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ASSESSMENT OF STRESS AND ANXIETY BIOMARKERS PRESENT IN THE FOLLICULAR FLUID

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Dedicated to my parents

“There is no passion to be found playing small - in settling for a life that is less than the one you are capable of living”.

- *Nelson Mandela*

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List of Abbreviations and Acronyms

11ß-HSD - 11β-hydroxysteroid dehydrogenase

ACTH - Adrenocorticotropic Hormone

ART - Assisted Reproductive Technology

BMI - Body Mass Index

BSA - Bovine Serum Albumin

CHUC - Centro Hospitalar e Universitário de Coimbra

CRH - Corticotropin-releasing Hormone

COC - Cumulus-oocyte Complex

FF - Follicular Fluid

FSH - Follicle Stimulating Hormone

GnRH - Gonadotropin-releasing Hormone

GH - Growth hormones

hCG - Human chorionic gonadotropin

hMG - Purified human menopausal gonadotropin

HPA - Hypothalamic Pituitary Adrenal Axis

HPG - Hypothalamic-pituitary-gonadal Axis

HRP - Horseradish Peroxidase

H_2O_2 - Hydrogen peroxide

ICSI - Intracytoplasmic Sperm Injection

IVF - *In vitro* Fertilization

LH - Luteinizing Hormone

MI - Metaphase I

MII - Metaphase II

NAD+ - Nicotinamide adenine dinucleotide (oxidized state)

NADP+ - Nicotinamide adenine dinucleotide phosphate (oxidized state)

NADPH - Nicotinamide adenine dinucleotide phosphate (reduced state)

OHS - Ovarian hyperstimulation syndrome

OSE - Ovarian surface epithelium

PCOS - Polycystic ovarian syndrome

POF - Premature ovarian failure

RT - Room temperature

STAI - State Trait Anxiety Inventory

TGF - Transforming growth factor

TMB - 3,3',5,5'-tetramethylbenzidine

Assessment of Stress and Anxiety Biomarkers present in Follicular Fluid

Abstract

Anxiety and stress make part of the daily life of today´s society and seem to play a crucial role in what concerns a social major issue, that is fertility. Both negatively affect the physical and mental condition of an individual, in an imperative way. Research has been relying on the exploration and identification of reliable biomarkers for oocyte quality and potential in assisted reproductive techniques (ART). However, inconsistent and contradictory results are observed. The follicular fluid, due to its direct relationship with the oocyte and because it provides an ideal environment for maturation, maybe the ideal place to identify these biomarkers. One of these biomarkers may be cortisol as it plays a key role in understanding the relationship between the nervous system and the female reproductive system. Anxiety and depression of infertile couples undergoing ART treatments have not often been associated with the different pregnancy outcomes. Psychological oscillations, due to anxiety and depression, have been associated with high cortisol concentrations affecting reproductive functionality at different levels, but studies are inconsistent. This study included a group of 58 women undergoing *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) treatments, in order to assess the presence and the potential of cortisol and cortisone in follicular fluid (FF), as biomarkers for quality assessment and development of the cumulus-oocyte complex (COC), as well as pregnancy predictors, under different psychological states of anxiety and depression. The same hormones were assessed in blood plasm (BP) to determine whether these hormonal measurements could replace hormonal measurements in FF. Key findings: Higher levels of cortisol were observed in the follicular fluid and blood plasma of pregnant women. There are significant differences between age and depression levels, between higher fertilization rate and lower anxiety levels, and between higher fertilization rate and higher cortisol levels. There seems to exist a similar behaviour of the 11ß-HSD enzyme in both fluids.

Keywords: Stress, anxiety, depression, cortisol, female infertility, follicular fluid, 11ß-HSD

Resumo

A ansiedade e a depressão fazem parte do quotidiano da sociedade atual e parecem exercer um papel crucial no que diz respeito a uma grande questão social, a fertilidade. Ambas afetam negativamente a condição física e mental de um indivíduo, de forma imperativa. A pesquisa científica tem tido por base a exploração e identificação de biomarcadores fiáveis da qualidade e do potencial oocitário nos tratamentos de procriação medicamente assistida (PMA). Contudo, resultados inconsistentes e contraditórios são observados. O líquido folicular, devido à sua relação direta com o oócito e por providenciar um ambiente ideal à maturação, poderá ser o melhor local para a identificação desses biomarcadores. Um deles poderá ser o cortisol, visto desempenhar um papel fundamental na compreensão da relação entre o sistema nervoso e o sistema reprodutor feminino. A ansiedade e a depressão de casais inférteis submetidos a tratamentos de PMA não têm sido associadas, com frequência, aos diferentes resultados de gravidez. Oscilações psicológicas, devido a ansiedade e depressão, foram associadas a níveis elevadas de cortisol, afetando a funcionalidade reprodutiva a diferentes níveis, mas os estudos são incongruentes. Este estudo incluiu um grupo de 58 mulheres submetidas a tratamentos de fertilização in vitro (FIV) e injeção intracitoplasmática de espermatozoides (ICSI), de modo a avaliar a presença e o potencial do cortisol e da cortisona no líquido folicular, como biomarcadores na avaliação da qualidade e desenvolvimento do complexo do cumulus-oócito (CCO), assim como agentes de previsão de gravidez, perante diferentes estados psicológicos de ansiedade e depressão. As mesmas hormonas foram avaliadas no plasma sanguíneo para determinar se estas medições hormonais poderiam substituir as medições hormonais feitas no líquido folicular. Conclusão: Níveis mais elevados de cortisol estão presentes no líquido folicular e no plasma sanguíneo de mulheres grávidas. Foi observada uma associação significativa entre a idade e os níveis de depressão, entre elevadas taxas de fertilização e baixos níveis de ansiedade, assim como entre taxas altas de fertilização e níveis elevados de cortisol. Parece existir evidência de um comportamento similar da enzima 11ß-HSD em ambos os fluídos.

Palavras-chave: *Stress, ansiedade, depressão, cortisol, infertilidade feminina, líquido folicular, 11ß-HSD*

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Chapter I – Introduction

Psychological biology associated with female reproductive health is still a very unknown and unexplored field. A growing interest in this area is emerging since the 21st century has witnessed an increasing instability regarding women´s mental health that arises from anxiety and depression of different etiologies (Burt *et al.,* 2009).

Infertility is a common worldwide condition affecting 10 to 15% of couples of reproductive ages ("Infertility Workup for the Women's Health Specialist," 2019; Sharma *et al.,* 2013) with an incidence in both genders (Ilacqua *et al.,* 2018) and it can lead to phycological and physical issues ("Infertility Workup for the Women's Health Specialist," 2019). As more women delay childbirth and life expectancy rises, reproductive aging and its consequences are becoming a major health concern, a scenario mainly observed in developed countries (Steiner *et al.,* 2017).

Anxiety and depression can arise from the underlying stress derived from a range of lifestyle events and changes (e.g., job and marital life), and seem to favor infertility and contribute to its persistence (Palomba *et al.,* 2018). It bursts into an immediate disruption of mental or physical homeostasis to which the organism responds through nervous, endocrine, and immunological stimulus (Palomba *et al.,* 2018). The interpersonal variability also seems to favor distinct responses to stress, as there are more susceptible individuals with higher psychological instability than others (Ilacqua *et al.,* 2018). They represent negative risks to female reproductive health (Homan *et al.,* 2007) as, for example, a decrease in the ovarian reserve (Palomba *et al.,* 2018). This stress blocks the neurological system, specifically the HPA axis, by changing steroid hormone concentrations that impact fertility directly, and by altering glucocorticoid hormones that are pointed to have a role in fertility (Campagne, 2006). Alterations in the physiological levels of these hormones and the increase of cortisol may impact oocyte quality and consequently reproductive outcomes (Campagne, 2006).

Several studies have tried to elucidate the psychobiological pathways that link stress and infertility. Cortisol, also called the stress hormone, maybe one of the best markers to understand these pathways since it has a relevant role on acute and chronic stress (Campagne, 2006).

A growing body of research has demonstrated that anxiety and depression, as well as high cortisol levels at the time of ovulation contribute to a greater difficulty to achieve a pregnancy with ART procedures (Smeenk *et al.,* 2001, 2005). However, these results are not conclusive, due to contradictory results in different studies and thus, more research is needed (Campagne, 2006).

1.1. Hypotheses

The assumptions that are expected to be verified are the following:

- Women with less anxiety and depression have higher fertilization and implantation rate;
- Lower levels of cortisol and higher levels of cortisone are correlated with women with higher fertilization and implantation rate;
- Lower levels of anxiety and depression correspond to lower levels of cortisol in follicular fluid and blood plasma;
- Presence of higher levels of anxiety and depression in couples with higher levels of cortisol and with lower rate of fertilization and implantation.

1.2. Female Human Reproduction

1.2.1. Physiology of the Female Reproductive System – the Ovary

The human female gonads comprise two complex oval endocrine glands, the ovaries, lying in the pelvic cavity, on either side of the uterus by a fibrous cord known as the ovarian ligament. The mesovarium is the portion of the uterine broad ligament that covers the ovary, and through which the ovaries get a vascular and lymphatic nerve supply (Tresguerres, 2009). Ovaries sit next to the Fallopian tube, a tube with fimbriae in its extremities evolving the ovary and necessary to receive the oocyte at ovulation. Fertilization occurs in the Fallopian tube if the oocyte finds a sperm (Seeley *et al.,* 2008; Suarez, 2014). Having surpassed the bipotent phase during embryogenesis (Sasaki *et al.,* 2021) and after differentiated, ovaries house the follicle (Hennet *et al.,* 2012). Follicular cells perform two vital functions: protection of mature oocytes for reproduction, every month, and synthesis of female sex hormones, estrogen and

progesterone. Estrogen is responsible for the maturation and maintenance of the reproductive organs, as well as the appearance of secondary sex characteristics in females during puberty (Abbara *et al*., 2018; Cui *et al*., 2013; Holesh *et al*., 2022; Ross, 2014). Progesterone interacts with estrogen to trigger endometrial changes during the menstrual cycle (Abbara *et al*., 2018; Chang *et al*., 2016; Holesh *et al*., 2022). The ovary begins to act in a cyclical and non-continuous way at puberty by releasing one oocyte per month, normally. When menopause approaches there is a decrease in the number of oocytes available in the ovaries (Tresguerres, 2009).

At the cellular level, ovaries are composed of germ cells and somatic cells responsible for the intra-ovarian signaling cascades. The first cells are the oocytes and somatic cells comprise granulosa, theca, and *cumulus* cells. In turn, the signaling pathways involve signaling molecules such as cytokines and growth factors, e.g., the transforming growth factor (TGF), working in a paracrine/autocrine manner. These signaling factors mediate a bidirectional communication between oocytes and the surrounding somatic cells. Furthermore, they are responsible for ovarian maturation and function, follicular formation and development, the emergence of ovulation, and the subsequent corpus luteum genesis, characterized as a yellow endocrine-secreting structure needed to establish and bear a pregnancy (Chang *et al*., 2016; Edson *et al*., 2009; Knight *et al.,* 2006; Richards *et al.,* 2010; Virant-Klun *et al*., 2012). Besides estrogen and progesterone, some other hormones are involved in this succession of events. These include two pituitary gonadotrophin hormones whose role is to control the female menstrual cycle in a coordinated manner: the follicle-stimulating hormone (FSH) and the luteinizing hormone (LH). Both are secreted from the anterior pituitary gland under the release of tightly synchronized pulses of the hypothalamic gonadotropin-releasing hormone (GnRH) (Richards *et al.,* 2010).

1.2.2. Oogenesis and Folliculogenesis

A normal ovarian cycle progression occurs due to two synchronous biological processes: oogenesis and folliculogenesis. In oogenesis developing oogonia differentiate into mature oocytes while, in turn, folliculogenesis begins with the

recruitment of primordial follicles that develop to produce large antral follicles capable to ovulate (Hillier *et al*., 2010).

1.2.2.1. Oogenesis

In female human embryogenesis, primordial germline cells migrate to the fetal ovaries, where they undergo successive mitosis and give rise to about 6 to 7 million oogonia (Cox *et al.,* 2022; Knight *et al.,* 2006). Even before birth, many will degenerate by apoptosis (Knight *et al.,* 2006). The remaining oogonia proceed to meiosis and are called primary oocytes surrounded by granulosa cells and composed of a large nucleus denominated germinal vesicle (Gilbert, 2000; Stuart Ira Fox, 2001). At birth, these primary oocytes have their meiotic cellular cycle arrested at prophase I, which is set to be resumed at the beginning of puberty (Gilbert, 2000; Holesh *et al.,* 2022) *(figure 1)*.

At puberty, as ovulation approaches, prophase I is resumed and the primary oocyte completes meiosis I, which results in two haploid cells with half of the genetic material of the mother cell: a large secondary oocyte with a great cytoplasm and one first polar body that, in turn, is a small residual cell with little cytoplasm that degenerates (Edson *et al.,* 2009; Gilbert, 2000).

The formation of the COC is a phenomenon of the last interactions between the oocyte and its follicle that takes place shortly before the LH surge during oogenesis. It results from the differentiation of antral granulosa cells into mural granulosa cells and *cumulus* oophorus surrounding the oocyte. This interaction is essential to aid in oocyte maturation, transcriptional activity, and metabolic support (Hennet *et al.,* 2012). Ovulation normally occurs 28-36 hours after a rise in FSH and a spike in LH levels (Holesh *et al.,* 2022; Kerin, 1982). These significantly impact the granulosa and theca cells by influencing them to secrete progesterone. At this stage, the secondary oocyte enters the second division of meiosis and becomes quiescent in metaphase II (MII) until fertilization (Gilbert, 2000). Under hormonal stimulation, the oocyte is released from the matured follicle through the ovarian wall, which is already severely weakened at this point (Tresguerres, 2009). It is surrounded by corona radiata cells and is released into the pelvic cavity to the fallopian tubes, where it may eventually be fertilized by a spermatozoon (Tresguerres, 2009). The surface of the ovary normally heals by itself as the ovulatory phase is over. If fertilized, the secondary oocyte

completes meiosis II in the fallopian tube (Gilbert, 2000; Holesh *et al.,* 2022) and gives rise to a second polar body and a zygote (Gilbert, 2000) *(figure 1)*.

Little evidence exists but, when separated from the follicular fluid, the physiognomy of the COC may allow the prediction of embryo maturity and developmental competence (Rienzi *et al.,* 2012). A mature COC presents a matured oocyte at MII and has expanded corona radiata cells surrounded by *cumulus* cells. Contrarily, immature COC is normally retrieved from small follicles and present various layers of condensed corona radiata and *cumulus* cells adhered to the zona pellucida (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011; Rienzi *et al.,* 2012). The zona pellucida is a thin layer surrounding the oocyte and found between the oocyte and granulosa cells at prophase I (Rienzi *et al.,* 2012), with an important role in the binding of sperm to the oocyte at fertilization (Wassarman, 2008). However, other parameters shall be evaluated to assess oocyte maturity. These may include nuclear and cytoplasmic maturation processes, considered to be two non-synchronous phenomenons (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011; Rienzi *et al.,* 2012). More specifically, a mature oocyte morphology has a spherical shape surrounded by a uniform zona pellucida, a translucid cytoplasm that is composed of a small perivitelline space, a small-sized polar body, and no inclusions or any sort of granularities (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011). In turn, the presence of a polar body determines nuclear maturity and dictates that the oocyte is in MII. When immature, the oocyte can be in a germinal vesicle state or, it can be in metaphase I (MI) without a germinal vesicle or polar body (Rienzi *et al.,* 2012). These cytoplasmic and nuclear modifications are required for oocyte capacitation, a process in which the oocyte reaches fully developmental competence and maturation (Hennet *et al.,* 2012). This nuclear maturation is arrested before ovulation by the somatic cells of the follicle (Hennet *et al.,* 2012).

1.2.2.2. Folliculogenesis

Folliculogenesis begins in the early development with primordial germline cells migrating to the ovaries, where they undergo successive mitosis and give rise to about 6 to 7 million primordial follicles, each surrounded by pre-granulosa cells and endowed with an oogonium (Cox *et al.,* 2022; Knight *et al.,* 2006). Even before birth, many of these quiescent state follicles degenerate by apoptosis (Knight *et al.,* 2006). At birth, the ovaries contain around 2.5 million primordial follicles surrounded by a single layer of cells containing primary oocytes (Tresguerres, 2009). Then, roughly 400.000 primordial follicles and quiescent oocytes will remain arrested in prophase I until a woman reaches puberty. However, at puberty, only about 400 primordial follicle cells will be able to reach ovulation under a strict regulated physiological process (Knight *et al.,* 2006; Tresguerres, 2009). The controlled recruitment of primordial follicles precedes the emergence of primary follicles under the influence of high FSH levels. This transformation results from the proliferation of morphologically cuboid granulosa cells as well from the release of nutrients and proteins responsible for the secondary oocyte maturation and genesis of the zona pellucida. Around the developing follicle, theca cells produce androgens later converted to estradiol by granulosa cells (Williams *et al.,* 2012; Donahoe, 2009). The secondary follicle emerges (Gilbert., 2000). At this stage, little fluid-filled sacs connect and form a cavity that displaces the oocyte to the periphery of the follicle (Tresguerres, 2009). This large open cavity is called the follicular antrum and contains follicular fluid (Gilbert, 2000). The oocyte remains attached to the follicle through a specialized type of granulosa cells, named cumulus oophorus, and by a thin cell layer of corona radiata surrounding the oocyte (Gilbert, 2000; Sánchez *et al.,* 2012). *Cumulus* oophorus cells stay firmly linked to the oolemma of the oocyte through the synthesis of cytoplasmic gap junctions capable of crossing the zona pellucida (Rienzi *et al.,* 2012). This communication gives nutrients and chemical messengers to the oocyte necessary to control its growth and maturation (Kidder *et al.,* 2002) *(figure 1)*. Every secondary follicle will degenerate except for one, the antral follicle (Williams *et al.,* 2012). At this point, the remaining follicle is mature and is given the name of Graafian follicle (Gilbert, 2000) *(figure 1)*. This series of events takes about 14 days (Tresguerres, 2009).

At ovulation, the Graafian follicle ruptures and releases the mature oocyte into the reproductive tract carrying the *cumulus* oophorus and the zona pellucida with it (Tresguerres, 2009) *(figure 1)*. The follicular cells that remain in the ovary after ovulation expand under the influence of LH, generating a transient glandular structure, known as the corpus luteum, with a main role during the second phase of the menstrual cycle (Gilbert, 2000; Oliver *et al.,* 2022; Tresguerres, 2009). No fertilization within approximately two weeks causes corpus luteum to atrophy and drop estrogen and progesterone levels (Gilbert, 2000; Oliver *et al.,* 2022). A new structure named corpus albicans originates, marking the end of the menstrual cycle (Gilbert, 2000; Oliver *et al.,* 2022).

In a natural spontaneous cycle, generally, only one follicle is set for ovulation. However, in ART procedures, ovaries are artificially stimulated. It aims to induce the development and maturation of a cohort of growing follicles and oocytes found in the ovaries during the reproductive cycle, avoiding spontaneous and precocious ovulation (Gallos *et al*., 2017; M. Fatemi *et al.,* 2012; Pereira *et al.,* 2021; Rienzi *et al.,* 2012).

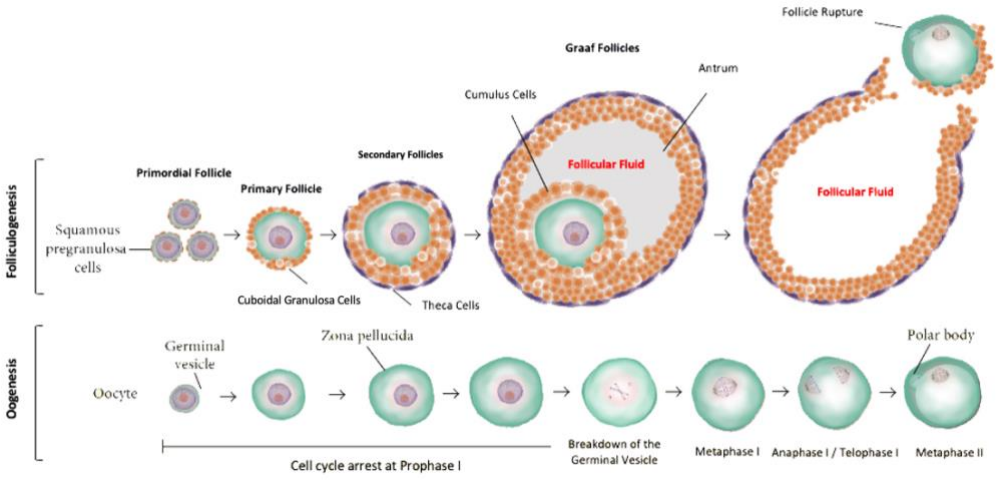


Figure 1 - Process of folliculogenesis and oogenesis maturation. Primordial follicles are surrounded by squamous pregranulosa cells and contain an immature GV (germinal vesicle) oocyte arrested at prophase I of meiosis. The acquisition of a cuboidal shape from the pregranulosa cells marks the transition from primordial follicles to primary follicles. A secondary follicle emerges by the time two or more layers of granulosa cells and theca cells are formed. The oocyte inside the secondary follicle is at this stage encapsulated by a zona pellucida. The Graafian or antral follicle dictates the final stage in folliculogenesis before ovulation. Graafian follicle is composed by an antrum filled with follicular fluid bathing the oocyte. The oocyte resumes meiosis I by surpassing the breakdown of VG, metaphase I, anaphase I and telophase I stages of development. The completion of meiosis I concurs with ovulation.

The first polar body is expelled and the ovulated oocyte is arrested at metaphase of meiosis II until reach fertilization. Adapted from Mihalas *et al*., 2017.

1.2.3. Follicular Fluid

Oocyte quality is under the influence of the nuclear and mitochondrial genomes as well as the surrounding microenvironment (Rienzi *et al.,* 2012). Inside the antrum is the follicular fluid (FF) bathing the oocyte. This fluid creates a propitious microenvironment low in oxidative agents (Dorado-Silva *et al.,* 2020) that is tailored to sustain oocyte growth, maturation, and integrity (Bartolomé *et al.,* 2021), as ovulation approaches (Hennet *et al.,* 2012).

