FACULTY OF MEDICINE OF SAARLAND UNIVERSITY

HOMBURG, GERMANY

DEPARTMENT OF OBSTETRIC, GYNECOLOGY & REPRODUCTIVE MEDICINE

Impact of Protein Concentration in the Follicular Fluid and Gene Expression in Mural Granulosa Cells on Oocyte Maturation, Fertilization Rate, and Embryo Quality in Intracytoplasmic Sperm Injection Cycles

Dissertation to acquire a degree of Doctor Rerum Medicinae (Dr. rer. Med.)

From the Faculty of Medicine

SAARLAND UNIVERSITY 2023

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ABSTRACT

Oocyte maturity, fertilization, and embryo quality are crucial to the ICSI procedure. Due to the Embryo Protection Act (EPA) in Germany and since the many published reports about follicular fluid biomarkers remain disputable, defining possible parameters that might be highly useful as non-invasive biomarkers of ICSI outcomes is crucial. Therefore, the current study objectives were to determine Amphiregulin, Luteinizing Hormone, and Insulin-Like Growth Factor II concentrations in the follicular fluid as well as to determine gene expression in the Mural Granulosa Cells (MGCs) of those (*AREG*, *LHCGR*, and *IGF-II)* genes and to explore their correlation to oocyte maturity, fertilization rate, and embryo development. A total of thirty-three women undergoing ICSI were recruited at the University Clinic of Saarland Fertility Centre (Homburg, Germany). Follicular fluid aspiration involved single/individual aspiration of follicles, enabling a 1:1 correlation with retrieved oocytes. Moreover, the oocytes retrieved after fertilization were followed and embryo quality was evaluated.

From the thirty-three patients, a total of 108 oocytes were included in the study, and for all 108 oocytes follicular fluid and MGC samples were available. Quantitative determination of Amphiregulin and Insulin-Like Growth Factor II release in the follicular fluid was performed by ELISA according to the Human Quantikine Assay. The concentrations of Luteinizing Hormone and Follicle-Stimulating Hormone release in the follicular fluid were determined by direct immunoassay with the use of an automated analyser Cobas 8000. Total RNA extraction of individual MGCs was carried out using the Roche High Pure RNA Isolation Assay. Expression of *AREG, LHCGR*, *IGF-II*, and Actine Beta (*ACTB*) was analysed by StepOnePlus™ Real-Time PCR System using TaqMan Fast Advanced Master Mix. All listed components were pipetted by the Liquid Handling Robot QIAgility™. The samples were normalized concerning the reference gene Actine Beta (*ACTB*) using the relative quantification $2^{-\Delta\Delta Ct}$ method where the mean value of mRNA transcripts from each probe was set as one for each gene.

Based on the oocyte maturity the ICSI procedure was performed with 93 oocytes. Out of the 61 fertilized oocytes, 13 zygotes were frozen, while 48 were cultivated. Thirty-four embryos (70.8%) reached good quality and fourteen (29.2%) had poor quality.

The results of the current study indicate that amphiregulin concentration in follicular fluid affects oocyte maturation, fertilization rate, and embryo quality. Additionally, the concentration of amphiregulin significantly differs between patients based on Body Mass Index.

The concentration of luteinizing hormone in follicular fluid affects oocyte maturation and significantly differs between fertilized and unfertilized oocyte groups as well as between good and poor embryo quality groups.

The results obtained for insulin-like growth factor II indicate that the concentration of this protein in follicular fluid does not affect oocyte maturity, fertilization rate, or embryo quality.

The gene expression of the studied genes (*AREG, IGF-II*, and *LHCGR*) indicates that only the expression of *AREG* significantly affects oocyte maturity, with *AREG* expression being downregulated in the immature oocyte group. *LHCGR* and *IGF-II* did not differ between groups and neither did they affect maturation, fertilization, or embryo quality.

The results on infertility indicate that the concentration of Amphiregulin and Insulin-Like Growth Factor II in follicular fluid statistically differ between healthy and infertile patients.

In conclusion, findings for studied proteins are of great importance to the future development of infertility treatment, especially in Germany due to its EPA. In addition, implementation of obtained results for Amphiregulin may offer prognostic information aiding the selection of the most viable oocytes and hence embryos because concentration affects oocyte maturity, fertilization rate, and embryo quality.

ZUSAMMENFASSUNG

Der Reifegrad der Eizelle, die Befruchtung und die Qualität des Embryos sind entscheidend für das ICSI-Verfahren. Aufgrund des Embryonenschutzgesetzes (EPA) in Deutschland und da die vielen Publikationen über Biomarker in der Follikelflüssigkeit umstritten sind, ist das Bestimmen möglicher Parameter, die als nicht-invasive Biomarker für das ICSI-Ergebnis nützlich sein könnten, von entscheidender Bedeutung. Daher waren die Ziele der aktuellen Studie die Bestimmung der Konzentrationen von Amphiregulin, Luteinisierendem Hormon und Insulinähnlichem Wachstumsfaktor II in der Follikelflüssigkeit, sowie die Bestimmung der Genexpression dieser Gene (*AREG, LHCGR* und *IGF-II*) in den muralen Granulosazellen (MGCs) und die Untersuchung einer möglichen Korrelation mit dem Reifegrad der Eizelle, der Befruchtungsrate und der Qualität der Embryonalentwicklung.

Insgesamt wurden in dieser Studie dreiunddreißig Frauen, die sich am Universitätsklinikum des Saarlandes (Fertilitätszentrum) (Homburg, Deutschland) einer ICSI Behandlung unterzogen haben, rekrutiert.

Bei der Entnahme der Follikelflüssigkeit wurden die einzelnen Follikel separat punktiert, so dass eine 1:1-Korrelation der punktierten Follikelflüssigkeit (einschließlich der muralen Granulosazellen) mit den gewonnenen Eizellen möglich war. Darüber hinaus wurden die nach der Befruchtung entnommenen Eizellen verfolgt und die Embryoqualität bewertet.
Von den dreiunddreißig Patientinnen wurden insgesamt 108 Eizellen in die Studie aufgenommen, und für alle 108 Eizellen waren die korrespondierenden Follikelflüssigkeits- und MGC-Proben verfügbar. Die quantitative Bestimmung der Freisetzung von Amphiregulin und dem Insulinähnlichem Wachstumsfaktor II in der Follikelflüssigkeit, erfolgte mittels ELISA unter Verwendung des humanen Quantikine Assays. Die Konzentrationen von Luteinisierendem Hormon und Follikelstimulierendem Hormon in der Follikelflüssigkeit wurden automatisiert durch einen direkten Immunoassay mit Hilfe eines Analysegerätes

(Cobas 8000) bestimmt. Die Gesamt-RNA-Extraktion einzelner MGCs wurde mit dem Roche High Pure RNA Isolation Assay durchgeführt. Die Expression von *AREG, LHCGR, IGF-II* und beta Aktin (*ACTB*) wurde mit dem StepOnePlus™ Real-Time PCR System unter Verwendung des TaqMan Fast Advanced Master Mix analysiert. Alle aufgeführten Komponenten wurden mit dem Liquid Handling Robot QIAgility™ pipettiert. Die Proben wurden in Bezug auf das Referenzgen beta Aktin (*ACTB*) unter Verwendung der relativen Quantifizierung $2^{-\Delta\Delta Ct}$ - Methode normalisiert, wobei der Mittelwert der mRNA-Transkripte von jeder Sonde für jedes Gen als eins festgelegt wurde.

Aufgrund des Reifegrades der Eizellen wurde das ICSI-Verfahren mit 93 Eizellen durchgeführt. Von den 61 befruchteten Eizellen wurden 13 Zygoten eingefroren, während 48 für den Embryotransfer weiter kultiviert wurden. Vierunddreißig Embryonen (70,8 %) erreichten eine gute Qualität und vierzehn (29,2 %) hatten eine schlechte Qualität.

Die Ergebnisse der aktuellen Studie zeigen, dass die Amphiregulin-Konzentration in der Follikelflüssigkeit den Reifegrad der Eizelle, die Befruchtungsrate und die Qualität der Embryonen beeinflusst. Außerdem unterscheidet sich die Amphiregulinkonzentration signifikant zwischen den Patientinnen in Abhängigkeit vom Body Mass Index.
Die Konzentration von Luteinisierendem Hormon in der Follikelflüssigkeit beeinflusst den Reifegrad der Eizelle und unterscheidet sich signifikant zwischen den Gruppen mit befruchteten von unbefruchteten Eizellen sowie zwischen den Gruppen mit guter und schlechter Embryoqualität.

Die Ergebnisse für den Insulinähnlichen Wachstumsfaktor II zeigen, dass die Konzentration dieses Proteins in der Follikelflüssigkeit keinen Einfluss auf den Reifegrad der Eizelle, die Befruchtungsrate oder die Embryoqualität hat.

Die Genexpression der untersuchten Gene (*AREG, IGF-II* und *LHCGR*) zeigt, dass nur die Expression von AREG die Eizellreife signifikant beeinflusst, wobei die *AREG*-Expression in der Gruppe der unreifen Eizellen herabreguliert ist. *LHCGR* und *IGF-II* unterscheiden sich nicht zwischen den Gruppen und hatten auch keinen Einfluss auf die Reifung, die Befruchtung oder die Embryoqualität.

Die Ergebnisse zur Unfruchtbarkeit zeigen, dass die Konzentration von Amphiregulin und Insulinähnlichem Wachstumsfaktor II in der Follikelflüssigkeit zwischen fruchtbar und unfruchtbaren Patientinnen statistisch unterschiedlich ist.

Zusammenfassend lässt sich sagen, dass die Ergebnisse für die untersuchten Proteine von großer Bedeutung für die zukünftige Entwicklung der Unfruchtbarkeitsbehandlung sind, insbesondere in Deutschland aufgrund der EPA. Darüber hinaus kann die Umsetzung der für Amphiregulin erhaltenen Ergebnisse prognostische Informationen liefern, die die Auswahl der lebensfähigsten Eizellen und damit der Embryonen unterstützen, da die Konzentration den Reifegrad der Eizellen, die Befruchtungsrate und die Qualität der Embryonen beeinflusst.

LIST OF ABBREVIATIONS

AMH: the anti-Mullerian hormone

AREG: amphiregulin

ART: assisted reproductive technologies

ASRM: American Society for Reproductive Medicine

BMI: body mass index

BMP 15: Bone Morphogenetic Factor 15

BTC: betacellulin

°C: Celsius degrees

cAMP: cyclic adenosine monophosphate

cDNA: complementary DNA

CGC: cumulus granulosa cells

cGMP: cyclic guanosine monophosphate

COC: cumulus-oocyte complex

Cx43: connexin 43

CYP11A: cholesterol side-chain cleavage enzyme

CYP19: cytochrome

CYP450scc: cholesterol side-chain cleavage enzyme

DHEA: dehydroepiandrosterone

DNA: deoxyribonucleic acid

EGF: epidermal growth factor

EGFR: epidermal growth factor receptor

EPA: Embryo Protection Act EREG: epiregulin

Erk1/2: extracellular signal-regulated kinases

ESHRE: European Society of Human Reproduction and Embryology

FSH: Follicle-stimulating hormone

GC: granulosa cells

GDF-9: Growth Differentiation Factor 9 (GDF-9),

GnRH: gonadotropin-releasing hormone

GQE: Good Quality Embryo

GV: Germinal Vesicle

HB-EGF: heparin-binding epidermal-like growth factor

hCG: human chorionic gonadotropin

HPG: hypothalamic-pituitary-gonadal

HSD17B: 17- hydroxysteroid dehydrogenase

HSD-3: hydroxysteroid dehydrogenase 3

ICSI: intracytoplasmic sperm injection

IGF: insulin-like growth factor

IUI: Intrauterine Insemination

IU: International Unit

IVF: In Vitro Fertilization

LHCGR: Luteinizing Hormone/Choriogonadotropin Receptor

LH: luteinizing hormone

M I: metaphase I oocyte

MAPK-p38: mitogen-activated protein kinases

MAR: mixed antiglobulin reaction

MGC: mural granulosa cells

MII: mature oocyte

MPF: maturation-promoting factor

mRNA: messenger-RNA

ng/ml: nanograms per milliliter

NTC: no template control

OSF: oocyte secretory factors

PB: polar body

PBS: phosphate buffer saline

PCOS: polycystic ovary syndrome

PCR: polymerase chain reaction

PDE3: 3 phosphodiesterase

pg/ml: picograms per millilitre

PGC: primordial germ cell stage

PGE2: prostaglandin E2

PKA: protein kinase A

POR: poor ovarian response

Pro-AREG: polarized glycoprotein precursor

rFSH: recombinant Follicle-stimulating hormone

rLH: recombinant luteinizing hormone

RNA: ribonucleic acid

ROC: receiver operating characteristic

StAR: steroidogenic acute regulatory protein

TGF ß: the transforming growth factor-beta

TGF-α: transforming growth factor alpha

TSH: thyroid-stimulating hormone

WHO: World Health Organisation

µL**:** microliter

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1. INTRODUCTION

1.1. Infertility

The World Health Organization (WHO) defines infertility as a disease of the female or male reproductive system that is characterized by the inability to achieve pregnancy within 12 or more months of regular unprotected sexual intercourse. Currently, no exact data are available concerning the prevalence of infertility around the world; however, the WHO estimates that 48 million couples and 186 million individuals struggle with infertility (WHO, 2020).

Based on the diagnosis, infertility can be primary or secondary. Infertility is considered to be primary when a pregnancy has never been achieved, and secondary when at least one pregnancy in the past has occurred (Mascarenhas *et al*., 2012).

Based on a European Society of Human Reproduction and Embryology (ESHRE) report, approximately 20–35% of infertility cases can be attributed to a female factor worldwide, and approximately 20–30% of cases can be attributed to a male factor. Furthermore, 25–40% of cases can be attributed to a problem in both partners; and for 10–20% of cases, there are no known relevant factors for infertility (ESHRE, 2014).

The current trend among women is to postpone the birth of their first child until they are older than 30 years, which has resulted in an increasing number of women seeking infertility-assisted treatments. The American Society for Reproductive Medicine (ASRM) reported that the number of *in vitro* fertilization (IVF) cycles that have been performed annually in the United States over the past decade have increased by more than 50% (Sunderam *et al*., 2015).

Concerning the female reproductive system, infertility can be related to a variety of factors, including ovarian disorders (e.g. polycystic ovary syndrome (PCOS) or follicular

disorder), tubal disorders (e.g. obstruction of fallopian tubes), and inflammatory uterine disorders (endometriosis). Additionally, many women suffer from different endocrine system disorders that lead to an imbalanced reproductive hormonal condition.

According to Ginsburg and Racowsky (2012), among infertile women, 11.5% have reduced ovarian reserves, 10.6% have multiple female factors, 7.7% have tubal factors, 6.8% have ovulatory dysfunction, 4.2% have endometriosis, and 1.4% have a uterine factor. On the other hand, dysfunctional or poor sperm quality such as abnormal morphology or decreased motility and concentration are the primary causes of infertility in the male reproductive system. In addition, testicular failure to create sperm due to certain medical treatments or varicocele, or certain hormonal disorders that result in unbalanced hormonal synthesis by the pituitary gland are common causes of infertility (Kumar *et al*., 2015).

When infertility is diagnosed, medical treatment cannot reverse the age-related reduction in fertility but can increase the chances of a successful pregnancy by using assisted reproductive technologies (ART). ART include different fertility treatments that involve *in vitro* manipulation of oocytes and sperms with the purpose of achieving successful pregnancy (Fitzgerald *et al*., 2017). The most common ART are

- Intrauterine insemination (IUI): In this procedure, based on the timing of a woman's ovulation through regular ultrasound monitoring, "processed" sperm is inserted using a sterile catheter directly into a woman's uterus. Due to the catheter's usage, the distance sperm must normally travel is significantly reduced (Trappe, 2017). To induce ovulation, IUI can be carried out with or without any hormonal stimulation. Since this procedure does not normally cause any pain, it can be carried out without anaesthesia. IUI is a relatively simple form of ART because fertilization occurs inside the woman's body (Diedrich *et al.*, 2013).
- IVF is a procedure that combines an oocyte and sperm in a laboratory environment. Once a woman is given hormones to induce ovulation, several cumulus–oocyte complexes

(COCs) are retrieved using a transvaginal method. Later, in the laboratory, the sperm is first purified and then incubated under controlled conditions with the COCs. Following insemination, fertilized oocytes are cultured in an incubator and later on developed embryo(s) are transferred into the patient's uterus (Wang *et al*., 2006). Fertilization and early embryo development occur *in vitro* under controlled incubator conditions inside a nutrient medium. IVF eliminates the need for the embryo to transit through the fallopian tube. The IVF procedure is the most fundamental type of all ART methods (Trappe, 2017).

- The intracytoplasmic sperm injection (ICSI) method was developed in the beginning of the 1990s. This method involves the microinjection of a single sperm into the cytoplasm of a mature oocyte. Therefore, the treatment's effectiveness does not depend on sperm motility or concentration (Palermo *et al*., 1992). ICSI was initially created to treat male infertility or sperm motility irregularities, and since then has become a significant advancement in the treatment of male subfertility. Worldwide, ICSI has been performed more frequently than standard IVF. Compared to IVF, the major disadvantage of ICSI is that the natural selection of sperm for microinjection is replaced by selection by an embryologist (Grainger *et al*., 2013).

1.2. Folliculogenesis

The ovary is the female gonad that promotes the maturation and development of female gametes and produces the hormones, including oestrogen and progesterone, required to coordinate the reproductive process. The ovary has three main sections: the hilum, the medulla, and the cortex. At the hilum, the ovarian ligament is linked to the ovary; moreover, blood vessels and nerves enter and exit the ovary in this region. The ovary's central part, the medulla, is composed of highly vascularized and nerve-rich stromal tissue. The outermost part of the ovary is the cortex, which consists of ovarian follicles containing oocytes and the stroma that lies between follicles (Mescher, 2018).

Follicles are the ovary's functional units and consist of highly organized germ cells (oocytes) and somatic cells (granulosa, theca, and stromal cells). The development of healthy, mature oocytes and the synthesis of steroid hormones are the two most important functions of the ovary, crucial for female fecundity and reproductive success (Holesh *et al*., 2022).

Folliculogenesis is a dynamic and highly controlled process that requires the coordinated actions of multiple systems in order to develop a viable gamete (Palma *et al*., 2012). A primordial follicle is a follicle that consists of an oocyte surrounded by a single layer of flattened granulosa cells (GC) (Konar, 2016). A relatively small number of follicles are continuously triggered throughout the reproductive lifespan, and the primordial follicle pool gradually decreases. According to Wallace and Kelsey (2010), women have approximately 2 million primordial follicles at birth, and by the initiation of puberty, this number has decreased to nearly 200,000. From primordial to large ovulatory follicles, development occurs in two phases: first, the preantral, gonadotropin-independent phase and, second, the antral gonadotropin-dependent phase (Figure 1) (Williams *et al*., 2012).

