the presence of OT, OT treatment produced higher beclin-1 expression compared to OTC treatment ($p<0.05$) and C treatment ($p<0.01$). Interestingly in PEO1 cells, treatment with OT+C produced significantly greater expression of beclin-1 than treatment with C alone ($p<0.01$) and produced significantly lower expression of beclin-1 than with OT treatment alone ($p<0.05$).

5.3.4 Effects of OT and C on Cell Migration in SKOV3, PEO1 and MDAH-2774 Cells

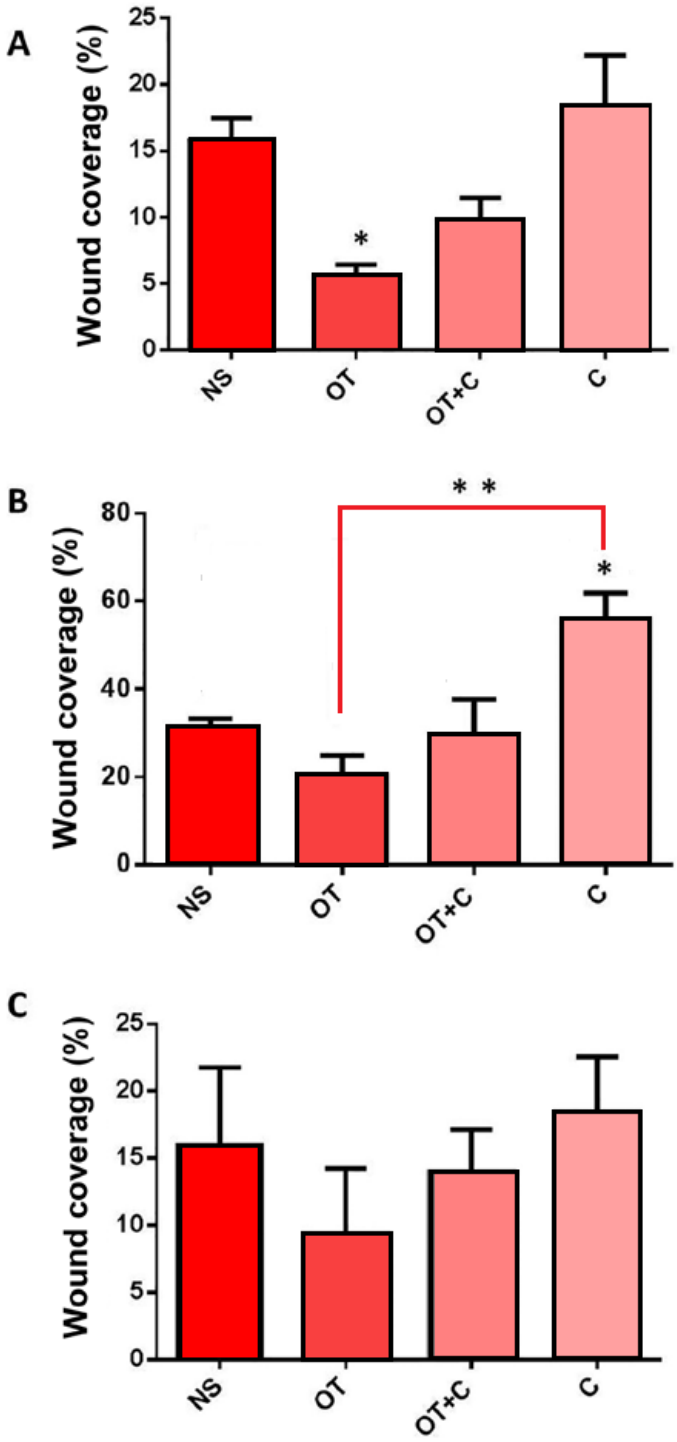

Figure 17. *Percentage coverage of wound for SKOV3 (A), PEO1 (B) and MDAH-2774 (C) cell lines treated for 18 hours in NS, 100nM OT, 100nM OT+C and 100nM C. All results are present ± SEM and statistical significance was calculated using the paired t-test; *p<0.05, **p<0.01. Cortisol seems to reverse the reduction in cell migration brought on by OT treatment.*

OT significantly decreased migration in SKOV3 cells ($p<0.05$), and although not at a significant level, it was observed that OT treatment in both PEO1 and MDAH-2774 cells also reduced the rate of cell migration. As seen previously with the viable cell data and WB data, OT partially mitigates the effects of C as for all three cell lines, wound coverage after 18h was lower in OT+ C treated cells compared to C treated, yet greater that OT treated, once again highlighting potential cross talk between these hormones *in-vitro*.

Cortisol treatment in PEO1 significantly drove up cell migration ($p<0.05$) and was significantly greater than OT treated PEO1 cells ($p<0.001$). Although data for MDAH-2774 did not yield statistical significance, all treatments produced the same trends seen in both SKOV3 and PEO1 cell lines.

5.3.5 Effect of OT and C on the GR Splice Variants

5.3.5.1 Effect of OT and C on GRα Expression

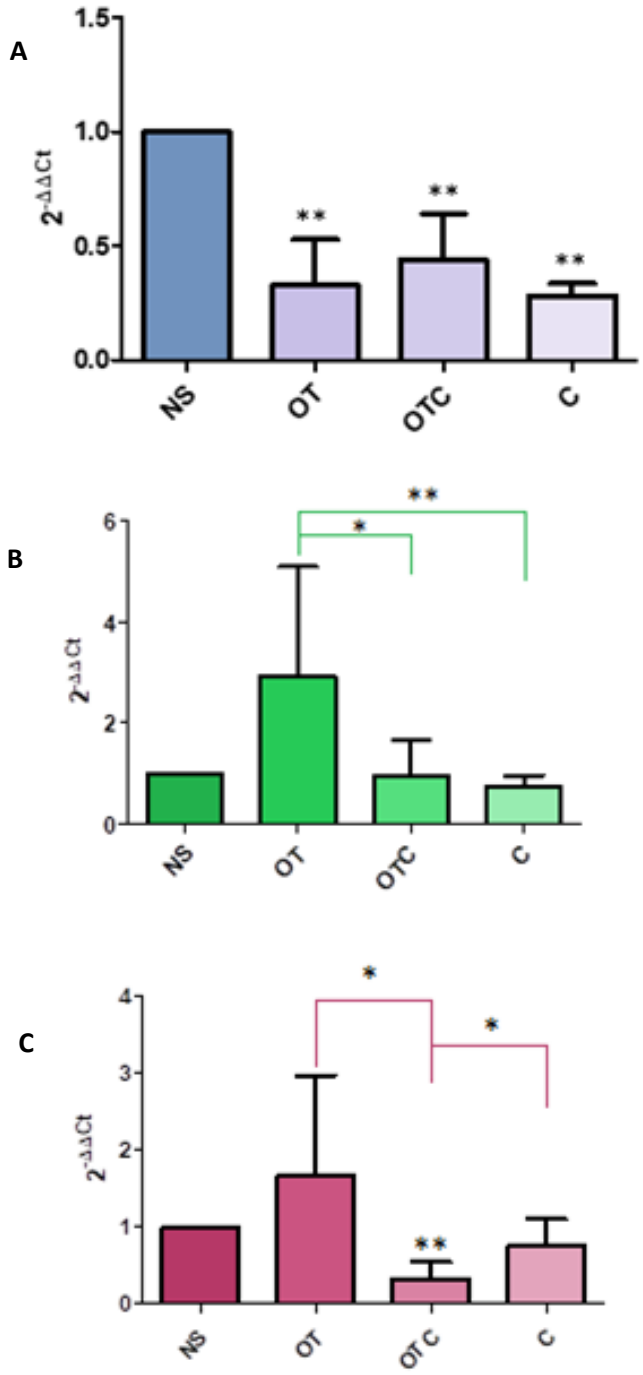

***Figure 18**. qPCR results showing change in expression (using $2^{-\Delta\Delta Ct}$) for GRα in SKOV3 (A), PEOI (B) and MDAH-2774 (C) cell lines after 48h treatment with 100nM OT, 100nM OT+C and 100nM C. All results are present ± SD and statistical significance was calculated using the paired t-test; *p<0.05, **p<0.01. Cortisol addition lowers GRα expression in both PEO1 and MDAH-2774 cell compared to OT treatment.*

5.3.5.2 Effect of OT and C on GRβ Expression

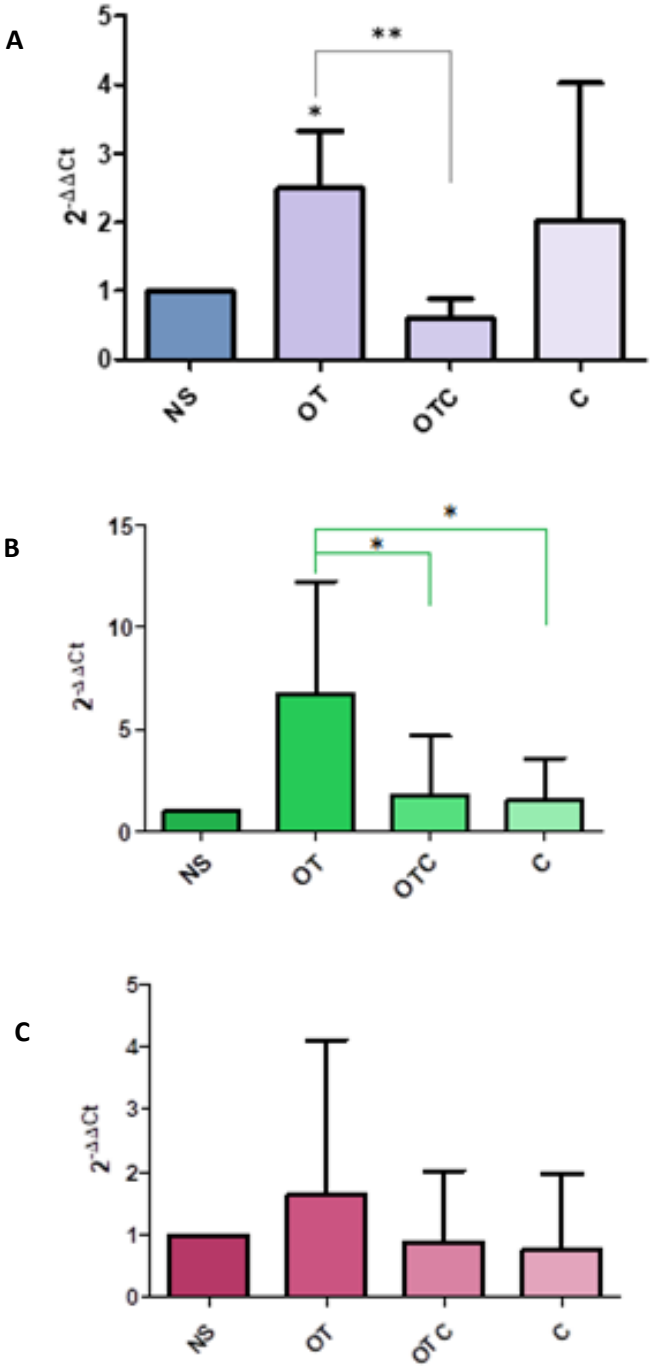

***Figure 19**. qPCR results showing change in expression (using $2^{-\Delta\Delta Ct}$) for GRβ in SKOV3 (A), PEOI (B) and MDAH-2774 (C) cell lines after 48h treatment with 100nM OT, 100nM OT+C and 100nM C. All results are present ± SD and statistical significance was calculated using the paired t-test; *p<0.05, **p<0.01. Addition of C to OT seems to lower expression of GRβ when compared to OT treated cells.*