Follicular fluid is a plasma exudate that is composed of components secreted by the oocyte, *cumulus* cells and granulosa cells, such as small soluble molecules and/or lipid signals (Bartolomé *et al.,* 2021; Hennet *et al.,* 2012; Zamah *et al.,* 2015). It mediates the transport of these substances, but also from other agents that arrive at it from the blood, through the blood-follicular barrier, all of which have a role in oocyte maturation (Hennet *et al.,* 2012). Follicular fluid components comprise metabolites (e.g., amino acids, lipids), enzymes, proteins, hormones (LH, FSH, growth hormone (GH)), human chorionic gonadotropin (hCG), estradiol and progesterone, antioxidants, reactive oxygen species, ionic compounds and molecules, whose roles can be benefic or deleterious during oogenesis, folliculogenesis and steroidogenesis in the ovary depending on their amounts (Dorado-Silva *et al.,* 2020; Hennet *et al.,* 2012; Revelli *et al.,* 2009). In a normal cycle, the volume of FF varies between 0.02 and 7 ml depending on the follicle size (Williams *et al.,* 2012).

In ART routine procedures, after follicular puncture, the follicular fluid is normally discarded after extraction of the oocytes, because it has no clinical relevance. This fact, together with the wide diversity of components in FF, has attracted attention from the scientific community as an interesting source of biomarkers to predict and improve ART outcomes (Chen *et al.,* 2016). It is also an attractive target for the development of non-invasive techniques to assess oocyte competence and quality, with benefits in selecting the greatest potential oocytes (Bianchi *et al.,* 2016). Some studies have already identified follicular fluid biomarkers with a direct link to oocyte

quality, which could be a valuable tool in the assessment of infertility. Cortisol is one of these potential biomarkers (Santa-Cruz *et al.,* 2020).

1.3. Ethiology of Female Infertility

Infertility is defined as a reproductive disorder by the World Health Organization and is characterized by the inability of a couple of childbearing age to conceive after at least 1 year of unprotected and regular sex ("Infertility Workup for the Women's Health Specialist," 2019; *World Health Organization*, 2022; Tyuvina *et al.,* 2019). Its etiology can be attributed to female and male factors, or even a combination of both gender features. There are several causes underlying this pathology, with the socioeconomic aspect, for example, having a significant impact on its origin (Barut *et al.,* 2016; de Berardis *et al.,* 2014). Regarding the female system, similarly to the socioeconomic aspect, some other lifestyle factors, such as the environmental exposure to biological, chemical and radioactive agents, weight, cigarette smoking, illicit drugs use or mental health issues may contribute to infertility and also stimulate and increase anxiety (Sharma *et al.,* 2013). Infertility itself also might cause anxiety due to societal pressure, family members, related to at which age couples might start a family, or even due to assisted reproductive treatments, for example. All these factors can negatively contribute to higher risks of women´s negative emotions, such as anxiety and depression, as well as can be deleterious to the reproductive health, which disrupts ovarian physiology (Prasad *et al.,* 2016; Liu *et al.,* 2021).

Infertility can be classified as primary infertility, when a couple has never been able to conceive, or secondary when there is difficulty in conceiving despite having had a pregnancy previously. This classification facilitates the diagnosis of infertility when evaluating the most appropriate method for ART (Mascarenhas *et al.,* 2012; Vander Borght *et al.,* 2018).

Ovarian dysfunction is the most common physiological cause of female infertility and is usually based on the hormonal deficit of gonadotropins. It includes early ovarian failure, luteal phase abnormalities, endometriosis, polycystic ovary syndrome (PCOS), premature ovarian failure (POF), ovarian hyperstimulation syndrome (OHS), ovulation defects, poor oocyte quality and, in a worse scenario, cancer (Gameiro *et al.,* 2015;

Richards *et al.,* 2010). Population studies have revealed that PCOS is one of the most common reproductive abnormalities (Li *et al.,* 2021; Wu *et al.,* 2020). It is a pathology of the female tract marked by abnormal pituitary hormone release and uterine function, as well as an unusual androgen production. In addition, it correlates with anxiety disorders (Valsamakis *et al.,* 2019). Endometriosis is another common female tract disease in which cells from the endometrium seep out and thrive outside of the uterine cavity, most commonly in the ovaries (Brosens *et al.,* 2011). Besides ovarian dysfunction, uterine pathology, tubal and peritoneal pathology, cervical factors, immune changes, genetic causes, or idiopathic infertility are among other factors that contribute to female infertility (Hornstein *et al.,* 2011).

1.4. Physiological Response to Anxiety and Depression and the Female Reproductive System

Mental stress is a neurological state of mind that challenges the homeostasis of an individual with many implications (Joseph *et al.,* 2017). It comprises anxiety, which is outlined as a biological stimulus that causes psychological tension representing a threat to the success of ART procedures, and depression, which is classified as a mental pathology (Campagne, 2006; Lewicka *et al*., 2003). Both occur in response to a certain past or present event that unbalances the organism (Hanson *et al*., 2017). Some studies point to a higher incidence of these mental disorders in women with infertility (Hanson *et al*., 2017).

The hypothalamic pituitary adrenal (HPA) axis is the neuroendocrine system composed of the hypothalamus, pituitary gland, and adrenal glands (Joseph *et al.,* 2017). It relies on endocrine feedback loops with different inputs needed for fertility and to reestablish homeostasis when the psychological system is disrupted for some reason (Joseph *et al.,* 2017).

The HPA axis is directly affected and activated by stressful events (Newton, 2000), which can be acute and chronic (Prasad *et al.,* 2016). This stimulation causes an excessive release of glucocorticoids (e.g., cortisol) into the bloodstream by the adrenal cortex and consequently affects and mediates several systems, such as the female reproductive system, that is regulated by the hypothalamic-pituitary-gonadal axis (HPG) (Massey *et al.,* 2014; Prasad *et al.,* 2016). High amounts of glucocorticoids block

gonadal function by decreasing the secretion of GnRH. Consequently, LH and FSH levels are diminished and oocyte maturation is affected (Joseph *et al.,* 2017; Young *et al.,* 2002). The stress stimulus causes an overexcitation of the hypothalamus, which response by releasing more amounts of corticotropin-releasing hormone (CRH) (Newton, 2000). CRH stimulates the corticotropic cells of the anterior pituitary gland to induce the synthesis and secretion of adrenocorticotropic hormone (ACTH) in a pulsatile pattern (El-Farhan *et al.,* 2017; Joseph *et al.,* 2017). In turn, ACTH acts on the adrenal cortex to release higher amounts of glucocorticoids into the bloodstream (Joseph *et al.,* 2017; Newton, 2000).

Following this acute activation of the HPA axis, a fight-or-flight response arises due to the excessive glucocorticoids released by the adrenal gland. GnRH is suppressed and a release inhibition of CRH and ACTH through a negative feedback loop arises (Joseph *et al.,* 2017; Whirledge *et al.,* 2010). All these events have a role in an individual´s self-preservation (Campagne, 2006; Yilmaz *et al.,* 2015). Regarding the female reproductive system, as no excessive cortisol continues to be released, the endocrine signaling mediated by the HPG axis is reestablished (Joseph *et al.,* 2017). The hypothalamic GnRH of the HPG axis is no longer inhibited and triggers the release of FSH and LH by the anterior pituitary, promoting follicular development, ovulation and oocyte maturation. Progesterone and estrogen are consequently released (Joseph *et al.,* 2017; Yilmaz *et al*., 2015; Young *et al.,* 2002).

Little research exists regarding the role of stress, anxiety, or depression in the success of ART procedures (Rooney *et al.,* 2016). However, less anxiety and depression have been pointed to better female fertility (Campagne, 2006; Rooney *et al.,* 2016).

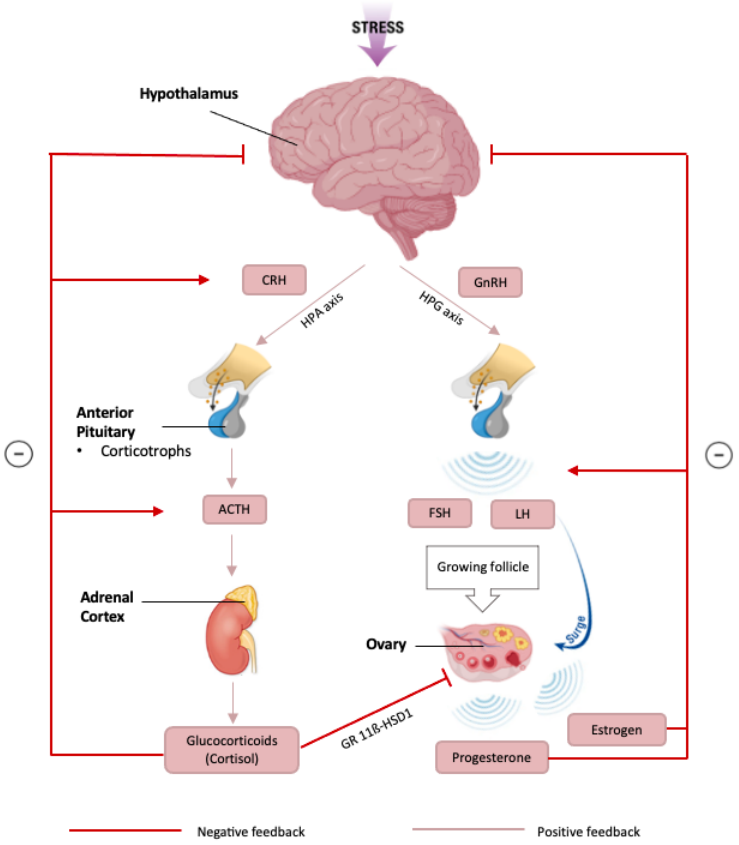


Figure 2 - Schematic representation of the interactions between Hypothalamic-Pituitary-Adrenal (HPA) and Gonadal Axis (HPG). HPA axis: CRH (corticotropin-releasing hormones) released from the hypothalamus drives the release of ACTH (adrenocorticotropic hormones) from the anterior pituitary gland. ACTH stimulates the release of glucocorticoids from the adrenal cortex. HPG axis: hypothalamus releases GnRH that stimulates the secretion of LH (luteinizing hormone) and FSH (follicle-stimulating hormone) from the anterior pituitary gland. LH and FSH contribute to oocyte maturation in the growing follicle. Progesterone and estrogen are released from the ovaries. The red line is negative feedback. The pink line is positive feedback. Adapted from Sominsky *et al.,* 2017; Young *et al.,* 2002.

1.5. Corticosteroids on Female Reproductive System

Corticosteroids are endocrine hormones, tightly regulated by the HPA-axis, and modulate physiological processes required for an individual´s survival (Joseph *et al.,* 2017). Their lipophilic nature allows them to diffuse easily across biological membranes so they can reach intracellular receptors (Chapman *et al.,* 2013; Ramamoorthy *et al.,* 2016). They are produced by the adrenal glands, which are triangular small glands located above the kidneys and composed of the adrenal medulla and the adrenal cortex (Burford *et al.,* 2017; Kemppainen *et al.,* 1997). The adrenal cortex is composed of the zona glomerulosa, responsible to secrete the

mineralocorticoid aldosterone, and the zona reticularis and zona fasciculata, which secrete glucocorticoids, such as cortisol (Chapman *et al.,* 2013; El-Farhan *et al.,* 2017; Kemppainen *et al.,* 1997). At the cellular level, the effects of these corticosteroids are essentially the result of transcriptional processes mediated by their binding to two types of intracellular receptors: the glucocorticoid receptors (GR) and the mineralocorticoid receptors (MR) (Chapman et al., 2013; Dammann *et al.,* 2019; Odermatt *et al.,* 2008).

Glucocorticoids are produced in response to ACTH (Michael *et al.,* 2003) in a circadian pattern (Ramamoorthy *et al.,* 2016). Are composed of a total of 21 carbons, with a ketonic group (carbon 3) and hydroxyl groups (carbons 11 and 21) (Errante *et al.,* 2014). They are coupled to corticosteroid-binding globulin (CBG), which is a high-affinity protein carrier with a limit of binding sites. Glucocorticoids may be also linked to albumin, and around 5% are free in blood plasma (Chapman *et al.,* 2013). Other roles of glucocorticoids include the regulation of the cardiovascular and nervous systems, their action as anti-inflammatory and immunosuppressors in immunological therapies, their contribution to metabolic homeostasis, cognitive behavior, and the reproductive system (Errante *et al.,* 2014; Joseph *et al.,* 2017).

Regarding reproduction, there is an increased response of glucocorticoids on ovarian cells towards stressful events (Whirledge *et al.,* 2010). This regulation occurs through three distinct mechanisms at the hypothalamic-pituitary-gonadal axis level: i) the hypothalamus and pituitary, with a decrease in the release of GnRH; ii) the hypophysis, by inhibiting the production, release and action of gonadotrophins, LH and FSH; and, iii) the presence of glucocorticoid receptors in ovarian cell types, which allow glucocorticoids to directly regulate gametogenesis and steroid hormone biosynthesis (Joseph *et al.,* 2017; Whirledge *et al.,* 2010). Although glucocorticoid synthesis does not occur locally in the ovary (Whirledge *et al.,* 2010), glucocorticoids are expressed and active in the human oocyte under the regulation of the 11β-hydroxysteroid dehydrogenase (11β-HSD) enzyme (Michael *et al*., 2003; Ricketts *et al.,* 1998; Smith *et al.,* 2000; Tetsuka *et al.,* 1997). Overall glucocorticoids are needed for ovarian function, including oocyte maturation and steroidogenesis, playing either benefic and/or negative effects (Whirledge *et al.,* 2010) depending on their concentrations. Their exact function in oogenesis is still unknown (Xiao *et al.,* 2022).

As referred before, two types of receptors are involved and 11β-hydroxysteroid dehydrogenase (11β-HSD) mediates glucocorticoid access to them (Benediktsson *et al.,* 1992). Glucocorticoid receptors are present in follicles, corpus luteum and ovarian surface epithelium (OSE) cells within the ovary, suggesting these receptors have a direct impact and role on gonadal reproductive activity (Albiston *et al.,* 1994; Benediktsson *et al.,* 1992; Whirledge *et al.,* 2010). They belong to the nuclear steroid receptor superfamily of ligand-dependent transcription factors (Joseph *et al.,* 2017) and are composed of three different structural domains: an amino-terminal transactivation domain, a central DNA binding domain, and a ligand-binding domain (Joseph *et al.,* 2017; Newton, 2000). The active GR complex comprises several tissue-specific regulatory proteins capable of being modulated by multiple post-translational modifications (Odermatt *et al.,* 2008). Without a ligand, GR is mostly kept as an inactive multi-protein complex in the cytoplasm (Newton, 2000). Glucocorticoids activity and regulation in the ovary rely on binding to available GR receptors present in target cells (Whirledge *et al.,* 2010). The presence of GR in the corpus luteum and the well-known anti-inflammatory effect of glucocorticoids is possibly related to the maintenance of the corpus luteum or even the regulation of the immune processes along with the luteal phase (Whirledge *et al.,* 2010). In fact, luteinization is characterized by increasing amounts of glucocorticoids and the LH surge is accompanied by a switch of expressing MR in the follicle to express GR in luteal cells. By this time cortisol rise in the follicular fluid is indicative of the 11β-HSD ovarian activity and supports glucocorticoid metabolism based on the transition between receptors (Whirledge *et al.,* 2010).

On the other side, mineralocorticoid receptors, similarly to GR, are members of the nuclear receptor superfamily of ligand-dependent transcription factors that controls gene transcription by transactivation or transrepression in response to its MR-proteins interactions (Joseph *et al.,* 2017). Their location and expression, regarding the reproductive system, are more restricted in comparison to GR (Joseph *et al.,* 2017). They are limited tissue distributed, with a strong expression confined to conventional aldosterone target organs such as the kidney, colon, the salivary glands and some particular brain regions (Chapman *et al.,* 2013).

1.6. Anxiety and Depression Biomarkers

1.6.1. Cortisol and Cortisone as Stress Response Biomarkers on Female Reproductive System

Cortisol is secreted in response to daily stressing factors and situations (Peti *et al*., 2018). Is important that their levels are kept stable in the bloodstream because, when insufficient or overproduced, severe morbidity may arise. This homeostasis is regulated by their binding to corticosteroid receptors (GR and MR) (El-Farhan *et al.,* 2017). Cortisol follows a pulsatile circadian rhythm, with a higher amplitude between 3 and 9 a.m. (El-Farhan *et al*., 2017) and a surge approximately one to two hours before waking up in the morning, a period called *cortisol awakening response* (Law *et al.,* 2020). Its frequency is lowered between 6 p.m. and midnight. This is possible due to an increased ACTH response in the morning, which tends to decrease along the day and night, a fact that explains the low levels of cortisol during these periods (El-Farhan *et al*., 2017). Due to its characteristics, cortisol can be used to determine the HPA axis activity under stressful situations (Tomlinson *et al.,* 2001). Multiple factors also have an impact on its rhythm, such as age, ethnicity and the menstrual cycle (El-Farhan *et al.,* 2017).

The possible use of cortisol to study oocyte function was initially neglected, since the enzyme necessary for its synthesis *de novo* by the adrenal gland, the 21α-hydroxylase, is found at deficient levels in the ovaries (Dehennin *et al.,* 1987; Hillier, 1994). This idea had already been refuted when a relationship between 21α-hydroxylase and oogenesis was suggested (Michael *et al.,* 2003). Although ovarian cells do not significantly synthetize glucocorticoids *de novo*, there is the presence of the 11ß-HSD enzyme that is capable of converting cortisol in cortisone and vice-versa (Dehennin *et al.,* 1987; Smith *et al.,* 2000; Tetsuka *et al.,* 1997). CBG-bound cortisol, as well as other large proteins commonly found in blood, are also observed in follicular fluid, so it is likely that they cross the barrier into the preovulatory follicles and may act as a protection during the development of the follicle (Fateh *et al*., 1989; Jimena *et al.,* 1992). Once inside follicles, some cortisol is detached from the CBG protein, an event that can arise from the presence of high amounts of progesterone found in FF (Andersen, 2002; Andersen *et al.,* 1994; Michael *et al.,* 2003).

Cortisol requires a protein carrier to be transported across the body (El-Farhan *et al.,* 2017). However, it can exist either in a free state or attached to proteins in the serum and in the pre-ovulatory follicular fluid (Andersen *et al.,* 1994; Fateh *et al.,* 1989). There is a higher concentration of total cortisol (i.e., bound or free) in serum compared to the follicular fluid. This fact has already been observed by Andersen and his colleagues (1994), which inferred that follicular fluid has around 78% of the total cortisol present in serum. More specifically, in serum cortisol is 70-80% bound to CBG, 10-20% is bound to albumin and only a small fraction of cortisol (5-10%) in circulation is unbound (Andersen *et al.,* 1994; El-Farhan *et al.,* 2017; Moore *et al.,* 1985). However, in follicular fluid of pre-ovulatory follicles, the rate of free cortisol is 22%, around 48% of cortisol is bound to CBG, and a small percentage is bound in low affinity to albumin. It indicates that around one-quarter of free biologically active cortisol is in the follicle at the moment of ovulation, a fraction that is higher than in serum (Andersen *et al.,* 1994). The reason why there is a higher percentage of free cortisol close to ovulation is due to an increase in the hormone progesterone. Progesterone competes with cortisol for a CBP binding site, and due to its higher affinity towards CBP, cortisol is left free (Andersen, 2002; Andersen *et al.,* 1994; Michael *et al*., 2003). Free cortisol is available to bind albumin, but because albumin binds easier to progesterone and estrogen in the follicle before follicle rupture, cortisol is once more left free, with only an average of 30% attached to albumin (Andersen *et al.,* 1994). Taken all this, the high affinity of progesterone and estrogen to CBP and albumin or, the less affinity of cortisol to CBP and albumin, can explain the existence of greater amounts of free cortisol in the follicle (Andersen *et al.,* 1994). The cortisol available in the pre-ovulatory FF is also speculated to be necessary to decrease inflammatory factors at ovulation that is described as an inflammatory event (Andersen *et al.,* 1994). Even though free and bound cortisol interact physically, they act in a physiologically independent manner (Moore *et al.,* 1985).

So far it has not yet been confirmed whether cortisol influences reproductive outcomes. Three studies have demonstrated contradictory results. One study has associated, after oocyte collection, high levels of cortisol in blood plasma and follicular fluid with unsuccessfully fertilized oocytes and thus, lower reproductive success rates (Jimena *et al*., 1992). These findings indicate that glucocorticoids may negatively

interfere with oocyte maturation (Whirledge *et al.,* 2010). Although high levels of cortisol may be associated with worse reproductive outcomes, they also seem to have a role in oocyte maturation (Fateh *et al.,* 1989). Some studies found that mature oocytes were enclosed within follicles, whose follicular fluid was rich in cortisol and, in contrast, immature oocytes were within follicles, whose follicular fluid contained lower concentrations of cortisol (Keay *et al.,* 2002; Lewicka *et al.,* 2003). Significant associations between elevated concentrations of cortisol in follicular fluid and an increased likelihood of becoming pregnant were also observed (Keay *et al.,* 2002). Finally, contrarily to the studies referred to before, one study does not confirm an association of cortisol and cortisone nor cortisol and cortisone ratios to implantation potential in embryos (Andersen *et al.,* 1999).

Regardless of the contradicting outcomes, cortisol is frequently used as a biomarker in population studies, to objectively assess and estimate the effects of stress on the neuroendocrine system (Antonelli *et al*., 2019), as it reflects the circadian pattern of ACTH (El-Farhan *et al.,* 2017). Elevated serum cortisol is thought to be a sign of stress (El-Farhan *et al.,* 2017).

The 11ß-HSD enzyme is regulated in different tissues and has two isoforms: 11β-HSD1, and 11β-HSD2, that interconvert cortisol and the inert 11-ketosteroid metabolite cortisone along the menstrual cycle *(figure 3)*. The enzyme behaves differently throughout oocyte maturation and plays a critical role in cortisol metabolism (Lewicka *et al.,* 2003; Smith *et al.,* 2000; Tetsuka *et al.,* 1997). In humans, 11β-HSD1 has a reversible bidirectional action depending on the redox state of NADP(H), i.e., the quantity of nicotinamide adenine dinucleotide (NAD+) and nicotinamide adenine dinucleotide phosphate (NADPH) available (Michael *et al*., 2003; Whirledge *et al.,* 2010). The 11ß-HSD1 isoform acts predominantly as a reductase and is dependent on the reduced state NADPH as a cofactor, to convert cortisone to cortisol (Tomlinson *at al.,* 2001). However, the isoform can still act as a dehydrogenase, mainly in tissue homogenates, depending on the abundance of NADP+ in the oxidized state to generate cortisone from cortisol. This bidirectional activity of 11ß-HSD1 is not yet clear (Michael *et al.,* 2003; Tomlinson *et al.,* 2001). Increased cortisol significantly decreases the biological activity of cortisone which, in turn, has negligible affinity for the glucocorticoid receptor (Tomlinson *et al.,* 2001). The

expression of 11ß-HSD1 mRNA is stimulated by cortisol in various cell types and tissues (Wu *et al*., 2020). It includes the ovaries (Benediktsson *et al.,* 1992), the oocyte (Lewicka *et al.,* 2003), adipose tissue, muscle, inflammatory cells, and the adult brain (Chapman *et al.,* 2013). On the contrary, the ovarian 11β-HSD2 isoform acts exclusively as a high-affinity dehydrogenase and is dependent on the oxidized state of nicotinamide adenine dinucleotide (NAD+) as an enzyme cofactor to reversibly catalyze the inactivation of cortisol to cortisone (Michael *et al.,* 2003). Thus, cortisone binds to MR and consequently cortisol is kept away with low biological activity to these receptors (Ferrari, 2010; Stewart *et al.,* 1995). The human ovary has low levels of mRNA encoding type 2 11β-HSD (Michael *et al.,* 2003).