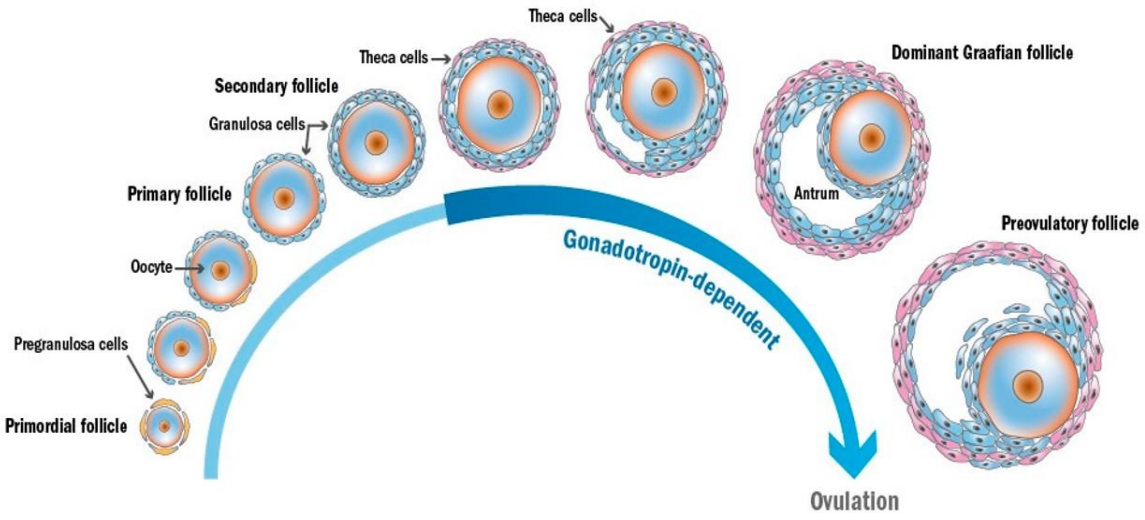

Figure 1: Schematic of Folliculogenesis (adopted from Hao *et al*., 2019).

All primordial follicles are created between the sixth and ninth months of foetal development. Primordial follicles have an average diameter of 29 μm and contain a small primary oocyte that is arrested in the diplotene stage of meiosis, a single layer of flattened GCs, and a basal lamina. Primordial follicles do not have an autonomous vascular system and only have limited access to the endocrine system (Gougeon *et al*., 1987).

The preantral stages of folliculogenesis can be divided into three categories according to the number of granulosa cell layers, the creation of theca tissue, and the development of a small cavity or antrum (Oktay *et al*., 1997).

Shortly after creation, some primordial follicles are recruited to grow. Histological signs of recruitment include a change in shape from squamous to cuboidal cells and the acquisition of mitotic potential in the GCs. This process is pituitary-independent and is controlled by autocrine/paracrine mechanisms (Yamoto *et al*., 1992).

Primary follicles consist of an oocyte that is surrounded by a single layer of cuboidal GCs. The cuboidal GCs start to express follicle-stimulating hormone (FSH) receptors at the same time that they undergo a change in shape and mitotic activity (Palma *et al*., 2012). Despite the fact that at this stage of folliculogenesis, FSH receptors exist in the GCs, it is assumed that during the regular menstrual cycle levels of FSH in the blood have no impact on granulosa responses due to the lack of an autonomous vascular system (Gougeon *et al*., 1994).

During the early growth phase, GCs expand, providing an oocyte with nutrients and various molecular signals. The GCs are connected to each other and with the oocyte cell membrane through gap junctions. Around the oocyte, primary GCs begin to release mucopolysaccharides to form a thick layer of glycoproteins and acid proteoglycans known as zona pellucida. Additional GC proliferation and follicular maturation result in the creation of a secondary follicle (Matzuk *et al*., 2002).

The increase in the diameter of the follicle that occurs during the transition process from a primary to a secondary follicle is one of the defining events that happen throughout the folliculogenesis process (from 0.1 mm to 0.2–0.4 mm). FSH, oestrogen, and androgen receptors that are associated with the secondary follicle are found in GCs (Speroff & Fritz, 2005).

FSH binds to its receptors on the surface of GCs, stimulating the aromatase enzyme and promoting the proliferation of GCs (Speroff & Fritz, 2005). Based on the widely accepted 'two-cell, two-gonadotrophin theory', FSH binding to the receptors in GCs stimulates cyclic adenosine monophosphate (cAMP) to induce aromatase which transforms androgens to oestrogens (Hillier *et al*., 1994). The proliferation of GCs is followed by a response from the theca cells to the luteinizing hormone (LH). Additionally, the interaction of LH with its receptors, which are located on the surface of theca cells, triggers the activation of cAMP which starts the synthesis of androgens from cholesterol (Speroff and Fritz, 2005). Androgens are subsequently converted to oestrogens, primarily estradiol-17ß, in the GCs. Oestrogens are secreted into the follicular fluid in order to regulate the further maturation of follicles (Erickson & Shimasaki, 2001).

A secondary follicle acquires a theca layer, and this is one of the most significant changes that occur during its development. This layer, composed of stroma-like cells surrounding the basal lamina, is divided into the inner layer – theca interna – and the outer layer – theca externa (Skinner, 2018). The development of the theca is accompanied by the neoformation of many small vessels. Blood circulates around the follicle, delivering hormones and nutrients and removing debris and secretory ingredients from the secondary follicle.

After the completion of the secondary development stage, a preantral follicle has five major structural units. These units are (I) a fully developed oocyte that is surrounded by a zona pellucida (II) GC (six to nine layers) (III) basal lamina (IV) theca interna (V) theca externa. The transition from secondary to tertiary follicles also called an antral follicle or a

Graafian follicle (Skinner, 2018), is only possible once a woman reaches puberty. The formation of a cavity in GCs is the initial sign that tertiary follicle development has begun. The accumulation of fluid between GCs, which over time results in the development of an internal cavity, is also known as cavitation or the early stage of antrum formation (Rodgers *et al*., 2010).

The GCs then proliferate and differentiate into mural granulosa cells (MGC) in the follicle's periphery and cumulus granulosa cells (CGC) nearest to the oocyte (Reynolds *et al*., 1992). The mature follicle (antral follicle), also called the Graafian follicle, is characterized by a large, fluid-filled antrum. In the antral stage, in response to available FSH, the majority of follicles undergo atresia, and only a few reach the preovulatory phase (Speroff & Fritz, 2005). The progression of the expansion and development of Graafian follicles can be categorized based on the size of the follicles as small (1–6 mm), medium (7–11 mm), and large (12–17 mm) to completely developed preovulatory stage (18–23mm) (Gougeon, 1983) (Figure 2).

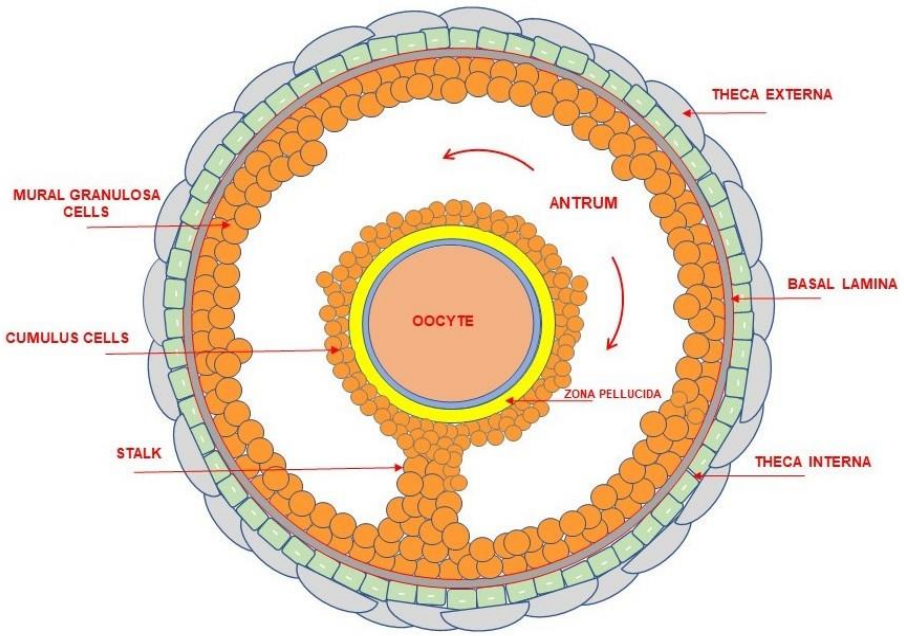

Figure 2: Schematic of mature Graafian follicles in the ovary with different types of surrounding cells.

The dominant follicle has an advantage over other follicles because it has a high rate of GC proliferation, resulting in a large number of FSH receptors, a high ability to aromatize, and increased production of oestrogen (Jones & Lopez, 2014). The preovulatory gonadotropin surge is the result of an increased oestrogen-positive feedback effect on the pituitary gland. Additionally, it is assumed that progesterone plays a role in ovulation stimulation (Zalanyi, 2001).

Before the gonadotropin surge, progesterone production begins in GCs and increases in the corpus luteum after ovulation. The final maturation of the follicle is assumed to be primarily stimulated by LH (Sullivan *et al*., 1999). In immature follicles, LH receptors have been identified to be expressed in theca cells, while in antral follicles, they are found in MGCs (Camp *et al*., 1991).

In response to the preovulatory gonadotropin surge, the dominant follicle produces a mature oocyte (Gougeon, 1998). In addition to gonadotrophins and oestrogens, the intraovarian regulation of folliculogenesis involves androgens, progesterone, and several growth factors (Hsueh *et al*., 2015).

Numerous members of the transforming growth factor-beta family of proteins, including bone morphogenetic factor 15 (BMF15), growth differentiation factor 9 (GDF-9), and anti-Mullerian hormone (AMH), play a crucial role as local regulators of follicular development and oocyte maturation (Knight & Glister, 2006). In addition, it has been confirmed that insulin-like growth factors (IGFs) as well as members of the epidermal growth factor (EGF) family have a crucial role in the maturation process (Shimada *et al*., 2006).

1.3. Oogenesis

Oogenesis is the transformation from the primordial germ cell (PGC) stage into a completely developed mature oocyte (MII) capable of being fertilized (Figure 3). The differentiation of oocytes during folliculogenesis is controlled by the activity of GCs, whose

functions are in turn controlled by different hormones and factors. The proliferation of PGC, oocyte growth, and oocyte maturation are three stages of oocyte development (Guraya, 2008).

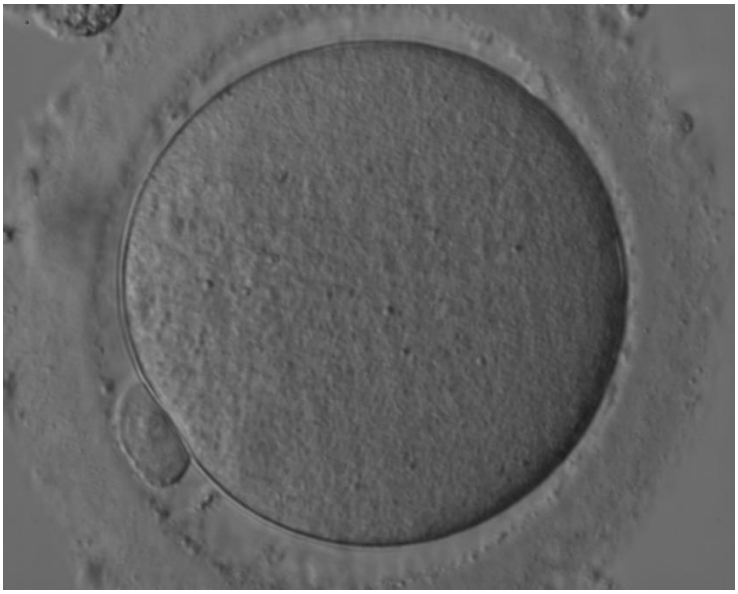

Figure 3: Mature Oocyte (MII) with an extruded Polar Body.

Approximately 3 to 4 weeks post-conception, PGCs can be recognized in the yolk sac wall, from week 4 in the hindgut epithelium, and from early week 5 in the gonadal area. In female embryos, PGCs differentiate into oogonia and continue to divide by mitosis. Just before and at the time of primordial germ cell (PGCs) arrival, there is an increase in the proliferation of the epithelium in the genital ridge and the epithelial cells penetrate the underlying mesenchyme, forming primitive sex cords, which surround the oogonia (Mamsen *et al*., 2012).

In the inner cortex of the ovary, proliferating oogonia undergo multiple mitotic divisions before beginning to differentiate into oocytes. Then, between the 11th and 12th week of gestation and after multiple rounds of mitotic germ cell division, oogonia begin to condense and form individual primordial follicles. Subsequently, the mitotic activity of the oogonia is

interrupted and the process of meiosis begins, which leads to the formation of a primary oocyte (Goto *et al*., 1999).

Oocytes undergo two meiotic cell divisions during their development (Figure 4). Each meiotic division comprises four phases: prophase, metaphase, anaphase, and telophase. The duration of the first meiotic prophase in oocytes is significantly longer than in any other cell type and can be divided into the transitory phase (leptotene, zygotene, and pachytene) and the stationary phase (diplotene).

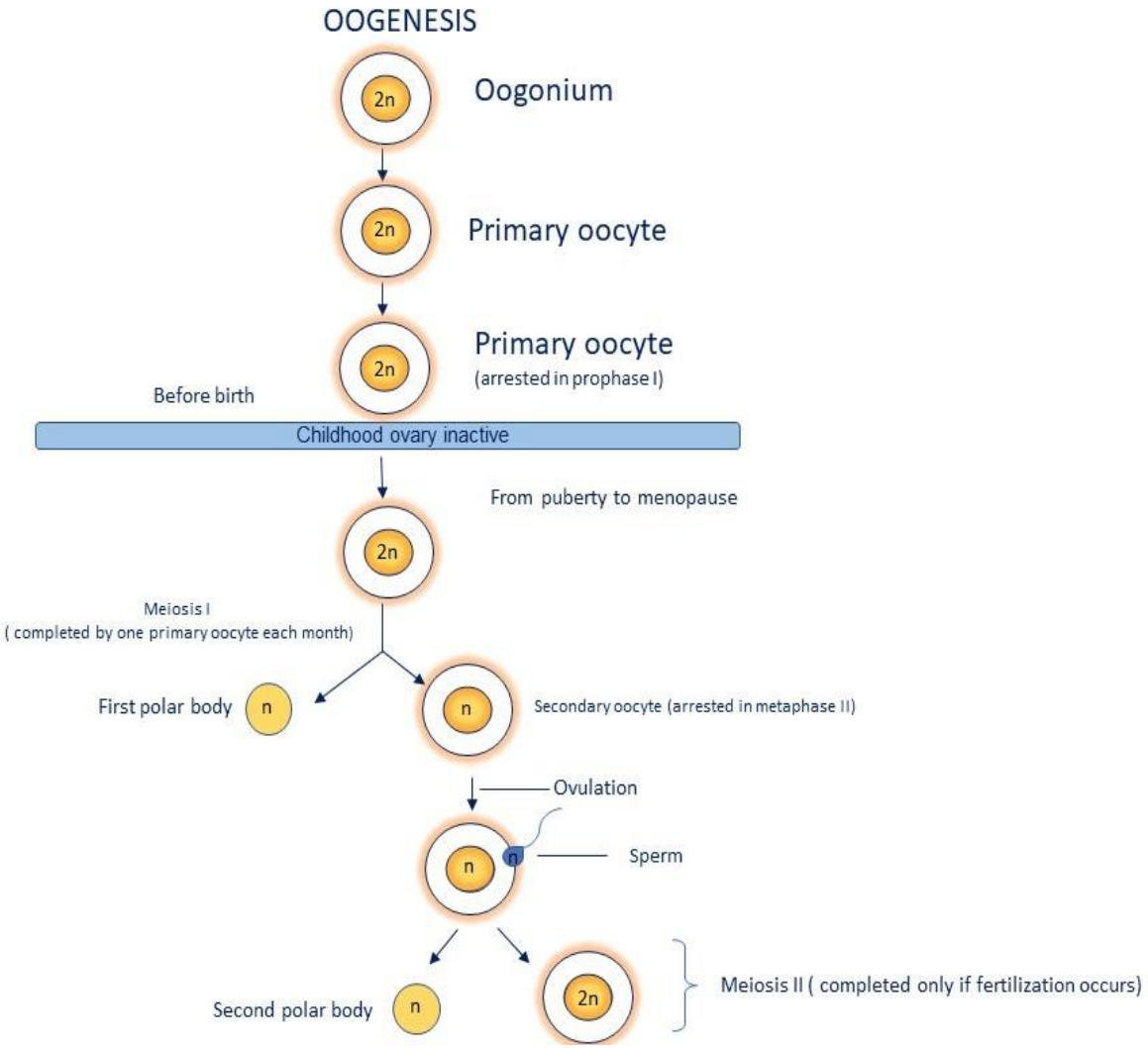

Figure 4: Schematic of Human Oogenesis.

As the oocyte gets into the diplotene stage, it enters a phase where it remains until just before ovulation occurs. A significant amount of intracellular cyclic adenosine monophosphate (cAMP) is released from GCs and transferred to the oocyte to maintain the arrest phase (Zhang & Xia, 2012). The production of cAMP is under the control of FSH, which enables the oocyte to be held in the arrested stage until ovulation. The cells that have been stopped in the prophase of meiosis I are morphologically in the germinal vesicle (GV) stage, which is characterised by an expanded nucleus (Figure 5) (Anirudh & Agarwal, 2013).

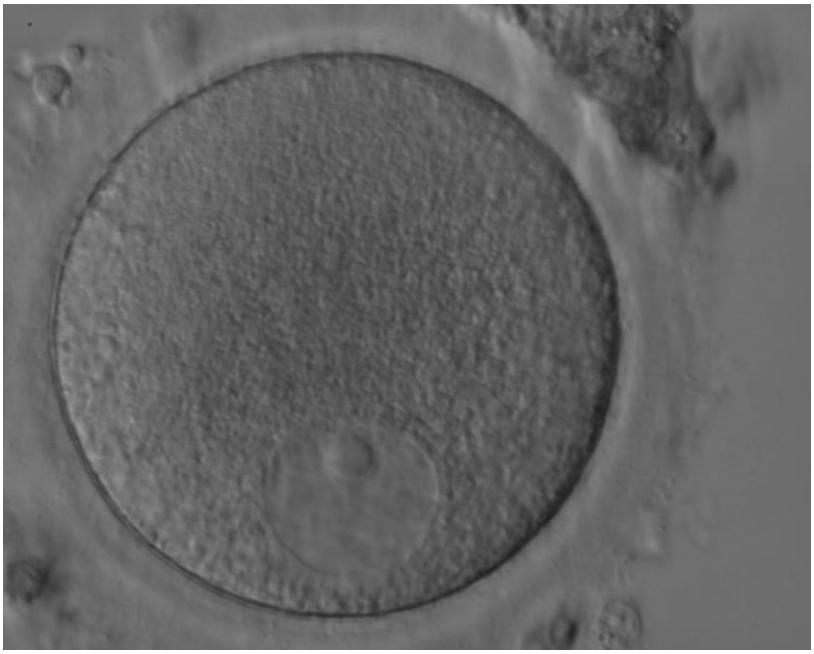

Figure 5: The Germinal Vesicle (GV) Oocyte with an eccentrically placed Nucleus and a prominent single Nucleolus. Adapted from Magli *et al* (2012).