5.3.5.3 Effect of OT and C on GRγ Expression

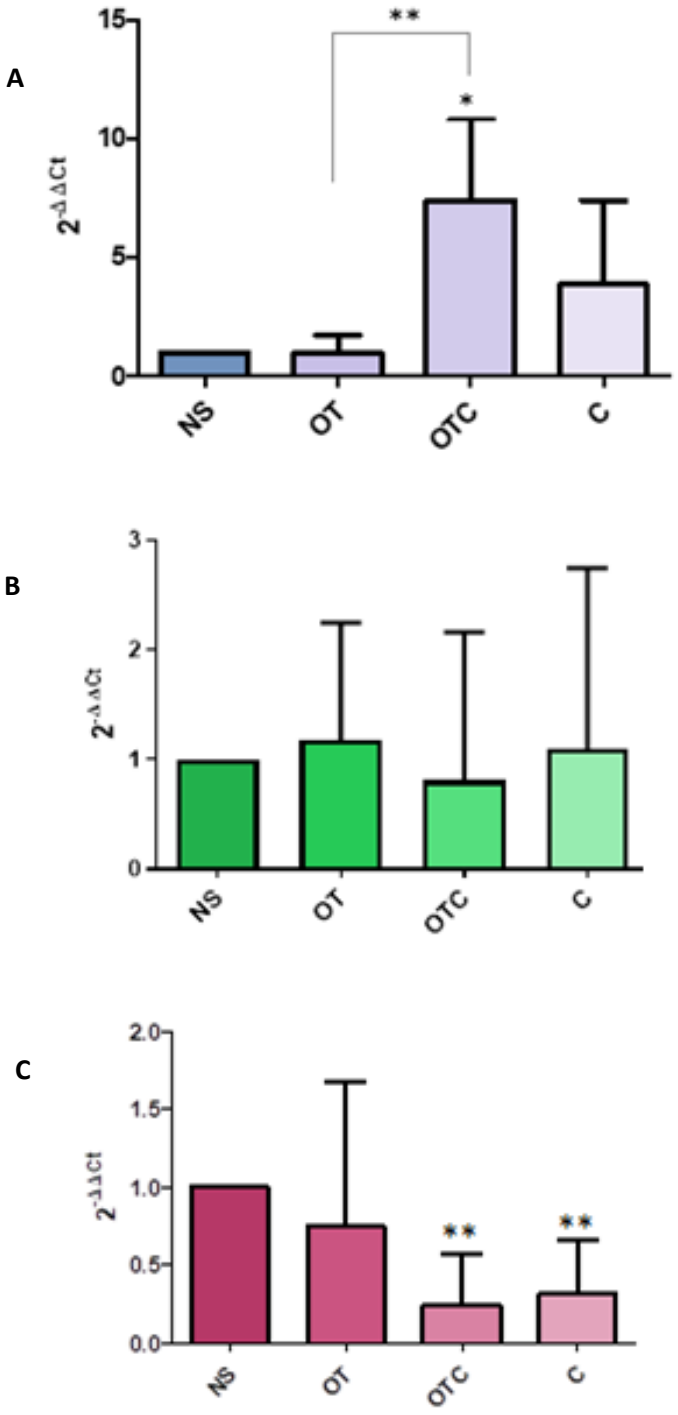

***Figure 20**. qPCR results showing change in expression (using $2^{-\Delta\Delta Ct}$) for GRγ in SKOV3 (A), PEOI (B) and MDAH-2774 (C) cell lines after 48h treatment with 100nM OT, 100nM OT+C and 100nM C. All results are present ± SD and statistical significance was calculated using the paired t-test; *p<0.05, **p<0.01. Treatments caused cell specific responses in GRγ expression.*

5.3.5.4 Effect of OT and C on GR-P Expression

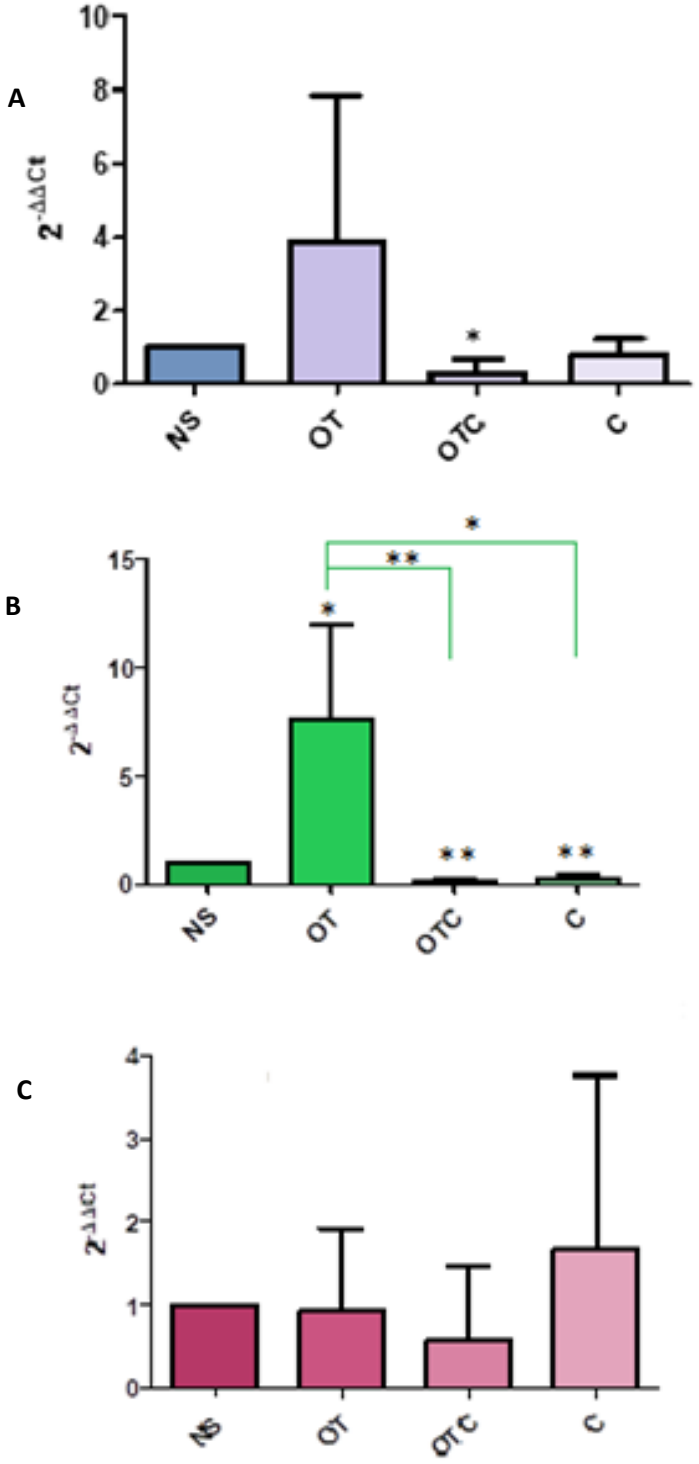

***Figure 21**. qPCR results showing change in expression (using $2^{-\Delta\Delta Ct}$) for GR-P in SKOV3 (A), PEOI (B) and MDAH-2774 (C) cell lines after 48h treatment with 100nM OT, 100nM OT+C and 100nM C. All results are present ± SD and statistical significance was calculated using the paired t-test; *p<0.05, **p<0.01. PEO1 cells increased GR-P expression in the presence of OT, and C addition was able to reverse and lower GR-P expression.*

Cells were treated for 48 hours in OT, combined OT+C and C, RNA was extracted and the relative fold change in GR's was measured using qPCR. Relative fold change after treatment was calculated using the ΔΔC*t* method. For SKOV3 cells, treatment with OT, OT+C and C significantly reduced expression of GRα (p=0.0001 for all three). Although not statistically significant, OT treatment seems to upregulate expression of GRα in both PEO1 and MDAH-2774 cell lines, and interestingly for both PEO1 and MDAH-2774 cells expression of GRα after OT treatment was significantly greater than treatment with OT+C (p=0.0183, p=0.0182 respectively) and C (p=0.0081, p=0.02 respectively).

Treatment with OT upregulated expression of GRβ in SKOV3 cells (p=0.0135). There also seems to be upregulation of GRβ after OT treatment in both PEO1 and MDAH-2774 cells however the data is not statistically significant. For both SKOV3 and PEO1 cells, combined OT+C treatments significantly reversed the effects OT had on GRβ expression (p=0.0002 and p=0.0292 respectively). The data for MDAH-2774 cells was inconclusive.

It is noted that all three cell lines have cell specific response to the hormone treatments. For GRγ expression in SKOV3 cells, treatment with combined OT+C increased expression (p=0.0143) whereas in MDAH-2774 treatment with OT+C and C decreased GRγ expression (p=0.0062 and p=0.009). Data for PEO1 cells was inconclusive.

It was very interesting to see that in PEO1 cells, OT treatment drove up expression of the GR-P splice variant (p=0.032). Treatment with both OT+C and C significantly drove down expression of GR-P (p<0.001 for both). Although not significant, OT seemed to also increase fold change in GR-P expression and significantly OT+C treatment lowered GR-P expression. Again data for MDAH-2774 cells was inconclusive.

5.3.6 Effect of OT and C on GAS5 Expression

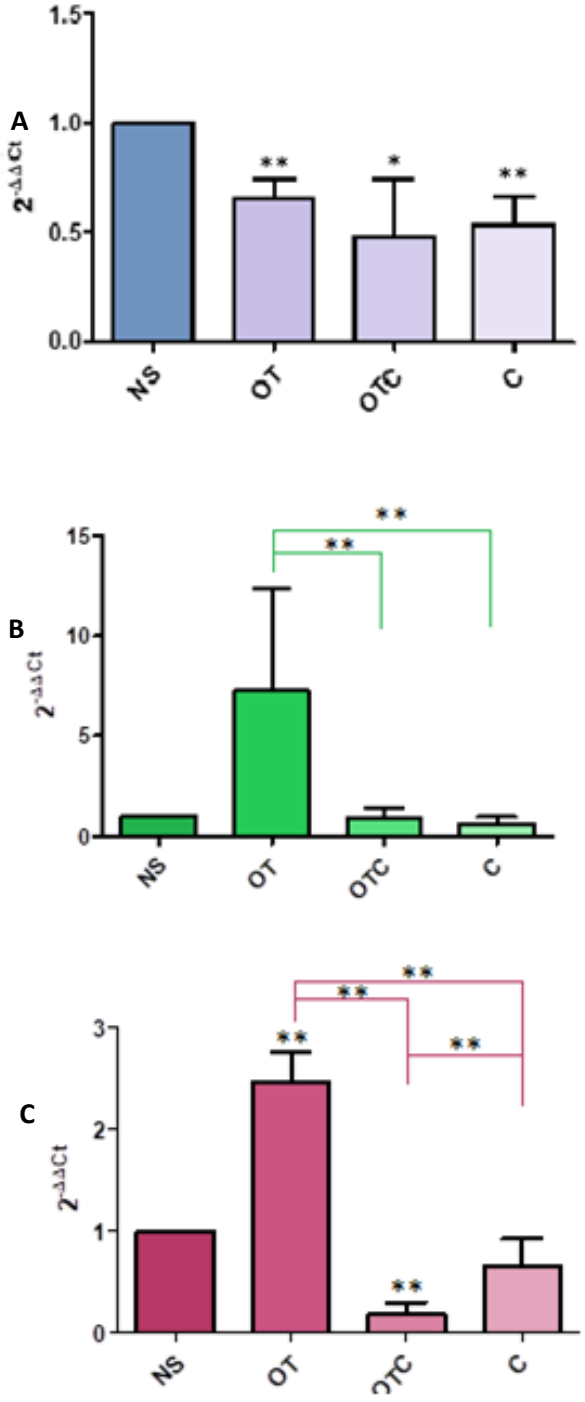

***Figure 22**. qPCR results showing change in expression (using $2^{-\Delta\Delta Ct}$) of GAS5 in SKOV3 (A), PEOI (B) and MDAH-2774 (C) cell lines after 48h treatment with 100nM OT, 100nM OT+C and 100nM C. All results are present ± SD and statistical significance was calculated using the paired t-test; *p<0.05, **p<0.01. GAS5 expression is elevated in the presence of OT for both PEO1 and MDAH-2774 cells and C addition is able to significantly lower GAS5 expression.*

The results of hormone treatments on the expression of GAS5 in all three cell lines mirrors the results seen in Figure 19 for the effects of hormone treatment on GRα expression. In SKOV3 cells all three treatments significantly lowered GAS5 expression in the same way GRα expression was lowered by all three treatments ($p=0.0005$, $p= 0.0134$, $p=0.0005$). Again what is seen in both PEO1 cells and in MDAH-2774 cells is C ability to reverse the effects of OT on GAS5 expression. In PEO1 cells C treatment did not significantly lower GAS5 expression compared to control, however OT+C and C treatments showed significantly lower expression compared to OT treated cells ($p=0.0021$ and $p=0.009$ respectively), meaning C presence counteracted the effect OT had. This is backed up by data from MDAH-2774 cells. The significant fold increase in GAS5 expression caused by OT treatment ($p<0.0001$) was reversed with the addition of C as both OT+C and C treated cells had significantly lower GAS5 expression than OT treated cells ($p<0.0001$ and $p<0.001$).