The 11β-HSD1 and 11β-HSD2, belong to the short-chain alcohol dehydrogenase superfamily of enzymes and are transcribed by *HSD11B1* and *HSD11B2* genes, respectively. There is low homology between those genes (Michael *et al*., 2003). Both isoforms were demonstrated to exist in the uterus (Chapman *et al.,* 2013) and in some other ovarian cell types, including the oocyte, *cumulus* cells, theca cells, granulosa cells, corpus luteum and the OSE (Whirledge *et al.,* 2010; Wu *et al.,* 2020).

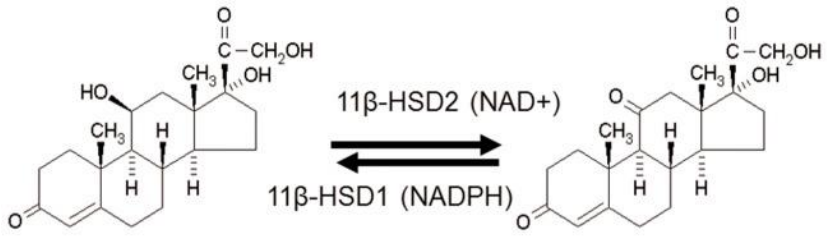
The ovarian cycle is differentially regulated by both enzymes depending on whether it is the follicular phase or the luteal phase (Whirledge *et al.,* 2010). Just before ovulation, there is a transition from the 11β-HSD2 to the 11β-HSD1 isoform expression and, a shift between the expression of both receptors is also observed. The 11β-HSD1 activation and GR expression prevail in the luteal phase (Wu *et al*., 2020), a phenomenon likely initiated by the LH surge and boosted by IL-1 and prostaglandins (Chapman *et al*., 2013). Contrarily, glucocorticoids are inactive during the follicular phase, a period characterized by follicular maturation and 11β-HSD2 expression (Michael *et al*., 2003; Whirledge *et al.,* 2010; Wu *et al*., 2020). At this stage, 11β-HSD1 is found inhibited probably for gonadal steroidogenesis protection (Michael *et al*., 2003). During the follicular phase, although glucocorticoids show affinity towards MR, when 11β-HSD2 is being expressed, glucocorticoids are kept away from MR. The 11β-HSD2 allows the binding of aldosterone (Joseph *et al.,* 2017). An example of these observations is human granulosa cells, which only expressed 11β-HSD2 and MR, before reaching the LH surge (Michael *et al*., 2003). In women undergoing *in vitro* fertilization,

the presence of 11β-HSD2 activity in cultured granulosa cells predicted pregnancy failure, but its absence predicted pregnancy success (Chapman *et al.,* 2013).

It has been admitted that the two isoforms may be in part regulated by sex hormones. During follicular maturation, estrogen may modulate 11β-HSD2 expression, while progesterone may regulate the expression of 11β-HSD1 from the LH surge onwards, until the end of the luteal phase, characterized by a drop in progesterone and thus, in the expression of 11β-HSD1 (Michael *et al*., 2003; Whirledge *et al.,* 2010). Nevertheless, one study said that the 11β-HSD1 expression does not depend on progesterone production (Thurston, 2003).

Acute cellular changes (Whirledge *et al.,* 2010), cytokines, interleukins (e.g., IL-1 family) and prostaglandin production (Michael *et al*., 2003), triggered by the ovarian inflammation around follicle rupture, are inhibited by the OSE cells action at this site. OSE cells form a simple cuboidal-cell layer in the epithelium surrounding the surface of the ovary and upregulate the production of anti-inflammatory glucocorticoids in granulosa cells. These glucocorticoids, such as cortisol, increase 11β-HSD1 expression, by the time of ovulation, as a repair mechanism against the inflammatory process (Michael *et al.,* 2003; Whirledge *et al.,* 2010). This inflammatory response has a role in the recovery of the epithelial surface of the damaged ovary after oocyte rupture (Tetsuka *et al.,* 1997; Tomlinson *et al.,* 2001; Zhu *et al.,* 2016), as well as the remodulation of the extracellular matrix (Donahoe, 2009).

Due to the increasing glucocorticoid synthesis at ovulation, it is expected that cortisol concentrations are higher in the follicular fluid at this time (Michael *et al*., 2003). This increase appears to be related to the promotion of final oocyte maturation and hence increases a potential pregnancy (Harlow *et al.,* 1997; Smith *et al.,* 2000).



Cortisol Cortisone

Figure 3 - **Interconversion of cortisol and cortisone by the two enzyme isoforms, 11ß-HSD1 and 11ß-HSD2, of the 11ß-hydroxysteroid dehydrogenase (11ß-HSD).** Adapted from Hu *et al*., (2013)

1.7. Influence of Psychological Assistance in ART treatment

It is not part of the doctrine of the European Society for Human Reproduction and Embryology to request psychological assistance during treatment for patients with infertility (Gameiro *et al.,* 2015).

Some studies state that anxiety causes women to give up before starting treatment, during treatment or even before reaching a pregnancy, due to the uncertainties about the treatment, viability of a pregnancy, social stigma, diagnosis, and age (Gameiro *et al.,* 2015; Rooney *et al.,* 2016; Smeenk *et al.,* 2001).

Arising from the preliminary indication that the psychological disposition of a couple acts upon their fertility, with a consequent influence on the reproductive outcomes (Campagne, 2006), it is important to implement psychological monitoring practices. These would involve a clinical psychologist, before and throughout the treatment, since ART treatments are sensitive procedures easily disturbed under psychological oscillations and disorders (Campagne, 2006; Hasanpoor-Azghdy *et al.,* 2014; Rooney *et al.,* 2016).

Current studies have limited validity regarding the efficacy of these therapies. It is probably due to a scarcity of standardized psychometric assessments or because studies only rely on stable variables (size of the sample, study design or even population characteristics) turning results hard to compare (Smeenk *et al.,* 2001). Apart from stable variables, some variable factors, such as psychological distresses, may be included in these type of studies (Campagne, 2006; Smeenk *et al.,* 2001).

1.8. Objetives

The present dissertation aims to explore the association between anxiety and stress biomarkers in follicular fluid and blood plasma, with the obtained reproductive outcomes of women undergoing ART treatments. Since the FF reflects the hormones present close to human oocytes, its analysis may allow an association with oocyte maturation and fertilization. The most related hormone to stress, cortisol, is pointed as

one of these biomarkers (Campagne, 2006) and its quantification may allow an indirect analysis of the 11ß-HSD activity effects. Thus, this study has the purpose of determining, at first, the concentrations of cortisol and cortisone, that reflect and allow to predict better ART outcomes. Finally, the same hormones are measured in blood plasma to see if they can replace the hormonal concentrations measured in FF.

The hypothetic conclusion that the values obtained in FF are similar to the cortisol and cortisone levels obtained by routine analysis in blood plasma, would be of great interest in the improvement of ART procedures. It would aid in the selection of patients with more anxiety and depression levels to be directed to psychological therapy, before and during treatment. Additionally, it would avoid high rates of waiver from women before treatment or already submitted to treatment.

Chapter II – Materials and Methods

The review of the state of the art, which served as the basis for the construction of this thesis, used PubMed database as the main search source.

Each stage stipulated for carrying out the following study was applied individually to each woman.

2.1. Laboratorial Project Design

2.1.1. Type of Study

Along the present laboratory study, several phases were elaborated, following a logical temporal execution order, namely:

i. Bibliographic and/or existing literature search;
ii. Design of the stages for the execution of the project;
iii. Acquisition of necessary reagents and materials;
iv. Execution of the steps stipulated in the project design;
v. Analysis and processing of the research data obtained;
vi. Writing of the final document;
vii. Thesis/dissertation defense;

Steps ii and iv comprise the following phases:

a) Completion of the stress and anxiety assessment questionnaire by women undergoing ART treatments;
b) Processing and storage of the patient FF that was collected and provided by the embryologists;
c) Processing and storage of the patient blood plasma that was collected and provided by the nurses;
d) Measurement of stress biomarkers, i.e., cortisol and cortisone, present in follicular fluid and in blood plasma;

e) Assessment and analysis of the clinical reports from the embryology database, which contains the evaluation of the oocytes and embryos;

Step v comprises the following steps:

a) Comparison of the cortisol and cortisone levels, obtained in follicular fluid with the levels of the same biomarkers measured in blood plasma in each patient;
b) Association of the biomarkers levels obtained in the follicular fluid and blood plasma with the levels of anxiety and depression of each patient and, also, with the clinical outcomes after the ART treatment.

2.1.2. Limitations

The fact that the volume of follicular fluid used corresponds to the total volume aspirated from the dominant follicles of the two ovaries of each patient, at the time of follicular puncture, is a limitation of the experiment. Ideally, FF hormonal measurements should be taken individually in each follicle. The fact that the collected follicles are from the same woman is not indicative that their composition is identical, and sometimes aspirated follicles do not contain oocytes, an aspect that would negatively contribute to an unreal correlation between the measured variables. Specifically, this limitation makes it impossible to associate an oocyte with its correspondent follicular fluid.

2.1.3. Sample Characterization

A survey was conducted with a group of women who started a cycle of assisted reproductive techniques treatment (IVF/ICSI) at the Reproductive Medicine Unit of the Coimbra Hospital and University Centre (CHUC) (Coimbra, Portugal) from September 2021 to April 2022.

Women who met the inclusion criteria, expressed interest to participate in the study, and agreed to provide follicular fluid and blood plasma samples, filled in a written informed consent. Additionally, all participants were solicited to complete the

Hospital Anxiety and Depression Scale (HADS) questionnaire, which assesses anxiety and depression symptoms experienced during the past week. The questionnaire was delivered to women in the Portuguese version (Pais-Ribeiro *et al.,* 2007; Appendix I) and its assessment was carried out in collaboration with the clinical psychologist at Reproductive Medicine Unit of CHUC. According to the classification of the HADS questionnaire, the study control corresponded to women with normal clinical background, i.e., those who do not suffer from anxiety or depression.

Eligibility criteria included women aged ≥ 18 to ≤ 40 years of age, from which two groups were made: i) women from 18 to 34 years old and ii) women from 35 years to 40 years of age. This division is important as 35 is considered the turn point age, where there is observed a notorious decrease in oocyte potential (Kahveci *et al.,* 2018). There is a decrease in the ovarian reserve by the age of 35 (Amanvermez et al., 2016) and, age-related fertility factors may decrease gamete quality (Duncan *et al.,* 2018). Furthermore, age has also been pointed out as a factor that may influence the secretory pattern of cortisol (El-Farhan *et al.,* 2017).

Of the 108 invited women, 102 agreed to participate in the study, and 6 declined or gave up. Some of the reasons for not participating included Covid-19 issues, missing questionnaires, no eligibility, or due to unknown reasons. Of the women who consented to participate, 58 met the inclusion criteria. Sixteen were aged between 18 and 34 years old and 42 were over 35 years old.

Women were assured that their participation would not, in any case, affect the ART treatments. The laboratory experiment described in the present thesis, the completion of the questionnaire, as well the participation in the study was granted ethical approval by the Ethics Committee of CHUC (Research Project Proc. Nº CHUC-170-20), to protect the rights, ensure confidentiality and the well-being of all included participants. Patients did not get any additional compensation because of their participation in the study.

2.1.3.1. Sample Size Determination

A power analysis (G*Power v3.1.9, Universität Dusseldorf, Germany) using an effect size of 0.8 and a power of 0.8 (generic input) was performed to estimate the

appropriate number of participants for a two-tailed difference between two independent means statistical test (t tests). The number of samples necessary to give the study significance corresponds to 52.

2.1.4. Hospital Anxiety and Depression Scale Questionnaire

The growing concern of physicians to discern whether their patients, with long-term illnesses, have anxiety or depression symptoms related to the disease, or if it persists as an independent factor, led to the construction of a questionnaire, entitled *The Hospital Anxiety and Depression Scale* (HADS) *(figure 4)*. It is a quick, simple, and reliable tool designed to indicate the presence of anxiety and/or depression, over the previous week (Snaith, 2003; Zigmond *et al.,* 1983). Early intervention and detection are relevant since these pathologies may influence the effectiveness of ART treatments (Beekman *et al.,* 2018).

The questionnaire takes 2 to 5 minutes to fully fill out and comprises 14 statements pertinent to either generalized anxiety or depression, the latter containing, in its majority, reflections of the state of anhedonia. The 14 questions are subdivided in 7 reflecting anxiety and 7 reflecting depression questions. Each of these questions present four possible answering categories (0 to 3). The sum of these categories led to the assignment of a final rate ranging from 0 to 21 for anxiety and 0 to 21 for depression, in which lower final scores indicate low incidence of the condition, and *vice-versa*. Within the 0 to 21 rate scale, 0 to 7 (normal) belong to patients in the normal range, 8 to 10 (borderline) correspond to those patients in which there is a suspicion of psychological pathology and finally, 11 to 21 score (abnormal) indicates the presence of anxiety and/or depression (Snaith, 2003).

The HADS is suitable for research purposes and clinical practices although, in any of the previous categories should be used as a definitive diagnostic tool, but rather to identify the presence of psychiatric symptoms (Beekman *et al.,* 2018). The complete Portuguese version validated by Pais-Ribeiro *et al.,* (2007) that was delivered and fulfilled by the women at CHUC is attached on the complementary data (appendix I).

Hospital Anxiety and Depression Scale (HADS)

Tick the box beside the reply that is closest to how you have been feeling in the past week.
Don't take too long over you replies: your immediate is best.

D	A		D	A	
		I feel tense or 'wound up':			**I feel as if I am slowed down:**
	3	Most of the time	3		Nearly all the time
	2	A lot of the time	2		Very often
	1	From time to time, occasionally	1		Sometimes
	0	Not at all	0		Not at all
		I still enjoy the things I used to enjoy:			**I get a sort of frightened feeling like 'butterflies' in the stomach:**
0		Definitely as much		0	Not at all
1		Not quite so much		1	Occasionally
2		Only a little		2	Quite Often
3		Hardly at all		3	Very Often
		I get a sort of frightened feeling as if something awful is about to happen:			**I have lost interest in my appearance:**
	3	Very definitely and quite badly	3		Definitely
	2	Yes, but not too badly	2		I don't take as much care as I should
	1	A little, but it doesn't worry me	1		I may not take quite as much care
	0	Not at all	0		I take just as much care as ever
		I can laugh and see the funny side of things:			**I feel restless as I have to be on the move:**
0		As much as I always could		3	Very much indeed
1		Not quite so much now		2	Quite a lot
2		Definitely not so much now		1	Not very much
3		Not at all		0	Not at all
		Worrying thoughts go through my mind:			**I look forward with enjoyment to things:**
	3	A great deal of the time	0		As much as I ever did
	2	A lot of the time	1		Rather less than I used to
	1	From time to time, but not too often	2		Definitely less than I used to
	0	Only occasionally	3		Hardly at all
		I feel cheerful:			**I get sudden feelings of panic:**
3		Not at all		3	Very often indeed
2		Not often		2	Quite often
1		Sometimes		1	Not very often
0		Most of the time		0	Not at all
		I can sit at ease and feel relaxed:			**I can enjoy a good book or radio or TV program:**
	0	Definitely	0		Often
	1	Usually	1		Sometimes
	2	Not Often	2		Not often
	3	Not at all	3		Very seldom

Please check you have answered all the questions

Scoring:
Total score: Depression (D) _____ Anxiety (A) _____
0-7 = Normal
8-10 = Borderline abnormal (borderline case)
11-21 = Abnormal (case)

Figure 4 - **Hospital Anxiety and Depression Scale questionnaire.** Original version that comprises 14 anxiety and depression questions from Zigmond *et al.,* (1983)

2.1.5. Medical Procedures

Ovarian stimulation followed the protocol adopted at CHUC Reproductive Medicine Unit. Women were clinically monitored using a transvaginal pelvic

ultrasound. As soon follicles reached a diameter of about 17 mm, an injection of the hCG hormone was administrated, inducing oocyte maturation and ovulation. Several stimulated follicles were retrieved by follicular puncture 36 hours after the hCG injection (Georgiou *et al.,* 2018; Rienzi *et al.,* 2012). Follicles content is collected and later manipulated *in vitro* by the embryologists that extract the oocytes present within the follicular fluid, which is usually discarded and that was used for biomarkers evaluation. This follicular fluid corresponds to the total volume aspirated from the dominant follicles of both ovaries of each patient, at the time of oocyte collection. Oocyte collection follows the protocol adopted at CHUC Reproductive Medicine Unit.

2.1.6. Laboratory of Embryology Procedures

2.1.6.1. Assessment of Oocyte Maturity and Quality

The maturity and quality of oocytes were evaluated by embryologists and some of the obtained data included the number of GV, MI, and MII oocytes. The number of oocytes inseminated or injected allowed to calculate the fertilization rate of oocytes and the cleavage rate of embryos.

Maturation rate (MR) was calculated as follows, where MII is the number of oocytes in metaphase II and $N_{oocytes}$ is the number of total oocytes:

$$Maturation\,rate = \frac{MII}{Noocytes}$$

2.1.6.2. Assisted Reproductive Techniques

In vitro fertilization or intracytoplasmic sperm injection were performed, depending on the type of treatment required in each case, and followed the protocol adopted at Reproductive Medicine Unit of CHUC. The results obtained from IVF or ICSI, regarding fertilization and embryo implantation rates, were compared to the FF and BP cortisol and cortisone levels, and then with the anxiety and depression levels obtained from the HADS questionnaire.

In an *in vitro* fertilization procedure, the oocytes separated from the follicular fluid, are incubated with a controlled concentration of sperm (Baldi *et al.,* 2020). It increases the chances of fertilization and, consequently, pregnancy (Bayer *et al.,* 2011) *(figure 5)*.

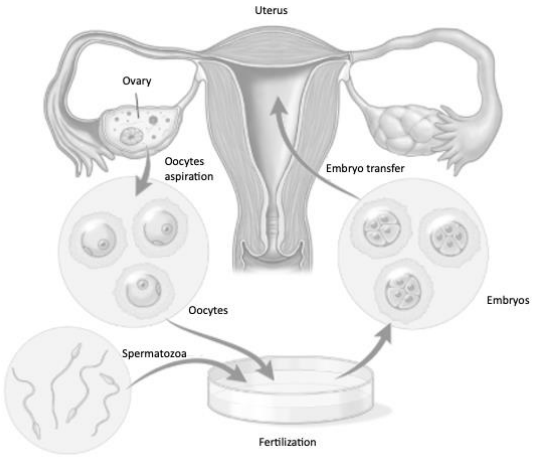


Figure 5 - *In vitro* Fertilization Procedure. Adapted from Jonathan Dimes, from BabyCenter (2021)

The Intracytoplasmic sperm injection is used in clinical cases of severe male factor, in which there is low sperm motility and/or low sperm numbers and in cases where IVF happens to fail. It involves the microinjection of only one normal sperm directly into the cytoplasm of a previous hyaluronidase denuded mature oocyte (Rienzi *et al.,* 2012; Bayer *et al.,* 2011; Gardner *et al.,* 2017) *(figure 6)*.

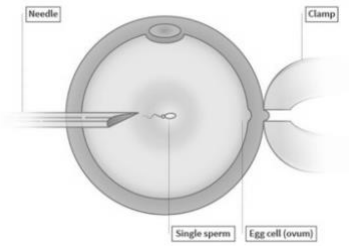


Figure 6 - Intracytoplasmic Sperm Injection Procedure. Taken from Fertility.com (2019)

2.1.6.3. Evaluation of Fertilization, Implantation and Pregnancy

The analysis of the fertilization and embryo development, from the day of fertilization up to day 5 of the embryo stage, was also carried out using the data provided by the embryologists. *Table 1* represents an adaptation of the Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, (2011) used by the embryologists, used for the analysis of embryonic development. It aims to normalize the evaluation of embryonic development.

Table 1 - **Types of observation, times and developmental stages expected in fertilized oocytes and cultured embryos, over time, in IVF (*In vitro* fertilization) and ICSI (Intracytoplasmic sperm injection).** Adapted from the Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, (2011)

		Type of observation	Hours post-insemination	Expected stage of developmental
Pre-zygote	Day 1	Fertilization check	17±1	Pronucalear stage
		Early cleavage check	26±1 (ICSI) and 28±1 (FIV)	2-cell stage
Embryon	Day 2	Embryo assessment	44±1	4-cell stage
	Day 3	Embryo assessment	68±1	8-cell stage
	Day 4	Embryo assessment	92±1	Morula
	Day 5	Embryo assessment	116±1	Blastocyst

On day 1, between the following 16 to 18 hours, after incubating the oocytes with sperm, fertilization is assessed and confirmed through the presence of two pronuclei.

Fertilization rate (FR) was determined by using the following formula where F is the number of fertilized oocytes that present two pronuclei and two polar bodies (2 = 2) and MII is the number of total oocytes in metaphase II:

$$Fertilization\ rate = \frac{F}{MII}$$

Early embryo cleavage is expected to occur 26 hours after incubation in ICSI and 28 hours after incubation in IVF, and indicates the division of the zygote in two cells. Day 2 (4-cell stage) is also indicative of early cleavage parameters. A later embryo development, considers the evaluation of the embryo's ability to divide and the number and shape of the blastomeres. It also comprises higher levels of differentiation and control checkpoints, which are mainly observed on day 3 (8-cell stage), day 4 (morula) and day 5 (blastocyst). The development of *in vitro* embryos allows the determination of the cleavage rate (CR) that was obtained by the following formula, where $N_{embryos}$ is the number of embryos obtained at day 2 of development and F is the number of fertilized oocytes that present two pronucleus and two polar bodies:

$$Cleavage\ rate = \frac{\text{Nembryos}}{F}$$

Embryo development is normally assessed on day 2 at the 4-cells stage because at this early cleavage phase is possible to associate between embryo visible morphological features more effectively (e.g., pronuclear and cytoplasmic morphology) and to predict favorable outcomes. The evaluation of an early embryonic cleavage pattern improves the chances of selecting high potential embryos, by excluding those zygotes that cleaved precociously many times (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011).