The primary oocyte, which is diploid (2N; 46 chromosomes or 23 pairs of chromosomes), undergoes a reductional meiotic division, resulting in the formation of 2 haploid cells (N = 23 chromosomes): the secondary oocyte and the first polar body (PB). Thereafter, the second meiotic division is initiated and goes on until metaphase II, where the secondary oocyte remains arrested. Ovulation is a process where the oocyte is released from the ovary, and if fertilization happens, the secondary oocyte finalizes the second meiotic

division by decreasing the genetic material to 23 single chromatids and producing the second PB, which contains the other pair of 23 single chromatids (Peters *et al*., 1980).

1.3.1. Oocyte Growth

The oocyte growth process is strictly regulated by the activity of cumulus cells which surround the oocyte during follicular development and assist it in acquiring competence (Eppig, 2001). During the growth process, the oocyte expands in size (approximately a hundredfold in volume) and accumulates additional ribonucleic acid (RNA) and metabolic substances such as carbohydrates and lipids. These components are necessary for the oocyte's ongoing growth and maturation process (Marteil *et al*., 2009). Additionally, during the growth process, the oocyte is responsible for the production, reorganization, and replication of new organelles (mitochondria) as well as the secretion of glycoproteins that will contribute to the formation of the zona pellucida and the cortical granules, which are essential for fertilization (Picton *et al*., 1998).

Upon reaching puberty, a woman's follicles are subjected to higher amounts of gonadotropins (LH and FSH) than they were previously. As a result of an increase in gonadal hormones, the oocyte is able to conclude the growth phase (achieving a final size of 60–120 µm). Additionally, oocytes undergo GV breakdown, which is followed by chromosome condensation and alignment of the chromosomes at the metaphase plate in order to prepare for the completion of the first meiotic division. These oocytes are referred to as metaphase I (MI) oocytes (Figure 6) (Zhang & Xia, 2012).

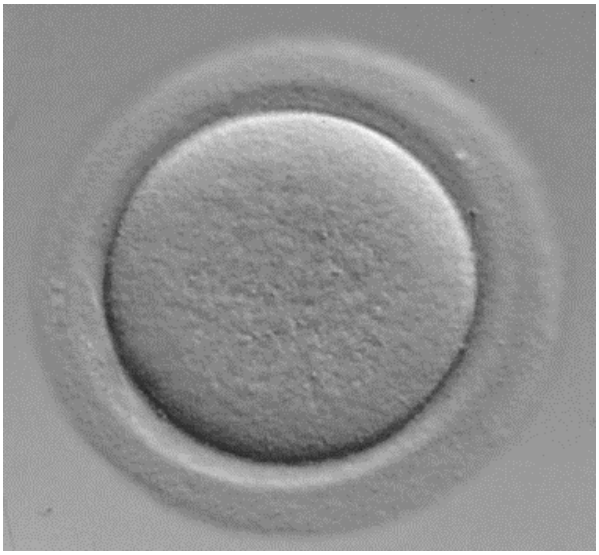

Figure 6: An Immature (MI) Oocyte with no visible Nucleus and the absence of an extruded Polar Body. Adapted from Magli *et al. (2012).*

Following the initial rise of LH, oocytes begin to control the expression of genes in the CGCs which are responsible for growth. Additionally, the synthesis of the mucinous matrix that consists of hyaluronic acid causes further enlargement. Due to this growth process, the gap junctions detach when the cumulus cells continue to expand, which later enables the resumption of meiosis and ovulation (Combelles *et al*., 2004).

1.3.2. Oocyte Maturation

The maturation process prepares the oocyte to complete meiosis and undergo fertilization. This is one of the most essential processes during oogenesis, directly affecting the quality of oocytes and defining developmental competence.

The developmental competence of an oocyte is its capacity to support embryonic development until the embryo can activate its own genome (Vassena *et al*., 2011). Two factors determine the level of developmental competence:

1. Nuclear maturation: This refers to the oocyte's capacity to complete meiosis. The quantity and position of crossovers during meiosis I can affect nuclear competence. The LH surge initiates nuclear maturation by causing the oocyte's GV to dissolve and meiosis to resume. Moreover, during nuclear maturation, the first PB is created and extruded from the cytoplasm. The created PB has a minimal amount of cytoplasm and 50% of the genetic material (Chaube, 2001).

2. Cytoplasmic maturation: This process can be divided into three distinct but interconnected stages: (I) the dispersion of organelles, (II) the reorganization of the cytoskeleton, and (III) the maturation of molecules. Changes in the concentration of hormones secreted by cumulus cells directly affect these processes (Ferreira *et al*., 2009). Cytoplasmic competence is determined by a number of factors, and the majority of them can only be evaluated on molecular and cellular levels (Coticchio *et al*., 2004).

1.4. Two-Cell Theory

The production of ovarian steroids is a highly controlled process that requires numerous distinct signalling pathways in different cell types. Steroidogenesis seems to occur according to the two-cell, two-gonadotropin model in which androgens are produced from cholesterol in LH-stimulated theca cells, followed by transformation to oestrogens in FSH-exposed GCs (Jamongjit *et al*., 2006). The synthesis of oestrogen requires the presence of both cells and gonadotropins. In the preovulatory follicle, the theca layer exhibits a well-developed vascular network and the mural layer of GCs interfaces with surrounding blood vessels. Therefore, these cells are well-positioned to respond to fluctuations in circulating hormones and to release the produced oestrogen directly into the venous effluent of preovulatory follicles (Wood & Strauss, 2002).

Gene expression of steroidogenic enzymes is regulated and modulated during the menstrual cycle, which causes fluctuations of the steroidogenic enzymes that are expressed in theca and/or GCs (Yong *et al*., 1994) (Figure 7).

The conversion of cholesterol to pregnenolone is the first step in the *de novo* production of all steroid hormones (Altmäe *et al*., 2011). This conversion is carried out in the mitochondrial inner membrane by an enzyme known as the cholesterol side-chain cleavage enzyme (CYP450scc; CYP11A). After pregnenolone has diffused to the smooth endoplasmic reticulum, it can be either transformed to progesterone by 3-hydroxysteroid dehydrogenase (3-HSD; HSD3B) or to dehydroepiandrosterone (DHEA) by 17-hydroxylase (CYP45017) and 17,20-lyase (CYP17A1) (Penning, 1997).

According to Hanukoglu (1992), the conversion of DHEA and progesterone to androstenedione is catalysed by HSD3B and CYP17A1, respectively. Within the theca cell, androstenedione can either be converted to testosterone by 17-hydroxysteroid dehydrogenase (HSD17B) or it can diffuse into the granulosa cell. After formation, 17-estradiol can undergo the additional process of metabolization to produce either 2-hydroxy estradiol or 4-hydroxy estradiol via cytochrome CYPA1/2 and cytochrome CYP 3A4 or cytochrome CYP1B1, respectively (Tsuchiya *et al*., 2005). For the success of this process within the follicle, granulosa and theca cells must interact with each other in a coordinated way.

In the presence of the enzyme CYP17, the conversion of pregnenolone and progesterone to DHEA and androstenedione occurs (Strauss & Penning, 1999). Additionally, certain receptors on the outer membrane of the theca cell are activated in response to LH signalling. The FSH signal is received by GC receptors, which induces an increase in the expression of the enzyme CYP19 and the transformation of androgens to oestrogens (Jamnongjit *et al*., 2006).

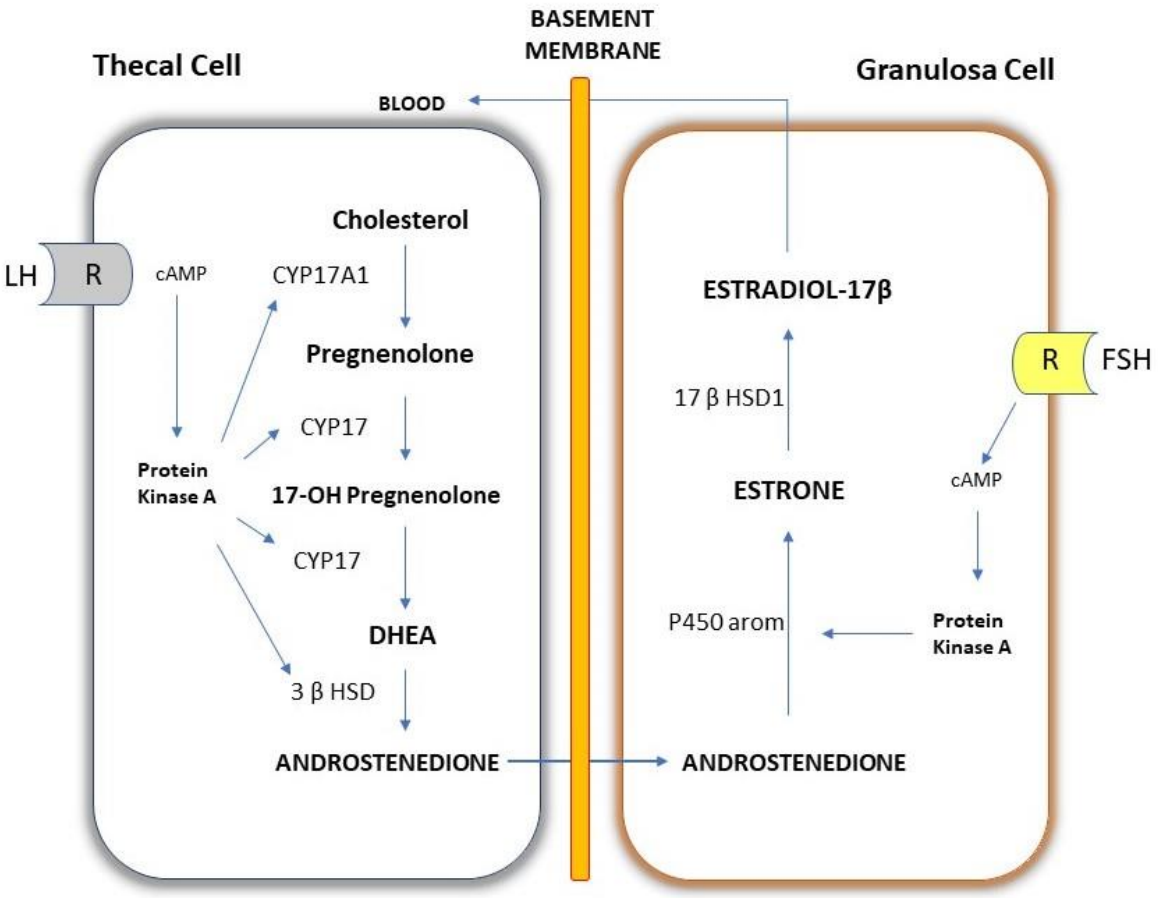

Figure 7: Schematic of principle pathways of the Two-Cell ovarian steroidogenesis model.

Additionally, both granulosa and theca cells produce a steroidogenic acute regulatory protein (StAR), P450 side-chain cleavage enzyme, and 3'-hydroxysteroid dehydrogenase (3-HSD). Therefore, both of these cell types have the potential to generate pregnenolone and/or progesterone from cholesterol (Christenson & Devoto, 2003). Additionally, during the follicular phase, avascularised GCs interact with a very small amount of oxygen or cholesterol; and, consequently, very low concentrations of the steroids are created. On the other hand, when GCs are exposed to gonadotropins, they undergo a process known as 'luteinisation', enabling them to produce prodigious amounts of pregnenolone and progesterone from cholesterol (Wood & Strauss, 2002).

Interestingly, due to the absence of CYP17 in GCs, GCs are unable to transform progestins into androgens. Therefore, this process normally takes place in theca cells that express CYP17. Due to the aforementioned processes, it seems that GCs are more responsible than theca cells for ovarian progesterone synthesis (Havelock *et al*., 2004).

Understanding the pathways by which LH-induced signalling in theca cells leads to progesterone synthesis in GCs is crucial. It is well known that progesterone is essential for ovulation and seems to be responsible for improving oocyte maturation (Jamnongjit *et al*., 2006).

1.5. Mural Granulosa Cell

GCs are a specialized type of ovary cells that regulate folliculogenesis and oocyte maturation. During the transition from preantral to antral follicles, these cells differentiate into two functional groups: the cumulus cells which are in direct contact with the oocyte and mural granulosa cells (MGCs) which surround the follicular wall around the antrum (De los Santos *et al*., 2012).

Although there are many similarities between these cell groups, there are also significant differences, especially in gene expression and protein production. The surrounding oocytes are connected to MGC through a small area, where all three types of cells communicate with each other through gap junctions (Richards *et al*., 2018).

The receptor of LH (luteinizing hormone/choriogonadotropin receptor; LHCGR) is only expressed in theca and MGC (Arroyo *et al*., 2020). Activation of LHCGR induces the production of androgens. Later on, these androgens are converted to oestrogens by GCs. The presence of LHCGR in MGC is essential for the activation of signals that initiate paracrine and autocrine pathways responsible for ovulation.

Pituitary LH induction is a signal that initiates and organizes the last phases of oocyte maturation and follicular rupture. The binding of LH to the LH-r receptor on MGC initiates the

activation of many different intracellular pathways, including protein kinase A (PKA), protein kinase C, and extracellular signal-regulated kinases (Erk1/2) (Figure 8) (Arroyo *et al*., 2020).

Evidently, meiotic arrest in prophase I and the resumption of meiosis are affected by follicle microenvironment conditions. cAMP, which is produced in an oocyte by G-protein-coupled receptors, acts as the main factor in oocyte meiotic regulation and resumption (Liu *et al*., 2013). By inhibiting the activity of the maturation-promoting factor (MPF) and stimulating cAMP-dependent PKA, increased intra-oocyte cAMP levels maintain the oocyte in the meiotically arrested stage (Gilchrist, 2011).

Tsafriri (1996) noted that an oocyte has highly active type 3 phosphodiesterase, an enzyme that dissolves cAMP. Furthermore, the cyclic guanosine monophosphate (cGMP) that the follicle's somatic cells deliver to the oocyte decreases PDE activity (Tornell *et al*., 1991). Increased amounts of cAMP in the oocyte during folliculogenesis sustain meiotic arrest and mediate the meiotic-inducing effects of gonadotropins. One possible explanation for this occurrence might be that a brief increase in cAMP levels results in the production of a signal that induces meiosis.

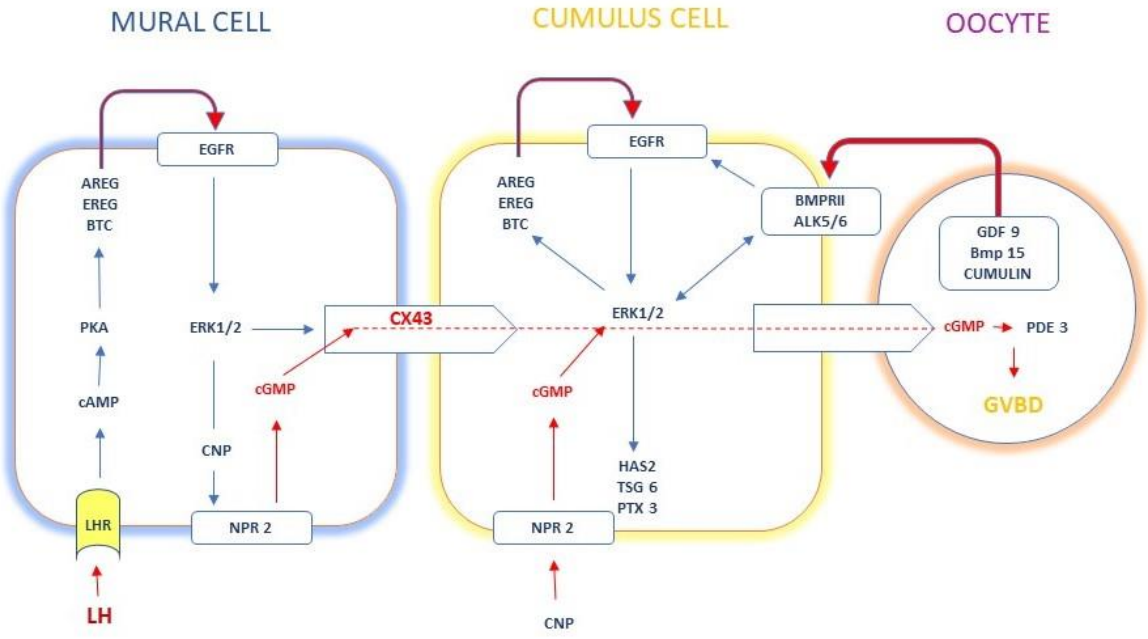

Figure 8: Schematic of the epidermal growth factor network, and transmission of the LH signal from the mural cells of the follicle to the oocyte to promote oocyte meiotic resumption and ovulation**.** Adapted from Gilchrist (2011).

A second crucial discovery is that the preovulatory increase of LH causes a fast cascade of EGF-like peptides in somatic cells (Park *et al*., 2004). These peptides include amphiregulin (AREG), epiregulin (EREG), and betacellulin (BTC). In response to LH, these peptides are primarily synthesized by MGCs; furthermore, these peptides, through EGF receptors, have autocrine effects on MGC and paracrine effects on cumulus cells (Gilchrist, 2011). The EGF receptor is a critical link in the distribution of the ovulatory LH signal from MGC to cumulus cells and the oocyte (Hsieh *et al.,* 2007). EGF-like peptides activate the EGF receptor and ERK1/2 which are responsible for transmitting intracellular signals. Additionally, EGF-like peptides are essential components of the MGC/CGC signal transduction pathway (Park *et al.,* 2004). Prostaglandin E2 (PGE2) and p38 mitogen-activated protein kinases (p38 MAPKs) are both induced as a result of the ERK1/2 activation, which leads to increased production of EGF-like peptides by MGC and cumulus cells (Park *et al*., 2004).

Oocyte meiotic resumption can be effectively induced by EGF-like peptides through a mechanism that requires ERK1/2 (Fan *et al*., 2009). The details of ERK1/2 cumulus cells that induce meiotic resumption are not fully understood, but it is hypothesised that this process may require phosphorylation of connexin 43, which would lead to the closure of granulosa–cumulus and cumulus–cumulus gap junctions and subsequent loss of the inhibitory activity of follicular cAMP and cGMP or synthesis of a stimulatory meiosis-inducing factor by cumulus cells (Gilchrist, 2011; Norris *et al*., 2009; Sela-Abramovich *et al*., 2006).

Oocytes control the proliferation and differentiation of GCs by secreting soluble paracrine growth factors. Folliculogenesis cannot occur without GDF9 and BMP15 (Gilchrist *et al.,* 2008), which are the two most important oocyte secretory factors (OSFs). It has been shown that OSF signalling can have various effects on the function of cumulus cells, and further investigation into this topic is ongoing (Alam & Miyano, 2019).

OSFs have various effects on cumulus cells, including promoting growth and controlling apoptosis (Vanderhyden *et al*., 1992), suppressing luteinisation (Eppig *et al*., 1997; Vanderhyden *et al*., 1993), controlling metabolic activity (Sutton *et al.,* 2003a), sterol biosynthesis, and cumulus cells expansion (Hussein *et al*., 2006).

Several studies on gene expression in MGCs during the IVF procedure have reported a significant correlation between gene expression and the ovarian stimulation protocol (Kaneko *et al*., 2000), oocyte maturation (Uyar *et al*., 2013), fertilization (Karabulut *et al*., 2020), and embryo development (Jiang *et al*., 2010). Additionally, Chin *et al*. (2002) reported that gene expression of MGCs was significantly different between normal and poor responders, suggesting that the MGC transcriptome can effectively determine ovarian reserve in IVF patients. Moreover, based on the aforementioned research, the AREG pathway and transmission of the LH signal from mural cells to the oocyte are the most crucial steps that promote oocyte meiotic resumption and ovulation (Liu *et al*., 2022).