5.3.7 Effect of OT and C on OTR Expression

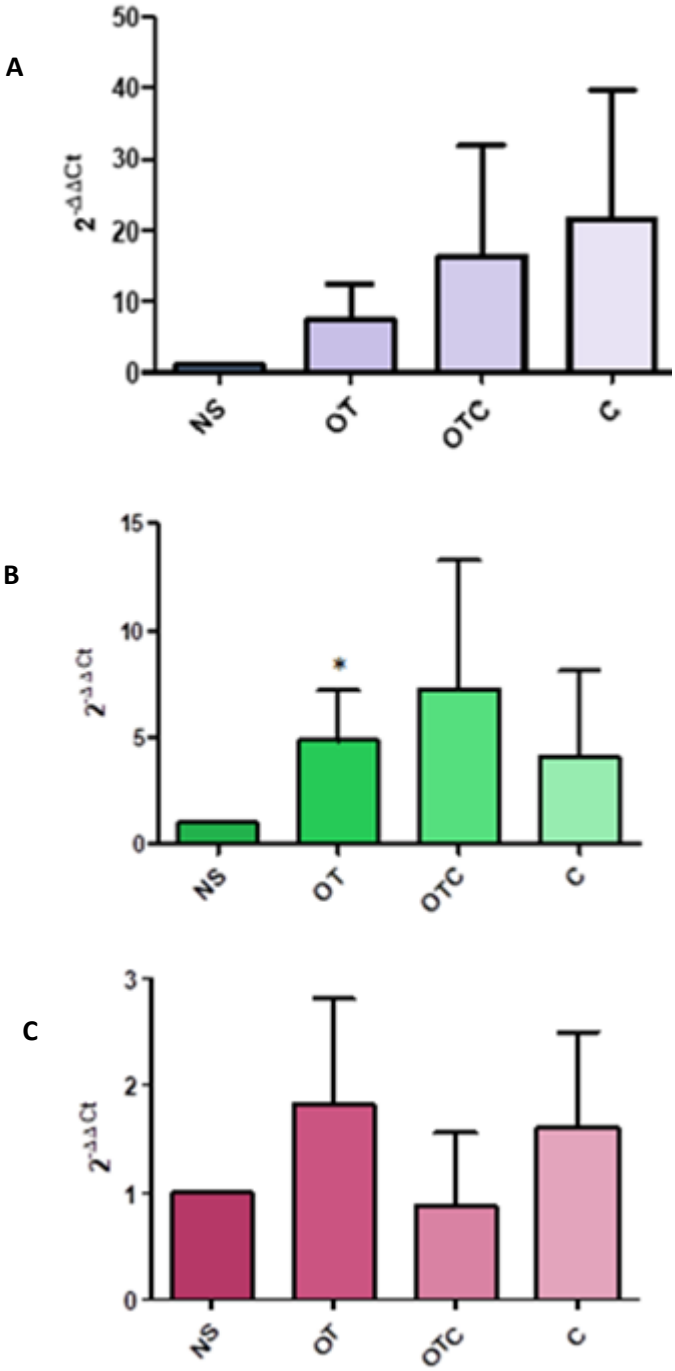

***Figure 23**. qPCR results showing change in expression (using $2^{-\Delta\Delta Ct}$) of the OTR in SKOV3 (A), PEOI (B) and MDAH-2774 (C) cell lines after 48h treatment with 100nM OT, 100nM OT+C and 100nM C. All results are present ± SD and statistical significance was calculated using the paired t-test; *p<0.05, **p<0.01. All cells produce a cell specific not significant response.*

Disappointingly, no real conclusion could be drawn for the expression of OTR in treated cells. PEO1 was the only cell line to show significant increase in OTR expression after treatment with OT, although data for SKOV3 and MDAH-2774 cells does show an increase though not statistically significant.

Our data and findings were published in 2016 in collaboration with Dr Yefei Pang and Dr Peter Thomas from the University of Texas at Austin, who carried out a GR luciferase reporter assay for the publication. SKOV3 cells co-transfected with GRE-Luc vector were incubated for 16h in 100nM OT, C and combined OT+C. OT treatment had no effect of GRE activity, C was able to significantly increase GRE activity ($p<0.01$) and interestingly, C in the presence of OT increased GRE activity significantly higher than C treatment alone ($p<0.01$) (Mankarious *et al.*, 2016).

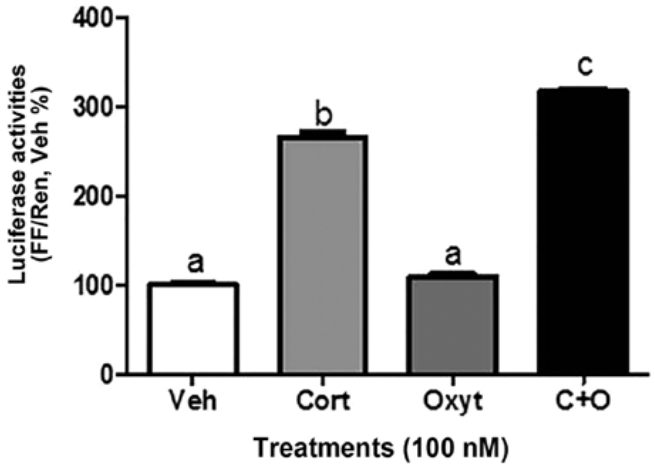

***Figure 24**. Effects of 16-h treatment with 100nM cortisol (Cort), 100nM oxytocin (Oxyt) alone and in combination (100nM C+O) on transactivation of human GR using a GRE-luciferase reporter system in SKOV3 cells. Data shown as mean ± SEM, n=3. Different letters denote values significantly different from each other (P<0.01) analysed by one-way ANOVA and Tukey post hoc test.*

As luciferase assays are extremely sensitive, they are used to determine repression or activation of target genes, even at very low changes in transcription. The data in figure 24 provides evidence that OT can transactivate the GR, therefore we hypothesis that OT drives differential splicing of GR's (as seen in figures 18-21) which may compromise GR signalling. GRβ was upregulated after OT treatment in all three cell lines (significant in SKOV3 $p<0.05$) which would act in a dominant negative manner and reduce GR signalling.

5.4 Discussion

The aim of this chapter was to investigate how oxytocin and cortisol are able to affect the mechanisms responsible for the regulation of cell proliferation and cell death. SKOV3, PEO1 and MDAH-2774 cells were treated for 48h in either oxytocin (O), cortisol (C) or combined OT+C and viable cell count, cell migration, expression of Caspase-3 and Beclin-1 were assessed. Data showed OT treatment was able to reduce viable cell count by increasing the activity and expression of pro-apoptotic proteins. OT may also potentially slow down cell migration in ovarian cancer cells (Mankarious *et al.*, 2016).

The results produced agrees with previously published data, which showed that OT treatment in SKOV3 cells reduces cell proliferation as well as reducing cell migration and invasiveness (Morita *et al.*, 2004). Its known for example, that OTR coupling to the Gqα subunit induces cell proliferation, whereas OTR coupling to the Gi α subunit inhibits cell growth in certain cell types in vitro (Reversi, Cassoni and Chini, 2006). The cell viability data in figure 14 and data produced by Morita (2004) suggests that in the three ovarian cancer cells, SKOV3, PEO1 and MDAH-2774, the OTR must couple with the Gi α subunit in the presence of OT which results in growth inhibition.

In figure 14, it can be seen that combining cortisol to oxytocin produced in all three cell lines a significantly greater number of viable cells compared to OT treatment alone, and a significantly lower viable cell count than treatment with C alone (seen in data for SKOV3). This was the first indication of a potential cross talk between the two hormones as the presence of C in the combined treatment reduced the effect of OT. Although cortisol has been shown to increase VEGF expression in SKOV3 cells (Lutgendorf *et al.*, 2003a) and increases the invasiveness of this cell line (Sood *et al.*, 2006), it does not stimulate cell proliferation in ovarian cancer cells and this was reaffirmed in figure 14.

The reduction in viable cell count for OT treated cells could have been brought on by the activation of caspase-3. For both SKOV3 and MDAH-2774 cells OT treatment increased expression of activated caspase-3 (figure 15) and in these cell lines, treatment with C and combined OTC was able to significantly reduce this activation ($p<0.001$ for all three cell lines).

As seen with the viable cell count data, combining OT with C seems to repress the effects OT treatment alone, furthering the notion of a potential cross talk between the two hormones. Runnebaum and Bruning (2005) found that cell death (brought on by apoptosis) was inhibited in cortisol treated ovarian cancer cell lines because of the up-regulation of a caspase inhibitor called cIAP2 (Runnebaum and Bruning, 2005). Looking at figure 15, for both the SKOV3 and the MDAH-2774 cell line, OT treatment significantly increases the expression of cleaved caspase 3, thus inducing apoptosis which would account for the low viable cell count witnessed in figure 14. Potentially, C treatment may have up-regulated cIAP2, which inhibited the cleavage of caspase3, preventing apoptosis and therefore explaining how OT+C treatment and C treatment produced significantly lower activated caspase-3 expression compared to OT treated, and greater viable cell counts.

For all cell lines Beclin-1 expression was significantly increased in cells treated with OT and this increases is reversed when treated with C (figure 16). The Beclin-1 protein is required for the formation of the autophagosome in autophagy. Beclin-1 is inhibited by the expression of BCL-2 in the cell (Erlich *et al.*, 2007). BCL-2 is from a family of apoptotic regulators that can be either anti-apoptotic or pro-apoptotic. The BCL-2 protein has anti-apoptotic properties and if BCL-2 is inhibited in the cell, the cell undergoes growth arrest and death, usually via the activated caspase 3 pathway (Zhu, Zhao and Liu, 2010). It is possible that in the presence of OT, BCL-2 may be inhibited and this would stop the BCL-2 from having an inhibitory effect on the Beclin-1 and so caspase 3 cleavage is initiated and cell growth inhibited as seen in figure 14. Sasson and Amsterdam (2002) published a study which showed that Human granulosa cells treated with dexamethasone had elevated levels of BCL-2 (Sasson and Amsterdam, 2002). It can be assumed that cortisol will have similar effects on the cell as dexamethasone as both glucocorticoids are able to bind to the glucocorticoid receptor, so it is possible that treatment with C may have increased expression of BCL-2 anti-apoptotic protein. This increase would inhibit Beclin-1 expression which was seen in figure 16. This also means it would prevent programmed cell death which is also seen in the cell viability assay in figure 14. Inhibition of Beclin-1 by BCL-2 would then result in the reduced cleavage of caspase 3 and

this was seen in figure 15 as cells treated with cortisol has reduced expression of the cleaved caspase 3.

Data in figure 17 did show that OT treatment caused slower rate of cell migration in all three cell lines which corresponds to what is already known; OT is able reduce cell migration in ovarian cancer cells (Morita *et al.*, 2004). It's now understood that OT is able to repress the activation of matrix metalloproteinase-2 (MMP-2) and VEGF which inhibits cell migration (Ji *et al.*, 2018). MMP-2, an enzyme involved in the breakdown of the extracellular matrix enabling cancerous cells to migrate, had lower expression and activation in OT treated SKOV3 cells. Their data proved OT treatment significantly reduced viable cell count and migration as their wound healing assay showed reduced rate of wound coverage after 48h ($p<0.05$) (Ji *et al.*, 2018). This corresponded with our findings. PEO1 cells were the only cell line where C treatment induced faster cell migration. The stress hormones norepinephrine and epinephrine increase migration and invasiveness in the three ovarian cancer cell lines; EG, 222 and SKOV3, yet cortisol has only been proved to effect invasiveness in SKOV3 and not in any other cell line (Sood, et al., 2006). Wound healing data results in figure 17 show that C treatment only produced a significant increase in the rate of cell migration in PEO1 cells although for both SKOV3 or MDAH-2774 cells and non-significant increase can be seen. It has previously been proposed that norepinephrine increases expression of MMP-2 in SKOV3 and EG cells (Sood, et al., 2006), but no data is currently available for MMP-2 expression in PEO1 or MDAH-2774 cell line. It can be hypothesised, that C treatment may increase MMP-2 expression/activation in PEO1 cells, and OT treatment may repress activation of MMP-2 which would explain why cell migration is faster in C treated cells compared to OT treated cells ($p<0.01$). Combined OT+C treatment in all three cell lines produced greater migration (though not significant) than OT treatment and lower migration compared to C treated cells (again non-significant) which may suggest potential cross talk between the two hormones on the effects they play on MMP-2 expression.

Quantitative PCR data for the effects of the three treatments OT, C and OT+C on expression of the GR's, GAS5 and the OTR in ovarian cancer cells produced cell specific results. In order to look for trends, cell specific responses, and patterns, the data was summarised as to display

overall changes. Tables 11, 12 and 13 present a simplified summary of fold changes per cell line with increases presented as (↑), decreases presented as (↓) and If no change was witnessed presented as (-). Statistical significance has been omitted for the purpose of simplifying and comparing the data.