Follicular fluid and blood plasma concentrations of cortisol, cortisone and the cortisol/cortisone ratio were compared with the ART outcomes and the HADS questionnaire. The cortisol/cortisone ratio allowed the evaluation of the expression of the two isoforms of the 11ß-HSD enzyme in the ovary. Analysis was further divided into four groups where: i) oocytes that did not fertilize (n = 3); ii) oocytes that fertilized but were not transferred (n = 38); iii) oocytes fertilized and transferred that did not result in pregnnacy (non-pregnant women) (n = 14); iv: oocytes fertilized and transferred that resulted in pregnancy (pregnant women) (n = 3).

2.1.7. Hormonal Measurements

The measurement of cortisol and cortisone in follicular fluid allowed to indirectly evaluate the 11ß-HSD enzyme activity in the granulosa cells. Measure the same hormones in blood plasma of the same patients allowed to verify if the hormonal levels in blood plasma could replace, or not, follicular fluid measurements. The BP was collected in the morning and at the same time as the FF. It is important due to the circadian rhythm of cortisol and to avoid results disparities. Hormonal concentrations in FF and blood plasma were measured in duplicate, and both measures were averaged to increase results reliability.

The number (N) of samples quantified and evaluated in the study was 58.

2.1.7.1. Processing of Follicular Fluid Samples

Following follicular puncture, the oocytes and follicular fluids recovered from follicles were separated by the embryologists.

Follicular fluid is classified into four categories: cystic, serous, serous-hematic, and hematic *(figure 7)*. This division is made accordingly to the color appearance of the fluid that is contaminated with red blood cells. Cystic fluid is removed from cysts present in the ovary at the time of follicular puncture and these samples are excluded. Serous samples have a small amount of red blood cells while, serous-hematic samples have higher quantities of red blood cells. Both are selected for hormonal analysis. Finally, hematic samples have the greatest volume of red blood cells giving it a reddest color and are excluded from further analysis.

Then, follicular fluid was stored either in endotoxin-free 50 ml sterile conical tubes (CL474, Biosigma) or in 15 ml sterile conical tubes (CL482, Biosigma), depending on the volume of the sample, and then centrifuged at 300 g for 15 minutes (Hettich Rotina 46R Centrifuge) at 4ºC to remove live cells. The formed pellet after the centrifugation was discarded and the supernatant obtained was centrifuged at 2000 g for 20 minutes (MicroCL 17 Microcentrifuge, Thermo Scientific) at 4ºC to remove any cellular debris present in the solution. Again, the supernatant was recovered and it was immediately stored frozen at -80ºC for future hormonal analysis.

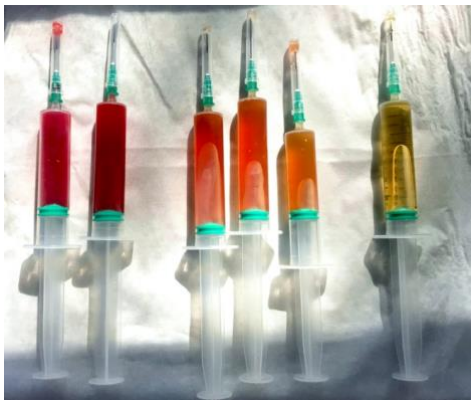


Figure 7 - Representation of follicular fluid samples characterization. From left to right: hematic, serous-hematic and serous

2.1.7.2. Processing of Blood Plasma Samples

Serum-free cortisol analysis is more suitable for clinical purposes as it is a more reliable indicator of adrenal function. It replaced total cortisol serum analysis because ill patients are often found with low plasma levels of CBG. Measuring total cortisol would negatively impact the ratio of CBG and thus, total cortisol concentrations. However, free cortisol analysis is limited as it requires complex protocols due to its low concentrations in serum. Also, due to the variable affinity of cortisol to CBG, it is not advised to quantify free cortisol based on both total cortisol and CBG measurements (Turpeinen *et al.,* 2013). Taking this into consideration, total cortisol was quantified in blood plasma, following the instructions of the cortisol kit, which measures total cortisol in serum or plasma. Cortisol can be quantified in serum or plasma with comparable results (El-Farhan *et al.,* 2017).

A volume of 7 ml of blood was collected into K_2EDTA plasma plastic tubes (Biosigma) from a group of 17 women admitted to the study. Blood sample tubes were gently inverted 8 times to avoid platelet aggregation. A volume of 3 ml of blood was transferred to 15 ml sterile conical tubes (CL482, Biosigma) and these were immediately centrifuged at 1100 g for 10 min at RT (18–25ºC). After centrifugation cell-free plasma was recovered with a sterile Pasteur pipette and placed in a 1.5 ml sterile eppendorf. Using a 1 ml syringe, plasma was aspirated and subsequently filtered with a 0.8 mm filter, drop by drop, to another 1.5 ml eppendorf. The eppendorf was

stored at 80ºC until further processing (Gaspar *et al.,* 2020; Stratton *et al.,* 2020; Thermo Scientific, 2007).

2.1.7.3. Enzyme-Linked Immunosorbent Assay

The Enzyme-Linked Immunosorbent Assay (ELISA) was the considered technique for detecting and quantifying cortisol in follicular fluid and blood plasma samples. One reason for this, is the fact that it is considered the immunoassay of choice to quantify substances of interest within biological fluids (Alhajj *et al.,* 2022; Aydin, 2015). Furthermore, the technique is applied in a variety of routine diagnostic assays and fields (Aydin, 2015; Burns *et al.,* 2005; Engvall, 2010).

ELISA is an immunoassay methodology capable of detecting and quantifying analytes of interest within biological fluids, even if present at low amounts (Aydin, 2015; Kinn Rød *et al.,* 2017). This fact is possible due to a high specificity between the substances in analysis (e.g., vitamins, hormones and proteins) and the antibodies immobilized in the polystyrene solid-phase microplate wells. This perfect match avoids nonspecific bindings (Aydin, 2015; Engvall, 2010).

It is a fast, easy, safe and low-cost specific assay (Aydin, 2015). It does not demand specific equipment and radioisotopes, avoiding health issues due to radiation (Aydin, 2015), and detects as low as a few picograms per milliliter of the analyte of interest in plate wells when using high-affinity antibodies (Kinn Rød *et al.,* 2017). Being less reliable comprises one of the limitations in comparison to RIA (Aydin, 2015). Differences in antibodies specificity towards cortisol and other steroid hormones (El-Farhan *et al.,* 2017), the need to sometimes release steroid molecules from its carrier protein before quantification (El-Farhan *et al.,* 2017) and, the requirement of qualified personnel to execute this sensitive assay (Aydin, 2015; Burns *et al.,* 2005; Engvall, 2010; Kinn Rød *et al.,* 2017), are some other disadvantages.

The competitive enzyme-linked immunosorbent assay (cELISA) uses a polystyrene plate composed of 96 microtiter wells, as the solid phase, and is pre-coated with a monoclonal goat anti-mouse IgG antibody. The assay is based on the competition for a limited number of binding sites to the capture antibody between an enzyme-linked antigen, (i.e., the enzyme-conjugate or labeled antigen) and an

untagged analyte to be measured in the sample. When the capture antibody is added to the wells, it immediately binds the monoclonal antibodies immobilized in the plate wells. Then, the labeled antigen competes with the analyte for a binding site to the capture antibody. An incubation period is followed and the non-specific bound agents, i.e., the unbound labeled and unlabeled antigens, are washed away. Afterward, the detection of the analyte is accomplished by adding a specific colorimetric substrate that measures the activity of the enzyme-conjugate bound to the capture antibody. The reaction is stopped by a stop solution and is read in a spectrophotometer. The signal is inversely proportional to the concentration of the analyte present in the sample. For example, a weaker signal occurs when higher concentrations of the analyte of interest are in the sample. It is accompanied by a low color intensity and low absorbance values after the read, meaning fewer capture antigens tagged the conjugate antibodies (EIAHCOR, Invitrogen, CA, USA).

2.1.7.4. Measurement of Cortisol and Cortisone in Follicular Fluid and Blood Plasma

As mentioned before, the determination of intrafollicular concentrations of cortisol and cortisone allows to obtain an indirect enzymatic expression of 11ß-HSD in the oocyte before follicular puncture. A cELISA was performed for the detection of cortisol and cortisone concentrations in, both, follicular fluid and blood plasma samples. For this purpose, an optimization of the Cortisol Competitive human ELISA kit (Catalog. No. EIAHCOR; Invitrogen, CA, USA) and Multi-Species Cortisone ELISA Kit (IMLEKT, Innovative research, MI, USA) was performed to measure cortisol and cortisone levels, respectively.

2.1.7.4.1. Cortisol in Follicular Fluid

1. Assay Preparation

The kit was shipped at -20ºC and once opened its components were either immediately used or were stored at 4ºC and used within 2 weeks. The components are the following:

- Cortisol Standard; 32,000 pg/ml cortisol in a stabilizing solution, 125 μl;
- Assay Buffer Concentrate (5X), 28 ml - Stable at 4ºC for 3 months;
- Clear 96 strip-well plate - Goat anti-Mouse IgG coated plate, 1X;
- Cortisol Antibody, 3 ml;
- Cortisol Conjugate, 3 ml;
- Dissociation Reagent, 1 ml;
- Wash Buffer Concentrate (20X), 30 ml - Stable at room temperature (RT) for 3 months;
- TMB (Tetramethylbenzidine) Substrate, 11 ml;
- Stop Solution - contains 1 M HCl, CAUSTIC, 5 ml;
- Plate Sealer (1X);

a) Reagents preparation

All kit components reached RT before the assay. To prepare 1X Assay Buffer, 14 ml of the Assay Buffer 5-fold concentrate (5x) were diluted in 56 ml of distilled water into a flask. To prepare 1X Wash Buffer, 15 ml of the Wash Buffer 20-fold concentrate (20x) were diluted in 285 ml of distilled water into a flask. The Assay Buffer is composed of a phosphate-buffered solution with bovine serum albumin (BSA). BSA acts as a diluent and blocking agent by passively adsorbing to all remaining binding sites in the microplate and thus, preventing non-specific binding of undesired biological agents. It is designed to improve the signal-to-noise ratio and reduce the background by not interfering between the antibody and the epitopes of the antigen already present in the wells. It also offers stabilization between ELISA samples and

standards. The wash Buffer is composed of Tris-buffered saline with 0.05% Tween-20, a suitable detergent in most applications. It is used to rinse and remove non-bound materials from plate wells without destabilizing antigen-antibody binding interactions. Therefore, it increases the sensitivity and signal-to-noise ratio and reduces the background in the wells.

b) Samples preparation

Since cortisol in follicular fluid is bound to proteins (Andersen *et al.,* 1994), even though in less extent than in blood plasma, the optimization of this ELISA kit for FF followed the procedure steps stablished for serum and plasma samples described in the kit, after the necessary centrifugations, as previously described.

In a 1.5 ml Eppendorf, were added 5 μl of dissociation reagent, and 5 μl of the FF sample previously centrifuged. The solution was vortexed and incubated at RT for at least 5 minutes. In the same Eppendorf, 490 μl of 1X Assay Buffer were added to prepare a 1:100 dilution of FF sample. The manufacturer recommends doing further dilutions higher than 1:100. The 1:200 dilution was prepared with 25.5 μl of the 1:100 sample and 25.5 μl of Assay Buffer and, the 1:400 dilution was prepared with 12.5 μl of the 1:100 sample and 37.5 μl of Assay Buffer. These volumetric proportions were done to each sample and allowed the pipetting of 50 μl to the appropriate plate well. Since extremely high dilutions were obtained using the 1:200 ratio and the 1:400 ratio, further samples were only diluted with a 1:100 ratio.

c) Standards preparation

The standard stock solution, i.e., the standard with known cortisol concentrations, was prepared through serial dilutions to plot the standard curve. Eight propylene Eppendorf tubes of 1.5 ml were placed on a rack and labeled with different cortisol standard concentrations and ratios, as follows: 3200 pg/ml, 1600 pg/ml, 800 pg/ml, 400 pg/ml, 200 pg/ml, 100 pg/ml, 50 pg/ml and 0 pg/ml and, 1:1, 1:2, 1:3, 1:4, 1:5, 1:6, 1:7 and 1:8, respectively. To each of the Eppendorf´s was added Assay Buffer.

First, 450 µl of Assay Buffer was dispensed in the 3200 pg/ml labeled eppendorf and 250 µl of the same buffer was added into each of the remaining labeled eppendorfs, from 1600 pg/ml to 0 pg/ml.

Then, 50 µl of the cortisol reconstituted standard, previously pipetted up and down three times to ensure accurate delivery, was added with a micropipette to the 3200 pg/ml eppendorf with Assay Buffer and the solution was mixed gently. This reagent is the cortisol stock solution and is composed of an organic solvent. Serial dilutions were made, always mixing thoroughly between steps. It started with the transference of 250 µl of the previous dilution, 3200 pg/ml, to the next eppendorf, 1600 pg/ml, generating the first standard point. The process was repeated up to the 50 pg/ml eppendorf, using the same pipette head between each transfer *(figure 8)*. The zero standard, 0 pg/ml, corresponds to the maximum binding (B0) wells. It allows the determination of the maximum color development in competitive ELISAs and helps to regulate the progression and feasibility of the assay.

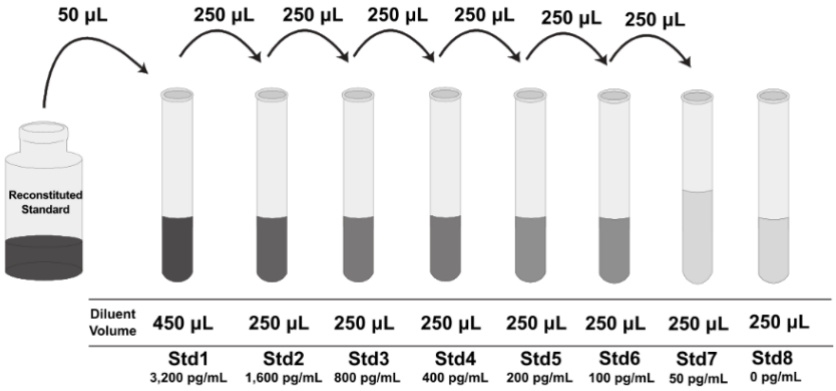


Figure 8 - Representation of the standard serial dilutions from the stock solution to generate a standard curve from 0 – 3200 pg/ml of cortisol. Taken from Cortisol competitive human ELISA kit (Catalog. No. EIAHCOR; Invitrogen, CA, USA) (96 tests))

2. Assay Performance

The 96-well plate has twelve removable strips composed of eight wells each and may be divided according to the researchers´ needs. It contained, in duplicate, the standards, the samples, and the negative control, which is the non-specific binding

(NSB) wells. NSB wells have no concentrations of cortisol and are used to determine NSB-corrected values for controls, standards, and samples, i.e., to determine the background due to unspecific binding of the conjugated enzyme.

The correspondent volumes of each sample, standard and reagents were added to the appropriate wells. A volume of 50 μl of the previous prepared standards, 3200 pg/ml, 1600 pg/ml, 800 pg/ml, 400 pg/ml, 200 pg/ml, 100 pg/ml, 50 pg/ml and 0 pg/ml, was added to the appropriate standard wells in the plate. Similarly, 50 μl of the previous prepared samples was added to the respective wells. After, 25 μl of the cortisol conjugate was added to every standard, samples and NSB wells. Cortisol conjugate is an antigen conjugated to a peroxidase, the horseradish peroxidase (HRP), necessary for the reading step and that competes with the analyte of interest for a binding site with the capture antibody. This is the enzyme of choice for researchers because it allows more molecules to be coupled to antibodies and eases the boost of signal. Finally, 25 μl of the Cortisol Antibody was added to all wells except for the NSB wells, as done before for the cortisol conjugate wells. The cortisol antibody acts as a capture antibody allowing the peroxidase-conjugate antigen or the analyte to bind. The NSB also had 75 μl of Assay Buffer.

The plate was sealed to prevent evaporation during 1h incubation time. It was incubated with shaking in a universal microplate mixer (LBX Instruments MM1500 series), allowing antigens and antibodies to bind accordingly to their affinity.

After the incubation period, the solution was aspirated, and the wells were washed four times with 300 μl of 1X Wash Buffer. It ensures that the unbound biological materials are cleared away from the wells, avoiding contaminations and non-specific binding.

Next, 100 μl of a colorimetric substrate, the 3,3',5,5'-tetramethylbenzidine (TMB) substrate, was added to every well and the plate was incubated for 30 minutes at RT, without shaking. TMB has high sensitivity and allows the detection of the conjugate due to an enzyme-substrate reaction, inducing the formation of a soluble colored blue product that accumulates over time relative to the quantity of enzyme present in the well (Burns *et al.,* 2005). It is possible because after adding TMB and in the presence of hydrogen peroxide (H_2O_2), the HRP catalyzes the oxidation of the substrate (TMB is

colorless) turning it blue. The more intense the color, the less the amount of cortisol present in the sample. Combining the HRP enzyme with TMB is considered a reliable and sensitive association (Burns *et al.,* 2005). The enzymatic reaction was quenched by adding 50 μl of the Stop solution (1M hydrochloric acid (HCl)) to each well, allowing the solution to change from blue to yellow.

For signal detection, the colorimetric substrate was read at a wavelength of 450 nm in a microplate reader (BioTek Synergy HT), and the Gen5 2.00 software was used within 10 minutes after adding the stop solution. The values obtained in each well correspond to the absorbance or optical density (O.D.), a property that describes a material´s capacity to absorb a given light intensity. In a competitive assay, the higher the O.D., the more intense the blue color showed and, thus, the lower the concentration of cortisol present. The samples O.D. values obtained, together with the cortisol standard concentrations, were used to obtain hormonal concentrations.

2.1.7.4.2. Cortisol in Blood Plasma

Cortisol levels in blood plasma were evaluated accordingly to the steps previously described for the analysis of cortisol in follicular fluid. Reagents preparation and the cortisol standards, used to plot the standard curve, were prepared as described in 2.1.7.4.1.

Regarding sample preparation, after blood plasma processing, the procedure steps described in the cortisol kit, for serum and plasma samples, were performed. The volumes of 5 μl of warm dissociation reagent and 5 μl of the blood plasma sample were added to a 1,5 ml eppendorf. The solution was vortexed and incubated at RT for at least 5 minutes. In the same eppendorf 490 μl of 1X Assay Buffer were added to prepare a 1:100 dilution of blood plasma sample. This dilution was enough to perform the assay and was done, individually, for every blood plasma sample.

2.1.7.4.3. Cortisone in Follicular Fluid

1. Assay Preparation

The Competitive Enzyme-Linked Immunosorbent Assay protocol was at first optimized to obtain valuable results.

The kit was shipped at -20ºC and once opened its components were either immediately used or were stored at -4ºC and used within 2 weeks. The components are the following:

- Cortisone Standard, 125 μl;
- Cortisone Antibody, 3 ml;
- Cortisone Conjugate, 3 ml;
- Assay Buffer Concentrate (5X), 28 ml – Stable at 4ºC for 3 months;
- Dissociation Reagent, 1 ml;
- Wash Buffer Concentrate (20X), 30 ml - Stable at RT for 3 months;
- TMB Substrate, 11 ml;
- Stop Solution - contains 1 M HCl, CAUSTIC, 5 ml;
- Plate Sealer (1X);
- Clear 96 strip-well plate - Goat anti Rabbit IgG coated plate, 1X;

a) Reagents preparation

All kit components were mixed and allowed to reach RT previous to use. To prepare 1X Assay Buffer, the Assay Buffer Concentrate 1:5 was diluted by adding one part of the concentrate to four parts of distilled water. For that, 14 ml of the Assay Buffer were diluted in 56 ml of distilled water into a flask. To prepare 1X Wash Buffer, the Wash Buffer Concentrate 1:20 was diluted by adding one part of the concentrate to nineteen parts of distilled water. For that 15 ml of the Wash Buffer were diluted in 285 ml of distilled water into a flask.

b) Samples preparation

Follicular fluid samples for cortisone analysis were prepared as described before for cortisol analysis in FF. A volume of 5 μl of the dissociation reagent and 5 μl of the centrifuged FF sample, were added to a 1.5 mL eppendorf. The mixture was vortexed and incubated at RT for at least 5 minutes. A volume of 490 μl of 1X Assay Buffer was added to the same eppendorf to prepare a 1:100 dilution of the FF sample and that was adequate to perform the assay. This preparation step was executed for each sample.

c) Standards preparation

The cortisone standard solution was prepared by performing serial dilutions that allowed to plot a standard curve.

Seven propylene Eppendorf tubes of 1.5 ml were placed on a rack and labeled with different cortisone standard concentrations and ratios, as follows: 3200 pg/ml, 1600 pg/ml, 800 pg/ml, 400 pg/ml, 200 pg/ml, 100 pg/ml and 50 pg/ml and, 1:1, 1:2, 1:3, 1:4, 1:5, 1:6, and 1:7, respectively. Primarily, 450 μl of Assay Buffer was dispensed in the 3200 pg/ml eppendorf and then, 250 μl of the same buffer was added into eppendorfs 1600 pg/ml to 50 pg/ml.

After, 50 μl of the reconstituted standard, previously pipetted up and down three times, was added and mixed, by vortexing, with the Assay Buffer in the 3200 pg/ml eppendorf. Serial dilutions were made, always vortexing thoroughly between steps. A volume of the 250 μl dilution from eppendorf 1, 3200 pg/ml, was transferred to eppendorf 2, 1600 pg/ml, generating the first standard point. The serial dilutions were repeated for eppendorfs 3, 800 pg/ml, through 7, 50 pg/ml. *Figure 8* is a schematic representation of the procedure, from eppendorf 1 to 7.

2. Assay Performance

The plate was composed of 96 removable striped wells. It contained, in duplicate, the standards, the samples, and the NSB wells. The correspondent volumes of each standard, sample and reagents added to the appropriate wells are detailed as follows. A volume of 50 μl of the prepared standards (3200 pg/ml, 1600 pg/ml, 800 pg/ml, 400 pg/ml, 200 pg/ml, 100 pg/ml and 50 pg/ml), was added to the appropriate standard wells in the plate. Similarly, 50 μl of the prepared samples were added to the respective sample wells. Then, 25 μl of the cortisone conjugate, which is coupled to a peroxidase, was added to every standard, sample and NSB wells and, 25 μl of the cortisone antibody was added to all wells except the NSB wells. The NSB also contained 75 μl of Assay Buffer. Finally, 50 μl of Assay Buffer was pipetted only into the B0 wells.

The plate was gently tapped to ensure adequate mixing and was subsequently sealed to prevent evaporation during the incubation time. It was placed to incubate for 2 hours at RT with shaking in a universal microplate mixer (LBX Instruments MM1500 series).