1.6. Luteinizing Hormone (LH)

The primary role of human gonadotropic cells in the anterior pituitary is the production of glycoprotein hormones known as gonadotropins. In addition to FSH and LH, there are three other glycoprotein hormones: human chorionic gonadotropin (hCG), thyroid-stimulating hormone, and inhibins. The primary role of gonadotropins is folliculogenesis regulation (Bergendah & Veldhuis, 2001).

The two main gonadotrophins are LH and FSH, which are heterodimeric glycoproteins composed of non-covalently coupled alpha and beta subunits. The beta subunit is hormone-specific and contains the receptor-binding domain, while the alpha subunit is similar to the beta subunit (Pierce *et al*., 1981).

The alpha subunit is encoded by a gene located on chromosome 6 (6q12-q21) while the genes for the beta chains of chorionic gonadotropin and LH are contiguous on chromosome 9q13.3. The transcription of the LH beta subunit is especially significant because it is the process that regulates LH production levels (Dias *et al*., 2002). The composition of the amino acid sequence in the beta subunit imparts to LH its essential biochemical activity and purpose (Choi & Smitz, 2014).

The anterior pituitary is stimulated by gonadotropin-releasing hormone (GnRH) to release FSH and LH into circulation, where they influence the gonads. The pulsatile secretion of LH, as compared to continuous secretion, is essential because GnRH pulses and corresponding LH pulses fluctuate in frequency (amount produced per minute) and amplitude (amount produced per pulse), which ultimately affect cellular responses and the regulation of the reproductive cycle at its different stages (Choi & Smitz, 2014). This process is called the hypothalamic–pituitary–gonadal (HPG) axis (Figure 9).

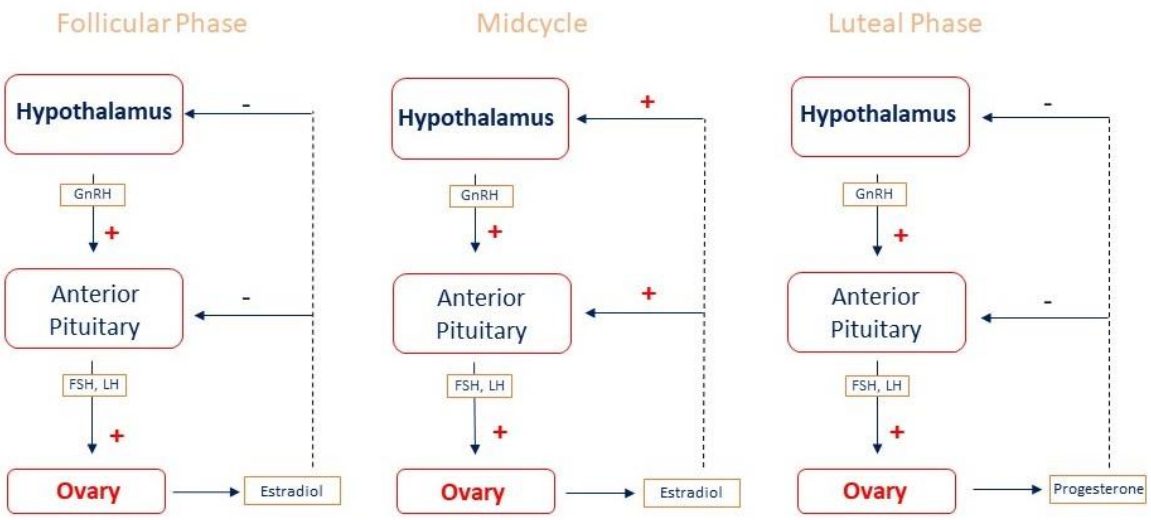

Figure 9: Schematic of the hypothalamic-pituitary-gonadal axis and relationship among the hypothalamus, the pituitary gland, and the ovary. The hypothalamic GnRH positively regulates the pituitary's activity. Additionally, oestradiol and progesterone exert variable feedback directly on the hypothalamus, allowing its correct functioning. (+) stimulatory effect; (-) inhibitory effect; GnRH-Gonadotropin-releasing Hormone; FSH-Follicle-stimulating Hormone; LH-luteinizing hormone.

The action of LH is mediated by its ability to bind to the trans-membrane LHCGR, which is found in developed GCs, theca cells, and luteal cells of ovaries. This binding leads to a structural change of the receptor which triggers a stimulatory G protein that later activates different enzymes such as adenylate cyclase, phospholipase C, and kinase. Additionally, LH affects theca cells, causing them to synthesize androgens, initiate ovulation, and sustain the corpus luteum (Richards *et al*., 2010).

Moreover, LH assists FSH in activating follicles and plays a crucial role in triggering ovulation and the release of the ovum, achieved via a massive LH surge caused by the positive feedback activity of increasing oestrogen on the pituitary, which results in the

completion of meiosis I in the primary oocyte, disruption of the follicular wall, and subsequent ovulation 9 h prior to the peak LH level (Olive *et al*., 2007).

The human LHCGR is a transmembrane G-protein coupled receptor activated by LH and hCG found primarily in the ovary, testis, uterus, and breast. Additionally, LHCGR is expressed in the endometrium, where it is involved in the regulation of decidualization and implantation (Ascoli *et al*., 2002).

The human *LHCGR* gene is composed of 11 exons and 10 introns. It is situated on the short arm of chromosome 2 and encodes for a highly glycosylated mature glycoprotein receptor containing 699 amino acids with a molecular weight range from 85 to 95 KDa (Troppmann *et al*., 2013).

The main function of *LHCGR* is to control steroidogenesis. The process is initiated by increasing cytochrome CYP450 which leads to the transformation of cholesterol into androgen precursors. In response to FSH, oestradiol, and other paracrine factors, the levels of *LHCGR* expression rise with follicle growth, reaching their peak just before ovulation (Menon *et al*., 2005).

In response to the preovulatory surge in LH that occurs during the transition of oestrogen-producing GCs into luteal cells, the expression of *LHCGR* is down-regulated (Hoffman *et al*., 1991). Due to the desensitization of the G protein-coupled response system that occurs during this period, the growing GCs continue to be resistant to LH. Afterward, there is a complete recovery of both *LHCGR* and the responsiveness of the developed GCs to LH (Herrlichh *et al*., 1996). During the mid-luteal phase, when progesterone synthesis has increased, *LHCGR* expression reaches maximum levels. The receptor levels then decrease as the corpus luteum regresses (Peegel *et al*., 1994). *LHCGR* expression appears to be controlled by transcriptional and, significantly, post-transcriptional processes. Segalof *et al*. (2009) reported that mutations of *LHCGR* in females can lead to infertility.

1.7. Amphiregulin (AREG)

Over the past few years, members of the epidermal-like growth factor family have increasingly gained attention as possible mediators of the activity of LH in follicles (Schneider *et al*., 2008). It has been demonstrated that the epidermal growth factor receptor (EGFR) and its ligands, which are expressed in female reproductive organs, influence numerous essential reproductive functions (Fang *et al*., 2016). EGFR is activated by epidermal growth factor (EGF), amphiregulin (AREG), betacellulin (BTC), epiregulin (EREG), heparin-binding EGF-like growth factor (HB-EGF), and transforming growth factor α - (TGF-α) (Harris *et al*., 2003).

The production of AREG, EREG, and BTC proteins is an essential component of the paracrine function of the LH ovulatory signal. These three proteins initiate a large network of gene activation in MGC and cumulus cells, which regulate processes such as oocyte maturation, cumulus development, and ovulation (Park *et al*., 2004). After being released into the antral fluid by the proteolytic process, these ligands bind EGFR on the MGC and cumulus cells in either an autocrine or paracrine manner (Richani *et al*., 2018). The activation of EGFR on cumulus cells causes an increase in the autocrine production of EGF-like peptides, which in effect amplifies the signal in these cells and helps to transmit and mediate the LH stimulus that comes from the periphery and passes to the oocyte (Holbro & Hynes, 2004; Yarden & Sliwkowski, 2001).

During the peri-ovulatory phase, the granulosa and cumulus cells produce EGF-like peptides periodically. AREG, EREG, and BTC are not observable before the LH surge but are rapidly elevated to maximal levels prior to oocyte GV breakdown and decrease immediately thereafter to basal levels (Conti *et al*., 2006). Following hCG administration, AREG can be found in the follicular fluid. Despite the fact that BTC and EREG can potentially be up-regulated by hCG, their protein levels in human follicular fluid are minimal. Accordingly, Zamah *et al*. (2010) reported that AREG is the most significant EGFR ligand in human follicular fluid.

Based on published data, the presence of AREG in follicular fluid might be used as a predictor of follicle growth. Inoue *et al*. (2009) reported that oocyte quality and pregnancy outcome were affected by the AREG concentration in follicular fluid. Additionally, Ben-Ami *et al*. (2011) demonstrated that human GV-stage oocytes treated with AREG via medium supplementation can considerably increase the rate of maturation compared to controls. Concerning IVF patients, Humaidan *et al*. (2011) compared the concentration of AREG based on different triggering protocols (GnRH and hCG) and suggested that different oocyte maturation outcomes could be achieved through alternative protocols.

A recent study indicated a significant connection between LH-induced *AREG* messenger-RNA (mRNA) expression in MGC and cumulus cells and IVF parameters, such as the number of retrieved oocytes and embryo quality (Huang *et al*., 2015). The results of a proteomic study on the follicular fluid of patients with PCOS and normal-ovulatory patients who were undergoing IVF showed that the amount of AREG in PCOS patient follicular fluid was significantly lower; consequently, these results support the hypothesis about a strong correlation between the follicular accumulation of AREG and oocyte developmental competence (Ambekar *et al*., 2015).

Nevertheless, the signalling pathway of the LH receptor involved in regulating *AREG* expression and release has not yet been completely characterized (Wang *et al*., 2019). The *AREG* gene is located on human chromosome 4q13–4q21. *AREG* is transcribed as a 1.4 kb mRNA composed of 6 exons that code for a transmembrane polarized glycoprotein precursor (Pro-AREG) composed of 252 amino acids (Sisto *et al*., 2017). Although there are many published reports that link *AREG* and oocyte competence, the number of studies that investigate *AREG* gene expression and IVF outcomes is insufficient.

1.8. Insulin-like growth factor II (IGF-II)

Insulin-like growth factors (IGF-I and IGF-II), which are proinsulin-like short peptides with a molecular weight of approximately 7.5 kDa, are essential mitogens that significantly affect cell development, differentiation, and metabolism (Wang *et al*., 1992). Besides the different endocrine effects, locally produced IGFs have paracrine and autocrine effects (Wang *et al*., 1999).

The IGF system in the ovary is composed of IGF-I and IGF-II as well as their two corresponding receptors in specific cells, six IGF binding proteins (IGFBP-1 to IGFBP-6), and IGFBP proteases (Wang *et al*., 1999).

In contrast to the IGF-I that is found in the ovarian follicles of other species, the IGF-II in the human ovarian follicle is the primary IGF that acts as a mediator of FSH action and stimulates steroidogenesis in MGCs (Zhou *et al*., 1993). Additionally, IGF-II enhances gonadotropin action, granulosa cell growth, aromatase activity, and steroidogenesis (Ipsa *et al*., 2019).

During the process of folliculogenesis, theca cells produce IGF-II, which then acts in an autocrine manner on theca cells and in a paracrine manner on GCs. Acting alongside FSH during the follicular phase of the menstrual cycle, IGF-II promotes granulosa cell proliferation (Mazerbourg *et al*., 2003). Following ovulation, together with LH, IGF-II stimulates progesterone secretion during the luteal phase of the menstrual cycle following ovulation (Baumgarten *et al*., 2015). IGFs are also present in the follicular fluid of human antral follicles, and their concentrations have been quantified in many studies. While circulating levels of IGF-I and IGF-II are unaffected by the menstrual cycle, follicular fluid levels fluctuate during follicle maturation (Mazerbourg *et al*., 2018). Van Dessel *et al.* (1996) reported that there is no correlation between changes in the amount of IGF I found in the follicular fluid and follicular size. On the other hand, levels of IGF II were positively correlated with follicle size and the obtained concentrations were high in dominant follicles.

The human *IGF-II* gene is found on chromosome 11p15.5 and contains approximately 30 kb of deoxyribonucleic acid (DNA). IGF-II has 4 promoters and 10 exons. However, only the last three exons contain coding sequences (Nordin *et al*., 2014). Nearly all human embryonic and foetal tissues express the *IGF-II* gene (Chao *et al*., 2008). Studies that examine interactions between steroid hormones and IGF II at the level of gene expression or signalling have yet to be conducted in MGCs. Understanding these interactions is essential for comprehending FSH activity and follicle development, given the location of the IGF system during human folliculogenesis.

Although Wang *et al*. (2006) reported on the different concentrations of IGF II in follicular fluid as well as their impact on post-ovulatory events, including fertilization and embryo development, further research is necessary to improve treatments for infertile patients with abnormal folliculogenesis.

1.9. The Purpose of The Study

Oocyte maturity, fertilization, and embryo quality are crucial to the ICSI procedure. Due to the Embryo Protection Act (EPA) in Germany and since the many published reports about follicular fluid biomarkers remain disputable, defining possible parameters that might be highly useful as non-invasive biomarkers of ICSI outcomes is crucial, especially in countries with restrictive reproductive medicine laws.

Therefore, in this study, we had the following aims:

I. First, to determine AREG, LH, and IGF II concentrations in the follicular fluid and to explore their correlation to oocyte maturity, fertilization rate, and embryo development.

II. Second, to determine gene expression in the MGCs of the *AREG*, *LHCGR*, and *IGF-II* genes and their correlation with protein concentration, oocyte maturity, fertilization rate, and development of embryos.

III. Third, to investigate the possible difference in protein concentration and gene expression between infertile and fertile patients.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Patients

After the implementation of inclusion criteria, primarily forty-five patients were approached for the study, out of these number three patients had follicular fluid aspiration without oocytes, five patients developed Ovarian Hyperstimulation Syndrome (OHSS) after the COS, while for four patients essay analysis were missing. Due to the aforementioned reasons finally thirty-three women undergoing ICSI were recruited at the University Clinic of Saarland Fertility Centre located in Homburg, Germany, between May 2020 and October 2021. Patients' ages ranged between 23 and 40 years. Anti-Mullerian blood levels ranged from 0.48 to 17.9 ng/mL. Body mass index (BMI) measurements ranged from 18.7 to 40.4 kg/m^2. The patients presented various infertility causes, and out of the 33 patients, 45.5% (N=15) had PCOS, endometriosis, or tubal disorder diagnosis while 55.5% (N=18) did not have any infertility diagnosis. Additionally, for 66.7% (N=22) of patients included in the study, this was the first-time cycle, while for 33.3% this was the second or third-time cycle (N=11). Male infertility diagnosis was confirmed in 21.2% (N=7) cases.

Patients were recruited based on the following criteria: age between 18 and 40, inability to achieve natural pregnancy during 12 months, normal uterus and fallopian tubes, and normal menstrual cycle. Specific exclusion criteria were: primary ovarian failure; two episodes of poor ovarian response (POR) after maximal stimulation; history or presence of tumors; the presence of an ovarian cyst >25 mm; use of testicular or epididymal sperm; cryptozoospermia. Due to the impact on embryo quality, couples where a male partner was diagnosed with strict teratospermie (<2% of normal sperm morphology), were excluded from the study.

2.1.2. Ethical Approval

The study received ethical approval from the Ethics Committee of the Medical Association of Saarland (reference number: 146/19). Every study participant provided written informed consent. Adherence to the Helsinki Declaration (ethical principles for medical research) was ensured throughout the study process.

2.1.3. Chemicals and Instruments

Chemicals	Company
• G-IVF Plus (Culture medium)	Vitrolife, Sweden
• G MOPS Plus (Culture medium)	Vitrolife, Sweden
• G-1 Plus (Culture medium)	Vitrolife, Sweden
• ICSI Cumulase	Origio, Denmark
• Liquid paraffin	Origio, Denmark
• Nuclease-free water	Qiagen, Germany
• RNA later Stabilization	Qiagen, Germany
• Pure Sperm 100	Nidacon International, Sweden
• PVP Clinical Grade	Origio, Denmark
• Sodium chloride (NaCl) 0,9%	B.Braun, Germany

Kits	Company
• Amphiregulin Quantikine Kit	R&D Systems Inc. USA
• Elecsys Progesterone Gen III	Roche Diagnostics, German
• Elecsys Estradiol Gen III	Roche Diagnostics, German
• Elecsys FSH	Roche Diagnostics, German
• Elecsys LH	Roche Diagnostics, German

• High Pure RNA Isolation Kit	Roche Diagnostics, German
• High Capacity RNA-to-cDNA kit	Applied Biosystems, USA
• Human IGF-II/IGF2 Quantikine Immunoassay Kit	R&D Systems Inc. USA
• TaqMan fast advanced Master Mix	Applied Biosystems, USA
• TaqMan primer assay (200), LHCGR	Applied Biosystems, USA
• TaqMan primer assay (200), AREG	Applied Biosystems, USA
• TaqMan primer assay (200), IGF2B	Applied Biosystems, USA
• TaqMan primer assay (200), ACTB	Applied Biosystems, USA
• TaqMan primer assay (200), GAPDH	Applied Biosystems, USA

Disposables	**Company**
• Biosphere Filter Tips (10-20-200-100 ml)	Sarstedt, Germany
• Centrifuge tube (15ml)	Vitrolife, Sweden
• Centre Well Dish	Vitrolife, Sweden
• Collection Dish 90mm	Vitrolife, Sweden
• Cryo Tube	Thermo Scientific Nunc. Denmark
• Culture Dish 40mm	Vitrolife, Sweden
• Culture Dish 60mm	Vitrolife, Sweden
• Culture Dish 5 Well	Vitrolife, Sweden
• Embryo Transfer Catheter Set	Cook Medical, USA
• Flexipet denuding pipette (140 µm, 170 µm, 300 µm)	Cook Medical, USA
• ICSI micropipettes	Origio, Denmark
• ICSI Dish	Thermo Scientific Nunc, Denmark
• MicroAmp Fast Optical 96-Well Reaction Plate with Barcode	Applied Biosystems, USA

• Oocyte collection tube (14 ml)	Vitrolife, Sweden
• Oosafe sperm collection cup (80ml)	SparMed, Denmark
• Optical Adhesive Covers (DNA/RNase/Inhibitors Free)	Applied Biosystems, USA
• PCR Soft tubes, 0.2 ml (DNA, DNase, RNase free)	Eppendorf, Germany
• Serological Pipette (5 ml)	Vitrolife, Sweden
• Syringe sterile 5 ml	B.Braun, Germany
• Syringe sterile U-40 1 ml insulin	BD Medical, USA
• Spinal needle	BD Medical, USA
• Steripette (60 mm)	Minitüb, Germany
• Storage Boxes	Sarstedt, Germany

Instruments

• Aspirator 3 (13907)	Labotect, Germany
• Accu-jet pro pipette controller	BrandTech Scientific, USA
• „Assistent“ Counter AC-8	Karl Hecht KG, Germany
• Blockthermostat	Labotect, Germany
• Centrifuge Rotixa RP	Hettich, Germany
• Centrifuge Universal 16	Hettich, Germany
• Centrifuge 1-14K	Sigma, Germany
• Freezer, -20°C	Liebherr, Germany
• Freezer, -80°C	Thermo Scientific, USA
• Freezer, 8°C	Liebherr, Germany
• Incubator (C16)	Labotect, Germany
• Incubator (C200)	Labotect, Germany
• Hot Plate 062	Labotect, Germany
• Makler Counting Chamber	Sefi-Medica, Haifa, Israel

• Micromanipulation system	Narishige, Japan
• Microscope BH2	Olympus, Japam
• Microscope SMZ18 Normal	Nikon, Japan
• Microscope Wild M8	Wild Heerbrugg, Switzerland
• Microscope heating system	Minitüb, Germany
• Nanodrop spectrophotometer ND-2000c	Thermo Scientific, USA
• Nikon ECLIPSE Ti, Nikon	Nikon, Japan
• StepOnePlus™ Real-Time PCR System	Applied Biosystems, USA
• Thermal Cycler C100	Bio-Rad, Germany
• QIAgility™	Qiagen, Germany
• Vortex-Genie 2	Scientific Industries, USA

2.2. Methodology

2.2.1. Stimulation Protocol

On the second day of the menstrual cycle, baseline measurements of oestradiol, LH, and progesterone were obtained, additionally, on the same day, regular ultrasound scans were performed as well. For ovarian stimulation, in the following month on the second day of the menstrual cycle, the dosage of 150 to 225 IU of recombinant Follicle-stimulating hormone (rFSH) (Gonal-F®; Merck Serono, Switzerland) was administered based on age, BMI, Anti-Mullerian hormone (AMH) level, and basal antral follicle count. From the fifth day of rFSH treatment, 0.25 mg of a gonadotropin-releasing hormone (GnRH) antagonist (Cetrotide®; Merck Serono, Switzerland) was conducted daily. When at least three follicles were developed to a minimum mean diameter of 17 mm, follicular maturation was completed by

administering 250μg of recombinant human Chorionic Gonadotropin (hCG) (Ovitrelle®; Merck Serono, Switzerland).