Treatment	***GRα***	***GRβ***	***GRγ***	***GR-P***	***GAS5***	***OTR***
OT	↓	↑	-	↑	↓	↑
OTC	↓	↑	↑	↓	↓	↑
C	↓	↑	↑	↓	↓	↑

Table 11*. A Summary of expression changes for treated SKOV3 cells indicating which treatment increased fold change (↑), decreased fold change (↓), and had no effect (-) on expression of GR's, GAS5 and the OTR.*

Treatment	***GRα***	***GRβ***	***GRγ***	***GR-P***	***GAS5***	***OTR***
OT	↑	↑	-	↑	↑	↑
OTC	-	↑	↓	↓	-	↑
C	↓	-	↓	↓	↓	↑

Table 12. *A Summary of expression changes for treated PEO1 cells indicating which treatment increased fold change (↑), decreased fold change (↓), and had no effect (-) on expression of GR's, GAS5 and the OTR.*

Treatment	*GRα*	*GRβ*	*GRγ*	*GR-P*	*GAS5*	*OTR*
OT	↑	↑	↑	-	↑	↑
OTC	↓	-	↓	↓	↓	↓
C	↓	↓	↓	↑	↓	↑

***Table 13**. A Summary of expression changes for treated MDAH-2774 cells indicating which treatment increased fold change (↑), decreased fold change (↓), and had no effect (-) on expression of GR's, GAS5 and the OTR.*

The summary data presented in table 11 provides a clearer look at the effects, OT, OT+C and C had on the GR's, GAS5 and the OTR expression in SKOV3 cells. It was seen from the SKOV3 data that the changes brought about under OT treatment are reversed in cells treated with OT+C and C. Both OTC and C treatments lowered GRα, GR-P and GAS5, whilst increasing GRβ, GRγ and the OTR. As seen in WB data for caspase-3 expression (figure 15), the addition of C seems to reverse the initial effects brought on by OT. Regrettably, as some of the data does not show statistical significance no certain conclusions can be drawn from this observation, however it again points to potential cross talks. This trend was not observed in either PEO1 or MDAH-2774 cells.

Another interesting trend seen in the summary data for SKOV3, PEO1 and MDAH-2774 cells was that the resulting effect of each three hormone treatments on GRα and GAS5 changes. For all three cells lines, it was observed that as GR expressions increases/decreases, GAS5 expression also follows the same directional fold change. Table 14 groups GRα and GAS5 data for all three cell lines.

	SKOV3		PEO1		MDAH-2774	
Treatment	GRα	GAS5	GRα	GAS5	GRα	GAS5
OT	↓	↓	↑	↑	↑	↑
OTC	↓	↓	-	-	↓	↓
C	↓	↓	↓	↓	↓	↓

Table 14*. Summarized table showing the effects of OT, OT+C and C treatment on GRα and GAS5 for all three cell lines. GRα and GAS5 always respond in the same way in a cell specific manner*

As discussed in chapter 4, GAS5 is responsible for the suppression of activated GRα by acting as a decoy for the GRE's. Figures 11 and 12 for baseline expression of GR's and GAS5 in ovarian cancer cells indicated that SKOV3 cells had low GRα expression and also had low GAS5 expression. Both PEO1 and MDAH-2774 cells with relatively high GRα expression also had relative high expression of GAS5, leading to the suggestion that GRα expression is associated with GAS5 expression and they might be regulated in similar manner. This association seems to hold true when cells were treated with OT, OT+C and C as seen in table 14, in all treatments, GAS5 responded as GRα did.

A study in 2019 found GAS5 overexpression in nucleus pulposus cells (NPC's) leads to the increased expression of caspase-3 and leased to the decrease in Bcl-2 expression (Wang *et al.*, 2019). High expression of GAS5 in ovarian cancer cells also suppresses cell proliferation and migration. In ovarian tumour tissue, lower expression levels of GAS5 was associated with poor prognosis, deeper invasive depth and higher tumour stage (Li *et al.*, 2016).For both PEO1 and MDAH-2774 cells, OT treatments increased GRα and GAS5 expression, whereas OT+C and C treatments reversed these changes. Potentially, OT increases GAS5 expression in PEO1 MDAH-2774 cells, this leads to activation and higher expression of caspase-3 (verified in figure 15) which would then drive down viable cell count (complementing data in figure 14). Treatment with C will then reverse this by decreasing GAS5 expression in cells, driving up Bcl-

2 expression, supressing caspase-3 activity therefore reversing the apoptotic effect OT had on the cells.

The response in SKOV3 cells is different, OT drives down expression of both GRα and GAS5 and yet still produced similar WB and viable cell data as PEO1 and MDAH-2774 cells. It's known that in certain cells, upon OT binding to the OTR, the receptor couples with G_q to promote cell proliferation, and in others, coupling to G_i inhibits cell growth. Potentially, OTR couples with G_i in SKOV3 cells to reduce viable cell count and activate the pro-apoptotic mechanisms within the cells without the need for high GAS5 expression.

Evidently from the data collected, it was observed that all three ovarian cancer cell lines had cell specific responses to each of the treatments. The variation seen in each cells response was due to variations in the differential state of each cell line, of the known mutations found within each cell line and with the subtype. SKOV3 human ovarian clear cell adenocarcinoma were derived from the ascites of a 64 year old Caucasian female. These cells have mutations in TP53 and PIK3CA genes, and these cells have high migratory potential (Bai *et al.*, 2015). The PEO1 cell line was derived from a malignant effusion from the peritoneal ascites of a patient with a poorly differentiated serous adenocarcinoma. MDAH-2774 cells are of human ovarian endometrioid adenocarcinoma origin, isolated from the ascites of an untreated female patient which have mutations in TP53, PIK3CA, KRAS, BRCA1 (silent) and BRCA2 (silent) (Beaufort *et al.*, 2014).

6.0 OvC Tissue express higher levels of GR-P and OTR, and high GR expression reduces survival in OvC patients.

6.1 Introduction

It is essential that *in-vitro* studies carried out on cells are supplemented with clinical data. Though established as ideal models for ovarian cancer, the cell lines SKOV3, PEO1 and MDAH-2774 have originated from three individuals respectively and do not account for ovarian cancer patient's different stages, subtypes and grades, as well as ages, backgrounds and social support status. The use of clinical samples allows better insight into elevated, or downregulated levels of key genes and proteins that can be later used as tools for earlier diagnosis and better prognosis. There is very little, if any, published data on expression of the GR's, GAS5 and the OTR in ovarian cancer tissue. One study in 2009 measured GR expression in ovarian tissue taken from patients undergoing exploratory laparotomy for suspected ovarian cancer. Patients were either administered dexamethasone (a synthetic glucocorticoid) or saline solution 30 minutes before the laparotomy and qPCR was used to asses expression of GR, glucocorticoid-regulated kinase 1 (SGK1) and map kinase phosphatase 1 (MKP1) in collected tissue. No changes in GR expression was detected however there was upregulation of SGK1 and MKP1 in dexamethasone treated tissue compared to controls. The study concluded that potentially the use of pharmacologic doses of glucocorticoids in chemotherapy may decrease its effectiveness in ovarian cancer patients through increasing expression of anti-apoptotic genes (Melhem *et al.*, 2009). Another study in 2015 reached the opposite conclusion. They proved glucocorticoid treatments induced expression of miR-708, a microRNA with the ability to regulate expression of other regulatory genes (Jang *et al.*, 2012), which in turn inhibited ovarian cancer cell migration and invasion in an orthotopic xenograft mouse model. Their clinical data showed lower expression of miR-708 in late stage ovarian cancer tissue compared to early stage and normal ovarian tissue, causing them to propose the novel use of glucocorticoids in conjunction with chemotherapy as a more effective treatment for ovarian cancer (Lin *et al.*, 2015).

Oncomine is a cancer microarray database which houses online genome expression analysis from published data. This platform contains expression analysis data on millions of genes that have been analysed in cancerous and normal tissue and allows the user to actively compare

differential expression of selected genes between different cancers, different subtypes of the same cancers and in normal tissue (Rhodes *et al.*, 2004). Oncomine will be used in this study to determine if OTR, GAS5 and GR expression in published data supports our findings.

Kaplan-Meier Plots, better known as KM plots, are graphs used to estimate the probability that a patient will survive after a period of time. Its main use in medical research is to measure the probable fraction of patients alive after treatment at certain time points (Kleinbaum and Klein, 2012). The data represents the probability of survival for a hypothetical population not the actual current percentage of patient's surviving. The plots are used in this study to assess if higher/lower expression of GR and OTR increase/reduce risk of survival in patients with ovarian cancer.

6.2 Objectives

1. To compare expression of GRs, GAS5 and the OTR present in ovarian cancer tissue and normal ovarian tissue.
2. To measure protein expression of GRα in 70 ovarian cancer samples (tissue microarray) and to assess if tissue from patients diagnosed at different stages of ovarian cancer have different levels of GRα expression.
3. To compare gene expression of OTR, GR and GAS5 in normal and ovarian cancer tissue using *in silico* analyses based on microarray and RNAseq data.
4. To compare the probability of patient survival in patients exhibiting high/low gene expression of GR and OTR using KM plots.

6.3 Results

6.3.1 Relative expression of GR's in Normal and Ovarian Cancer tissue

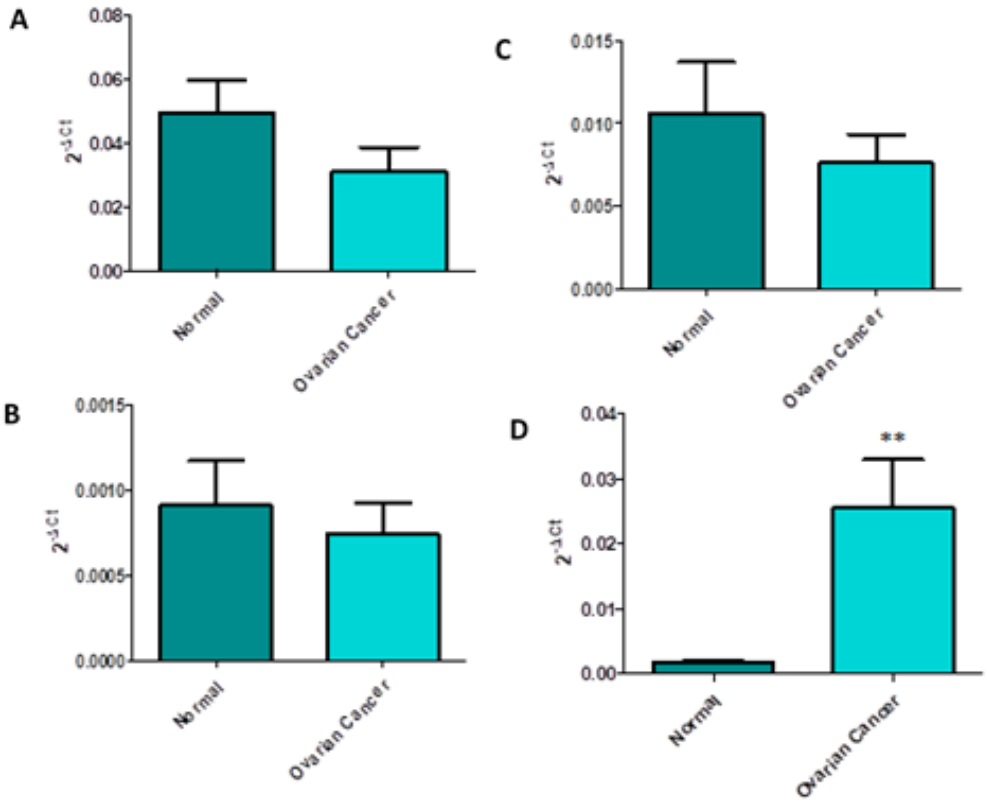

Figure 25. *Base line receptor expression presented as $2^{-\Delta Ct}$ in both normal and ovarian cancer clinical tissue for GRα (A), GRβ (B), GRγ (C) and GR-P (D). All results are present ± SD and statistical significance was calculated using the unpaired t-test; *p<0.05, **p<0.01. Ovarian Cancer patients have elevated levels of GR-P.*

GRα expression in ovarian cancer tissue was lower than expression in normal ovarian tissue, though just outside of the statistical parameters (p=0.56). No statistical difference was seen in expression of GRβ and GRγ between normal and ovarian cancer tissue, however it should be noted that these receptors have very low expression compared to expression of GR-P and GRα, seen by the very small Δ*Ct* values. As with data from GRα, although not significant there seems to be less expression of GRβ and GRγ in ovarian cancer tissue compared to normal. The receptor that really proved to differ between normal and cancerous tissue was GR-P. The receptor is greatly expressed in ovarian cancer tissue compared to normal tissue with more than a 6-fold increase in GR-P mRNA levels (p<0.001).