After the incubation period, the plate was aspirated and each well was washed four times with 300 μl of 1X Wash Buffer.

Then, 100 μl of the TMB substrate was added to every well and the plate was incubated for 30 minutes at RT, without shaking.

The enzymatic reaction was stopped by pipetting 50 μl of the Stop solution (1M hydrochloric acid (HCl)) to each well, allowing to change the color of the solution from blue to yellow.

The O.D. generated from each well was read in a microplate reader (BioTek Synergy HT) at 450 nm, within 10 minutes after adding the stop solution. The Gen5 2.00 was the software used. The higher the O.D. in a well, the more intense the blue color showed and the lower the concentration of cortisone present. The samples O.D. values obtained, together with the cortisone standard concentrations, were used to determine cortisone concentrations.

2.1.7.4.4. Cortisone in Blood Plasma

Cortisone levels in blood plasma were evaluated accordingly to the steps previously described for the analysis of cortisone in follicular fluid. Reagents and cortisone standards preparation, used to obtain the standard curve necessary to calculate cortisone concentrations in blood plasma samples, were prepared as described in point 2.1.7.4.3.

After blood plasma processing the procedure described in the cortisone kit, for plasma samples preparation, was performed. In a 1,5 ml eppendorf, were added 5 μl of the dissociation reagent together with 5 μl of the blood plasma sample. The solution was vortexed and incubated at RT for at least 5 minutes. In the same Eppendorf, 490 μl of 1X Assay Buffer were added to prepare a 1:100 dilution of blood plasma sample.

2.1.8. Statistical Analysis

Statistical analysis was performed using Excel (Microsoft, Seattle, WA, USA) and GraphPad Prism version 8.0 (GraphPad Software, San Diego, CA, USA) software. Data were expressed as mean ± standard deviation (SD) in nmol/l. A t-test was performed for comparison between two populations. Multiple comparisons were performed using one-way ANOVA. Correlations between variables were analyzed using Pearson´s correlation coefficient (r) for parametric data. A p-value of < 0.05 was considered statistically significant. N.S. corresponds to not significant statistics.

Chapter III - Results

3.1. Hormonal Measurements

3.1.1. Measurement of Cortisol and Cortisone in Follicular Fluid and Plasma

Three standard curves (*figure 9*) were plotted using the cortisol standard concentrations of 3200, 1600, 800, 400, 200, 100, 50 and 0 pg/ml and their respective absorbances. With these values, the cortisol and cortisone samples concentrations in FF and BP were calculated using GraphPad Prism and the Y = bottom + ((top – bottom) / 1 + (IC50/x) ^ hillslope)) equation. Graphics follow a sigmoidal curve in a four-parameter logistic pattern, where the X-axis is cortisol concentration in pg/ml and the Y-axis is the O.D. in nm.

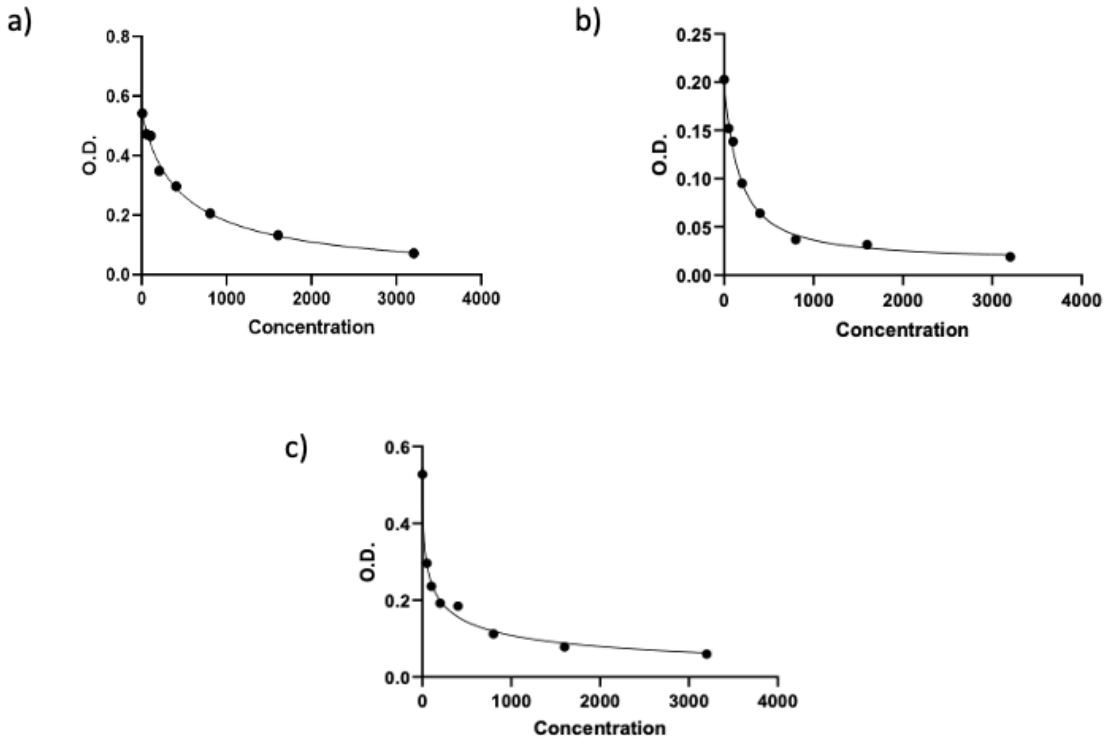


Figure 9 - Standard curves to determine the concentrations of cortisol in follicular fluid (*graphic a*), cortisol in follicular fluid and blood plasma (*graphic b*), and cortisone in follicular fluid and blood plasma (*graphic c*). **a)** $R^2 = 0.9922$, **b)** $R^2 = 0.9941$ and **c)** $R^2 = 0.9912$

From the 58 women enrolled in the study, cortisol concentrations were measured in 58 follicular fluid and 17 blood plasma samples. Cortisone concentrations were measured in 21 follicular fluid and 17 blood plasma samples.

3.1.2. Cortisol Concentration

Pearson's correlation coefficient (r) allowed to determine the correlation between cortisol concentration (nmol/l) in blood plasma and cortisol in follicular fluid with an r of 0.4. A $p = 0.06$ was obtained (*figure 10*).

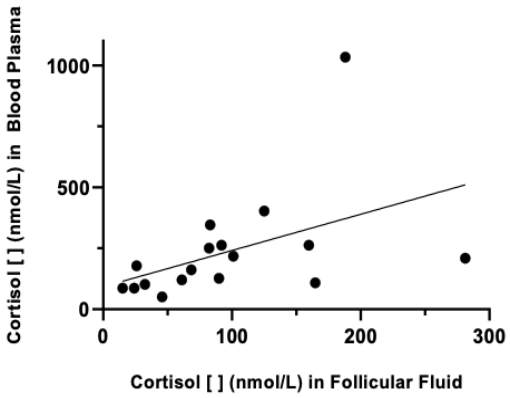


Figure 10 – Representation of the correlation of cortisol concentration between follicular fluid and blood plasma (r = 0.4; $p = 0.06$) (n=17)

Table 2 - Follicular fluid and blood plasma concentrations of cortisol and their cortisol and cortisone ratios in the unfertilized, fertilized, not pregnant (hCG-) and pregnant (hCG +) groups. Values are shown as mean ± SD

	Mean ± SD														
Groups	*n*	Cortisol FF (nmol/l)	*p*	*p*	*n*	Cortisol BP (nmol/l)	*p*	*p*	*n*	Cortisol/cortisone FF	*p*	*p*	*n*	Cortisol/cortisone BP	*p*	*p*
Unfertilized	3	54.24±50.68	n.s. (0.2053)		1	178.7			1	6.33±0.0			1	4.13		
Fertilized	36	124.9±61.35	<0.0001	n.s. (0.1886)	12	264.1±264.8	0.0054	n.s. (0.2957)	11	5.37±6.26	0.0182	n.s. (0.7477)	11	10.72±10.45	0.0070	n.s. (0.5730)
hCG -	14	124.9±73.15	<0.0001		2	94.55±10.85	0.0516		2	1.46±1.12	n.s. (0.3272)		2	1.84±0.58	n.s. (0.1438)	
hCG +	5	160.9±82.94	0.0123		2	236.0±37.39	n.s. (0.0710)		2	7.3±0.03	0.0023		2	4.55±4.08	n.s. (0.3632)	

The comparison between the cortisol concentrations (nmol/l) in follicular fluid (*figure 11* and *table 2*) and blood plasma (*figure 12* and *table 2*) with the unfertilized, fertilized, not pregnant (hCG -) and the pregnant (hCG +) groups was performed. The cortisol concentrations and the cortisol/cortisone ratio values, in both fluids, were

normally distributed with an approximate mean (± SD) value (*table 2*). Horizontal bars represent mean value (± SD) (*figures 11* and *12*).

As observed in *table 2*, there is no significance (n.s.) between the follicular fluid cortisol concentrations and the unfertilized group. The fertilized (p = <0.0001), not pregnant (p = <0.0001), and the pregnant (p = 0.0123) groups show significance with cortisol levels in FF. Cortisol in FF was not significantly different between the groups (n.s.). Follicular fluid cortisol of women, whose embryos were transferred but that did not achieve a clinical pregnancy, had a mean value of 124.9 (±73.15) nmol/l. This value was significantly lower (p < 0.0001) than the mean value for the pregnant group, whose cortisol mean value was 160.9 (±82.94) nmol/l (*table 2* and *figure 11*). The fertilized group obtained a similar cortisol mean value (124.9±61.35) to the group that did not achieve pregnancy and thus, a similar significance (*table 2*). Fertilized groups comprise the oocytes that only fertilized but were not transferred into the uterus, the oocytes that fertilized but did not reach a pregnancy (hCG -), and the oocytes that fertilized and implanted (hCG +). These groups have more oocytes fertilized and higher cortisol levels, in comparison to the unfertilized group (*54.24±50.68*) (*figure 11* and *table 2*).

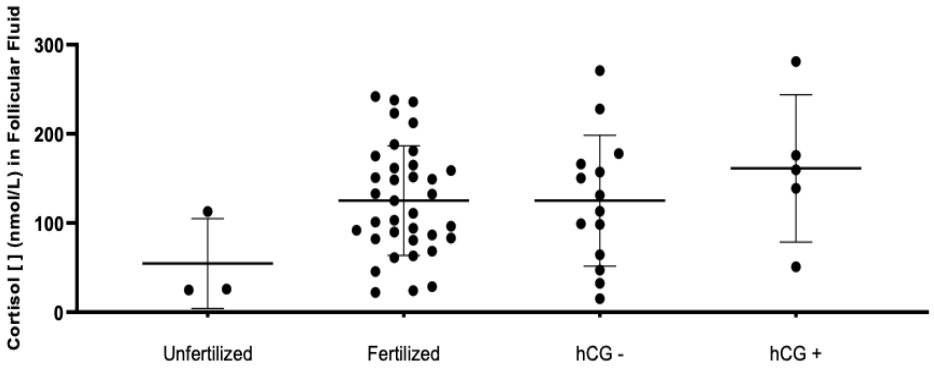


Figure 11- Comparison of cortisol concentrations in follicular fluid with the four reproductive outcome groups: unfertilized oocytes (n=3), fertilized oocytes without embryo transfer (n=36), fertilized oocytes without pregnancy (hCG-) (n=14) and fertilized oocytes with implantation (hCG+) (n=5)

There was obtained a significant value between the blood plasma cortisol concentrations and the fertilized group (p = 0.0054) and with the group that did not result in pregnancy (p = 0.0516), with concentrations of 264.1 (±264.8) nmol/l and 94.55 (±10.85) nmol/l, respectively. Cortisol in BP was not significant with the group that did achieve pregnancy (n.s.), and which concentration was (236.0±37.39) (*table 2* and *figure 12*). Cortisol in BP was not significantly different between the groups (n.s.).

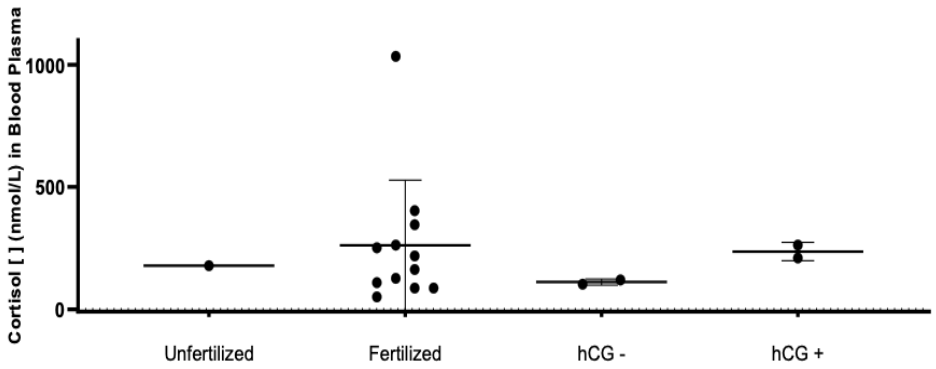


Figure 12 - Comparison of cortisol concentrations in blood plasma with the four reproductive outcome groups: unfertilized oocytes (n=1), fertilized oocytes without embryo transfer (n=12), fertilized oocytes without pregnancy (hCG-) (n=2) and fertilized oocytes with implantation (hCG+) (n=2)

3.1.2.1. Cortisol concentrations and ART outcomes

In the 58 women receiving ART treatment, 8.6% (5/58) achieved pregnancy and 24.1% (14/58) did not achieve pregnancy.

- **Assessment of Oocyte Maturity**

The number of total oocytes recovered at follicular puncture were correlated with the cortisol concentrations (nmol/l) in follicular fluid and blood plasma. Significant results were obtained for cortisol concentrations in FF with a p = 0.03 (*figure 13 a*) but not in BP (p = 0.9) (*figure 13 b*).

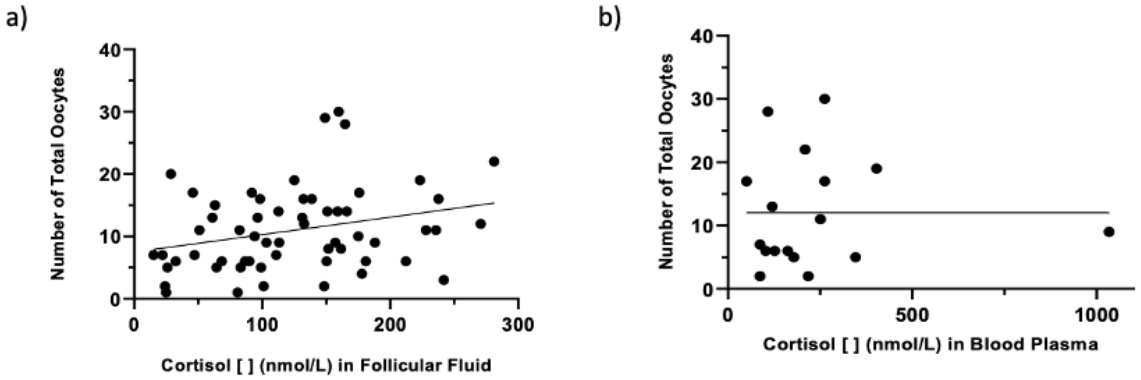


Figure 13 - a) Correlation of cortisol concentrations in follicular fluid with the number of oocytes (r = 0.2; *p* = 0.03) (n=58); **b)** Correlation of cortisol concentrations in blood plasma with the number of oocytes (r = - 0.0008; *p* = 0.9) (n=17)

The cortisol concentrations (nmol/l) in follicular fluid and blood plasma were correlated with the maturation rate. A significant association, *p* = 0.05, was obtained between the maturation rate and the cortisol in follicular fluid *(figure 14 a)*. The same correlation was not observed in blood plasma (*p* = 0.2) (*figure 14 b*).

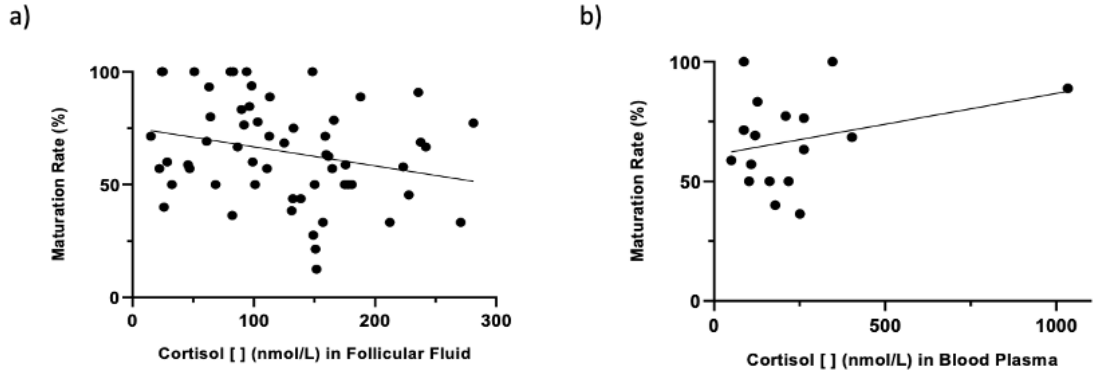


Figure 14 - a) Correlation of cortisol concentrations in follicular fluid with the maturation rate (r = - 0.2; *p* = 0.05) (n=58); **b)** Correlation of cortisol concentrations in blood plasma with the maturation rate (r = 0.3; *p* = 0.2) (n=17)

- **Assessment of Fertilization and Implantation**

The same approach was made between the fertilization rate with the cortisol concentrations (nmol/l) in follicular fluid and blood plasma. A significant association was demonstrated between the fertilization rate and the cortisol concentrations in follicular fluid, $p = 0.008$, *(figure 15 a)*. The same correlation was not observed in blood plasma ($p = 0.09$) (*figure 15 b*).

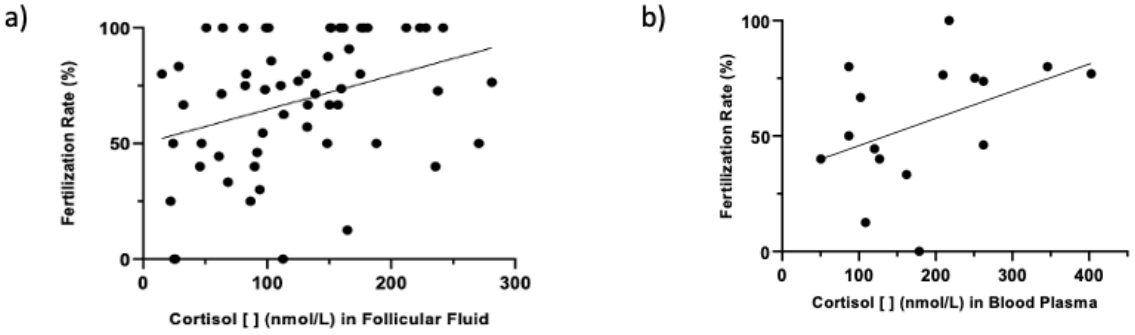


Figure 15 - a) Correlation of cortisol concentrations in follicular fluid with the fertilization rate (r = 0.3; $p = 0.008$) (n=58); **b)** Correlation of cortisol concentrations in blood plasma with the fertilization rate (r = 0.4; $p = 0.09$) (n=16)

The cleavage rate on different days of embryo development was correlated with the cortisol concentrations (nmol/l) in follicular fluid and blood plasma. No significance and no correlation were observed between cortisol concentrations in follicular fluid on days 2, 3, 4 and 5 of embryo development, with $p = 0.3$, $p = 0.8$, $p = 0.2$, and $p = 0.9$, respectively (*figure 16 a, c, e, g,* respectively). The same correlation was not observed in blood plasma on days 2, 3, 4 and 5 of embryo development, with $p = 0.3$, $p = 0.7$, $p = 0.8$, and $p = 0.6$, respectively (*figure 16 b, d, f, h,* respectively).

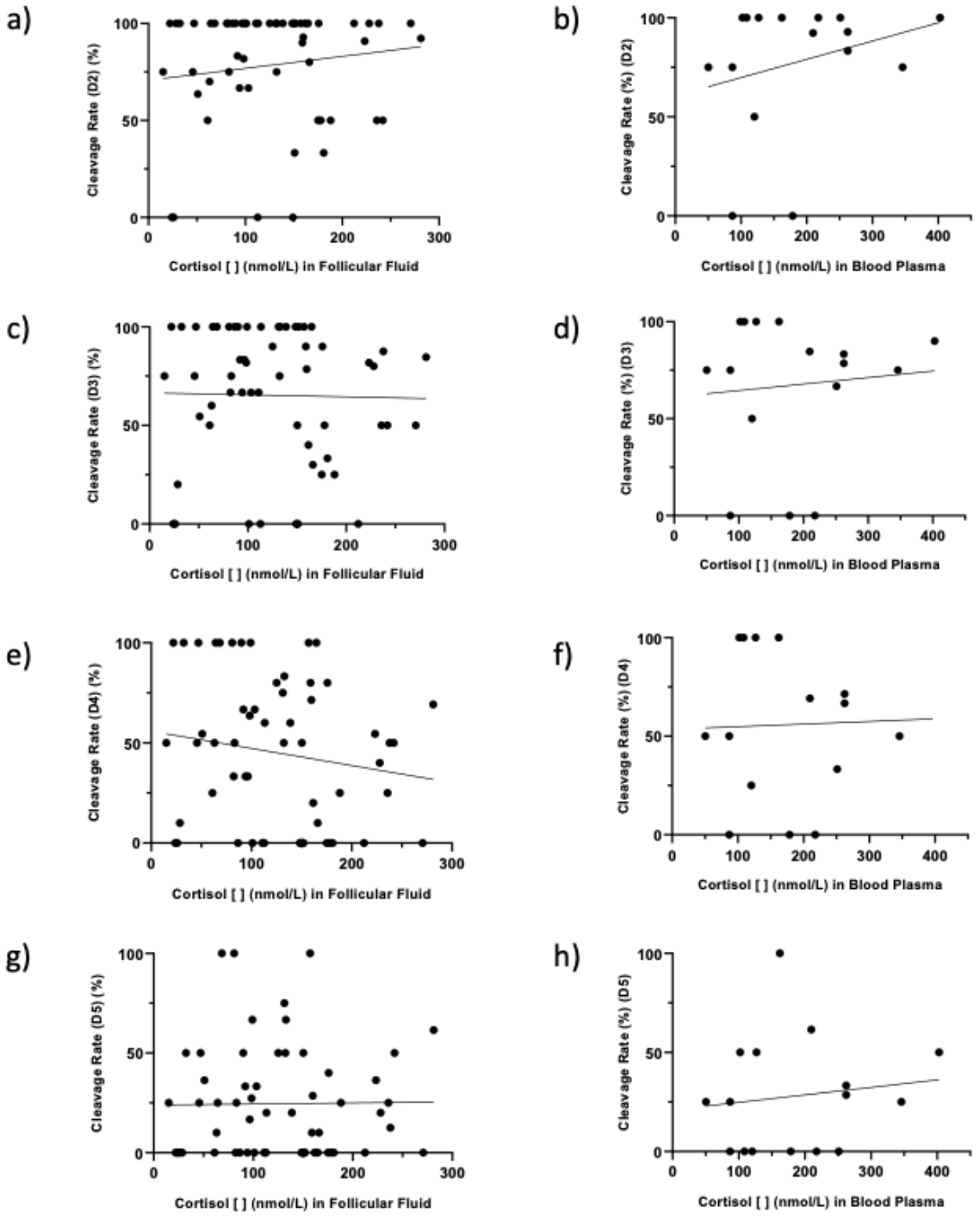


Figure 16 - Correlation of cortisol concentrations in follicular fluid and in blood plasma with the cleavage rate on day 2, **a)** r = 0.1; *p* = 0.3 (n=58) and **b)** r = 0.2; *p* = 0.3 (n=16), respectively; on day 3, **c)** r = - 0.01; *p* = 0.8 (n=58) and **d)** r = 0.09; *p* = 0.7 (n=16), respectively; on day 4, **e)** r = - 0.1; *p* = 0.2 (n=58) and **f)** r = 0.03; p = 0.8 (n=16), respectively; on day 5, **g)** r = 0.01; *p* = 0.9 (n=58) and **h)** r = 0.1; *p* = 0.6 (n=16), respectively

3.1.2.2. Cortisol concentrations and HADS

In the 58 women receiving ART treatment, 65.5% (38/58) had anxiety on the day of starting the treatment, and the percentage of those with moderate (borderline), and severe (abnormal case) anxiety was 31% (18/58), and 34.4% (20/58), respectively. 22.4% (13/58) had depression on the day of starting the treatment, and the percentage of those with moderate (borderline), and severe (abnormal case) anxiety was 15.5% (9/58), and 6.8% (4/58), respectively.