For patients older than 35 years or with AMH levels lower than 1.0 ng/ml different type of stimulation protocol was used. Ovarian stimulation was initiated on the second day of the menstrual cycle by administering 150–300 IU per day of a combined recombinant follicle-stimulating hormone (rFSH) and recombinant luteinizing hormone (rLH) (Pergoveris, Merck Serono, Aubonne, Switzerland). From the fifth day of stimulation therapy, a daily 0.25 mg of GnRH antagonist (Cetrotide®; Merck Serono, Switzerland) was administered. At the point where at least three follicles had acquired a minimum mean diameter of 17 mm, follicular maturation was accomplished by administering 250μg of hCG (Ovitrelle®; Merck Serono, Switzerland). Following the day of oocyte retrieval, support for the luteal phase was begun with vaginal progesterone suppositories 200mg (Utrogest™ 200; Besins Healthcare, Germany) three times per day until the β-hCG assay two weeks later.

Oocyte retrieval was performed transvaginal, 34 to 36 hours after hCG dosing. The follicles were identified using transvaginal ultrasonography with a high-frequency transducer (5 to 7 MHz) and needle guide. The follicles were then retrieved under negative pressure (100 to 120 mm Hg), where due to the specific study design flushing for the follicles included in the study was not performed. The follicular fluid contained in each follicle was collected independently. For each patient, from one up to six follicles were aspirated individually in a 14-ml polystyrene Oocyte Collection tube (Vitrolife, Goteborg, Sweden). For patients with more than six follicles due to the possible risk of bleeding and the prolonged amount of time required for the procedure, individual aspiration was done maximally for up to the six follicles. Additionally, for those patients, follicles were chosen based on the size (≥18 mm), and always were aspirated two or three follicles from each ovary. After the aspiration, every single tube

with follicular fluid was decanted into a 90-mm collection dish (Vitrolife, Goteborg, Sweden), and the cumulus-oocyte complex (COC) was harvested.

2.2.2. Follicular Fluid Aspiration and Mural Granulosa Cells Isolation

In the time following COC harvesting, follicular fluid was replaced with a sterile spinal needle (Becton Dickinson, Madrid, Spain) and syringe (Braun, Melsungen, Germany) from the dish to the sterile 15ml tube (Vitrolife, Sweden) and centrifuged at 2000 revolution per minute (rpm) for 5 minutes to isolate mural granulosa cells. The supernatant containing purified follicular fluid was stored at − 80 °C in CryoTube™ vials (Nunc, Denmark) in aliquots until assayed. In addition, 2 ml of phosphate buffer saline (PBS) was added to the pellet, and the diluted solution was slowly layered upon a 40:80% of Pure Sperm (Sigma Aldrich, Germany) density gradient and centrifuged at 2500 rpm for 30 min.

After centrifugation, three layers were detected: the top layer containing the PBS, the bottom layer containing erythrocytes, and a ring-like layer in the middle containing MGC. This middle layer was collected, resuspended in 2 ml of PBS, and washed two times by centrifugation for 10 minutes at 3000 rpm. The supernatant was discarded and the pellet was resuspended with 200µL of RNA later Stabilization Reagent (Qiagen, Germany) and cryo-stored at − 80 °C in CryoTube™ vials (Nunc, Denmark) until RNA isolation.

2.2.3. Semen Collection and Analysis

Ejaculatory abstinence was performed for a period of three to five days by all male patients. A semen sample was produced on-site by masturbation and collected into a sterile cup for analysis. Following collection, the sample was liquefied for 20-30 min before analysis at 37 ° C, and 6% CO_2 in ambient air and maintained throughout the evaluation at the same temperature. In accordance with the fifth edition of WHO guidelines for the analysis of human

sperm, volume, color, pH, viscosity, viability by eosin, and mixed antiglobulin reaction (MAR) tests were processed within an hour of collecting.

With the assistance of a Makler chamber, routine sperm analyses including concentration and motility were conducted as well as in accordance with WHO criteria.

The standard swim-up method was utilized to collect motile and viable sperm. The sperm sample was centrifuged at 2300 rpm for 10 minutes. After discarding the supernatant, 1.0 ml of pre-warmed G-IVF Plus (Vitrolife, Goteborg, Sweden) culture medium was gently layered over the pellet.

The tube was inclined at an angle of 45° and incubated at 37°C, 6% CO_2 and in ambient air for 90 minutes. A sterile pipette was used to remove the supernatant containing actively motile sperms. The supernatant was then aspirated and transferred into an empty tube. Before dispatching a 10µL of prepared sample was examined for motility, concentration, and vitality. Prepared specimens were kept in the incubator (at 37°C, 6% CO_2) until the ICSI procedure.

2.2.4. ICSI and Embryo Culture

After the harvesting of COC from follicular fluid, COCs were washed with G-MOPS medium (Vitrolife, Goteborg, Sweden) and cultured individually at 37°C, 6% CO_2, and ambient air in 50 µl culture medium droplets G-IVF Plus (Vitrolife, Goteborg, Sweden) under liquid paraffin oil (Origio, Måløv, Denmark). Oocyte denudation was performed 2.5 hours after retrieval using ICSI Cumulase® (recombinant human hyaluronidase (conc.80IU/ml); Origio, Måløv, Denmark). Each COC was denuded separately and the maturation status of the contained oocyte was determined as MII, MI, GV, or empty zona. After the denudation oocytes were incubated additionally for maximally 1 hour in G-IVF Plus medium (Vitrolife, Goteborg, Sweden) under paraffin oil (Origio, Måløv, Denmark) at 37°C, 6% CO_2, and in ambient air. Immobilization of motile sperms with normal morphology was performed in an 8-

µL drop of 5% polyvinylpyrrolidone (PVP) Clinical Grade (Origio, Måløv, Denmark) by scoring the tail. ICSI was then performed in a G-MOPS medium (Vitrolife, Goteborg, Sweden) at ×200 magnification using an inverted microscope (Nikon ECLIPSE Ti, Nikon, Tokyo, Japan) with micromanipulators (Narishige, Tokyo, Japan).

After fixation of the oocyte with the polar body at the 6 or 12 o'clock position onto the holding pipette (Origio, Virginia, USA), the injection micropipette MIC-50-35 (inner diameter,5.0–5.7 µm; (Origio, Virginia, USA)) was inserted through the zona pellucida and the oolemma into the cytoplasm at the 3 o'clock position. Once the cytoplasm was aspirated to confirm oolemma penetration, the spermatozoon was gently ejected. After that, the injection pipette was carefully withdrawn, and the oocyte was released from the holding pipette. Every single injected oocyte was replaced into a single 50µl culture droplet containing culture medium G-1 Plus (Vitrolife, Goteborg, Sweden) and incubated at 37°C, 6% CO_2 covered with paraffin oil. The oocytes were examined for fertilization within 16 – 20 hours after the injection on the following day, and only normally fertilized oocytes (those with two pronuclei) were further cultivated in a 50 µl droplet of G1 Plus (Vitrolife, Goteborg, Sweden) culture media until the embryo transfer.

2.2.5. Embryo Evaluation and Transfer

On day 2 (44–48h after injection) and day 3 (68–72h after injection), the embryos were evaluated based on the following characteristics:

1. Number of blastomeres.
2. The degree of fragmentation: 0= no fragmentation; 1=<10% fragmentation; 2= 11–25% fragmentation; 3= 26–50% fragmentation and 4= >50% fragmentation.
3. The size of the blastomeres: 0= equally sized blastomeres; 1= slightly unequal blastomeres (25–50% size difference); 2= unequal blastomeres (>50% size difference) (Paternot *et al*., 2011).

A Good Quality Embryo (GQE) was defined on day 2 as a 4-cell stage embryo with less than 25% of fragmentation and with equally or slightly unequally sized blastomeres. On day 3 as a 7–9 cell stage embryo with less than 25% of fragmentation and with equally or slightly unequally sized blastomeres (Figure 10). Embryo transfer was performed on day 2, or day 3 depending on the patient's age and embryo quality.

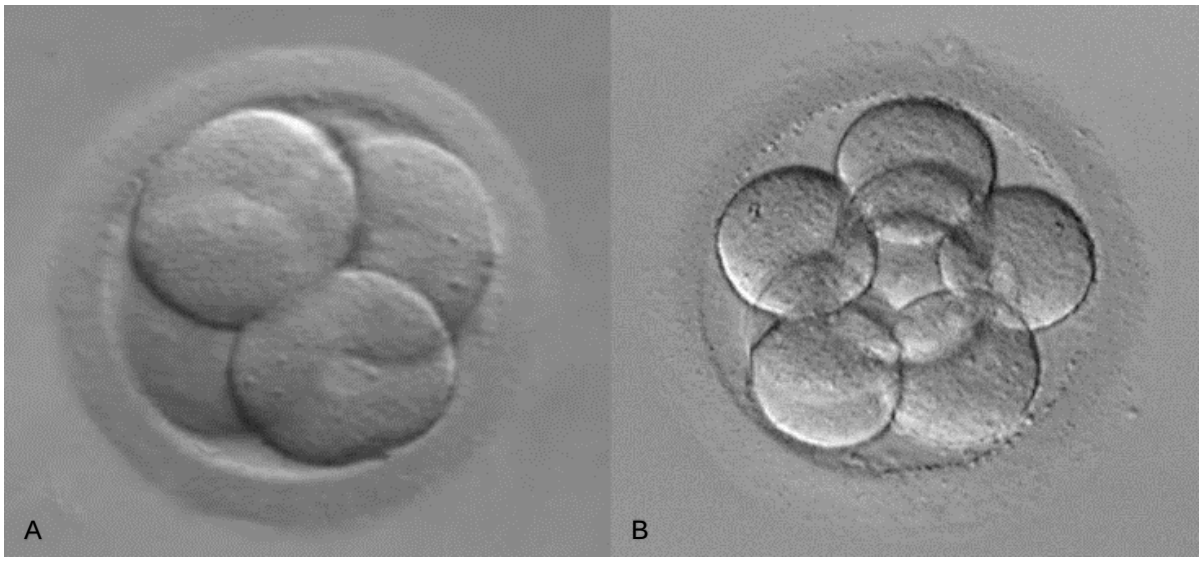

Figure 10: Good quality embryo; (A) 4-cell stage embryo with equal blastomere and no fragmentation on day 2; (B) 8-cell stage embryo with equal blastomere and no fragmentation on day 3. Adopted from Magli *et al*. (2012)

2.2.6. Enzyme-Linked Immunosorbent Assay (ELISA)

2.2.6.1. Amphiregulin (AREG)

Quantitative determination of AREG release in the follicular fluid was performed by ELISA according to the Human Amphiregulin Quantikine Kit (R&D Systems Inc., Minneapolis, MN). Samples were replaced from − 80 °C to -20°C, 24h before the ELISA procedure. On the day of the analysis, samples were liquefied on ice for 20 minutes. All the samples were analysed in duplicate, and all reagents were prepared according to the manufacturer's instructions. The follicular fluid samples were diluted 1:625 with Calibrator Diluent RD5-18. Fifty microliters of assay dilution buffer RD1-14 were added to each microplate well. In addition, fifty microliters of standard or sample were then added and incubated for 2 hours at room temperature on a horizontal orbital microplate shaker set at 500 rpm. After four washes with 400µL of wash buffer, wells were incubated for 2 hours, at 4 °C, with 200µL of Human Amphiregulin Conjugate. At the end of incubation process, wells were again washed four times with 400µL of wash buffer. Two hundred microliters of substrate solution (containing 100µL of stabilized hydrogen peroxide and 100µL of stabilized chromogen tetramethylbenzidine mixture) were added to each well and incubated for 30 minutes at room temperature (protected from light). The reaction was terminated by adding a 50µL stop solution (2 N sulfuric acid). The optical density was measured at 450nm and referenced to 570nm on a 96-well microplate reader (Sunrise-Tecan, Life Science). Amphiregulin levels were obtained with a four-parameter logistic curve fitted against a standard curve and multiplied by the dilution factor using Magellan 7.2 Ink Data Analysis Software (Life Science-Tecan). Amphiregulin concentration in picograms per milliliter (pg/ml) were normalized and depicted as nanograms per milliliter (ng/ml).

2.2.6.2. Insulin-like Growth Factor-2 (IGF-II)

Quantitative determination of IGF-II release in the follicular fluid was performed by ELISA according to the Quantikine Human IGF-II/IGF2 Immunoassay (R&D Systems Inc., Minneapolis, MN). 24h before the ELISA procedure, samples were replaced from − 80 °C to -20°C. On the day of the analysis, samples were liquefied on the ice for 20 minutes. All reagents were prepared according to the manufacturer's instructions and all analyses and calibrations were performed in duplicate. Quantikine Human IGF-II/IGF2 Immunoassay was developed for use with human serum or urine samples since the concentration of IGF-II in follicular fluid is bigger than in urine or serum, samples were diluted 1:100 with Calibrator Diluent RD5-42. Fifty microliters of assay dilution buffer RD5-42 was added to each microplate well. In addition, fifty microliters of standard or sample was added and incubated for 1 hour at room temperature on a horizontal orbital microplate shaker at 500 rpm. After four washes with 400µL of wash buffer, wells were incubated for 2 hours at room temperature, with 200µL of Human IGF-II Conjugate. Following, wells were washed four times with 400µL wash buffer at the end of the two-hour incubation process. Two hundred microliters of substrate solution (containing 100µL of stabilized hydrogen peroxide and 100µL of stabilized chromogen tetramethylbenzidine mixture) were added to each well and incubated for 30 minutes at room temperature (protected from light). The reaction was terminated by adding a 50µL stop solution (2 N sulfuric acid). On a 96-well microplate reader (Sunrise-Tecan, Life Science), optical density was measured at 450nm and referenced to 570 nm. Using Magellan 7.2 Ink Data Analysis Software (Life Science- Tecan), IGF-II levels were calculated with a four-parameter logistic curve fitted to a standard curve and multiplied by the dilution factor. IGF-II concentration in pg/mL was normalized and depicted as ng/ml.

2.2.7. The Hormonal Assessment

Blood samples were taken as a routine procedure, three days before follicle aspiration between 7 and 10 am, in standard SST gel tubes (Becton Dickinson, Plymouth, UK) and were centrifugated at 4000 *g* for 10 min in order to separate the serum. Within one hour of collection, samples were transported to the laboratory and evaluated on the same day. In each individual serum sample Progesterone, LH, and Oestradiol levels were determined by direct immunoassay with the use of an automated analyser Cobas 8000 (Module e801; Roche Diagnostics). On the day of the follicle aspiration, blood was taken again a few minutes before anaesthesia, and serum was analysed for LH, FSH, and Oestradiol concentration with the use of an automated analyser Cobas 8000 (Module e801; Roche Diagnostics, Mannheim, German). All measurements were performed according to the manufacturer's instructions using a commercially available Elecsys® kit, intended for in vitro quantitative determination in human serum. Measuring ranges for assays (defined by the lower limit of detection and the maximum of the master curve) were as follows:

Elecsys® Progesterone Gen III, 0.159–191 nmol/L (0.050–60.0 ng/mL)

Elecsys® Estradiol Gen III, 18.4–11,010 pmol/L (5.0–3000 pg/mL);

Elecsys® FSH, 0.100–200 mIU/mL

Elecsys® LH, 0.3–200 mIU/mL

For LH and FSH the analyser automatically calculated the analyte concentration of each sample in mIU/mL.

Additionally, the same automated analyser Cobas 8000 (Module e801; Roche Diagnostics), and the Elecsys® kits were used to measure the concentration of LH and FSH from the follicular fluid. All of the hormonal measurement procedures, including setting up the preparation, diluting the sample, conducting the assay regulation, and quality assurance, were carried out in accordance with the instructions provided by the manufacturer. Follicular

fluid samples were replaced from − 80 °C to -20°C, 24h before the procedure. On the day of the analysis, samples were liquefied on ice for 20 minutes.

2.2.8. RNA Isolation

The MGC of each oocyte was separately numbered, frozen, and cryo-stored at − 80 °C in CryoTube™ vials (Nunc, Denmark) until RNA isolation as described (2.2.2). Total RNA extraction of individual MGCs was carried out using the High Pure RNA Isolation Kit (Roche Applied Science, Mannheim, Germany). After being thawed on ice, samples were centrifuged at 10000*g* for 10 minutes, the supernatants were discarded, and the pellets were resuspended in 200µl PBS and 400µl of lysis buffer. 700 µl of lysates sample were transferred to spin filter columns containing glass filter fleece that binds nucleic acid and were centrifugated at 8000 *g* for 15 seconds. DNase I was added to the column to digest DNA directly on the filter fleece and the column was incubated at room temperature for 15 min. After the incubation process, the column was washed with wash buffer (supplied by the manufacturer) and centrifuged at 8000*g* for 15 seconds three times to remove digested DNA and contaminants. As recommended by the manufacturer final elution of the total RNA from the column into a sterile tube was performed using 50µl of Elution buffer. Therefore, an elution buffer was used for the blank calibration. The spectrophotometric ratio with absorbance measurements at wavelengths of 260 and 280 nm on a NanoDrop-2000 (Thermo Fisher, Darmstadt, Germany) was used to confirm the RNA concentration and purity. RNA samples were stored at − 80 °C until the reverse-transcribed complementary DNA (cDNA) process.