6.3.2 Relative expression of GAS5 and the OTR in Normal and Ovarian Cancer

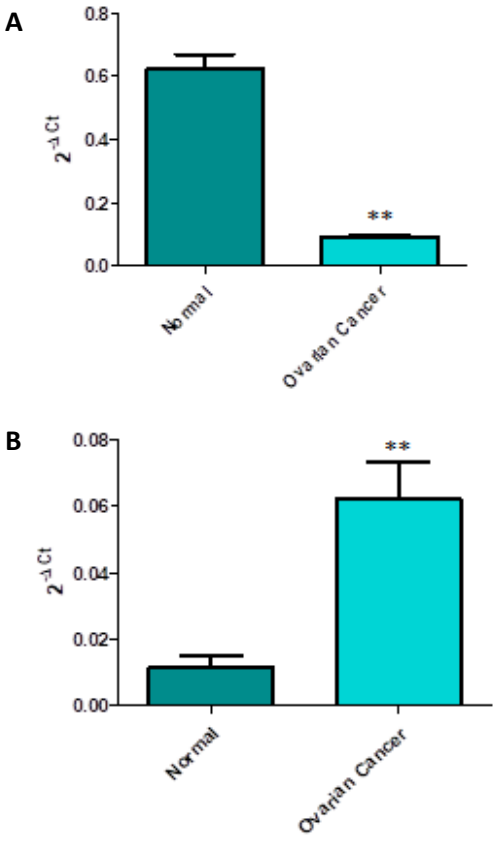

Figure 26*. Base line receptor expression presented as* $2^{-\Delta Ct}$ *in both normal and ovarian cancer clinical tissue for GAS5 (A) and OTR (B). All results are present ± SD and statistical significance was calculated using the unpaired t-test; *p<0.05, **p<0.01. Ovarian Cancer patients have significantly lower GAS5 expression and much higher OTR levels.*

The data for GAS5 expression in figure 26 produced similar Δ*Ct* values as data for PEO1 and MDAH-2774 cells in figure 11. GAS5 expression in MDAH-2774 cells had a Δ*Ct* value of 0.109, and in ovarian cancer tissue the Δ*Ct* value was 0.09, indicating that MDAH-2774 cell lines might be better ovarian cancer tissue model than the other two cell lines. GAS5 is significantly under expressed in ovarian cancer tissue compared to normal ovarian tissue ($p<0.001$) with levels in normal tissue being 7 times greater than that in ovarian cancer tissue. Patients with ovarian cancer also had a 6 fold increase in OTR expression when compared to normal ($p<0.001$). Interestingly, data from qPCR data for baseline expression of the OTR in the three cell lines (figure 11) did not collaborate the clinical sample data. OTR Δ*Ct* values in all three cell lines (SKOV3=0.018, PEO1=0.004 and MDAH-2774=0.005) was similar to base line levels for OTR in normal ovarian tissue (0.011) and dramatically less than the Δ*Ct* value for the expression of OTR in ovarian cancer tissue (0.06).

6.3.3 Immunohistochemistry on tissue array for the expression of GRα

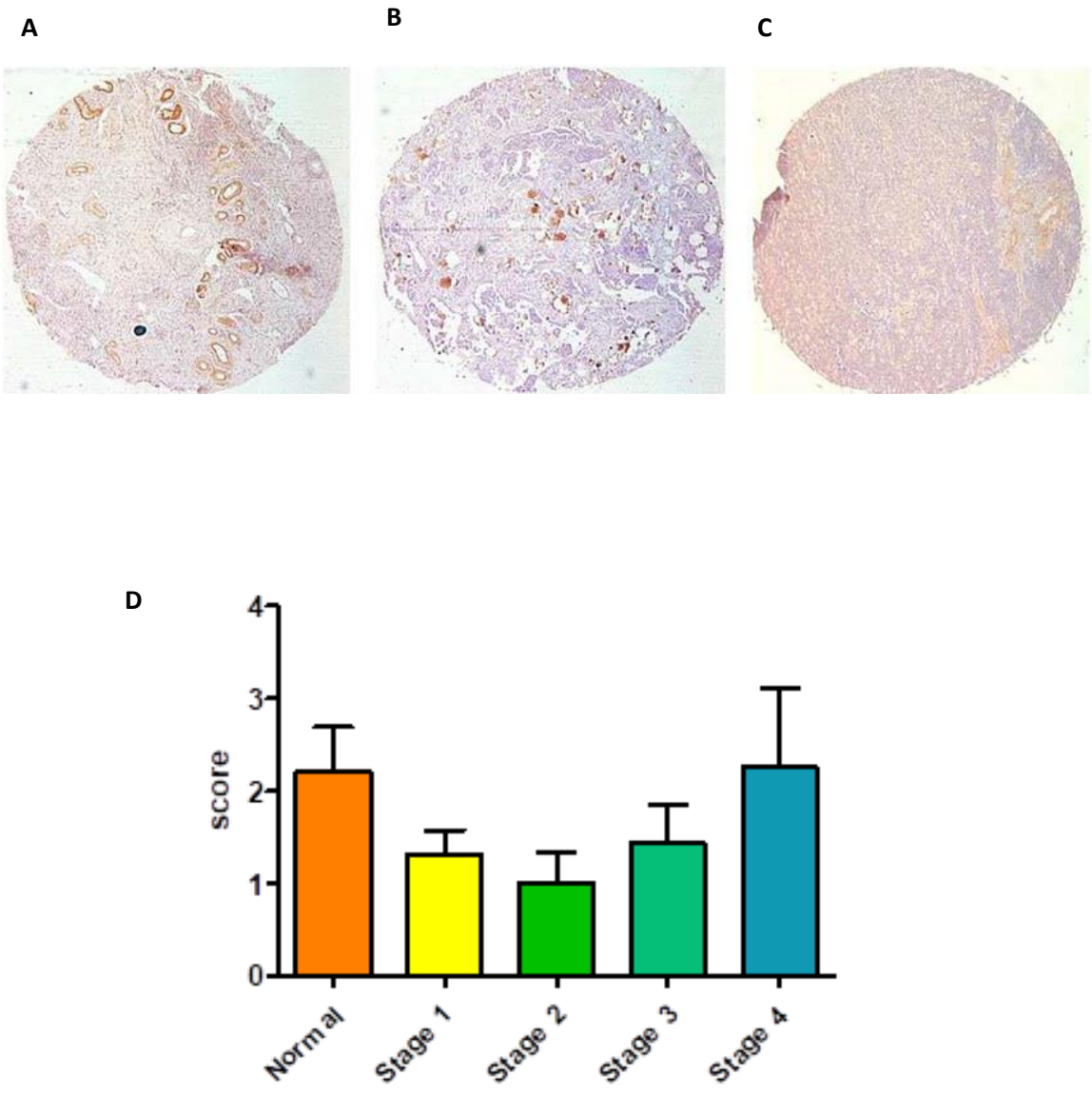

***Figure 27**. Images for protein expression of GRα in Normal controls (A), in early stage serous papillary carcinoma (B) and in late stage serous papillary carcinoma (C). Average score (calculated according to Table 9 in section 3.7.1) for expression of GRα in Normal and different staged ovarian cancer tissue. All results are present ± SD and statistical significance was calculated using the unpaired* t*-test; *p<0.05, **p<0.01. Though statistically*

insignificant GR-α expression appears to be lower in early stage OvC and increases as the disease progresses.

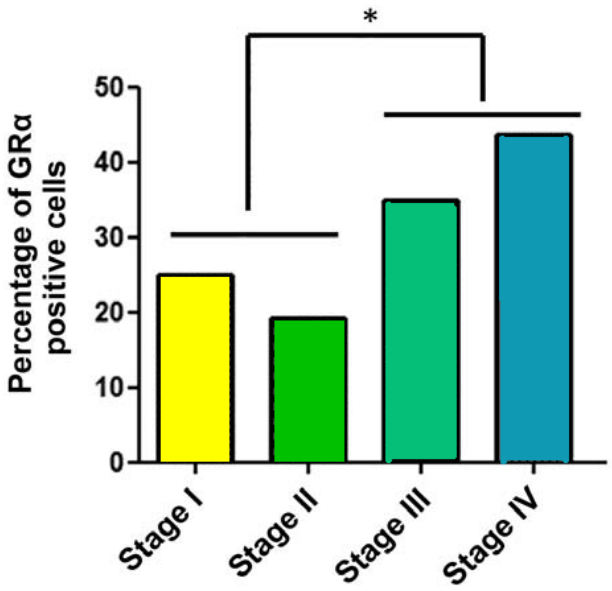

***Figure 28**. Data for GRα expression in different staged ovarian cancer tissue, this time protein expression is presented at percentage of positive staining (calculated by counting all stained cells/total cells in field of view and multiplying by 100). In order to increase statistical power, data was grouped in early stages (I and II) versus late (III and IV). Log transformation of GRα expression showed late stage patients had significantly higher GRα expression (1.2) than early stage patients (0.4) * p=0.015*

Figure 28 presents the immunohistochemistry results for the expression of GRα in ovarian cancer tissue (n=70) and normal tissue (n=5). Expression was calculated (see table 9) as a score based on the percentage of cells per case staining positive for GRα. GRα expression appears lower in ovarian cancer tissue than in normal tissue, especially in stage I, II and III cancers. It should be noted that off the ovarian cancer cases present on the tissue micro-array slide, 43.9% were stage I, 21.1% were stage II, 28.1% were stage III and only 7% were stage IV. For this reason, the SD bars drawn on stage 4 are greater than the other plots. The same

data was presented again in figure 28 without using the scoring system set out in table 9. Data was plotted as percentages of positively stained cells per tissue sample and grouped together; I with II and III with IV, to show early and late stages of ovarian cancer. This increased data population in both groups allowing for a fairer and more effective statistical analysis. The results indicate a significant difference in the expression of GRα between early stages of ovarian cancer and the late stages of ovarian cancer (p=0.015).

6.4 *in-silico* analysis using Oncomine

6.4.1 Expression of the OTR in Ovarian Cancer and Normal tissue

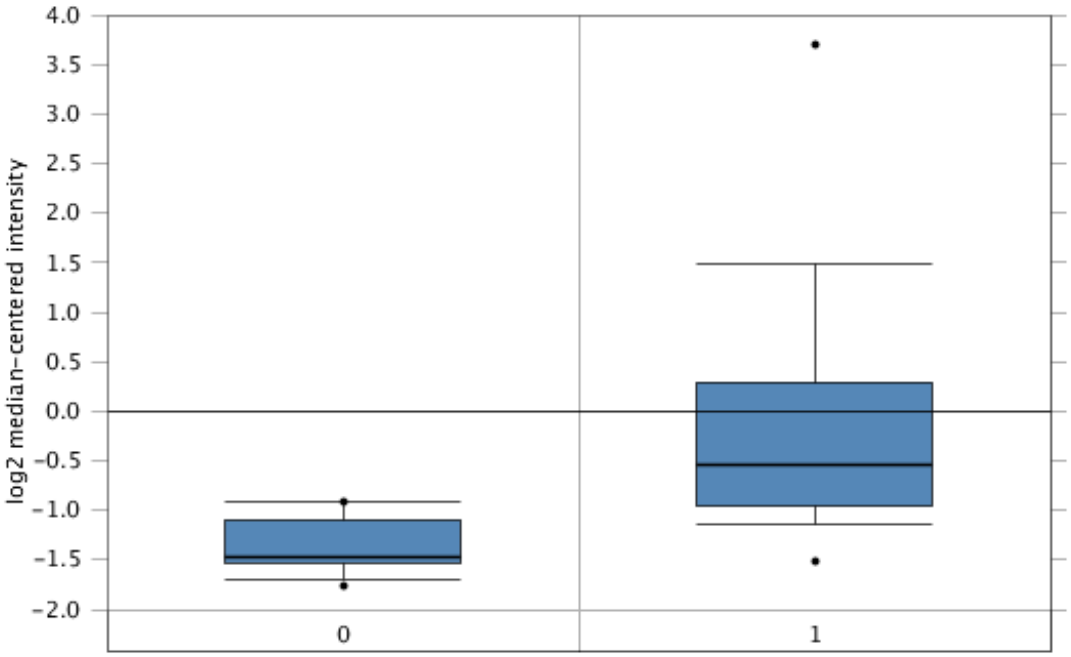

***Figure 29**. OTR expression data from the Bonome et al., 2008 dataset plotted by Oncomine. Graph shows the mean OTR gene expression in Normal (0, n=10) and Ovarian Carcinoma (1, n=185). Box plots show the 25th, the median and 75th percentile, outer bars show the 10th and the 90th percentile. The complete spread of data is highlighted with a *. There is a 2.347 fold change in OTR expression in ovarian carcinoma versus normal (p<0.0001).*