An analysis to determine whether there is an association between cortisol concentrations (nmol/l) in follicular fluid and blood plasma with the anxiety levels was performed. No significant correlations were observed in follicular fluid (p = 0.3) (*figure 17 a*) and blood plasma (p = 0.6) (*figure 17 b*).

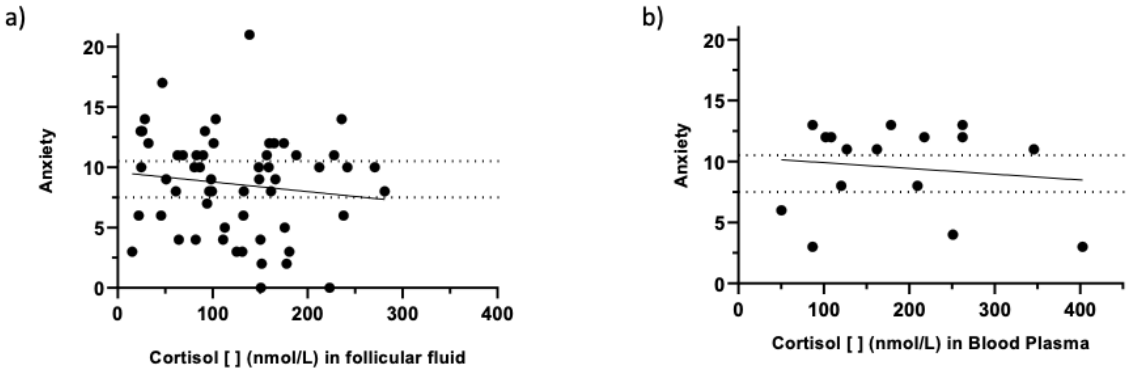


Figure 17 - a) Correlation of cortisol concentrations in follicular fluid with anxiety (r = - 0.1; p = 0.3) (n=58); **b)** Correlation of cortisol concentrations in blood plasma with anxiety (r = -0.1; p = 0.6) (n=16)

The same analysis was performed for depression. The cortisol concentrations in follicular fluid (p = 0.3) (*figure 18* a) and the cortisol concentrations in blood plasma did not indicate an association with depression (p = 0.3) (*figure 18* b).

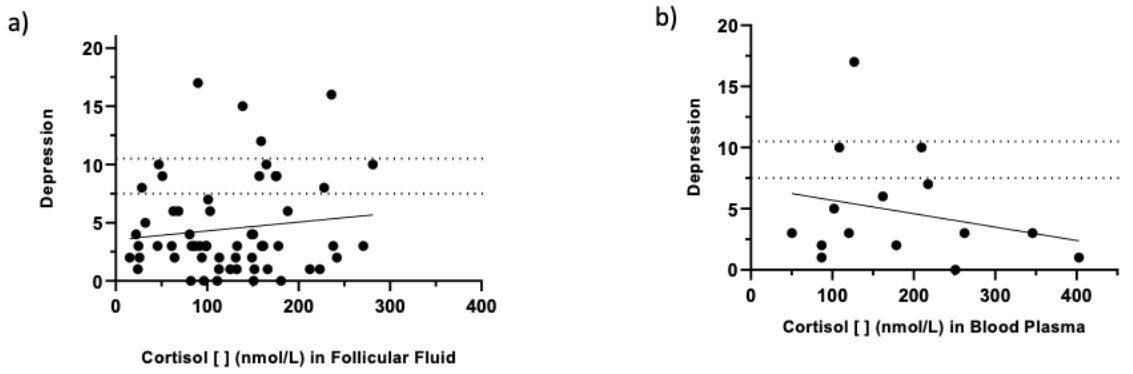


Figure 18 - a) Correlation of cortisol concentrations in follicular fluid with depression (r = 0.1; *p* = 0.3) (n=58); **b)** Correlation of cortisol concentrations in blood plasma with depression (r = - 0.2; *p* = 0.3) (n=16)

3.1.3. Cortisol/Cortisone Ratios

The correlation between the cortisol/cortisone ratio in blood plasma and in follicular fluid was obtained. Cortisol/cortisone ratio reflect the behavior of the 11ß-HSD enzyme that is responsible to modulate local glucocorticoid activity in the ovary. A *p* = 0.01 was obtained indicating a significance between the two biological fluids. An r = 0.6 is indicative of a positive correlation between both parameters (*figure 19*).

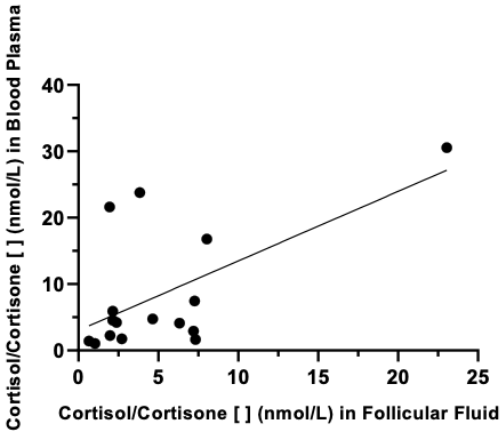


Figure 19 - Representation of the correlation of the cortisol/cortisone ratio between follicular fluid and blood plasma (r = 0.6; *p* = 0.01) (n=16)

The cortisol/cortisone ratio in follicular fluid and blood plasma were correlated with different ART outcomes. The number of oocytes retrieved at follicular puncture did not demonstrate a significant correlation, r = 0.09 and *p* = 0.7, with the

cortisol/cortisone ratio in follicular fluid (*figure 20 a*). The same correlation was not observed for cortisol/cortisone in blood plasma, where the r = 0.2 and *p* = 0.4 (*figure 20 b*).

The maturation and fertilization rate of oocytes were correlated with the cortisol/cortisone ratio in follicular fluid and blood plasma. No significant correlation was observed between the maturation rate and cortisol/cortisone in FF, r = 0.3 and *p* = 0.2, (*figure 21 a*), and in BP, r = 0.2 and *p* = 0.2, (*figure 21 b*). The same observations were obtained for the fertilization rate and the cortisol/cortisone in FF, r = -0.1 and *p* = 0.6, (*figure 22 a*), and for the cortisol/cortisone in BP, r = -0.3 and p = 0.1, (*figure 22 b*).

An association between cortisol/cortisone ratio in follicular fluid and blood plasma with embryo development, i.e., the cleavage rate of embryos on different days, was performed. No correlation and significance were observed in follicular fluid on days 2, 3, 4 and 5 of embryo cleavage, with *p* = 0.2, *p* = 0.2, *p* = 0.5, and *p* = 0.9 (*figure 23 a, c, e, g*). The same correlation was not observed in blood plasma on days 2, 3, 4 and 5 of embryo development, with *p* = 0.9, *p* = 0.9, *p* = 0.6, and *p* = 0.6, respectively (*figure 23 b, d, f, h*).

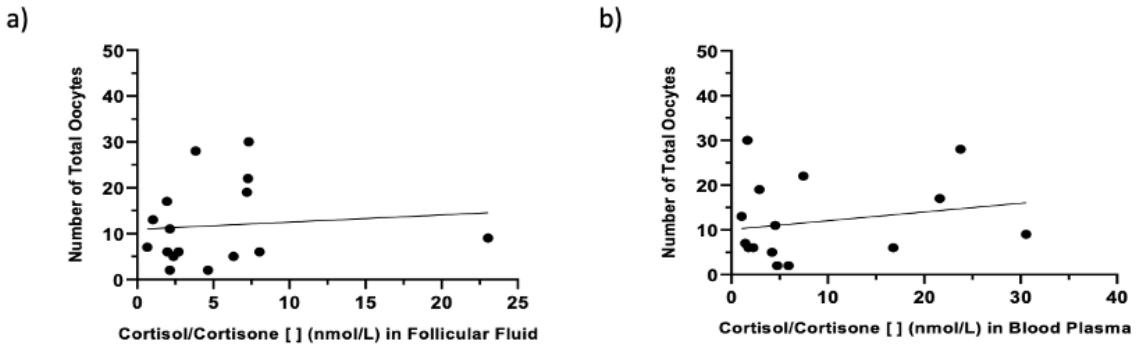


Figure 20 - a) Correlation of the cortisol and cortisone ratio in follicular fluid with the number of total oocytes (r = 0.09; *p* = 0.7) (n=16); **b)** Correlation of the cortisol and cortisone ratio in blood plasma with the number of total oocytes (r = 0.2; *p* = 0.4) (n=16)

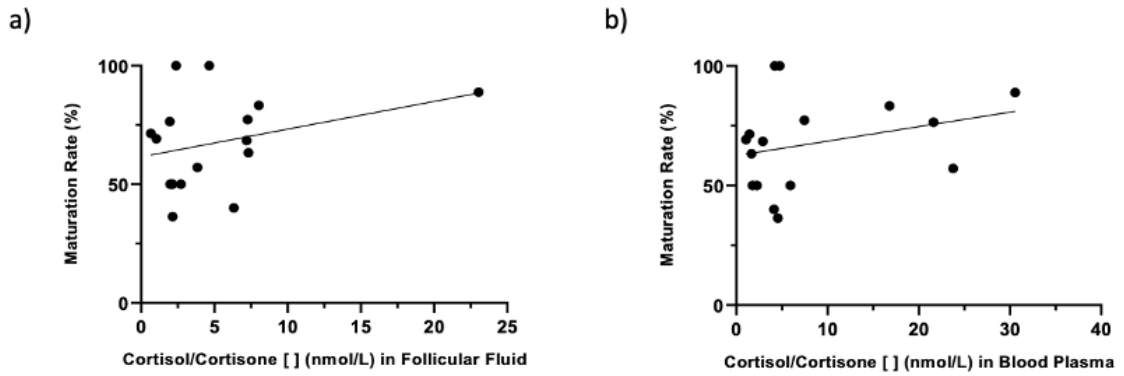


Figure 21 - a) Correlation of the cortisol and cortisone ratio in follicular fluid with the maturation rate (r = 0.3; p = 0.2) (n=16); **b)** Correlation of the cortisol and cortisone ratio in blood plasma with the maturation rate (r = 0.2; p = 0.2) (n=16)

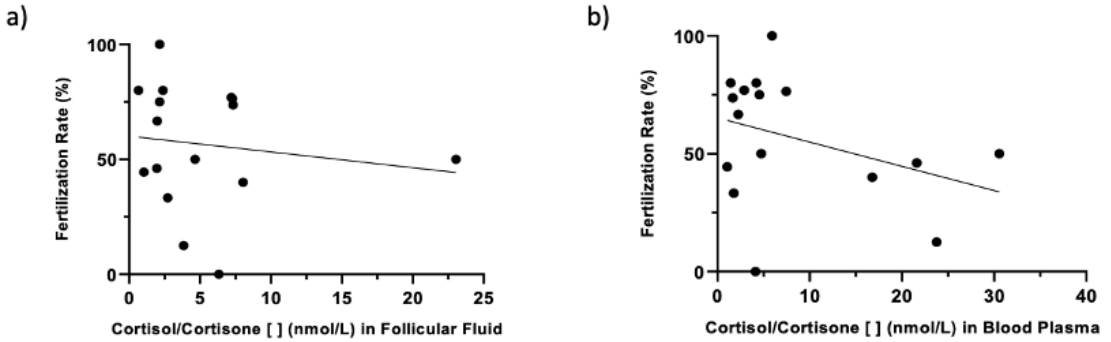


Figure 22 - a) Correlation of the cortisol and cortisone ratio in follicular fluid with the fertilization rate (r = -0.1; *p* = 0.6) (n=16); **b)** Correlation of the cortisol and cortisone ratio in blood plasma with the fertilization rate (r = -0.3; *p* = 0.1) (n=16)

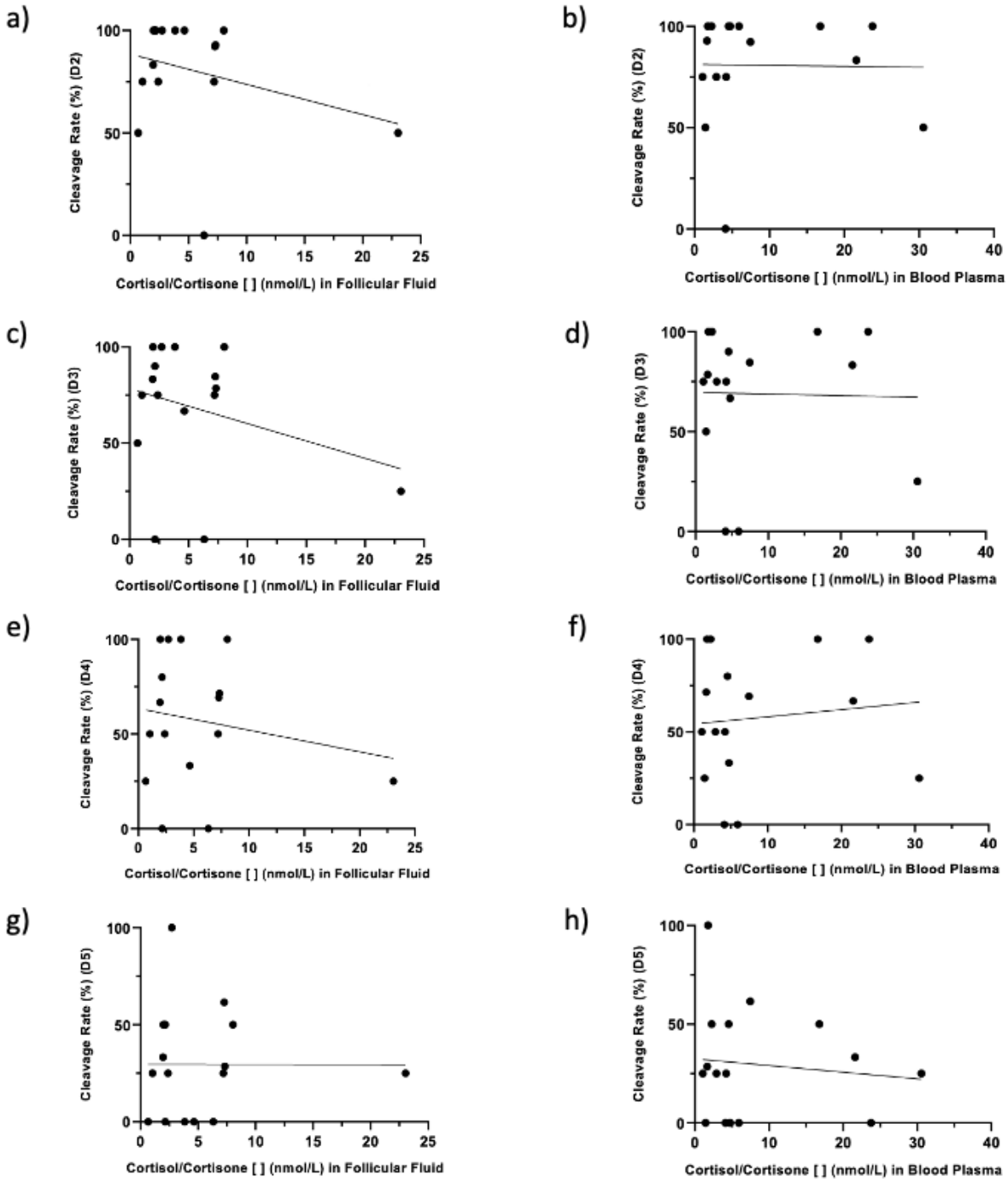


Figure 23 - Correlation of the cortisol and cortisone ratio in follicular fluid and in blood plasma with the cleavage rate on day 2, **a)** r = - 0.2; p = 0.2 (n=16) and **b)** r = - 0.01; p = 0.9 (n=16), respectively; on day 3, **c)** r = - 0.2; p = 0.2 (n=16) and **d)** r = - 0.02; p = 0.9 (n=16), respectively; on day 4, **e)** r = - 0.1; p = 0.5 (n=16) and **f)** r = 0.1; p = 0.6 (n=16), respectively; on day 5, **g)** r = - 0.002; p = 0.9 (n=16) and **h)** r = - 0.1; p = 0.6 (n=16), respectively

The cortisol/cortisone ratio in follicular fluid was compared between the unfertilized, fertilized, not pregnant (hCG -) and pregnant (hCG +) groups (*table 2*). Horizontal bars represent mean value (± SD) (*figures 24* and *25*).

A higher ratio was observed in the pregnant group, 7.3±0.03 (p = 0.0023), in comparison to the unfertilized, fertilized (p = 0.0182) and not pregnant (p = n.s) groups, whose ratios are 6.33±0.0, 5.37±6.26, and 1,46±1.12, respectively (*figure 24*). Cortisol/cortisone in FF was not significantly different between the groups (p = n.s.) (*figure 24* and *table 2*).

The same analysis was made for blood plasma where a statistically significant and higher cortisol/cortisone ratio, 10.72±10.45, was observed in fertilized oocytes (p = 0.0070). The not pregnant group, 1.84±0.58, and the pregnant group, 4.55±4.08 did not obtain a significant value with the BP cortisol/cortisone ratio. Cortisol/cortisone in BP was not significantly different between the groups in analysis (p = n.s.) (*figure 25* and *table 2*).

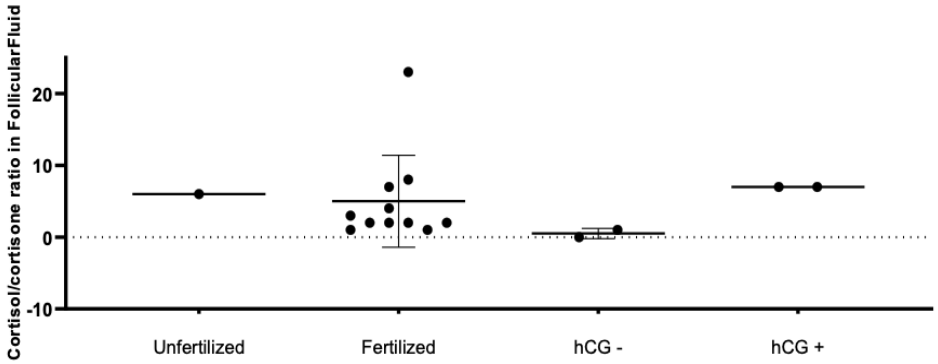


Figure 24 - Comparison of the cortisol/cortisone ratio in follicular fluid with the reproductive outcome results: unfertilized oocytes (n=1), fertilized oocytes without embryo transfer (n=11), fertilized oocytes without pregnancy (hCG-) (n=2) and fertilized oocytes with implantation (hCG+) (n=2)

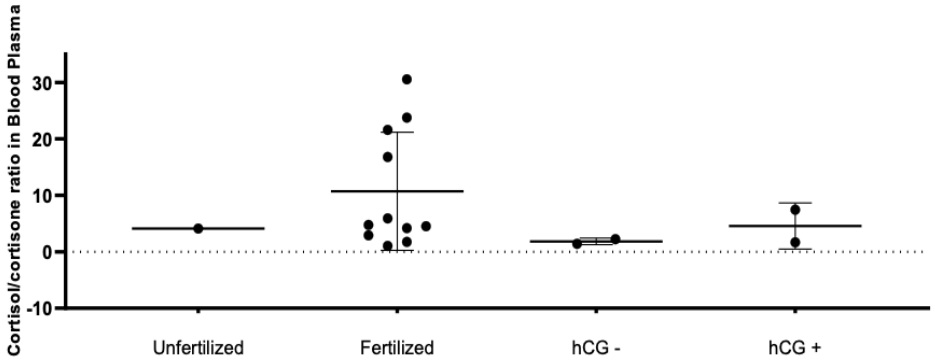


Figure 25 - Comparison of the cortisol/cortisone ratio in blood plasma with the four reproductive outcome results: unfertilized oocytes (n=1), fertilized oocytes without embryo transfer (n=11), fertilized oocytes without pregnancy (hCG-) (n=2) and fertilized oocytes with implantation (hCG+) (n=2)

Table 3 - Concentrations of cortisone, cortisol and the cortisol and cortisone ratio in the follicular fluid and blood plasma (mean values ± SD). $Cortisone_{bp}$ = blood plasma cortisone concentration; $cortisone_{ff}$ = follicular fluid cortisone concentration; $cortisol_{bp}$ = blood plasma cortisol concentration; $cortisol_{ff}$ = follicular fluid cortisol concentration; n.s. = not significant

	ART outcome (IVF/ICSI	*n*	Mean ± SD	P (pregnant versus not pregnant)
Follicular Fluid				
Cortisone (nmol/l)	Pregnant	3	28.08±9.21	n.s. (0.1684)
	Not pregnant	3	17.2±5.21	
Cortisol (nmol/l)	Pregnant	5	161.3±82.56	n.s. (0.4175)
	Not pregnant	14	125.1±73.36	
Cortisol/cortisone	Pregnant	2	7.3 ±0.036	n.s. (0.0692)
	Not pregnant	2	1.3± 0.92	
Blood Plasma				
Cortisone (nmol/l)	Pregnant	2	92.87±91.55	n.s. (0.6479)
	Not pregnant	2	52.94±10.86	
Cortisol (nmol/l)	Pregnant	2	236.0±37.39	0.0359
	Not pregnant	2	94.55±10.85	
Cortisol/cortisone	Pregnant	2	4.5±4.0	n.s. (0.5188)
	Not pregnant	2	1.8±0.5	
$Cortisone_{bp}/cortisone_{ff}$	Pregnant	2	3.97±4.59	n.s. (0.7341)
	Not pregnant	2	2.71±0.06	
$Cortisol_{bp}/cortisol_{ff}$	Pregnant	2	1.194±0.63	n.s. (0.2138)
	Not pregnant	2	4.446±1.82	
$Cortisol/cortisone_{ff}$:cortisol/ $cortisone_{bp}$	Pregnant	2	2.68±2.42	n.s. (0.4547)
	Not pregnant	2	0.72±0.21	

Cortisol, cortisone and the cortisol/cortisone ratio in the follicular fluid were not statistically different between the pregnant and the non-pregnant groups (*table 3*).