2.2.9. Reverse Transcription and Quantitative Real-Time Polymerase Chain Reaction

From each sample, cDNA was synthesized from 57.60 ng of total RNA using the High Capacity RNA-to-cDNA kit (4387406; Applied Biosystems, Foster City, CA, USA). Reverse

transcription reactions were performed in Bio-Rad S1000 (Bio-Rad, USA). The reaction mix was incubated at 37°C for 60 minutes, and then the reaction was stopped by heating it to 95°C for 5 minutes and hold at 4°C. Samples were stored at -80 °C until a real-time quantitative polymerase chain reaction (RT-qPCR).

Expression of *LHCGR*, *AREG, IGF-II*, Actine Beta (*ACTB*), and Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was analysed by StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, United States) using TaqMan Fast Advanced Master Mix (Applied Biosystems). The TaqMan gene expression assays for:

LHCGR (Hs00174885_m1);

AREG (Hs00950669_m1);

IGF2 (Hs00538954_g1);

GAPDH (Hs02786624_g1);

ACTB (Hs99999903_m1) was purchased from Applied Biosystems.

Briefly, 2.0µl cDNA was added in 18.0µl reaction mixtures comprised of 10.0µl TaqMan fast advanced Master Mix, 1.0µl TaqMan assay primer, and 7.0µl Nuclease free water, which made up to the final volume of 20µl. All listed components were pipetted by the Liquid Handling Robot QIAgility™ (Qiagen) into the 96-well plate (MicroAmp ®, Applied Biosystems) and subsequently loaded into the StepOnePlus™ Real-Time PCR System using the the following program:

50°C for 2 minutes, 95°C for 20 seconds, followed by 40 cycles on 95 °C for 3 seconds and 60 °C for 30 seconds. After completion of the PCR run (approximately 40 minutes), the expression levels of the genes were calculated. Each sample was analysed in triplicate, with no template control (NTC) included in each run. Results were evaluated using the StepOnePlus software version 2.3 (Life Technologies GmbH, Darmstadt, Germany). The samples were normalized concerning the reference gene Actine Beta (*ACTB*) using the

relative quantification $2^{-\Delta\Delta Ct}$ method where the mean value of mRNA transcripts from each probe was set as one for each gene.

2.3. Statistical Analysis

All variables obtained in the current study were analysed using IBM SPSS version 27 (IBM Corp., Armonk, NY, USA). First data were tested for normality using the Kolmogorov-Smirnov and Shapiro-Wilk tests. Normally distributed data were presented as the mean ± SD (standard deviation from the mean), while non-parametric data were presented as the median (interquartile range). Mann-Whitney U test was used to compare the medians of the different group variables. Additionally, data were analysed based on patients' age, BMI, number of mature oocytes, fertilization rate, and the formed percentage of good-quality embryos. Univariate logistic regression analyses were used to detect a correlation between the concentration of proteins, and gene expression levels, according to the number of mature oocytes, fertilization rate, and the percentage of good-quality formed embryos. All parameters which showed significant correlation were included in a multiple regression analysis. Furthermore, receiver operating characteristic (ROC) analyses were used to investigate the diagnostic performance of the parameters considering the area under the curve AUC. Besides the logistic regression analysis, the Spearman correlation test was performed to determine the correlation between proteins from the follicular fluid and different studied parameters. Based on a reference Actine Beta gene, relative quantification was utilized for the analysis of the real-time qPCR data. Using the Livak or ΔΔCt technique, the relative quantity of each gene's mRNA was assessed (Livak & Schmittgen, 2001). The fold change was calculated using the equation $2^{-\Delta\Delta C}$. In the present study, a *p*-value of 0.05 was determined as statistically significant, while $p<0.001$ was highly significant, and $p<0.10$ was considered a trend.

3. RESULTS

3.1. Descriptive Statistics for Evaluated Parameters

From the thirty-three patients, a total of 108 oocytes were included in the study, and for all 108 oocytes FF and MGCs samples were available. Out of this number 84 (77.77%) were mature MII oocytes, 9 (8.33 %) were immature MI, 5 (4.62%) cells were in the GV stage and 10 (9.25 %) cells were degenerated. Therefore, the ICSI procedure was performed with 93 oocytes. The fertilization rate was 67,74 % (N=63), out of this number 61 oocyte, were correctly fertilized, while 2 oocytes had 3PN. Additionally, out of the aforementioned 9 MI oocytes, 2 were correctly fertilized and cryopreserved.

Due to the specific Embryo Protection Act (EPA), out of the 61 fertilized oocytes, 13 zygotes were frozen, while 48 were cultivated. Thirty-four embryos (70.8%) reached good quality and fourteen (29.2%) had poor quality. In the present study, two embryos were arrested on Day 2 so they were as well characterized as poor-quality embryos.

Embryo transfer was performed on Day 2 for six (18.2%) patients, and on Day 3 for twenty-one (63.6%) patients, while for six (18.2%) patients due to the unfertilized oocyte or arrested embryos, transfer was not performed.

For the study purpose patients were divided into groups based on age, BMI, and infertility. In addition, oocytes were divided into groups based on maturity and fertilization while embryos were divided based on quality.

The descriptive statistic of analyzed parameters is listed in Table 1.

Table 1: Descriptive statistics of studied parameters for all patients (N=33).

Parameter	Median	Minimum	Maximum	Mean	Std. Deviation
Age	33.54	23.00	43.00	33.17	5.16
BMI (kg/m^2)	26.10	18.70	40.40	28.60	7.09
Blood					
AMH (ng/ml)	3.39	0.48	17.90	3.70	3.11
LH * (mIU/mL)	2.06	0.16	7.49	2.36	1.40
Progesterone* (ng/ml)	0.58	0.12	10.28	0.94	1.65
Oestradiol* (pg/ml)	1690	624.80	8654.00	2333.42	1767.37
LH** (mIU/mL)	0.84	0.10	7.45	1.47	1.70
FSH** (mIU/mL)	7.60	2.70	13.20	8.20	2.86
Oestradiol** (pg/ml)	1158	263.00	3777.00	1230.01	782.14
Progesterone**(ng/ml)	10.20	2.57	23.10	10.93	6.50
Follicular fluid					
AREG (ng/ml)	110.89	25.08	236.83	117.98	47.80
IGF-II (ng/ml)	371.44	215.55	467.92	364.51	54.82
LH (mIU/mL)	0.88	0.10	9.97	1.62	1.76
FSH (mIU/mL)	5.90	2.00	14.90	6.41	2.72

*3 days before oocyte aspiration

** day of the oocyte aspiration

To determine the relative quantification of each target gene's expression (*AREG, IGF-II, LHCGR*), the normalized expression analysis method, which is reflected by the Delta Ct value (ΔCt) was used (Table 2). In this analysis, *ACTB* served as the reference gene.

Table 2: Descriptive statistic of the studied gene expression level (ΔCt) for all patients (N=33).

Parameter	Median	Minimum	Maximum	Mean	Std. Deviation
ΔCt *AREG*	3.18	0.33	6.10	3.21	0.81
ΔCt *IGF-II*	6.58	2.74	14.00	6.66	1.28
ΔCt *LHCGR*	10.11	5.15	14.41	9.96	1.24

3.2. Impact of Patients' age and Stimulation Protocol on Protein Concentration, and ICSI outcomes

Since it is very well known that females' age is one of the main important factors of reproductive outcome and fertility, and in our study patients older than 35 years had different stimulation protocols, for the study purpose patients were divided into two groups:

Group I - patients younger than 35 years (N= 20) / Stimulation with rFSH

Group II - patients older than 35 (N=13) / Stimulation with rFSH+rLH.

Obtained results showed that younger patients after the stimulation produce more MII oocytes compared to the older patients [58.3% (49/84) vs 41.7% (35/84)]. What is more, the fertilization rate was higher in group I, where 38 out of the 61 fertilized oocytes were obtained from younger patients [62.3% vs 37.7% respectively, $p<0.05$] (Figure 11). Additionally, although fertilized oocytes obtained from the younger patients more frequently formed good-quality embryos compared to the older patients [58.8% (N=20) vs 41.2 (N=14)], the difference was not statistically significant.

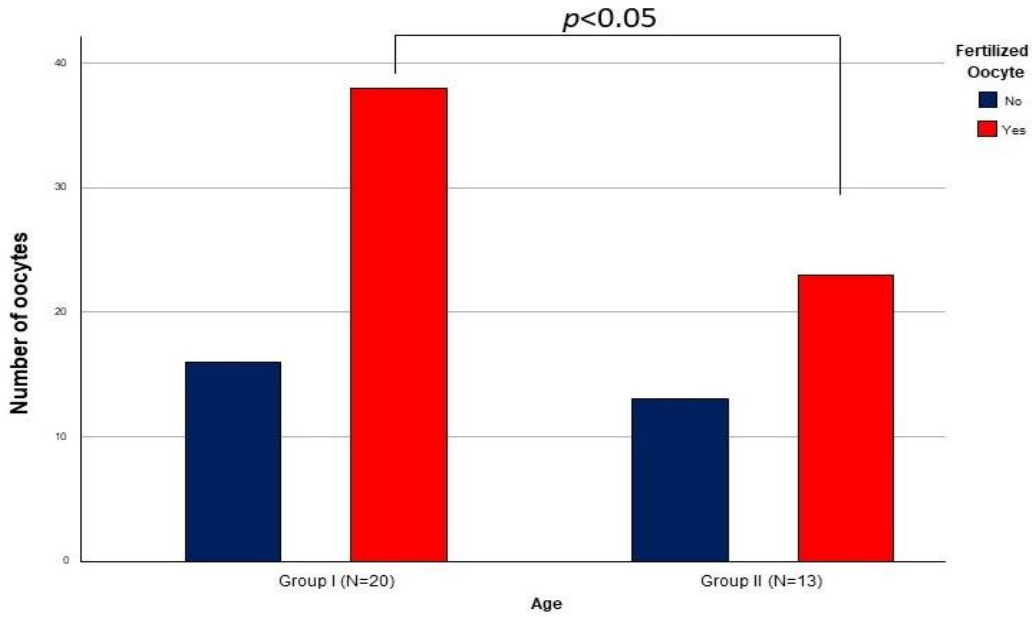

Figure 11: Difference between fertilization rates based on the patients' age.

Descriptive statistic for studied parameters confirms that only the concentration of the FSH in the blood was significantly different between groups while the concentration in the follicular fluid showed a trend (Table 3).

Table 3: Comparison of the studied parameters between the groups based on the patients' age.

Parameter	Group I (N=20)			Group II (N=13)			*p-value*
	Median	Minimum	Maximum	Median	Minimum	Maximum	
Blood							
AMH (ng/ml)	3.39	1.56	5.79	2.30	0.56	8.0	0.39
LH * (mIU/mL)	2.40	0.16	7.49	3.44	1.45	5.71	0.13
Progesterone* (ng/ml)	0.50	0.17	0.91	0.61	0.12	1.12	0.75
Oestradiol* (pg/ml)	1598	834.6	4186.0	2228.0	624.8	5298.0	0.19
LH** (mIU/mL)	1.0	0.10	5.67	0.71	0.10	4.45	0.57
FSH** (mIU/mL)	6.65	2.7	10.5	10.10	7.30	12.90	**0.0001**
Oestradiol** (pg/ml)	1133	616.0	2724.0	1226.0	426.20	1569	0.79
Progesterone**(ng/ml)	10.4	2.57	20.10	10.20	3.14	22.60	0.37
Follicular fluid							
AREG (ng/ml)	123.99	27.69	236.83	100.2	63.9	166.60	0.58
IGF-II (ng/ml)	367.92	215.55	467.92	385.40	314.69	450.97	0.25
LH (mIU/mL)	1.21	0.15	9.97	0.51	0.10	5.34	0.73
FSH (mIU/mL)	5.0	2.0	12.6	8.80	4.5	14.9	**0.05**

*3 days before oocyte aspiration

** day of the oocyte aspiration

3.2.1. Correlation between Patients' Age and Gene Expression

The relative amounts of the investigated genes mRNA (mean ΔCt) (*AREG, IGF-II, LHCGR*) were differentially expressed between the compared groups. This difference in the expression of *LHCGR* between the patients younger and older than 35 was highly significant (p<0.0001) (Table 4).

Additionally, linear regression analysis confirmed the impact of patients' age on the relative amount of mRNA of *LHCGR* (r= 0.251; p= 0.009).

Table 4: Comparison of the studied gene expression level (ΔCt) between groups based on the patients' age.

Parameter	Group I (N=20)			Group II (N=13)			*p-value*
	Median	Minimum	Maximum	Median	Minimum	Maximum	
ΔCt *AREG*	3.24	0.33	6.10	3.09	1.69	5.87	0.27
ΔCt *IGF-II*	6.66	4.96	14.00	6.72	4.39	7.86	0.46
ΔCt *LHCGR*	10.22	8.59	14.41	9.57	8.59	11.07	**0.0001**

3.3. Impact of BMI on Protein Concentration and ICSI Outcomes

For the purpose of the study, based on the BMI values patients were divided into two groups:

Group I: Women with a normal BMI of 18.5–24.9 kg/m^2

Group II: Women with a BMI over 24.9 kg/m^2

Out of the thirty-three patients, 54.5% (N=18) had normal BMI, while 45.6% (N=15) had BMI over 24.9 kg/m^2. In group II out of fifteen patients, 33.3% (N=5) were overweight and 77.7% (N=10) were obese. In group I mean age was 33.8 ± 4.83 while in group II mean age was 33.6 ± 5.42, and there was no significant difference in age within BMI groups.

Based on the total number of the cells included in the study, obtained results showed that patients with normal BMI after the stimulation produce more MII oocytes compared to the patients with raised BMI [59.5% (50/84) vs 40.5% (34/84)]. What is more, descriptive statistic data showed that the fertilization rate was higher in group I, where 40 out of the 61 fertilized oocytes were obtained from patients with normal BMI [65.5% vs 35.5%; $p<0.05$] (Figure 12). Additionally, although fertilized oocytes obtained from the patients with normal BMI more frequently formed good-quality embryos compared to the patients with raised BMI [55.8% (N=19) vs 45.2 (N=15)], the difference was not statistically significant.

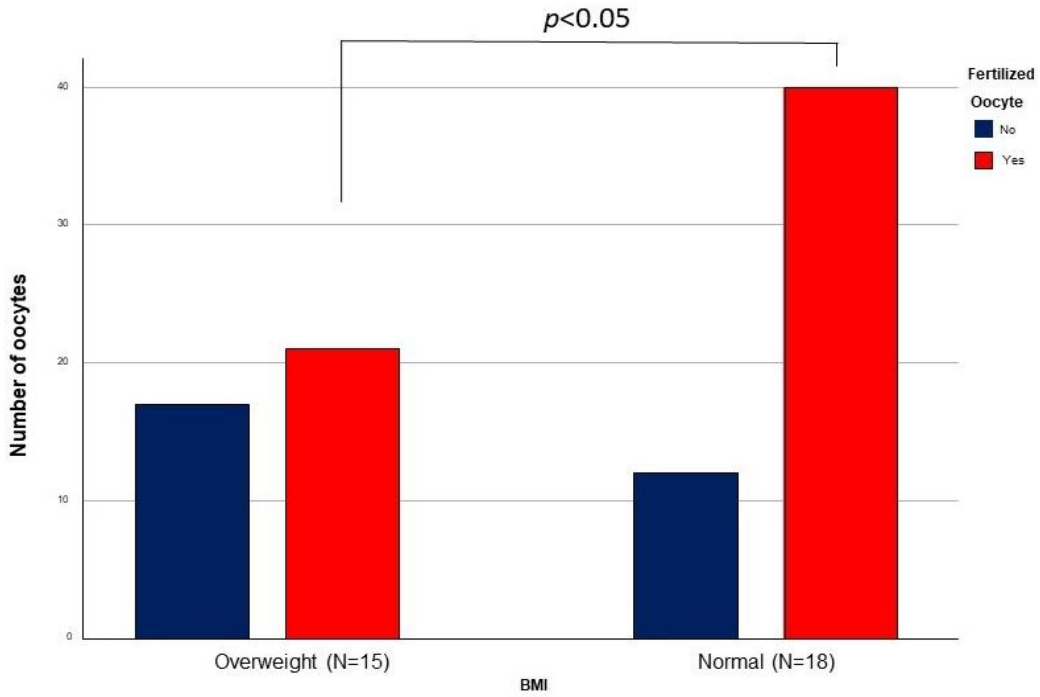

Figure 12: Difference between fertilization rate based on the BMI.

Differences in the BMI between the patients did not affect the concentration of the studied hormones in the blood. Additionally, a comparison between groups based on the protein concentration in the follicular fluid showed that only the concentration of AREG was significantly different between groups (Table 5).

Table 5: Comparison of the protein concentration in the follicular fluid between the groups based on the patients' BMI.

Parameter	Group I (N=18)			Group II (N=15)			*p-value*
	Median	Minimum	Maximum	Median	Minimum	Maximum	
AREG (ng/ml)	120.10	49.42	236.83	99.45	25.0	209.51	**0.048**
IGF-II (ng/ml)	361.76	236.39	467.92	374.08	215.55	458.20	0.44
LH (mIU/mL)	1.11	0.10	7.72	0.88	0.34	9.97	0.93
FSH (mIU/mL)	6.50	2.00	14.90	5.70	2.30	11.6	0.13

Therefore, logistic regression analysis confirmed the impact of BMI on AREG concentration (r=0.257; *p*=0.008), where patients with increased BMI had decreased concentration of amphiregulin in the follicular fluid.

3.3.1. Correlation between BMI and Gene Expression

The mean of the relative expression levels of *AREG* and *IGF-II* were significantly different between groups (Figure 13). Additional analysis indicates that the relative expression level of all three studied genes was lower in the overweight group (Table 6).

Due to the aforementioned difference in the relative expression level of *AREG* and *IGF-II* between the groups, linear regression analysis was performed, and obtained results confirmed that BMI affects the expression of *AREG* (r=0.287; *p*=0.003) and *IGF-II* (r=0.297; *p*=0.002).

Table 6: Comparison of the studied gene expression level (ΔCt) between groups based on the patients' BMI.