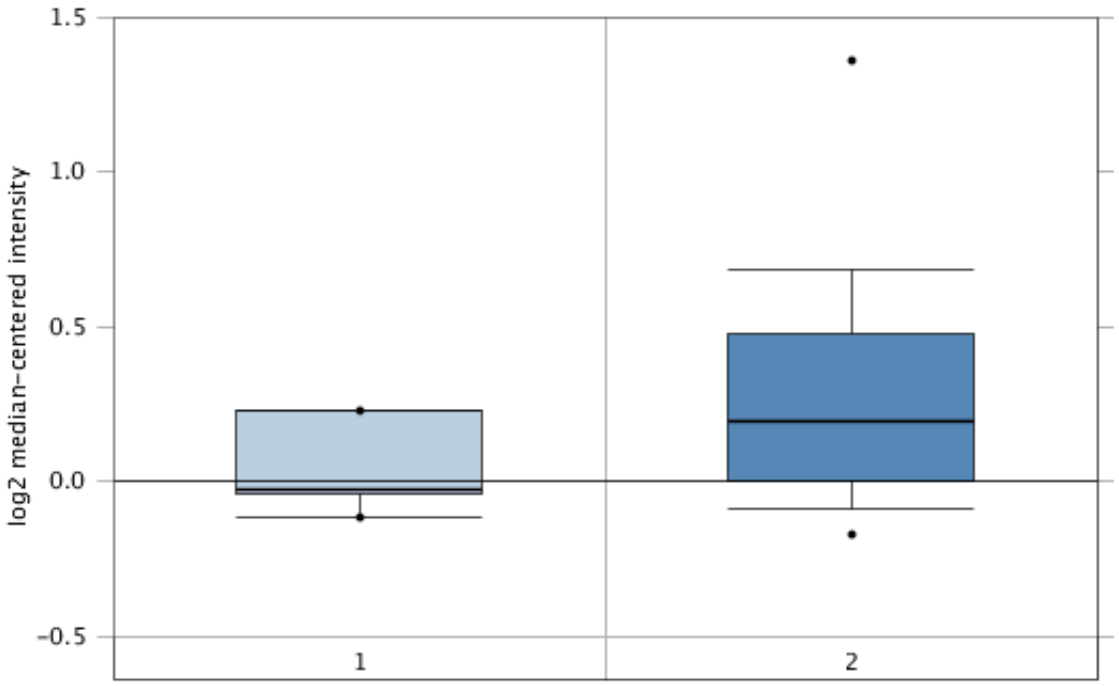

***Figure 30**. OTR expression data from the Hendrix et al., 2006 dataset plotted by Oncomine. Graph shows the mean OTR gene expression in normal ovary (1, n=4) and ovarian serous adenocarcinoma (2, n=41). Box plots show the 25th, the median and 75th percentile, outer bars show the 10th and the 90th percentile. The complete spread of data is highlighted with a *. There is a 1.190 fold change in OTR expression (p=0.015).*

In both figures 30 and 31, it can be seen that there is significant upregulation of the OTR in ovarian carcinoma and in ovarian serous adenocarcinoma. There is a 2.347 fold chance in OTR expression between normal ovarian surface epithelium and ovarian carcinoma ($p<0.0001$) and in ovarian serous adenocarcinoma the fold change is 1.19 ($p=0.015$) (Hendrix *et al.*, 2006; Bonome *et al.*, 2008). This reflects our findings in figure 27 where OTR was greatly expressed in ovarian cancerous tissue compared to normal ovarian tissue.

6.4.2 Expression of GR in Ovarian Cancer and Normal tissue

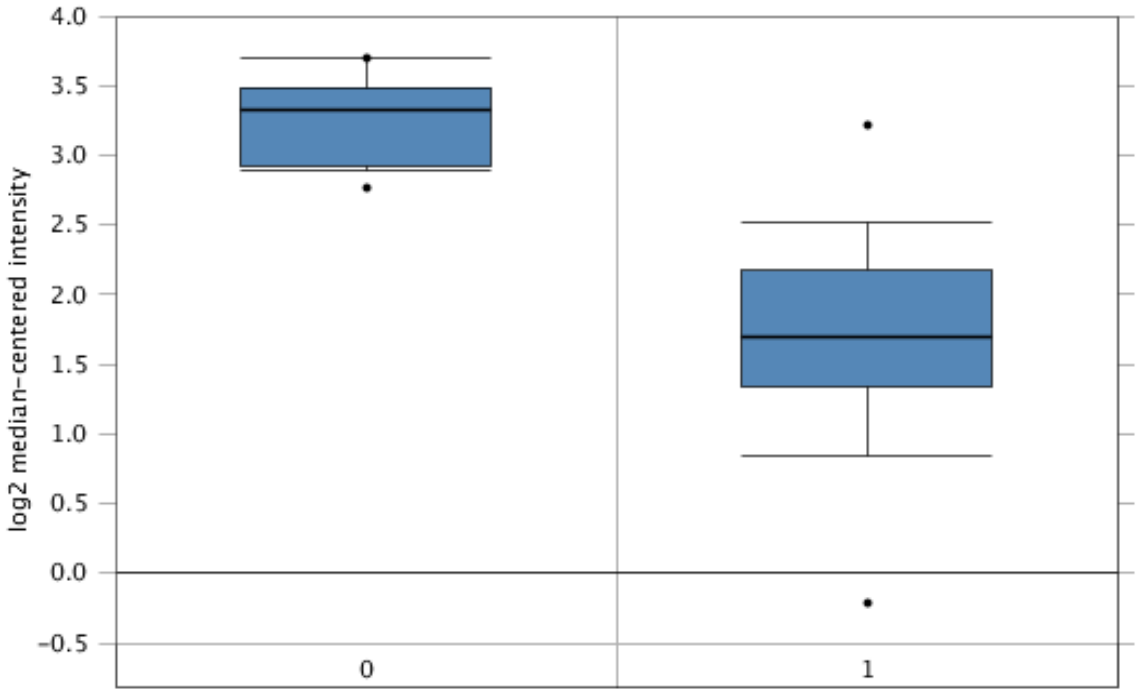

Figure 31. *GR expression data from the Bonome et al., 2008 dataset plotted by Oncomine. Graph shows the mean GR gene expression in Normal (0, n=10) and Ovarian Carcinoma (1, n=185). Box plots show the 25th, the median and 75th percentile, outer bars show the 10th and the 90th percentile. The complete spread of data is highlighted with a *. There is a -1.338 fold change in GR expression in ovarian carcinoma versus normal (not statistically significant, p=0.986).*

The expression data in figure 32 is for the expression of the GR gene (NR3C1) and doesn't differentiate between the different splice variants. As expression of GRβ and GRγ is relatively low in all cells and tissues (Yudt and Cidlowski, 2002) and GR-P spice variant is missing exon 8 and 9 (Turner *et al.*, 2010) it is assumed that figure 32 shows expression differences of the GRα splice variant between normal and ovarian carcinoma. There is a negative 1.338 fold

change, which although non-significant (p=0.986), is a reflection of the data in figure 26 where there was a reduction in GRα expression between normal and ovarian cancer tissue (p=0.056).

Interestingly, Hendrix et al, 2006 has data for the fold change in GR expression between normal ovarian tissue and ovarian clear cell adenocarcinoma (fold change of -1.215), ovarian mucinious adenocarcinoma (-1.245), ovarian serous adenocarcinoma (-1.135) and ovarian endometrioId adenocarcinoma (-1.246) which all display lower expression in cancerous tissue compared to normal, however none of the data is statistically significant (Hendrix *et al.*, 2006).

6.4.3 Expression of GAS5 in Ovarian Cancer and Normal tissue

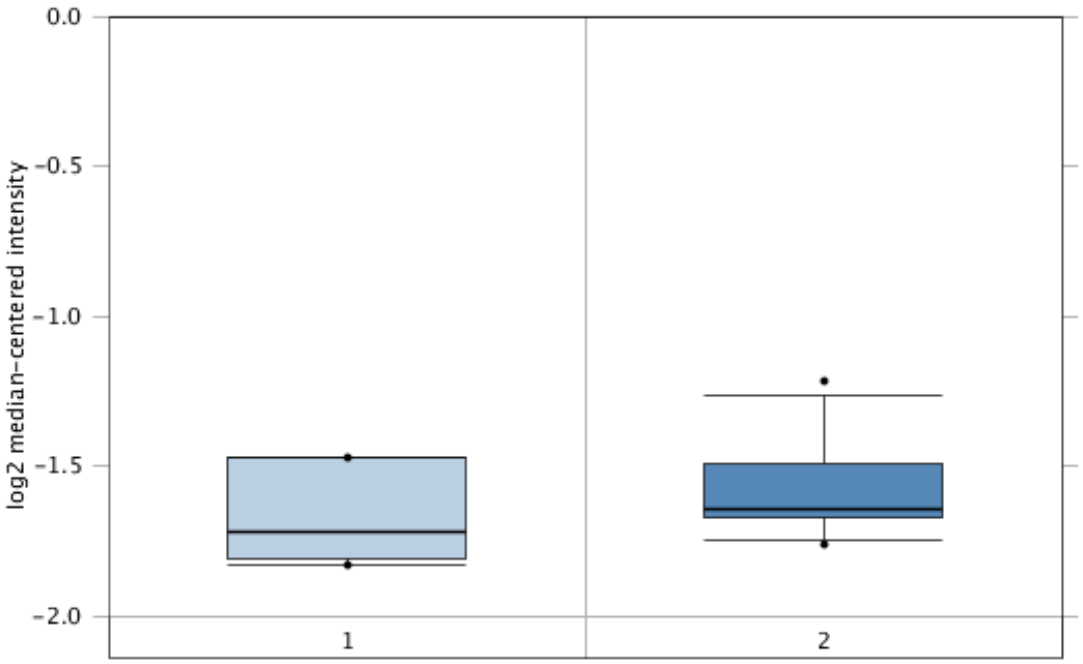

***Figure 32**. GAS5 expression data from the Lu et al., 2004 dataset plotted by Oncomine. Graph shows the mean GAS5 gene expression in Normal (1, n=5) and ovarian serous adenocarcinoma (2, n=20). Box plots show the 25th, the median and 75th percentile, outer bars show the 10th and the 90th percentile. The complete spread of data is highlighted with a *. There is a 1.071 fold change in GAS5 expression in ovarian serous adenocarcinoma versus normal (not statistically significant p=0.112).*

Data from figure 32 also supports our findings. GAS5 shows a 1.071 fold increase in ovarian serous adenocarcinoma compared to normal ovarian tissue, however the p value was greater than 0.05 (p=0.112). Lu et al, 2004, also provides data for GAS5 expression in ovarian clear cell adenocarcinoma (fold change of 1.093), ovarian mucinious adenocarcinoma (1.012) and ovarian endometrioId adenocarcinoma (1.110) where in each cancerous tissue there is an increase in fold change, although as before, no statistical significance was established (Lu *et al.*, 2004).

6.5 Kaplan-Meier Plots

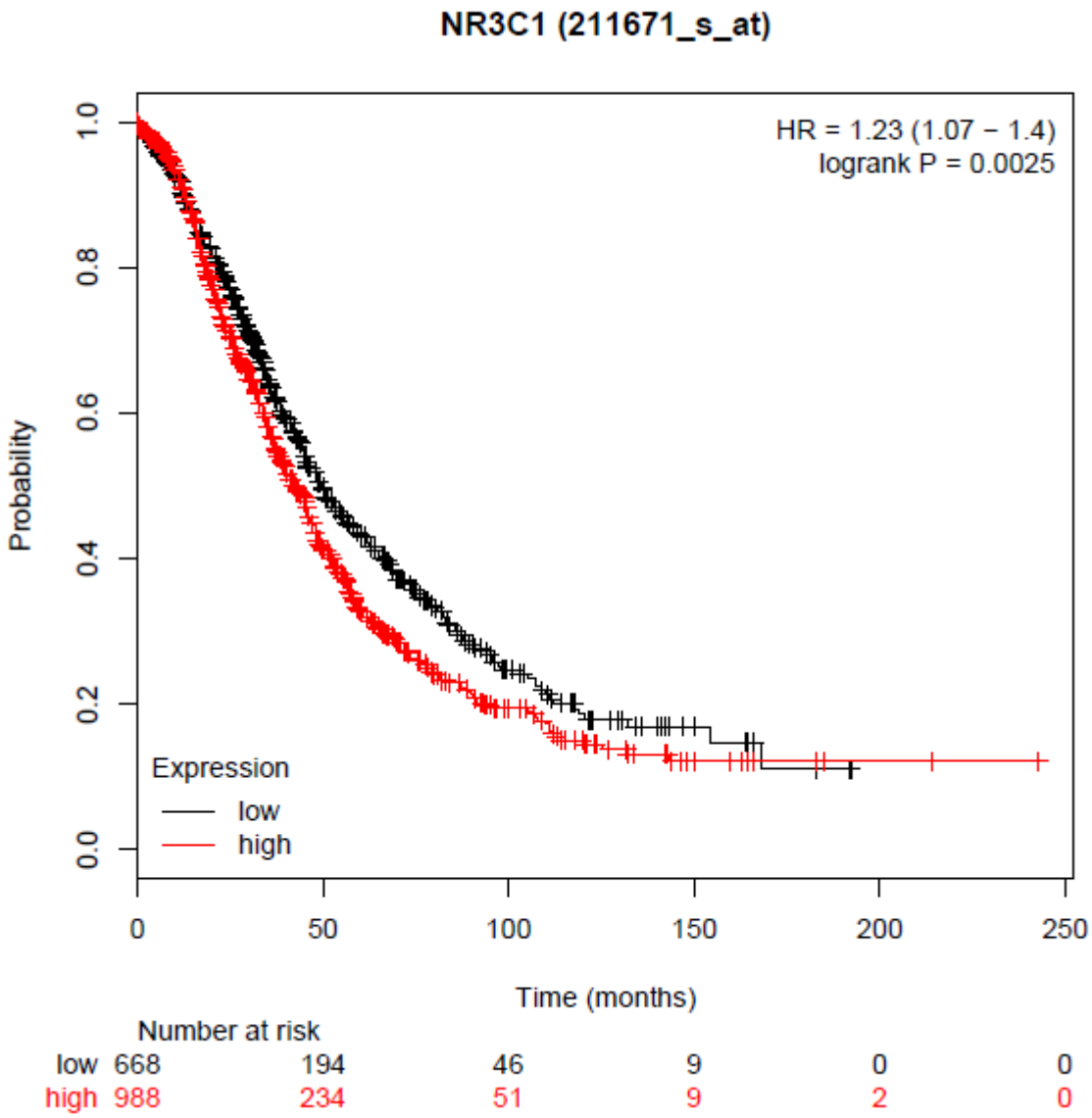

***Figure 33**. KM plot for the survival probability for ovarian cancer patients with low (black line) expression of the NR3C1 (GR) gene, and high (red line) expression of GR. Patients with high GR expression are less likely to survive past 50 months compared to patients with low GR expression (p=0.0025). Note that this expression does not differentiate between splice variants.*