Cortisol in blood plasma was significant in the conception cycles (p = 0.0359) and not in group that did not conceive, with concentrations of 236.0±37.39 and 94.55±10.85, respectively. The cortisone and cortisol/cortisone ratio in blood plasma were not statistically significant between women in whom treatment resulted in pregnancy and those who did not achieve pregnancy (*table 3*).

No significant differences were observed between the implantation outcomes and the $cortisone_{bp}/cortisone_{ff}$, $cortisol_{bp}/cortisol_{ff}$, and in the $cortisol/cortisone_{ff}$/$cortisol/cortisone_{bp}$ groups *(table 3)*.

The correlation of the cortisol/cortisone ratio in follicular fluid and blood plasma with the anxiety levels was also assessed. No correlation was found between the anxiety levels and the cortisol/cortisone ratio in follicular fluid, p = 0.6 and r = 0.1 (n=16) (*figure 26 a*). The same analysis was not observed in blood plasma, p = 0.2 and r = 0.3 (n=16) (*figure 26 b*).

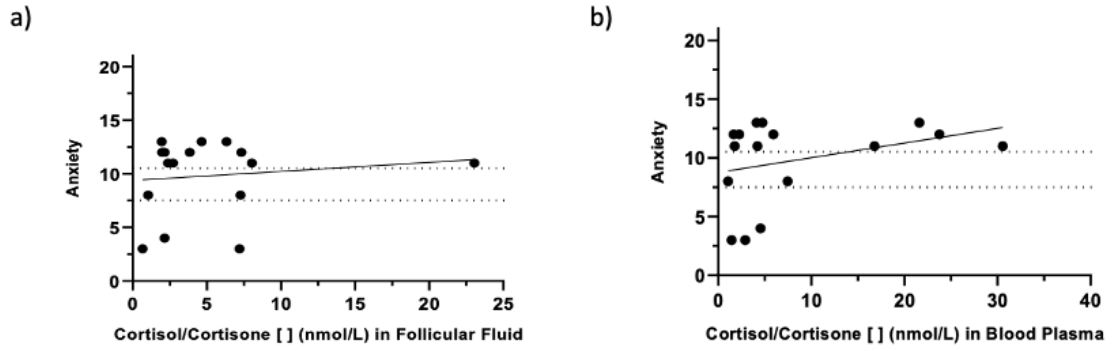


Figure 26 - a) Correlation of the cortisol/cortisone ratio in follicular fluid with anxiety (r = 0.1; p = 0.6) (n=16); **b)** Correlation of the cortisol/cortisone ratio in blood plasma with anxiety (r = 0.3; p = 0.2) (n=16)

The same analysis was performed for the depression levels. Depression levels showed no correlation, r = 0.2, and no significance, p = 0.3, with cortisol/cortisone in FF (*figure 27 a*). No correlation, r = 0.4, and no significance, p = 0.07, were observed for cortisol/cortisone in BP (*figure 27 b*).

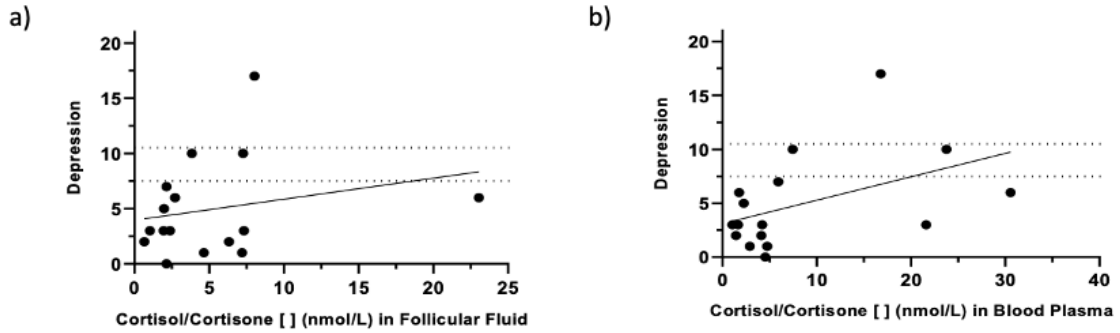


Figure 27 - a) Correlation of the cortisol and cortisone ratio in follicular fluid with depression (r = 0.2; p = 0.3) (n=16); **b)** Correlation of the cortisol and cortisone ratio in blood plasma with depression (r = 0.4; p = 0.07) (n=16)

3.1.4. ART outcomes and HADS

From the pregnant women, 80% (4/5) had anxiety, and 80% (4/5) had depression. From the women that did not achieve pregnancy, 57.1% (8/14) had anxiety, and 21.4% (3/14) had depression.

- **Assessment of Oocyte Maturity and Quality**

The number of total oocytes was correlated with the anxiety and depression levels. No significant correlations were observed between the number of total oocytes and anxiety (p = 0.9) (*figure 28 a*) or depression (p = 0.4) (*figure 28 b*).

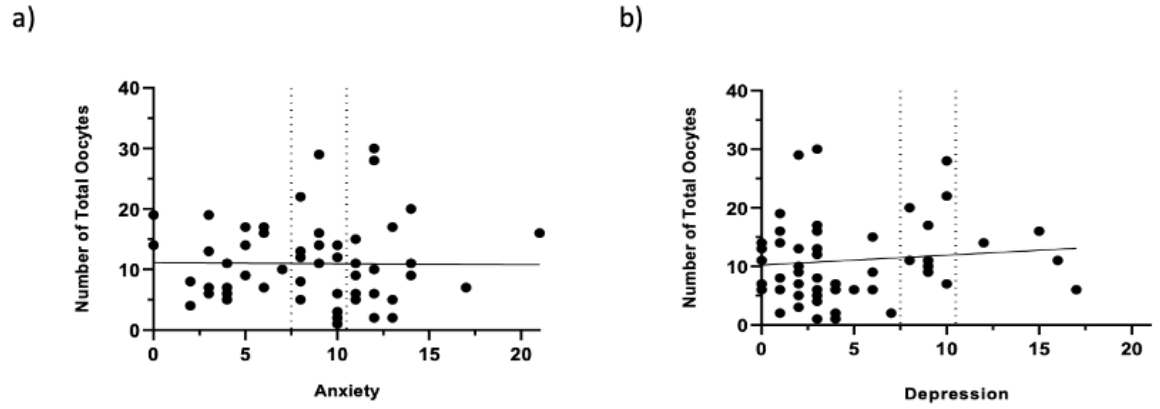


Figure 28 - a) Correlation of anxiety with the number of oocytes retrieved at follicular puncture (r = -0.009; p = 0.9) (n=58); **b)** Correlation of depression with the number of oocytes retrieved at follicular puncture (r = 0.1; p = 0.4) (n=58)

The same analysis was performed for the maturation rate with the anxiety and depression levels. No correlation was obtained between maturation rate and anxiety (p = 0.1) (*figure 29 a*) or depression (p = 0.3) (*figure 29 b*).

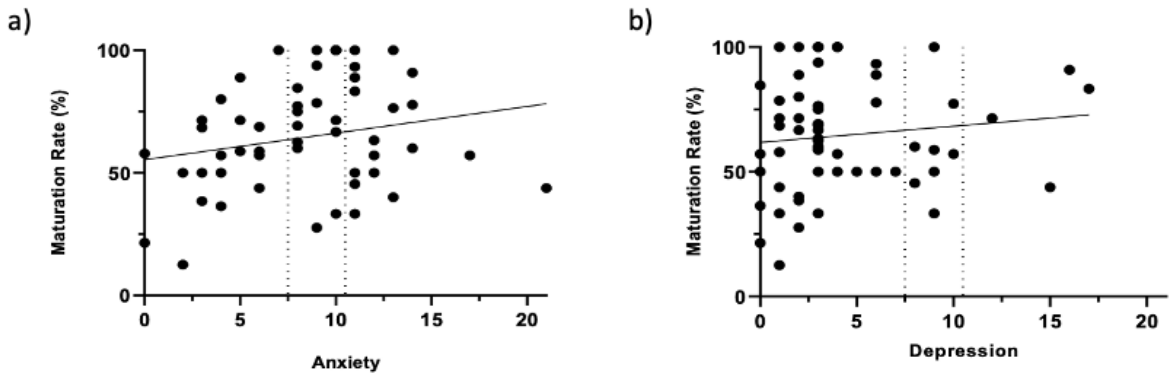


Figure 29 - a) Correlation of anxiety with the maturation rate (r = 0.2; *p* = 0.1) (n=58); **b)** Correlation of depression with the maturation rate (r = 0.1; *p* = 0.3) (n=58)

- **Assessment of Fertilization**

The same analysis was performed for the anxiety and depression levels with the fertilization rate. A significant correlation with a *p* = 0.04 was observed between the fertilization rate and anxiety (*figure 30 a*). The same was not observed in *figure 30 b)* (*p* = 0.5).

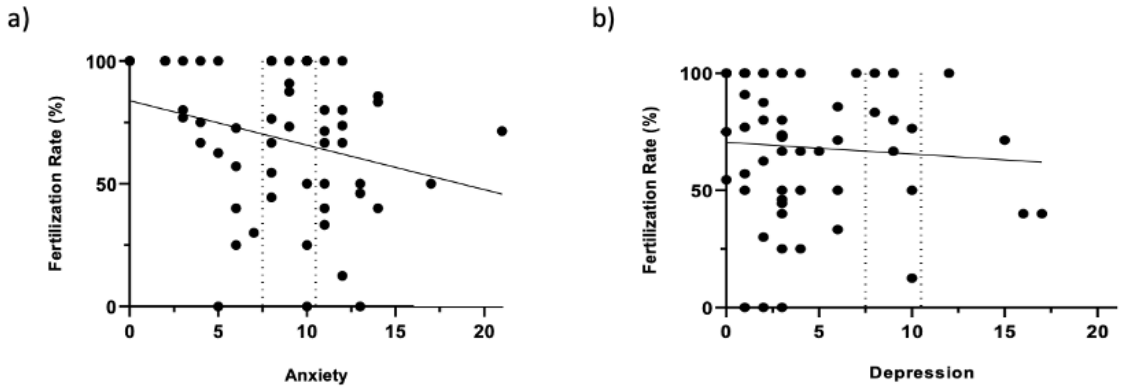


Figure 30 - a) Correlation of anxiety with the fertilization rate (r = - 0.2; *p* = 0.04) (n=58); **b)** Correlation of depression with the fertilization rate (r = - 0.07; *p* = 0.5) (n=58)

The cleavage rate on days 2, 3, 4 and 5 (*figure 31*) was compared with the anxiety and depression levels. Significant results were observed for depression on day 4 of embryo´s cleavage with a *p* = 0.006 (*figure 31 f*). No correlation and significance were observed in follicular fluid on days 2, 3, 4 and 5 of embryo cleavage, with *p* = 0.9, *p* = 0.7, *p* = 0.3, and *p* = 0.6 (*figure 31 a, c, e, g*). The same correlation was not observed in blood plasma on days 2, 3 and 5 of embryo development, with *p* = 0.1, *p* = 0.08, and *p* = 0.1, respectively (*figure 31 b, d, h*).

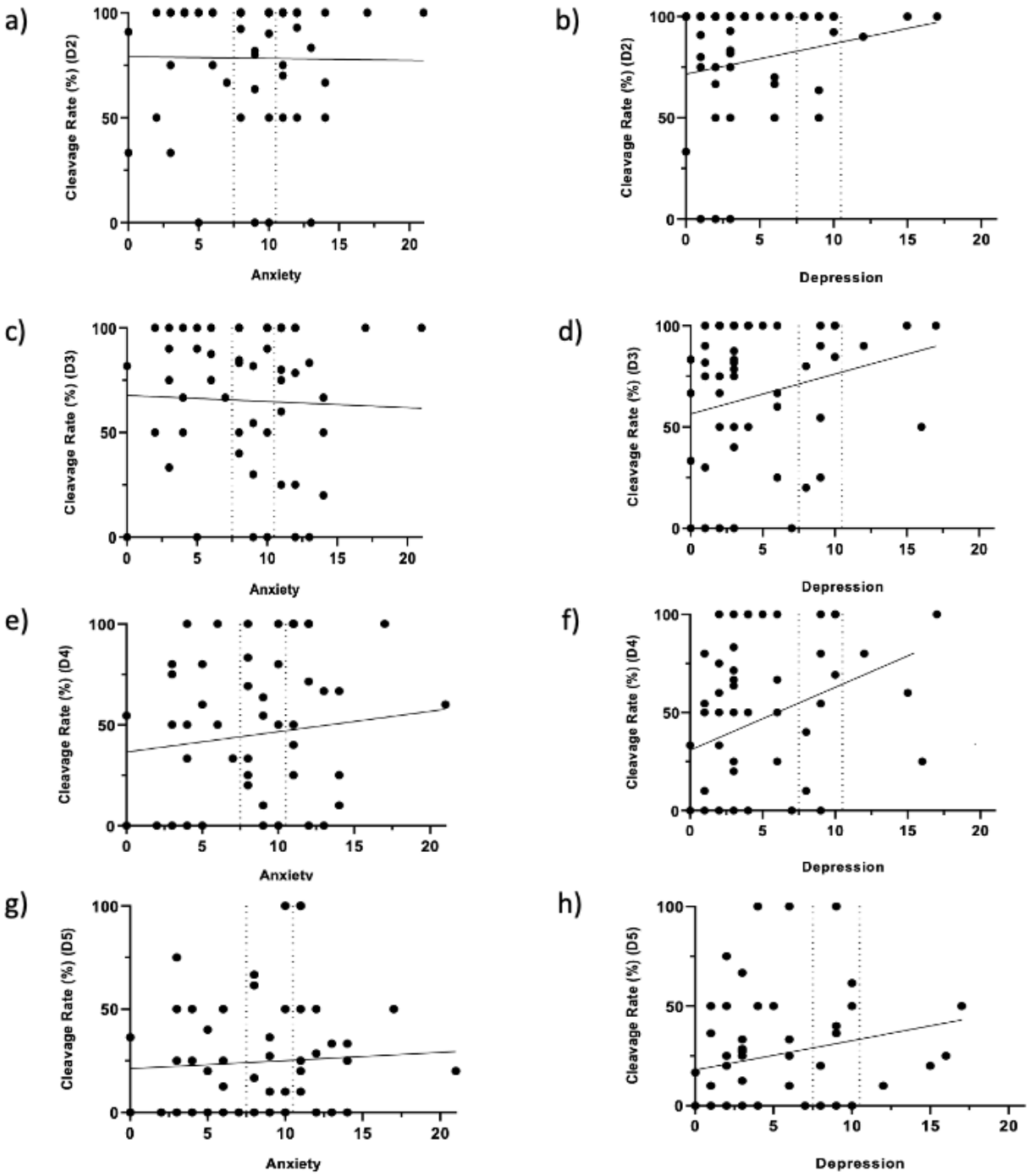


Figure 31 - Correlation of anxiety and depression with the cleavage rate on day 2, **a)** r = - 0.01; *p* = 0.9 (n=58) and **b)** r = 0.1; *p* = 0.1) (n=58), respectively; on day 3, **c)** r = - 0.03; *p* = 0.7 (n=58) and **d)** r = 0.2; *p* = 0.08 (n=58), respectively; on day 4, **e)** r = 0.1; *p* = 0.3 (n=58) and **f)** r = 0.3; *p* = 0.006 (n=58), respectively; on day 5, **g)** r = 0.05; *p* = 0.6 (n=58) and **h)** r = 0.2; *p* = 0.1 (n=58), respectively

3.1.5. Age

In this study, the average age of patients was 35.8 years. An analysis between the depression levels and the age of women was performed. For that purpose, two age groups were compared: a group from ≥18 – 34 and a group from ≥ 35 – 40.

A significant correlation with a p = 0.02 was observed for both age groups of women undergoing ART in comparison to depression (*figure 32*).

The same age groups were compared with the anxiety levels, the cortisol concentrations, the cortisol/cortisone ratio and with the ART outcome rates. However, no significance and correlation were found (data not shown).

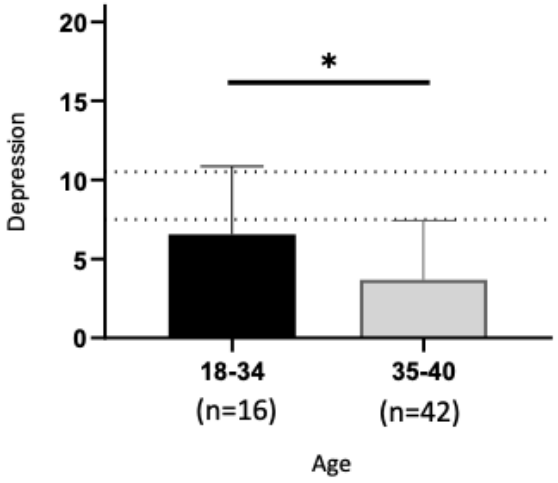


Figure 32 - Correlation of the age (≥ 18 - 34; ≥ 35 - 40) with depression (p = 0.02) (n=58). The group between ≥ 18 to 34 has 16 women. The group between ≥ 35 to 40 has 42 women. Statistical analysis was performed using One-way ANOVA ($p \leq 0.05$).

Chapter IV – Discussion

The present dissertation intended to evaluate whether there is a relationship between anxiety/depression and stress biomarkers in follicular fluid with the reproductive outcomes of women undergoing assisted reproductive techniques. The follicular fluid was the biological fluid chosen for this purpose as it is, normally, discharged and because it is the microenvironment in which the oocyte develops. Furthermore, its composition contains several hormones present in the human ovary before ovarian stimulation. Cortisol and cortisone were the analyzed hormones as they are expressed by the 11ß-HSD enzyme, which is of great interest due to its activity in the human ovary. For this reason, cortisol and cortisone are pointed to as potential intermediates to indirectly access the regulatory activity of the 11ß-HSD in the ovary. Hormonal measurement would allow to determine the cortisol and cortisone concentrations that better reflect anxiety and depression, as well to predict the reproductive outcomes after ART treatments. Additionally, the same hormones were evaluated in blood plasma to determine if their concentrations were correlated and, thus, possible to substitute the same measures in the FF. Some of the hypotheses that were also expected to observe are presented as follows. Lower levels of anxiety and depression correspond to lower levels of cortisol in follicular fluid and blood plasma and thus, to better pregnancy rates. The opposite was expected to see, where augmented levels of anxiety and depression correspond to a bad prognosis of oocytes fertilized and embryos implanted.

In this thesis, some results support previous literature, and some disagree. As an example, the evidence for a follicular fluid or blood plasma cortisol association with ART seems to be inconclusive, as reported before in some studies (An *et al.,* 2013; Demyttenaere *et al.,* 1991; Keay *et al.,* 2002; Michael *et al.,* 1999; Thurston, 2003). Other works suggest no association between cortisol levels and ART outcomes (Csemiczky *et al.,* 2000; Lewicka *et al.,* 2003).

The study confirms that the steroids cortisol and cortisone exist in the follicular fluid of the follicles recovered after artificial stimulation. The range of concentrations obtained for cortisol, cortisone and the correspondent cortisol/cortisone ratio are

presented in *table 3*. Values measured for cortisol, cortisone and cortisol/cortisone in follicular fluid agree with those from Lewicka *et al.,* (2003). Intrafollicular cortisol concentrations also agree with those from An *et al.,* (2011). However, follicular fluid cortisol levels are half those of Keay *et al.,* (2002). A possible explanation for the difference between hormonal concentrations in both biological fluids may be attributed to a different women population and the use of different techniques, as Keay *et al.,* (2002) used radioimmunoassay instead of the ELISA assay. Higher cortisone levels and lower cortisol levels were obtained in blood plasma (*table 2* and *3*), contradicting the values from Lewicka *et al.,* (2003) and Morineau *et al.,* (1997). Cortisol levels in BP agree with An *et al.,* (2011). A linear tendency for the concentrations of glucocorticoids and the cortisol/cortisone ratio in women who achieved a pregnancy and in those who did not conceive, seem to point to a relationship between the expression of the enzyme and the reproductive outcomes. Furthermore, it suggests that active glucocorticoid may have an important role for final oocyte maturation and embryo implantation.

Cortisol levels were lower in FF compared to the BP, confirming earlier reports (Andersen *et al*., 1999). More specifically, cortisol levels in the follicular fluid are, approximately, half those in blood plasma in the group that conceived (*table 2* and *3)*, agreeing with Lewicka *et al.,* (2003). Concentrations of cortisone in follicular fluid of pregnant and not pregnant women were approximately one third the concentrations observed in blood plasma. All these different hormonal levels between both fluids may result from the different blood-follicle penetration. As the ovaries are relatively deficient in 21-hydroxylase, the enzyme that induces 11ß-HSD enzyme´s expression, these hormones are originated from the blood (Smith *et al.,* 2000; Dehennin *et al.,* 1987; Hillier, 1994; An *et al.,* 2011). *Table 3* data seems to support that women with a high type 1 11ß-HSD activity in follicular fluid, reflected by a high cortisol/cortisone ratio, and that receive lower amounts of cortisol from blood to the FF, observed by lower glucocorticoid levels in FF, have increased chances to become pregnant. There seems to exist a tendency for increasing cortisol levels in FF as cortisol levels in BP increase, although no significance (p = 0.06) was found (*figure 10*), as already observed by Lewicka *et al.,* (2003). It suggests that the concentrations of cortisol in follicular fluid cannot be substituted by the same measurements in blood plasma.