Parameter	Group I (N=18)			Group II (N=15)			*p-value*
	Median	Minimum	Maximum	Median	Minimum	Maximum	
ΔCt *AREG*	3.41	1.24	6.10	3.03	0.33	5.11	**0.010**
ΔCt *IGF-II*	6.83	4.39	14.00	6.43	2.74	10.22	**0.005**
ΔCt *LHCGR*	10.16	8.41	14.41	9.99	5.15	13.01	0.374

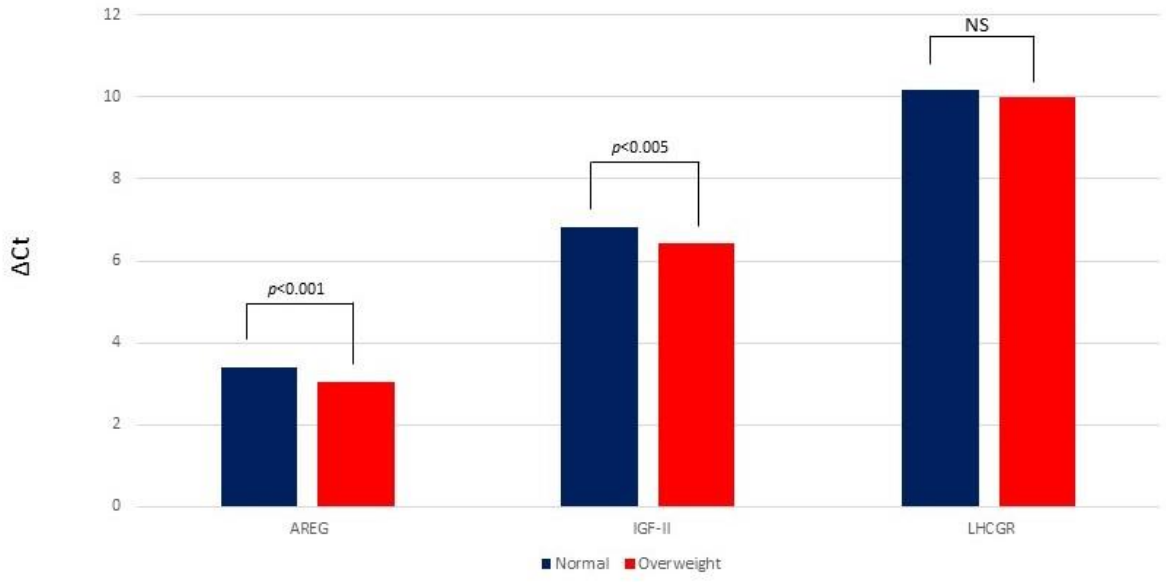

Figure 13: Differences in *AREG, IGF-II,* and *LHCGR* expression levels between groups based on BMI.

3.4. Impact of Infertility on Protein Concentration and ICSI Outcomes

Out of the thirty-three female patients included in the study, 45.5% (N=15) were diagnosed with infertility. Out of all infertile female patients, 60.0% (N=9) were diagnosed with Polycystic Ovary Syndrome, while 33.3% (N=5) patients had endometriosis and only 6.7% (N=1) had the tubal factor of infertility. On the other side, male infertility was diagnosed in 21.2% (N=7) patients, where out of those 7 patients, 42.8% (N=3) patients had Asthenozoospermia, and 57.2 % (N=4) patients had Teratozoospermia.

For the purpose of the study, female patients were divided into two groups,

Group I: patients without infertility diagnosis,

Group II: patients with a confirmed infertility diagnosis.

Based on the total number of the cells included in the study obtained results showed that the number of mature oocytes (58.3% vs 41.7%), fertilization rate (57.4% vs. 42.6%) and embryo quality (52.9% vs. 47.1%) were higher in group I, but the difference was not statistically significant. Additionally, descriptive statistics confirm a significant difference only for the progesterone and LH concentration in the blood (Table 7).

Table 7: Comparison of the protein concentration in the blood between healthy patients and those diagnosed with infertility.

Parameter	Group I (N=18)			Group II (N=15)			*p-value*
	Median	Minimum	Maximum	Median	Minimum	Maximum	
Blood							
AMH (ng/ml)	2.95	1.14	8.00	3.24	0.56	5.79	0.21
LH * (mIU/mL)	3.35	0.46	7.49	1.82	0.16	4.80	**0.008**
Progesterone* (ng/ml)	0.84	0.18	1.33	0.38	0.12	0.91	**0.02**
Oestradiol* (pg/ml)	2228	857.0	5298.0	1598.0	624.8	4186.0	0.63
LH** (mIU/mL)	0.81	0.10	4.45	0.99	0.10	5.67	0.65
FSH** (mIU/mL)	9.70	4.60	12.90	7.00	2.70	12.10	0.56
Oestradiol** (pg/ml)	1158.0	565.80	1834.0	1192.0	426.20	2724.0	0.69
Progesterone**(ng/ml)	10.20	3.26	23.10	10.85	2.57	20.10	0.17

Regarding the concentration of studied parameters from the follicular fluid, significant difference between the groups was observed for the concentration of AREG and IGF-II (Table 8). Further analysis confirms as well significant difference in AREG concentration between the patients diagnosed with PCOS and those without any infertility problems (p=0.002) (Figure 14). Additionally, although between healthy and infertile patients, difference in the LH concentration was not statistically significant, a comparison between healthy and PCOS patients indicated a significant difference (p=0.024).

Table 8: Comparison of the protein concentration in the follicular fluid between healthy (Group I) and infertile patients (Group II).

Parameter	**Group I (N=18)**			**Group II (N=15)**			***p*-value**
	Median	Minimum	Maximum	Median	Minimum	Maximum	
AREG (ng/ml)	162.29	25.08	236.83	95.99	43.19	191.0	**0.016**
IGF-II (ng/ml)	354.22	215.55	450.97	394.12	304.91	467.92	**0.037**
LH (mIU/mL)	0.82	0.10	5.34	1.20	0.15	9.97	0.46
FSH (mIU/mL)	6.80	2.3	14.9	5.90	2.0	12.6	0.86

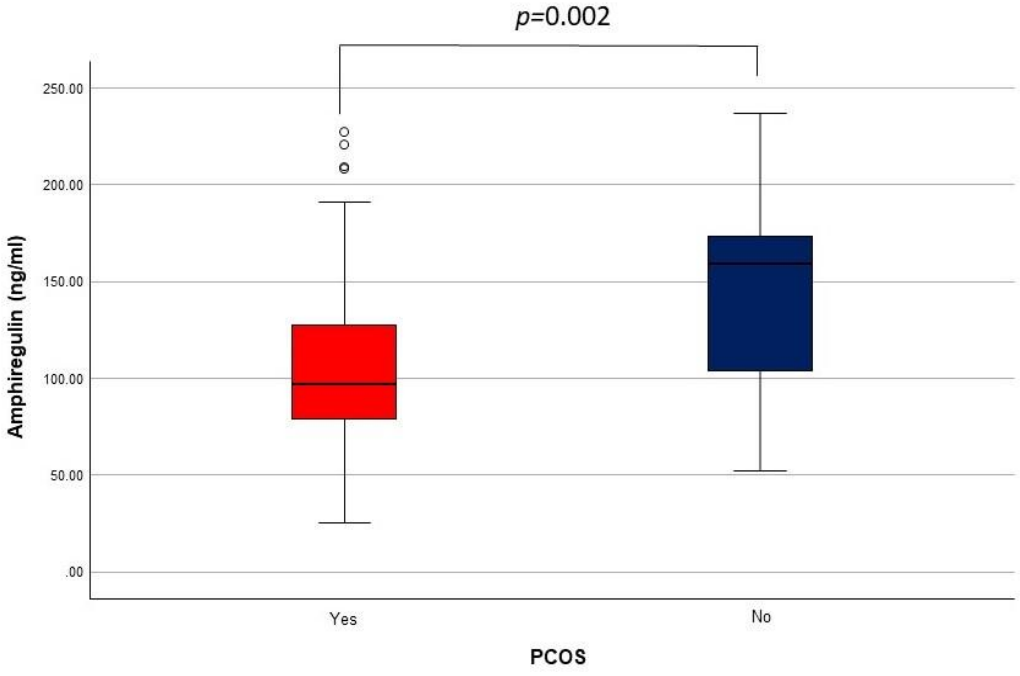

Figure 14: Difference in the amphiregulin concentration between healthy patients and patients diagnosed with PCOS.

Due to the aforementioned difference in AREG concentrations between the groups, linear regression was performed and the results confirmed that infertility diagnosis (r=0.279; *p*<0.003) and particularly PCOS (r=0.316; *p*<0.001) affects AREG concentration in the follicular fluid.

Obtained results demonstrated that PCOS can significantly affect the concentration of amphiregulin in the follicular fluid (OR: 1.01, CI:1.002-1.025 *p*=0.020). The predictive strength was assessed by quantifying the area under the curve (AUC) of the receiver operating characteristic (ROC). In this case, the area under the ROC curve was determined to be AUC = 0.695 (Figure 15).

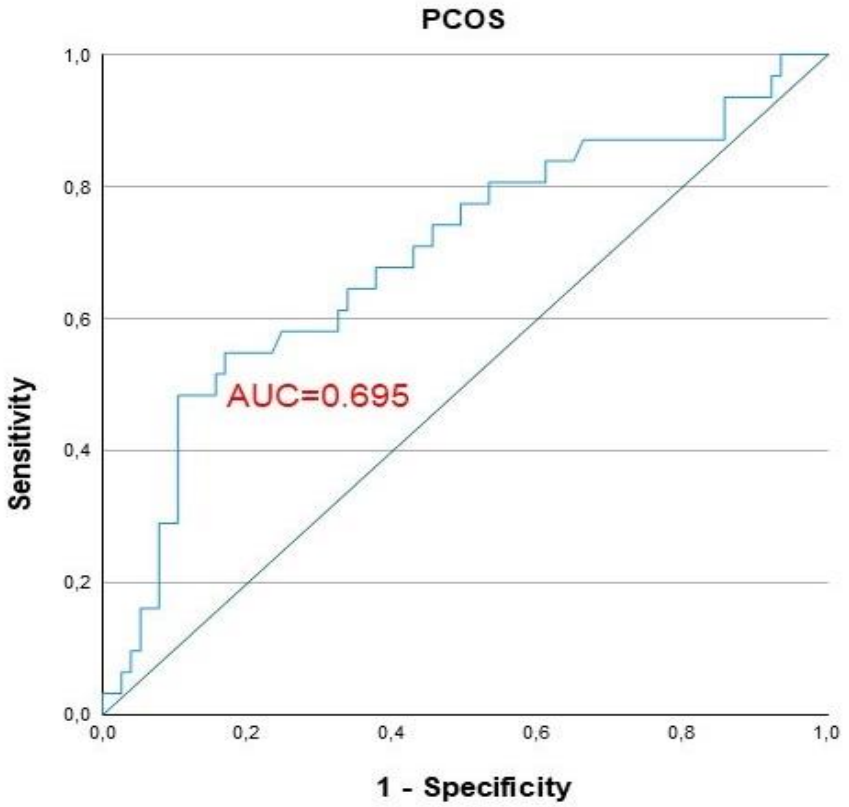

Figure 15: The ROC curve for amphiregulin concentration by the patients diagnosed with PCOS.

Since infertility, especially PCOS diagnosis as well BMI significantly affects AREG concentration multiple regression analysis was performed, and obtained results indicated that from the aforementioned parameters, only PCOS diagnosis significantly affected AREG concentration (Table 9).

Table 9: Multiple regression analysis -the impact of BMI, Infertility and PCOS on the AREG.

Model	B	Std. Error	Beta	t	Sig.
(Constant)	97.458	7.541		12.923	.000
BMI	13.674	9.110	.142	1.501	.136
Infertility	9.813	10.981	.102	.894	.374
PCOS	25.608	11.685	.243	2.191	.031

a. Dependent Variable: Amphiregulin (AREG)

3.4.1. Correlation between Infertility Diagnosis and Gene Expression

The obtained results indicated that the difference between the groups in the relative expression level of studied genes was not statistically significant.

On the other hand, the relative expression of the *AREG* compared between healthy and PCOS patients shows a trend (p=0.09).

Table 10: Comparison of the studied gene expression level (ΔCt) between groups based on the patient infertility diagnosis.

Parameter	Group I (N=18)			Group II (N=15)			*p-value*
	Median	Minimum	Maximum	Median	Minimum	Maximum	
ΔCt *AREG*	3.25	1.66	5.11	3.36	2.58	6.10	0.80
ΔCt *IGF-II*	6.72	4.96	11.73	6.63	4.39	14.00	0.50
ΔCt *LHCGR*	10.16	8.41	14.41	9.65	8.65	12.02	0.16

3.5. Impact of the Protein Concentration on Oocyte Maturity

The study included a total of 108 oocytes. Out of this number 84 (77.77%) were mature MII oocytes, 9 (8.33%) were immature MI, 5 (4.62%) cells were on GV stadium and 10 (9.25%) cells were degenerated (DEG). By comparing protein concentration in the follicular fluid between mature (MII) and immature oocytes (MI, GV, DEG) obtained results indicated that AREG, IGF-II, and FSH were elevated in the mature oocyte group, while LH concentration was decreased (Table 11). For the AREG and LH differences in the concentration between the mature and immature oocyte groups were statistically significant (Figure 16).

Table 11: Comparison of the protein concentration in the follicular fluid between mature and immature oocyte groups.

Parameter	Mature Oocyte (N=84)			Immature Oocyte (N=24)			*p*-value
	Median	Minimum	Maximum	Median	Minimum	Maximum	
AREG (ng/ml)	121.99	27.69	227.54	81.31	25.08	236.83	**0.004**
IGF-II (ng/ml)	371.93	264.63	467.92	358.02	215.55	441.01	0.197
LH (mIU/mL)	1.21	0.10	9.97	0.88	0.10	7.72	**0.046**
FSH (mIU/mL)	5.95	2.0	14.9	5.55	2.3	10.4	0.880

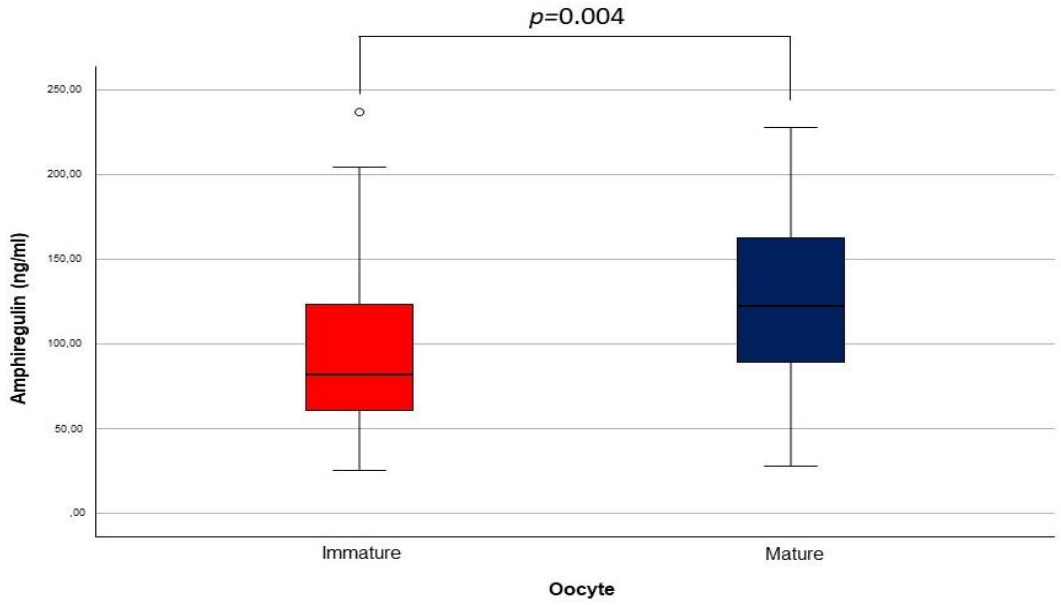

Figure 16: The amphiregulin concentration difference between follicles containing the mature or immature oocytes.

Furthermore, based on the fact that out of the 24 immature oocytes, 10 were from patients diagnosed with infertility, further statistical analysis (Fisher's Exact Test) was performed, and the obtained results indicated that the aforementioned analysis data were not biased with an infertility diagnosis (p=0.163).

In addition, univariate logistic regression analysis was performed in order to evaluate the impact of AREG and LH on oocyte maturity. Obtained results for AREG demonstrated that concentration in the follicular fluid could significantly affect oocyte maturity (OR: 1.01, CI:1.002-1.025 *p*=0.020). The predictive strength was assessed by quantifying the AUC of the ROC. In this case, the area under the ROC curve was determined to be AUC = 0.691 (Figure 17).

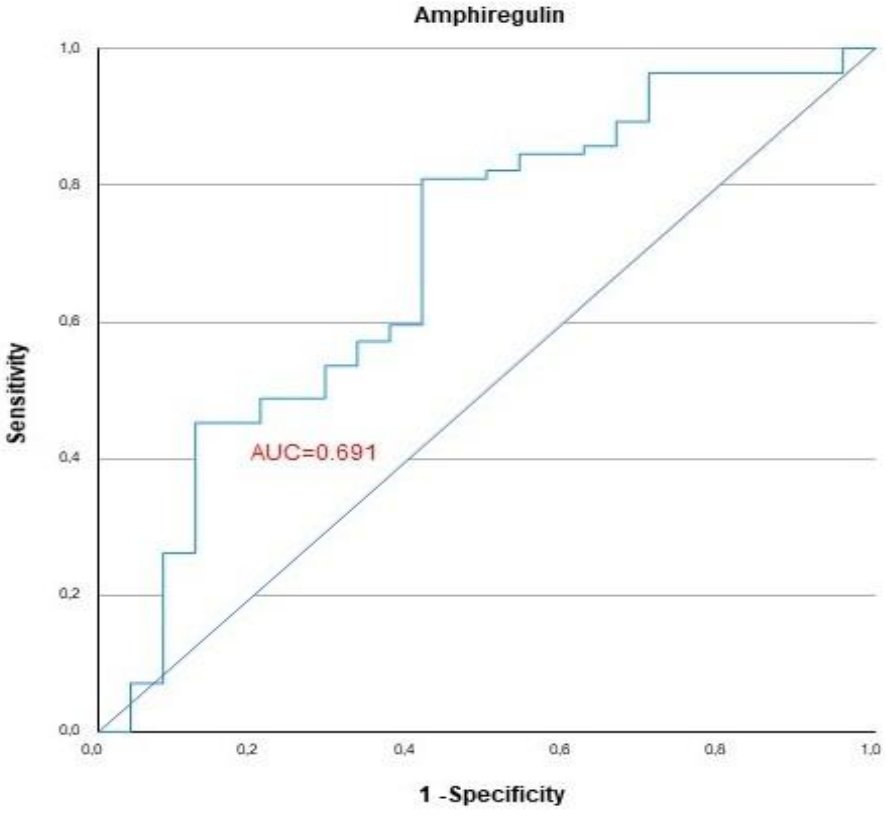

Figure 17: The ROC curve for oocyte maturity by the amphiregulin concentration.

Univariate logistic regression analysis for LH as well demonstrated that concentration in the follicular fluid could significantly affect oocyte maturity (OR:0.762, CI:0.600-0.967 *p*=0.026). Predictive strength was quantified using the area under the curve of the receiver operating characteristic, where the area under the ROC curve was AUC = 0.633 (Figure 18).