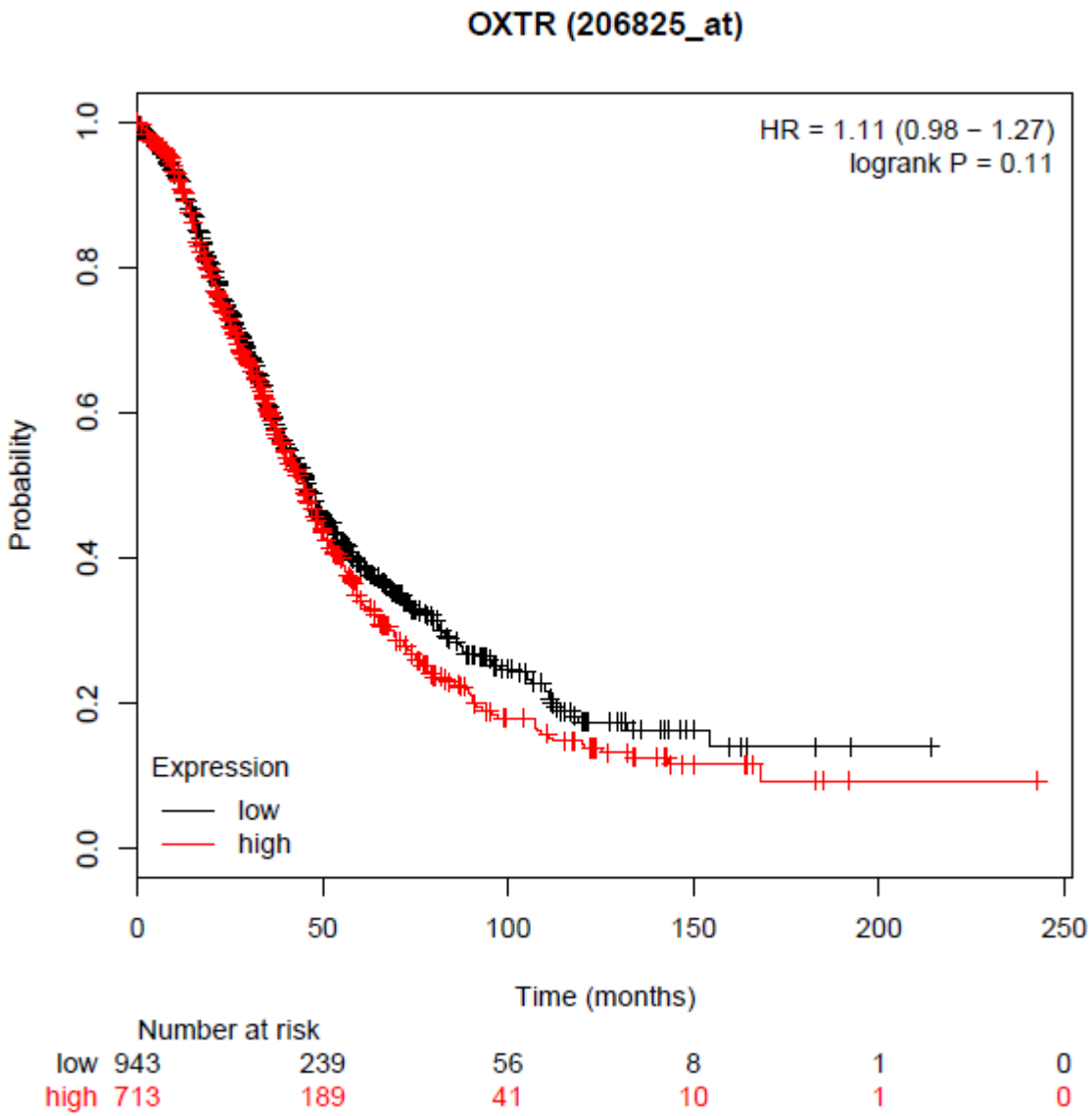

***Figure 34**. KM plot for the survival probability for ovarian cancer patients with low (black line) expression of the OTR gene, and high (red line) expression of OTR. Patients with high OTR expression seem less likely to survive past 80 months compared to patients with low GR expression however p value is greater than 0.05 (p=0.11)*

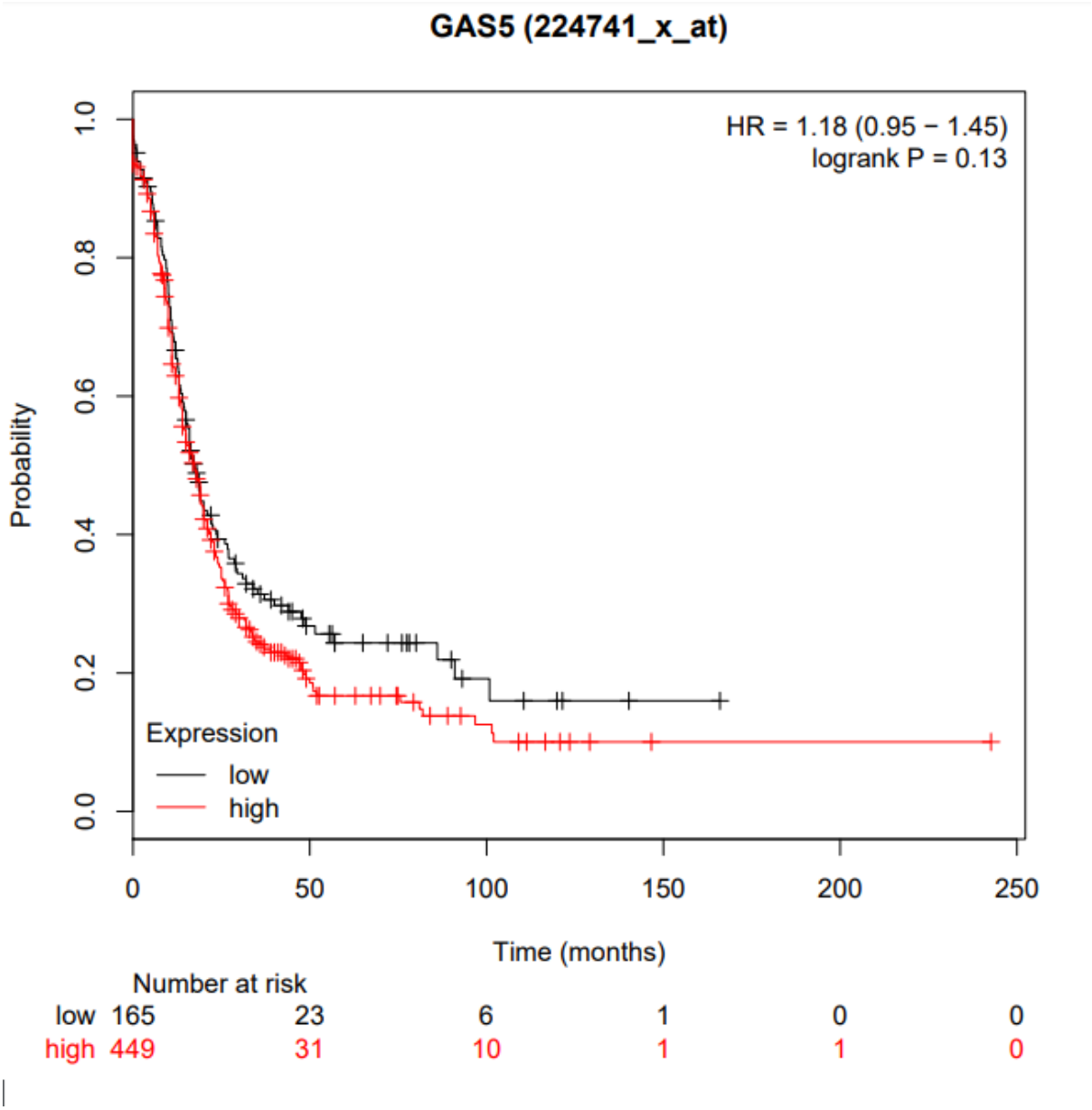

***Figure 35**. KM plot for the survival probability for ovarian cancer patients with low (black line) expression of the GAS5 gene, and high (red line) expression of GAS5. Probability of survival for patients expressing higher levels of GAS5 seems lower than the probability of survival for those expressing low GAS5 levels, but data is not statistically significant (p=0.13)*

Probability of survival begins to decrease as time extends after treatment for all ovarian cancer patients (figure 33). After the 50 month mark however, patients with higher GR expression have a lower probability of survival (approximately 0.4) compared to patients with lower GR expression (approximately 0.5). Around the 100 month mark, probability of survival for patients with high GR expression drops to 0.19 and for patients with lower GR expression the probability of survival is approximately 0.25. These differences are statistically significant with a p value of 0.0025.

The survival curve in figure 34 also shows the same trend, up until the 50 month mark both ovarian cancer patients with high and low OTR expression have the same decrease in survival probability. Around 80 months after treatment, probability for survival for patients exhibiting high expression of the OTR is around 0.25, and for patients exhibiting lower levels of the OTR the probability is higher, approximately 0.3 however this difference is not significant (p=0.11).

As seen with both figures 33 and 34, figure 35 for the probable survival of ovarian patients expressing high and low levels of GAS5 show the same patterns as before. At the 50 month mark, probability of survival for ovarian cancer patients expressing higher GAS5 is around 0.18, where in those expressing the lower levels of GAS5 the probability is approximately 0.22. This data is not statistically significant (p=0.13).

6.6 Discussion

Interestingly, the only GR splice variant to show and significant difference in expression in ovarian cancer tissue compared to normal tissue was GR-P, which was over expressed ($p<0.001$). GRα did show lower expression though not to a statistically significant degree ($p=0.056$). This data matched all oncomine data plots for GR expression differences in different types of ovarian cancerous tissue and in normal ovarian tissue, which repeatedly produced lower expression of GR in ovarian cancer tissue yet never statistically significant. Oncomine and KM plots both measure expression of the NR3C1 gene. This gene codes for the GR but does not specify which splice variants are investigated in these collective studies. Theoretically it can be assumed that all the data is a measurement for expression of GRα (and potentially GRγ) and not GRβ or GR-P. In section 2.2.2.4 the structure of the GR receptor was discussed in detail. It's known that the GRβ splice variant has an entirely different exon 9 (Turner *et al.*, 2010) at that end which causes GRβ not to be able to ligand bind to glucocorticoids and therefore cDNA for GRβ will not bind to NR3C1 chip on the microarray. GRγ, if expressed, may bind on the microarray as it's structurally similar to GRα except for the presence of an extra codon (McMaster and Ray, 2007) but as its expression is relatively low in both cancer cells, cancer tissue and normal tissue that we can assume the oncomine and KM plot data are for GRα not GRγ expression. Knowing that the splice variant GR-P lacks both exons 8 and 9 (Yudt and Cidlowski, 2002) it's almost certain that microarray data and the KM plots are not measuring the differences in expression of the GR-P splice variant between cancerous and normal ovarian tissue. High GR-P expression can increase the activity of GRα (Lange, Segeren and Koper, 2010). If GR-P is increasing GR-α activation but not GR-α expression it may explain why pooled microarray data shows lower levels of GRα in ovarian cancer tissue compared to normal, yet published papers find that cortisol and other glucocorticoids have proliferative, invasive migratory increases on cancerous cells (Lutgendorf *et al.*, 2003b; Thaker and Sood, 2008; Arranz *et al.*, 2010; Schrepf *et al.*, 2013).

A study in 2017 found higher GR expression (assuming GRα) in high grade tumours, serous tumours and advanced stage tumours than in other subtypes of ovarian cancers, and correlated high GR expression with poor prognosis (Venerisa *et al.*, 2017). This data supplements our findings in figure 28. There was a significantly greater expression of GRα in

late staged cancer tissues compared to GRα expressed in earlier stages (p=0.015). If GRα is expressed in high grade tumours and in more 'aggressive' subtypes, as well as expressed higher in late stage cancer tissues (figure 28), this will explain why patients with higher expression of GR are have lower survival probabilities seen in the KM plots (figure 33).