During the menstrual cycle, there is a switch in the expression of the type 2 11ß-HSD isoform to the type 1 11ß-HSD isoform (Yong *et al*., 2000). 11ß-HSD1 leads to the conversion of more cortisol. Higher levels of cortisol in the follicular fluid are described in the literature to have an important role in the maturation of oocytes (Keay *et al.,* 2002; Lewicka *et al.,* 2003), but this is not indicative that the oocyte fertilizes (Andersen *et al.,* 1994). This fact can be observed on *tables 2 and 3,* where higher cortisol concentrations belong to women that conceived. However, higher maturation rates were associated to lower cortisol levels (*figure* 14 a), not supporting what had been said before. Contrarily, and although no significant difference was observed, the unfertilized oocytes correspond to lower levels of cortisol (*table 2* and *figure 11*), an observation that can be explained by higher activity of the 11ß-HSD2 isoform during the follicular phase of the menstrual cycle, where immature oocytes prevail (Lewicka *et al.,* 2003). Moreover, during this phase, higher cortisol levels during the follicular phase may destabilize the normal function of the granulosa cells, whose development is stimulated by FSH. Thus, an expression of the 11ß-HSD2 is very important (Lewicka *et al.,* 2003). The oocytes that fertilized and the oocytes that fertilized but did not result in pregnancy, present similar concentrations of cortisol, although in lower amounts than the pregnant group (*table 2*). A similar observation is represented in *figure 12* for cortisol in blood plasma. Although this data is preliminary, a bigger sample number should be used in order to reduce the existence of outlier points, as observed in this graphic.

The cortisol concentrations and their relationship with the ART treatment outcomes were evaluated. The assessment of oocyte quality and maturity, evaluated the number of total oocytes retrieved at follicular puncture, as well as the maturation rate of oocytes. Higher cortisol levels in FF were related to a greater number of total oocytes obtained (*figure 13 a)*, contradicting previous data that did not associate cortisol levels with oocyte number (Andersen *et al.,* 1994). Lower concentrations of cortisol in follicular fluid are accompanied by higher maturation rates (*figure 14 a*). This data does not confirm previous studies (Fateh *et al.,* 1989; Keay *et al.,* 2002; Lewicka *et al.,* 2003) that associate higher cortisol levels with follicles containing mature oocytes, and does not support the study from Andersen *et al.,* (1994) that did not find an association between cortisol concentrations and oocyte maturity. Low cortisol content

in the follicle has already been associated with immature oocytes (Keay *et al.,* 2002; Lewicka *et al.,* 2003). The correlation of fertilization, cleavage and implantation rates with the cortisol concentrations was then analyzed. Higher cortisol levels in FF were associated with higher rates of fertilized oocytes (*figure 15 a*). These results oppose a study where unfertilized oocytes were associated with high cortisol levels (Demyttenaere *et al.,* 1991). Cortisol levels in BP were found to be associated with oocyte fertilization rates (*figure 15 b*), not supporting the study from Bider *et al.,* (1998). Later data found no correlation between the cortisol in FF and BP and the oocytes that cleaved on days 2, 3, 4 and 5 (*figure 16*), confirming earlier reports (Andersen *et al.,* 1999; Fateh *et al.,* 1989). Regarding implantation, cortisol concentrations in follicular fluid were significantly higher in the group obtaining a pregnancy compared to the non-conception group, which confirms the data from Keay *et al.,* (2002). The same was observed for blood plasma (*table 2* and *3*), which agrees with Thurston, (2003), but contradicts the data reported by Andersen *et al.,* (1999), Lewicka *et al.,* (2003) and Michael *et al.,* (1999) for FF and BP. Cortisol levels in BP and FF do not associate with unfertilized oocytes (*table 2*), which opposes other study (Jimena et al., 1992). Higher levels of cortisone were also present in the follicular fluid and blood plasma of women who achieved pregnancy compared to those who did not, contradicting results from Lewicka et al., (2003), who observed cortisone in the follicular fluid to be significantly lower in conception cycles. However, the same author did not find statistical difference in serum cortisone levels between women who became pregnant after treatment and those who did not.

One of the hypotheses to test was to determine the concentration range of cortisol and cortisone that are associated to higher levels of anxiety and depression. Regarding the cortisol concentrations and the HADS questionnaire, no significance was obtained for anxiety (*figure 17)* and depression (*figure 18*) with the levels of cortisol in the FF and BP. No data was previously reported comparing steroids in FF and BP with the HADS questionnaire.

When evaluating the cortisol and cortisone ratio, it was observed that a higher cortisol/cortisone$_{ff}$ ratio is related to a higher cortisol/cortisone$_{bp}$ ratio (*figure 19*), which may be indicative of similar behavior of the 11ß-HSD enzyme in both fluids. Contrarily, results suggest no significant correlation between cortisol/cortisone in FF

and BP with anxiety (*figure 26*) and depression (*figure 27*), and no literature is available for comparison. From the data obtained, there is also no association between the cortisol/cortisone ratio and the number of total oocytes (*figure 20*), maturation rate (*figure 21*) and fertilization rate (*figure 22*). Nonetheless, Simerman *et al.,* (2015) found a positive correlation of $cortisol_{ff}$ concentrations with the number of total and methaphase II oocytes collected at follicular puncture. The same ratio in both fluids has no significance or correlation with the embryo´s cleavage on days 2, 3, 4 and 5 (*figure 23*). The ratios of $cortisone_{bp}$/$cotisone_{ff}$, $cortisol_{bp}$/$cortisol_{ff}$, and cortisol/$Cortisone_{ff}$/cortisol/$Cortisone_{bp}$ do not show significance between the pregnant and the non-pregnant group (*table 3*). The cortisol/$cortisone_{ff}$ ratio was significantly higher in those women who became pregnant compared to the fertilized group. This observation was reported in the study from Lewicka *et al.,* (2003) and Keay *et al.,* (2002), where significantly higher cortisol/$cortisone_{ff}$ was associatied to pregnant women. This increased cortisol/$cortisone_{ff}$ ratio in conception cycles may suggest that cortisol have an important role for final oocyte maturation and embryo implantation. Moreover, high intrafollicular cortisol/cortisone ratios possibly reflect either decreased rates of cortisol oxidation, or increased rates of cortisone reduction to cortisol. No significant difference in cortisol/$cortisone_{ff}$ from women in whom ART treatment resulted in pregnancy and those who failed to achieve pregnancy, as observed before (Lewicka *et al.,* 2003). There is an association between the fertilized oocytes and the cortisol/$cortisone_{bp}$ (*table 2*). An increased cortisol/cortisone ratio in the follicular fluid of pregnant women compared to the group that did not conceive (*table 2* and *3*) supports findings from (Lewicka *et al.,* 2003; Michael *et al.,* 1999) and contradict results from Andersen *et al.,* (1999), who did not find an association. The same ratio was higher in BP of pregnant women (*table 2* and *3*) in comparison to the non-pregnant women. More specifically, in the pregnant group, the cortisol/cortisone ratio in blood plasma was approximately half the ratio in follicular fluid (*table 2* and *3*). The same ratio in the group that failed to become pregnant was not different between FF and BP (*table 2* and *3*). These last results do not support one of the hypotheses previously stated. It was expected a higher cortisol/cortisone ratio in the pregnant group, as it is suggested that this group have a lower number of immature oocytes caused by the expression of type 2 11ß-HSD enzyme (*table 3*).

The different ART treatment outcomes were compared with results obtained from the HADS questionnaire. The assessment of oocyte maturity included the analysis of the number of total oocytes retrieved at follicular puncture and the maturation rate of the oocytes. Oocyte maturation rate increased when the anxiety levels were higher, but differences between groups didn´t reach statistical significance (*figure 29*). Longer stress responses, are linked to higher cortisol levels, which can lead to estradiol inhibition, as evidenced by impaired granulosa cell function, and thus, potentially affect follicular maturation (Lancastle *et al.,* 2005). It was not possible to associate the total number of oocytes with anxiety and depression levels (*figure 28*). A previous study found that the number of retrieved oocytes was similar between women with low levels of anxiety and women with severe anxiety (Sallem *et al.,* 2021) thus, showing no correlation between the two parameters. Gürhan *et al.,* (2009) and Donarelli *et al.,* (2016) did not find an association between anxiety and depression with the number of oocytes retrieved. Lower number of oocytes collected were associated with increased depression (Gürhan *et al.,* 2009). After, fertilization, cleavage and conceiving rates were assessed. A higher cleavage rate on day 4 was found to be associated with higher depression levels (*figure 31 f*). A higher fertilization rate was observed for lower anxiety levels (*figure 30 a*), supporting one of the hypotheses previously stated. No association was suggested between fertilization rates and depression (*figure 30 b*). Limited data is available in the literature comparing ART outcome with the HADS questionnaire. However, some evidence suggests that emotional reactions may negatively interfere with fertilization (Klonoff-Cohen et al., 2001; Klonoff-Cohen and Natarajan, 2004; Ebbesen et al., 2009). Women who did not achieve pregnancy experienced lower levels of anxiety and depression. It was expected the opposite, where non-pregnant women would experience a higher incidence of psychological disturbances, as was reported in previous findings (Karlidere *et al.,* 2008). However, anxiety levels were higher than depression, corroborating the literature that associates anxiety to the most common state of psychological distress, after depression (Kloss *et al.,* 2015). Demyttenaere *et al.* (1994), Sallem *et al.,* (2021), Gürhan et al., (2009), Kee *et al.,* (2000), and Facchinetti *et al.,* (1997) significantly correlated anxiety with a negative outcome in ART. Kee *et al.* (2000), Demyttenaere *et al.* (1998), and Smeenk *et al.* (2001), also found a significant association of depression

with a decreased chance of successful outcomes in ART. One explanation for this outcome may be possibly due the low number of women achieving pregnancy, in this study. As well as previous studies (Liu *et al.,* 2021), was not found a solid correlation of the impact anxiety and depression exert on ART treatment outcome and thus, this relationship remains unclear (Bapayeva *et al.,* 2021). Other studies (Klerk *et al.,* 2008; Lovely *et al.,* 2003; Anderheim *et al.,* 2005) were unable to associate psychological distress with ART outcomes, even though Klerk *et al.,* (2008) had used the HADS questionnaire. An important fact to have into account is that the majority of the available studies comparing anxiety and depression with the ART outcome, is made in the Turkish and Chinese population, and these ethnicity differences may lead to discrepancies between the results. Furthermore, almost all psychological studies use the STAI (State and Trait Anxiety Inventory) questionnaire, which may also contribute to distinct outcomes. Taken all these contradicting reports, the question remains whether there is a reliable effect of anxiety and depression on the pregnancy rate, as studies comparing these factors are somewhat limited.

The study results indicated that 65.5% of the women had mild to severe anxiety, and 22.4% of the women had mild to severe depression. The prevalence rate of anxiety in this study was higher than the rate obtained by Huang *et al.,* (2019) and Liu *et al.,* (2021). The prevalence rate of depression was slightly lower than the depression rate in the study of Huang *et al.,* (2019). One explanation may be the low number of samples and possibly due to the presence of different ethnicities in the different studies.

Fell stress is a side effect of ART treatment. Women had blood tests, were given some ovarian hyperstimulation drugs, and were submitted to ultrasound scans on a regular basis to monitor the growth of follicles at the start of treatment (Huang *et al.,* 2019). All these steps may account to moderate and severe distress in women. Anxiety and/or depression during treatment arise regardless of whether it is the first cycle of ART treatment or a repeated cycle. Therefore, psychological intervention and clinical aid should be implemented effectively to decrease the psychological distress these women face.

When the age of women submitted to the ART treatment was assessed, were observed differences in the incidence of depression in terms of age in both age groups.

This data agrees with previous studies, as the experiments from Liu *et al.,* (2021) and Xu et al., (2017), that demonstrated an effect of age over depression in women submitted to ART treatment. It was observed that younger women have higher levels of depression, instead of older women, which was also observed in the study of Xu et al., (2017) (*figure 32*). In addition, a higher number of non-pregnant women were older, with the average age of 36 years, supporting findings from Huang *et al.,* (2019).

Regarding the ELISA assay, different formats can be employed depending on the features of the analyte and biological samples under analysis: direct, indirect, sandwich, or competitive assays (Aydin, 2015). Direct ELISA is a fast method mainly used for immunohistochemical staining of cells and tissues and uses an enzyme-labeled primary antibody that directly reacts with the antigen-coated to the well. Due to the inexistence of a secondary antibody, signal amplification may be affected when the labeled enzyme is attached to the primary antibody. The indirect method uses an enzyme-labeled secondary antibody and binds specifically to a primary antibody that is bound to an antigen- coated well. The primary antibody has various epitopes to which the labeled secondary antibody can be bound, increasing the signal amplification and consequently the sensitivity of the test. The disadvantage is the cross-reactivity of the secondary antibody, which lowers the specificity. The sandwich assay follows a similar methodology to the indirect assay; however, instead of an antigen coupled to the well, there is a capture antibody. It is one of the favorite methods due to its specificity and sensitivity. One disadvantage is the cross-reactivity that can occur between the capture antibody and the primary and secondary antibodies. Finally, the competitive ELISA, which was the technique employed in this thesis, is equated to the sandwich assay regarding its sensitivity and specificity (Aydin, 2015). The main reason for having chosen the competitive ELISA, is its effectiveness in detecting and measuring specific small-molecule antigens within complex biological samples, as it happens with follicular fluid. Due to its small size and low molecular weight, 362.5 Da, as well as its lipophilic nature, cortisol is a good candidate to be detected by a competitive assay (Aydin, 2015; Kohl *et al.,* 2017; Stalder *et al.,* 2020).

Several limitations can be addressed after the interpretation of the results. The main limitation was the small number (*n*) of follicular fluid and blood plasma samples collected from the women undergoing ART treatments. Numerous factors can explain

this low number of samples. These include women that did dropout from participating in the study or those women that had to be excluded from the study because were at the clinic for oocyte donation or fertility preservation purposes. Follicular punctures also did not occur every day, limiting oocyte collection. Furthermore, a small *n* may also decrease the significance of the study. For example, results can be affected because several samples or outlier points correspond to rates of 100%, as is observed for the cleavage rate on day 2 with the follicular fluid cortisol levels (*figure 16 a*). The opposite can be observed on day 5 of cleavage rate with the anxiety levels (*figure 31 g*), where samples mostly correspond to 0% rates. More time is, thus, required to significantly increase the number of samples obtained. Numbers close to previous studies, (*n* total = 387) (Lewicka *et al.,* 2003) would, possibly, represent a good approach, as higher effect sizes and power enable more reliable results.

Another limitation is the fact that in the end, the data obtained only reflects an indirect assessment of 11ß-HSD enzymatic behavior in the ovary, through the measurement of anxiety and stress biomarkers. Instead, and for better results, the activity of this enzyme should be evaluated directly in the oocytes recovered at follicular puncture. The reason for this relies on the fact that follicular fluid is merely a fluid surrounding the oocyte. It means that its cortisol and cortisone concentrations, possibly, only correspond to a lower activity representation of the 11ß-HSD activity in the ovary. However, as oocytes are targeted for treatment and due to ethical reasons, it is hard to tell whether a direct relationship between the oocyte potential and the prediction of the ART outcomes will ever be accomplished.

Another shortcoming of this research is the measurement of cortisol and cortisone in the total follicular fluid recovered and not in single follicles. It affects hormonal measurements because the composition of the follicular fluid may not be equal. As multiple embryos are, generally, transferred in each woman is impossible to determine which follicle and oocyte resulted in a pregnancy. Furthermore, some aspirated follicles do not contain oocytes, a fact that negatively correlates hormonal measurements in FF with the ART outcomes. Since it is not possible to follow the fate of single follicles, values were approximated by calculation of the mean, likely reducing the significance of the differences. In the future, follicular fluid extracted from each collected follicle should be stored individually and be associated with its respective

oocyte. The study of Mendoza, (2002), surpasses this limitation and increases the validity to the study.

Along the execution of this work was also not possible to measure cortisol and cortisone in the follicular fluid and blood plasma of the same number of women. One of the reasons was that blood only started to be collected later, decreasing considerably the number of samples available for hormonal measurements. It prevented the analysis of the *r* and the *p* values in the pregnant, not pregnant, and in both groups regarding their association with $cortisone_{bp}$ versus $cortisone_{ff}$, the $cortisol_{bp}$ versus the $cortisol_{ff}$ and, the cortisol/$cortisone_{bp}$ versus the cortisol/$cortisone_{ff}$.

Finally, sample mean age, mean duration of infertility and, whether it is the first or repeated ART cycle women are enrolled in, may also account to heterogeneous findings between the reports described in the thesis.

To the best of my knowledge, no scientific research has associated the HADS questionnaire with the glucocorticoid levels in follicular fluid and blood plasma and with ART outcomes. Moreover, it is the first Portuguese study reported to evaluate anxiety and depression with the ART outcomes and with the glucocorticoid levels in both fluids. These factors may be another reason for the wide outcome variety between the findings in this thesis and other authors.

Future research can build on what was found in this dissertation and also improve the findings present here by overcoming some of the limitations previously stated. First, the number of samples collected should be higher. Studying the connection between the follicular fluid and the oocyte from a single follicle would also be a major improvement. Another factor that may also account for heterogeneity within the literature is the type of study design and the wide variety of methods. However, other methodologies and collection methods of cortisol should be explored to study the connection between distress and ART. One of these could potentially include hair sampling that is increasing its popularity within the scientific community, as it allows for quantifying stress up to three months before the surge of a stress agent. It differs from the follicular fluid, blood plasma, urine and saliva samples that only measure actual cortisol levels (Massey *et al.,* 2014).

Chapter V – Conclusion

The first observation presented in this dissertation confirms the presence of the 11ß-HSD enzyme in the follicular fluid of the follicle, and its interconversion activity from cortisol to cortisone.

Some significant associations were found between the psychological variables, anxiety and depression, and ART treatment outcomes, more precisely the clinical pregnancy rate. The same observation was possible between cortisol concentrations in follicular fluid with the total number of oocytes collected at follicular puncture, and with the maturation and fertilization rates. In blood plasma, a significant association was also observed between cortisol levels and the fertilization rate. Pregnant and not pregnant women were significantly associated in the blood plasma cortisol. Age was correlated with depression. Results were non-significant for associations between anxiety and depression levels and the number of oocytes retrieved and/or the maturation rate, and between anxiety and depression with the levels of cortisol in follicular fluid and blood plasma. Additionally, this thesis does not give enough information that allows to say that the cortisol and cortisone in follicular fluid can be substitute by the same hormonal measurements in the blood plasma.

Associating anxiety and stress biomarkers present in the human ovary to the potential role that psychological distress may have in determining ART treatment outcomes, has undoubtedly been one of the most challenging barriers to overcome. The present findings suggest inconclusive evidence that cortisol has a role in predicting conception, total oocyte number, oocyte maturation, oocyte fertilization, and embryo cleavage in women submitted to ART treatments. Furthermore, it remains uncertain the follicular fluid and blood plasma glucocorticoid concentrations that allow to predict an optimal reproductive functioning. Knowing these values would allow women to move forward with ART treatment or not, depending on their psychological status.

The studies reported in the discussion section, are considered heterogeneous between them due to the contradictory results they report. Additionally, some are also more robust and reliable than others. However, almost every research article supports the implementation of psychological aid during treatment.

Nonetheless, the findings are encouraging for the general population of ART women, as the evidence from this study implies that psychological stress and distress may have only a minor impact on the chances of establishing a pregnancy following ART treatment.

The present research is preliminary, and a further large study is required to replicate and verify these findings.

Chapter VI - Bibliography

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Chapter VII - Complementary Data

Appendix I - Hospital Anxiety and Depression Scale questionnaire (Portuguese version)

ANSIEDADE E ESCALA DE DEPRESSÃO

Pedimos-lhe que leia cada uma das perguntas e faça uma cruz (X) no espaço anterior à resposta que melhor descreve a forma como se tem sentido na última semana. Não demore muito tempo a pensar nas respostas. A sua reação imediata a cada questão será provavelmente mais correta do que uma resposta muito ponderada. Por favor, faça apenas uma cruz em cada pergunta. HADS R.P., Snaith, & A. P., Zigmond, (1994) | Versão portuguesa: J. Pais-Ribeiro, I. Silva, T. Ferreira, A. Martins, R. Meneses & M. Baltar (2007)

1. Sinto-me tensa/nervosa
☐ Quase sempre
☐ Muitas vezes
☐ Por vezes
☐ Nunca

2. Ainda sinto prazer nas coisas de que costumava gostar
☐ Tanto como antes
☐ Não tanto agora
☐ Só um pouco
☐ Quase nada

3. Tenho uma sensação de medo, como se algo terrível estivesse para acontecer
☐ Sim e muito forte
☐ Sim, mas não muito forte
☐ Um pouco, mas não me aflige
☐ De modo algum

4. Sou capaz de rir e ver o lado divertido das coisas
☐ Tanto como antes
☐ Não tanto como antes
☐ Muito menos agora
☐ Nunca

5. Tenho a cabeça cheia de preocupações
☐ A maior parte do tempo
☐ Muitas vezes
☐ Por vezes
☐ Quase nunca

6. Sinto-me animada

☐ Nunca
☐ Poucas vezes
☐ De vez em quando
☐ Quase sempre

7. Sou capaz de estar descontraidamente sentada e sentir-me relaxada
☐ Quase sempre
☐ Muitas vezes
☐ Por vezes
☐ Nunca

8. Sinto-me mais lenta, como se fizesse as coisas mais devagar
☐ Quase sempre
☐ Muitas vezes
☐ Por vezes
☐ Nunca

9. Fico de tal modo apreensiva (com medo), que até sinto um aperto no estômago
☐ Nunca
☐ Por vezes
☐ Muitas vezes
☐ Quase sempre

10. Perdi o interesse em cuidar do meu aspeto físico
☐ Completamente
☐ Não dou a atenção que devia
☐ Talvez cuide menos que antes
☐ Tenho o mesmo interesse de sempre

11. Sinto-me de tal forma inquieta que não consigo estar parada
☐ Muito
☐ Bastante
☐ Não muito
☐ Nada

12. Penso com prazer nas coisas que podem acontecer no futuro
☐ Tanto como antes
☐ Não tanto como antes
☐ Bastante menos agora
☐ Quase nunca

13. De repente, tenho a sensação de pânico
☐ Muitas vezes
☐ Bastantes vezes
☐ Por vezes
☐ Nunca

14. Sou capaz de apreciar um bom livro ou um programa de rádio ou televisão

☐ Muitas vezes
☐ De vez em quando
☐ Poucas vezes
☐ Quase nunca