In clinical samples, GAS5 expression is significantly lower in ovarian cancer tissue compared to normal ovarian tissue ($p<0.01$). Oncomine plot for GAS5 expression changes between normal ovarian tissue and ovarian serous adenocarcinoma however gave a different result with ovarian serous adenocarcinoma having a 1.071 fold change, though not significant ($p=0.112$). The qPCR data in figure 25 fit observations made in the section 5.4. Overexpression of GAS5 leads to greater caspase-3 activity and lower Bcl-2 expression (Wang *et al.*, 2019), lower GAS5 expression was found in more aggressive ovarian cancer sub-types and in late tumour stages (Li *et al.*, 2016). All bar one of the patients providing ovarian cancer tissue in table 7 were at stage 3 (essentially a late stage) when diagnosed, which would explain why we found lower expression of GAS5 compared to normal controls. What is interesting is that KM plot data in figure 35 points to higher expression of GAS5 decreasing probability of survival than low level of GAS5 (though data is not statistically significant), which was not expected.

The overexpression of OTR in ovarian cancer tissue agreed with data from the oncomine plots which showed to a significant level that OTR expression was greater in ovarian carcinoma ($p=0.0001$) and ovarian serous adenocarcinoma ($p=0.015$) compared to control. This increase doesn't decreases survival probability as shown in the KM plot (figure 34) to a significant degree however from the KM plot in figure 34 it can be seen that there is lower survival probability for patients with high OTR expression compared to patients and it would be interesting to see as time goes on and more data is inputted, if the probability 'gap' increases and become more statistically significant. Not much is known about OTR expression in ovarian cancer tissue and the role it plays in prognosis. There is little to no published data stating the effects OT has on OTR expression in clinical tissue or in ovarian cancer cells. Published material focuses in the direct role of OT on ovarian cancer instead. qPCR data in figure 23 provided no real insight on how either OT or C presence in ovarian cancer cells effects expression of the OTR (although PEO1 did show a very small but significant increase in expression of the OTR in the presence of OT). What was interesting, is our cell line data supports the theory that OT

reduces cell proliferation, migration, and incudes pro-apoptotic mechanisms through increasing expression of caspase-3 and beclin-1 (figures 11-16) and published data also supports the understanding that OT brings about slower prognosis, reduction in cancer cell viability and tumour size (Reversi *et al.*, 2005; Gidron and Ronson, 2008; Benavente *et al.*, 2016; Reim *et al.*, 2019) yet higher OTR expression is found in ovarian cancer tissue compared to normal (Hendrix *et al.*, 2006; Bonome *et al.*, 2008), this is further backed up by our qPCR data in figure 23 ($p<0.01$), and KM plots predict that higher expression of OTR may potentially reduce probability of survival (though not statistically significant). If OT is not driving up expression of the OTR in ovarian cancer tissue, then potentially, glucocorticoids are.

It's therefore hypothesised that increasing cortisol levels, brought on by chronic stress in ovarian cancer patients, potentially augments OTR expression. Though data in the study was unable to conclusively prove this, figure 23(a) shows ever increasing expression of OTR in both OT+C and C treatments in SKOV3 cells, and C treatment increased fold change for OTR expression in both PEO1 and MDAH-2774 cells.

7.0 Discussion

The aim of this study was to dissect the molecular pathways taken by OT and C in ovarian cancer cells to better understand the roles these two hormones have in ovarian cancer and to see if potentially OT could be used to mitigate the proliferative role stress has on cancer cells. It was hypothesised that the activity of C in ovarian cancer cells might be compromised if OTR signalling was activated. What was discovered however, was an indirect interaction between the two hormones, with OT possibly controlling the differential splicing of the GR's, and C potentially augmenting OTR expression. The net result would be a compromised or augmented GR and OTR signalling respectively. Furthermore, we expanded our observations in clinical samples and concluded with in silico observations in terms of gene expression and overall survival of ovarian cancer patients.

Ovarian cancer cells exhibited cell specific relative quantities of the GR splice variants. SKOV3 cells expressed greater levels of GRα and OTR, whilst expressing lower levels of GR-P and GAS5, and PEO1 and MDAH-2774 cells expressed relatively lower levels of GRα and OTR, whilst expressing higher levels of GR-P and GAS5. OT not only brought about reduction in cell viability in all three cancer cell lines, but was able to partially do so in the presence of C indicating the first suggestion of cross talk between the two hormones. Western Blotting analysis then reaffirmed this suggestion of cross talk when the upregulation of both caspase-3 and Beclin-1 by OT was partially reversed by the addition of C in all three cell lines again. To further cement the notion of cross talk between OT an C, wound healing assays to measure migratory rates of treated cells showed OT to reduce cell migration and C mitigating this reduction (however P values were greater than 0.05).

It was observed in the clinical samples that ovarian cancer patients had elevated expression levels of the GR-P splice variant and the OTR, and lower levels of GAS5 compared to normal ovarian tissue. GRα expression was found to be increased in patients with late stage diagnosis compared to early stage. Clinical data was backed up findings from Oncomine, an online database which pools all microarray data from published material to produce a box plot diagram with statistical analysis enabling easy comparison between target genes in cancerous

and non-cancerous tissue. The above findings could have broader implications for ovarian cancer patients who frequently exhibit stress, depression and anxiety (Schrepf *et al.*, 2013).
A major finding of this study is the inhibitory role that oxytocin can exert over the cortisol effects in tumour cells. By inducing autophagy through the upregulation of Beclin-1, OT was able to reverse the effects of cortisol. Decreased expression of Beclin-1 is correlated with poorer outcomes in ovarian cancer patients (Lin *et al.*, 2013) and with epithelial ovarian tumour development (Shen *et al.*, 2008), so upregulation of Beclin-1 through OT treatments should improve outcome and reduce tumour development. Dexamethasone (a synthetic glucocorticoid) is usually administered to cancer patients to help with the side effects of chemotherapy, but it has been observed to significantly induce expression of SGK1 and MKP1, genes which play an important role in cancer cell survival (Melhem *et al.*, 2009). Even though the addition of dexamethasone before the start of chemo therapy has been reported to be beneficial for chemo patients by reducing the symptoms of nausea and vomiting (Tannock, 2012) , it counteracts the effectiveness of cytotoxic treatment and reduces the treatment-induced growth delay of ovarian cancer tissue.

OT was able to induce activation of caspase-3 in both SKOV3 and MDAH-2774 cells. This effect has also been observed in prostate cancer cells (Xu *et al.*, 2017), in human granulosa cells (Saller *et al.*, 2010) and in breast cancer cells (Alizadeh *et al.*, 2017). Cortisol however did not affect the activation of caspase-3 in the presence of OT in the three cell lines implying that OT exerts a direct apoptotic effect independent of cortisol. Past data has connected OT with both proliferative and suppressive effects *in-vitro* (Morita *et al.*, 2004; Reversi *et al.*, 2005; Reim *et al.*, 2019), yet our data revealed OT has cytostatic/cytotoxic effects in SKOV3, PEO1 and MADAH-2774 cells. This was in line with other published data showing the anti-proliferative effects of OT in cancer cells (Bussolati *et al.*, 1996; Thibonnier *et al.*, 1999; Copland *et al.*, 2013).

We observed also OT ability to transactivate the GR gene in the presence of cortisol, providing evidence for cross talk between the two hormones. This ability for OT to activate the GRE could have implications on GR splicing and OT could potentially compromise GR signalling by upregulating GRβ (observed in all three cell lines) which has a dominant negative effect. Data

shows high elevation of OT for prolonged periods (such as during breastfeeding) reduces risk of developing ovarian cancer (Colditz, 2000) and reduces the risk of endometrioid ovarian cancer more than any other subtype (Modugno, Ness and Wheeler, 2001; Danforth *et al.*, 2007).

The results obtained from this study, and the information available in published data provide evidence that links better social support to better diagnosis. High OT levels are a marker for better/positive social care (Swaab, Bao and Lucassen, 2005; Gidron and Ronson, 2008). It is observed that OT increases apoptotic effects and reduces/reverses the anti-apoptotic effects brought on by chronic stress and high C levels in ovarian cancer. Managing stress potentially by increasing social support, or by administration of OT in distressed ovarian cancer patients could help provide better prognosis and outcome. Chronic stress, low social support and depression increase levels of MMP-9 in tumour-associated macrophages, bringing about higher rates of on invasion and migration (Lutgendorf *et al.*, 2008). Our data shows OT reduces migratory rates in SKOV3, and OT also reduces migration in other ovarian cancer cell lines (Ji *et al.*, 2018), providing more reasons to why exploring the potential use of OT as a therapeutic agent is now needed more than ever.

In all three ovarian cancer cell lines used in this study, response to OT was observed to be the same in the viable assay and in WB data for Beclin-1 expression. Differences were seen in GR expression levels after treatment, caused by the difference in subtype, differential stage and origins of each of the cell lines. Though not statistically significant, both OT and C treatments brought increases in OTR expression. Although our study did not include direct evidence linking cortisol and the OTR, we hypothesis that C augments OTR expression and could be a compensatory defensive response to chronic stress (Mankarious *et al.*, 2016). OTR expression in ovarian cancer tissue was significantly greater than in normal ovarian tissue, and GRα expression was slightly decreased.

Potentially, the use of OT could have dual benefits. One; OT administration would reduce the feeling of loneliness and reduce stress in patients, and two; OT could partially reverse the anti-apoptotic effects brought on by glucocorticoids. Further studies into the relationship

between social support, OT, cortisol and ovarian cancer prognosis will produce a clearer understanding of how these two hormones interact at a cellular/tissue level to produce new therapeutic treatments that increase positive outcomes for highly stressed ovarian cancer patients.

7.1 Limitations of Study

Baseline expression of the GR splice variants, GAS5 and the OTR, and changes in their expressions after OT and C treatment in the ovarian cancer cell lines SKOV3, PEO1 and MDAH-2774 provided an insight into ovarian cancer cells response to stress and social support. The use of normal ovarian cells would have been an essential tool for comparison. Using Normal Ovarian Surface Epithelium (NOSE) cell lines as a control would have provided a clearer understanding of the role of different GR splice variants in ovarian cancer. Attempts were made to culture NOSE. Unfortunately cultured cells did not survive long enough for cell treatments and after a several attempts to culture NOSE the idea was abandoned.

Regrettably, OTR antibody purchased from Cell Signaling Technology produced no results. There is no data for OTR protein expression in the cell lines and in ovarian cancer tissue samples from the paraffin embedded slides. This would have been important to use as it could potentially have complemented data showing high OTR mRNA expression in ovarian cancer tissue.

As mentioned in chapter 4, immunofluorescence would have been better for measuring protein expression of GRα, GRβ and the OTR, plus producing images that show where in the cell these proteins are located. However with high levels of background signalling this had to be scrapped and the use of DAB staining was selected instead.

Also the lack of commercially available GRγ and GR-P antibodies meant protein expression of these two splice variants could not be used to back up data for mRNA expression in cell lines.

7.2 Future Experiments

Our Western blot data had shown that OT was able to reduce Cortisol induced proliferation by activation of Caspase 3 and Beclin-1. To better understand how the binding of OT to the OTR may reduce proliferation, Western Blot analysis of the phosphorylation of the Mitogen Activated Protien Kinases (MAPK) analysis should be carried out.

The MAPK pathway, also known as the Extracellular Signal- Regulated Kinases (ERK) pathway is a molecular pathway involving a chain of proteins that respond to extracellular signalling by phosphorylating subsequent proteins in the chain that ultimately regulate cell proliferation (Busca, Pouyssegur and Lenormand, 2016). Treating cells with 100nM OT, 100nM C and 100nM combined OT+C and then measuring the protein expression of phosphorylated ERK over total ERK would expose the role played by C in initiating proliferation, and potentially show if OT is able to reduce this activation by decreasing expression of phosphorylated ERK over total ERK.

It is still unclear from our data which G-protein (G_q or G_i) couples upon the binding of OT to the OTR. Co-Immunoprecipitation analysis could be used to measure coupling of the OT-bound-OTR to either the G_i or G_q trimeric G protein in ovarian cancer cells. This data could be used to predict how OTR in ovarian cancer tissue couples, which signalling pathway does it activate and how OT would affect growth, migration and invasiveness. OT treated cells would be lysed and incubated with anti-OTR antibodies. The mixture would then be mixed with G-protein G coupled beads that would bind to anti OTR antibodies, potential adhered to OTR carrying either G_q or G_i alpha subunits. Centrifugation should pellet the bead complex, allowing extraction followed by Western blotting of the proteins (*Co-immunoprecipitation Protocols And Methods*, 2020). The use of ant-G_q alpha antibody and anti- G_i alpha antibody (both available from Sigma-Aldrich) would indicate which G-protein couples with OTR in the presence of OT. These results could explain why OT is able to slow down proliferation in ovarian cancer cells if expression of G_i alpha is found to be greater than G_q.

References

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