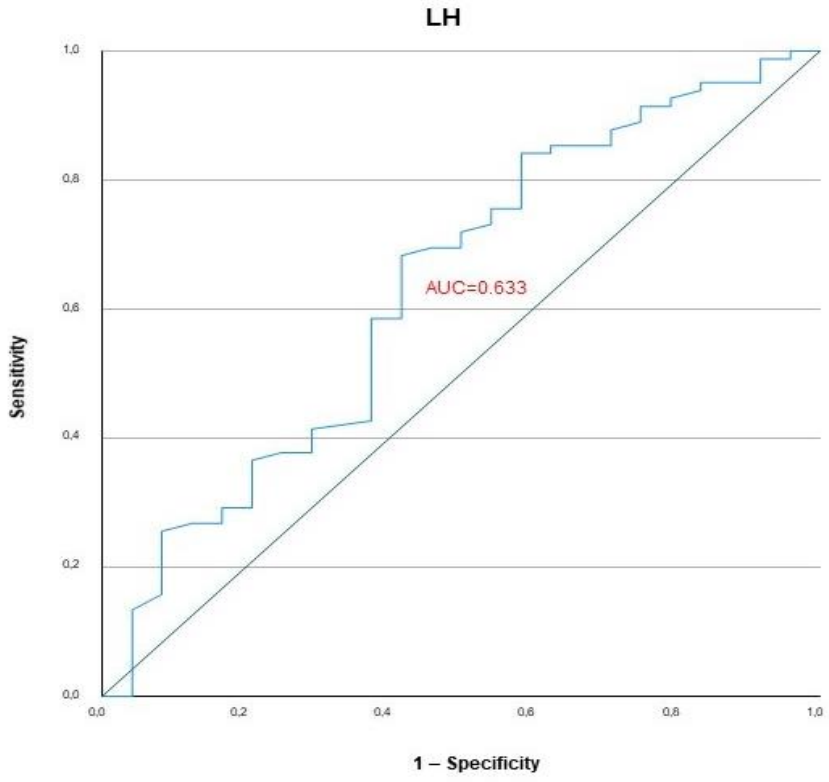

Figure 18: The ROC curve for oocyte maturity by the LH concentration.

Since the univariate logistic regression analysis confirms statistical significance, AREG and LH were included in a multiple regression analysis. The obtained data confirmed the significant impact of both studied proteins on oocyte maturity (Table 12).

Table 12: Multiple regression analysis: the impact of the amphiregulin and LH on oocyte maturity.

Model	B	Std. Error	Beta	t	Sig.
(Constant)	.612	.108		5.645	.000
AREG (ng/ml)	.002	.001	.246	2.652	.009
LH mIU/ml	.058	.022	.243	2.622	.010

3.5.1. Correlation between Gene Expression and Oocyte Maturity

The relative amounts of mRNA for the studied genes (mean ΔCt values) - *AREG, IGF-II,* and *LHCGR* - displayed differential expression between the compared groups (Table 13). This difference in the expression of *AREG* between the group of mature and immature oocytes was significant (p=0.031).

Additionally, univariate logistic regression analysis confirmed that the expression of *AREG* correlates the oocyte maturity (OR:0.49; Cl 0.26-0.91; p=0.024).

Table 13: Comparison of the studied gene expression level (ΔCt) between mature and immature oocyte groups.

Parameter	Mature Oocyte (N=84)			Immature Oocyte (N=24)			*p-value*
	Median	Minimum	Maximum	Median	Minimum	Maximum	
ΔCt *AREG*	3.07	0.33	5.87	3.37	2.24	6.10	**0.031**
ΔCt *IGF-II*	6.58	2.74	14.00	6.65	4.86	11.73	0.89
ΔCt *LHCGR*	9.94	5.15	13.01	10.21	8.01	14.41	0.39

Since the correlation between ΔCt and the level of gene expression is inverse, higher ΔCt values indicate a decrease in gene expression. Therefore, Table 14 illustrates that gene expression of all researched genes (*AREG, IGF-II*, and *LH*) was downregulated in the immature cell group.

Table 14: The mean expression level of and fold change of examined genes based on the oocyte maturity groups.

Genes	Mean ΔCt Mature Oocyte	Mean ΔCt Immature Oocyte	ΔΔCt	Fold Change	Log2Fold Change	Regulation
AREG	3.12	3.58	0.46	0.73	-0.46	Down
IGF-II	6.64	6.75	0.12	0.92	-0.12	Down
LHCGR	9.89	10.21	0.32	0.80	-0.32	Down

3.6. Impact of the Protein Concentration on Fertilization Rate

Out of the 108 oocytes included in the study, the ICSI procedure was performed with 93 oocytes (84 MII and 9 MI). The fertilization rate was 67,74 % (N=63), and 61 oocytes were correctly fertilized (2 PN). Out of the 9 MI oocytes, 2 were correctly fertilized. However, due to the possible bias in the result, MI oocytes were excluded from further statistical analysis. For all oocytes, a fertilization check was done exactly 18h after the ICSI procedure. By comparing protein concentration in the follicular fluid between fertilized and unfertilized oocytes obtained results indicated that AREG, LH, and FSH were elevated in the fertilized oocyte group, while IGF-II concentration was decreased (Table 15). Mann Whitney U test showed that AREG (Figure 19) and LH concentration were significantly different between groups.

Table 15: The difference in the protein concentration between fertilized and unfertilized oocyte groups.

Parameter	**Fertilized Oocyte (N=59)**			**Unfertilized Oocyte (N=23)**			***p*-value**
	Median	Minimum	Maximum	Median	Minimum	Maximum	
AREG (ng/ml)	126.98	71.63	227.54	89.05	43.19	170.96	**0.001**
IGF-II (ng/ml)	365.48	264.63	467.19	387.45	270.08	467.92	0.115
LH (mIU/mL)	0.93	0.15	5.77	0.64	0.16	3.45	**0.025**
FSH (mIU/mL)	5.90	2.1	12.6	5.80	2.0	14.9	0.723

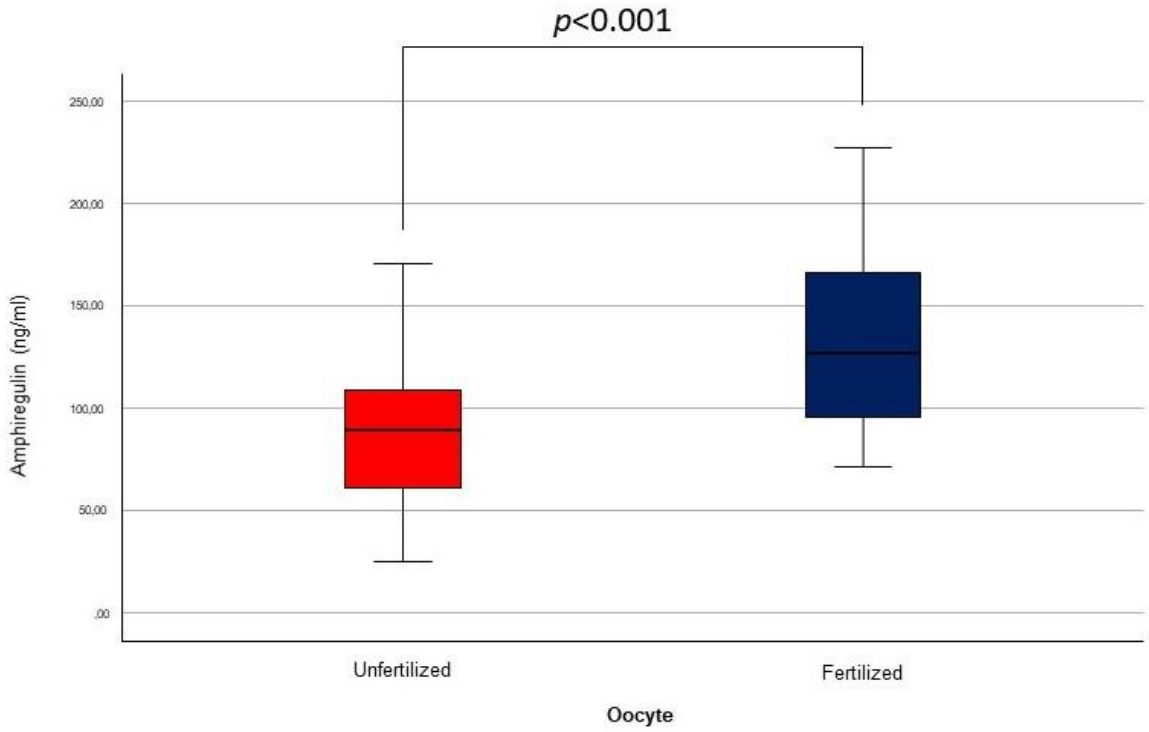

Figure 19: The difference in the amphiregulin concentration between fertilized and unfertilized oocytes.

In addition, univariate logistic regression analysis was performed in order to evaluate the impact of AREG and LH on fertilization rate. Obtained results demonstrated that amphiregulin concentration could significantly predict oocyte fertilization (OR: 1.02; CI:1.00-1.03; p<0.003). The predictive strength was assessed by quantifying the area under the curve of the receiver operating characteristic. In this case, the area under the ROC curve was determined to be AUC = 0.735 (Figure 20). On the other side, LH concentration based on the univariate logistic regression analysis results did not significantly affect oocyte fertilization (p=0.123).

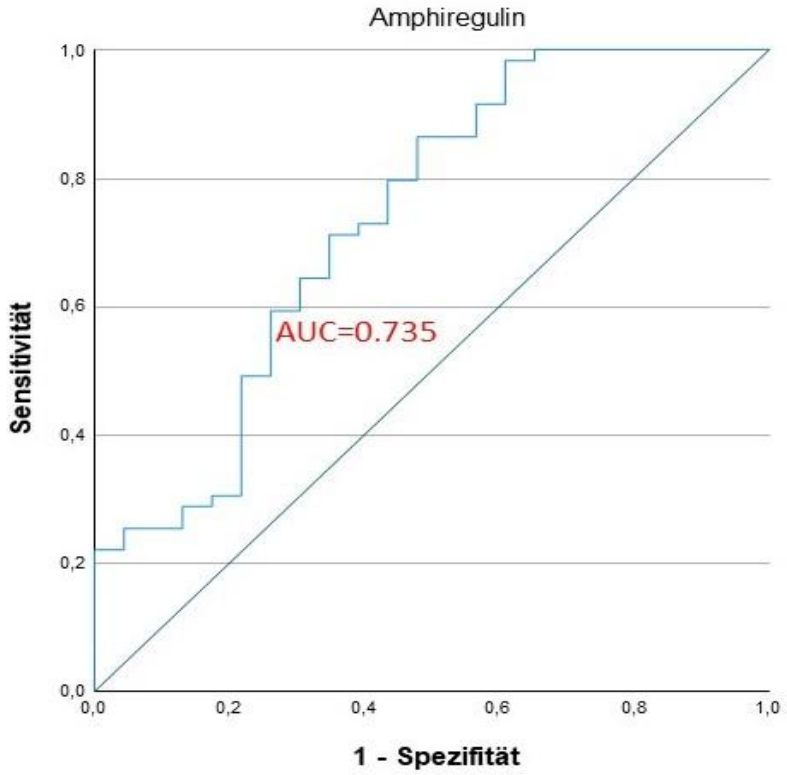

Figure 20: The ROC curve for oocyte fertilization by the amphiregulin concentration.

3.6.1. Correlation between Gene Expression and Oocyte Fertilization

Despite the fact that protein concentrations in the follicular fluid were different between fertilized and unfertilized oocyte group gene expression was not significantly different between groups (Table 16).

Table 16: Comparison of the studied gene expression level (ΔCt) between fertilized and unfertilized oocyte groups.

Parameter	Fertilized Oocyte (N=59)			Unfertilized Oocyte (N=23)			*p-value*
	Median	Minimum	Maximum	Median	Minimum	Maximum	
ΔCt *AREG*	3.09	0.33	5.36	3.22	1.69	6.10	0.342
ΔCt *IGF-II*	6.58	2.74	14.00	6.57	3.63	11.73	0.593
ΔCt *LHCGR*	10.23	5.15	13.01	9.71	7.27	12.02	0.278

Additionally, the mean ΔCt values and Fold change levels indicated that the expression of *AREG* was downregulated while the expression of *IGF-II*, and *LHCGR* was upregulated in the unfertilized oocyte group.

Table 17: The mean expression level and fold change of examined genes based on the oocyte fertilization.

Genes	Mean ΔCt Fertilized Oocyte	Mean ΔCt Unfertilized Oocyte	ΔΔCt	Fold Change	Log2Fold Change	Regulation
AREG	3.08	3.38	-0.30	1.23	0.30	Down
IGF-II	6.74	6.57	0.16	0.89	-0.16	Up
LHCGR	10.04	9.71	0.34	0.79	-0.34	Up

3.7. Impact of the Protein Concentration on Embryo Quality

As previously aforementioned the fertilization rate was 67,74 % where 61 oocytes were correctly fertilized. Due to the application of the specific Embryo Protection Act (EPA), out of the 61 fertilized oocytes, 13 zygotes were frozen, while 48 were cultured. Thirty-four embryos (70.8%) reached good quality (GQE) and fourteen (29.2%) had poor quality (PQE).

By comparing protein concentration in the follicular fluid between GQE and PQE obtained results indicated that AREG, LH, and IGF-II were elevated in the good-quality embryo group, while FSH concentration was decreased (Table 18). Mann Whitney U test showed that AREG (Figure 21) and LH concentration were significantly different between groups.

Table 18: The difference in the protein concentration between GQE and PQE groups.

Parameter	Good Embryo Quality (N=34)			Poor Embryo Quality (N=14)			*p*-value
	Median	Minimum	Maximum	Median	Minimum	Maximum	
AREG (ng/ml)	126.44	71.63	227.54	95.28	43.19	166.50	**0.016**
IGF-II (ng/ml)	366.03	264.63	467.19	359.27	231.72	467.92	0.838
LH (mIU/mL)	1.15	0.37	5.77	0.64	0.46	1.39	**0.017**
FSH (mIU/mL)	5.75	2.1	12.6	6.60	3.70	11.4	0.163

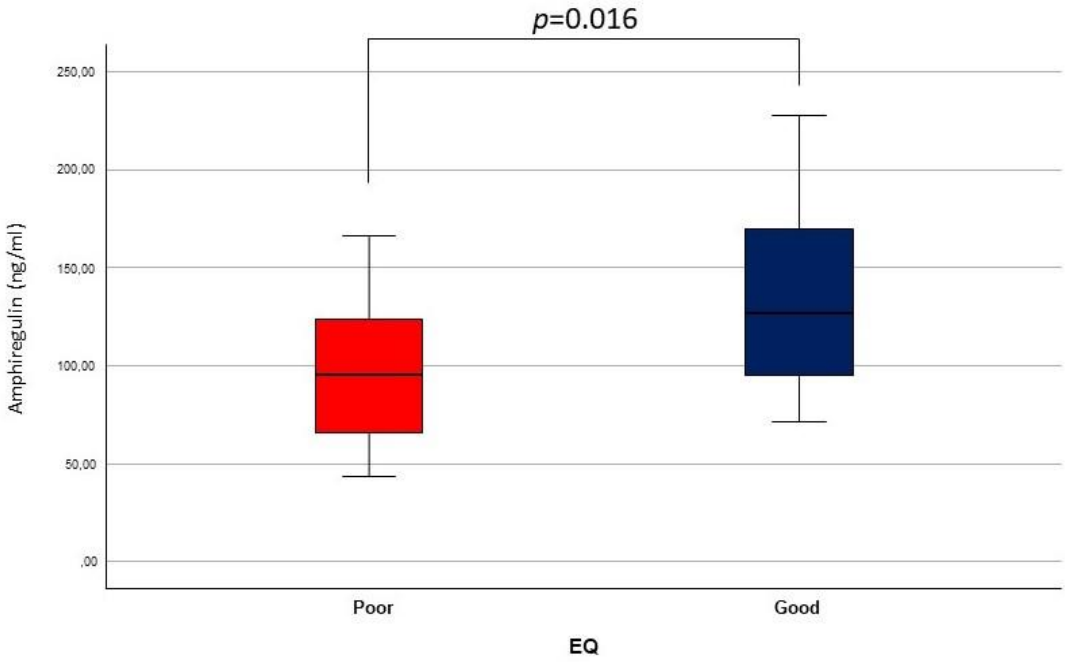

Figure 21: The difference in the amphiregulin concentration between GQE and PQE.

Furthermore, based on the fact that out of the 34 GQE, 10 were from patients diagnosed with infertility, further statistical analysis (Fisher's Exact Test) was performed, and the obtained results indicated that the aforementioned data were not biased with an infertility diagnosis (p=0.621).

In order to determine the impact of AREG and LH concentration on embryo quality, univariate logistic regression analysis was performed. Obtained results demonstrated that amphiregulin concentration could significantly affect embryo quality (OR: 1.02, CI:1.00-1.04; *p*=0.018). In this case, the area under the ROC curve was determined to be AUC = 0.723

(Figure 22). On the other side, LH concentration based on the univariate logistic regression analysis showed a trend (p=0.072) in the prediction of embryo quality.

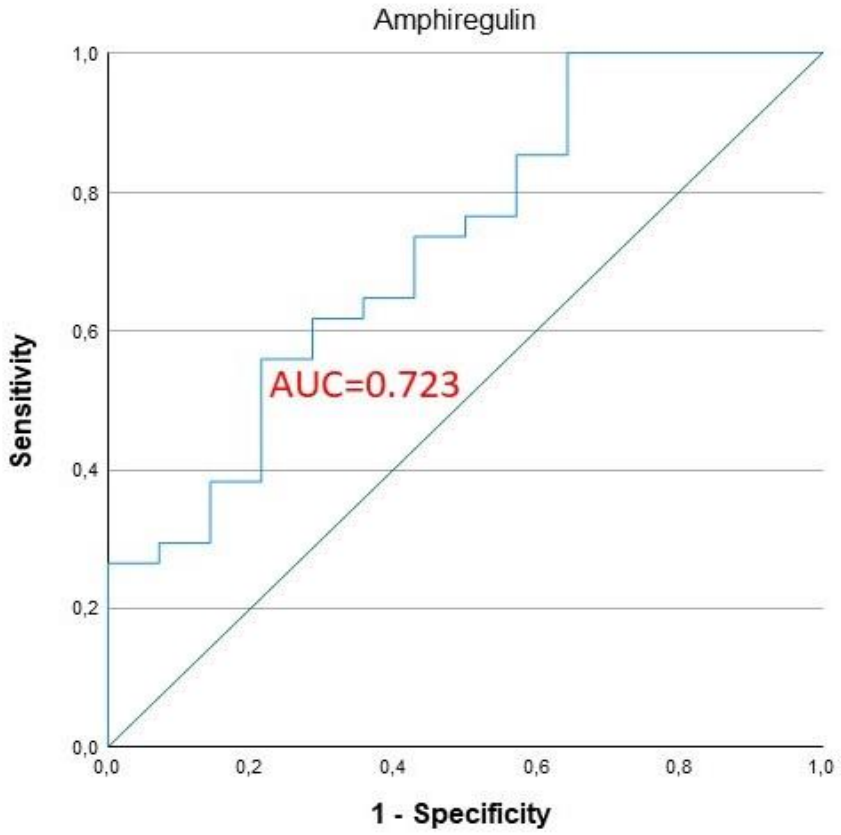

Figure 22: The ROC curve for embryo quality by the amphiregulin concentration.

3.7.1. Correlation between Gene Expression and Embryo Quality

Differences in the relative expression level of studied genes (*AREG, IGF-II, LHCGR*) did not reach statistical significance when compared between GQE and PQE.

Table 19: Comparison of the studied genes expression level (ΔCt) between GQE and PQE.

Parameter	Good Embryo Quality (N=34)			Poor Embryo Quality (N=14)			*p-value*
	Median	Minimum	Maximum	Median	Minimum	Maximum	
ΔCt *AREG*	3.32	0.33	4.36	3.28	2.49	5.36	0.650
ΔCt *IGF-II*	6.65	2.74	14.00	6.56	4.96	8.54	0.964
ΔCt *LHCGR*	10.27	5.15	11.75	9.81	8.57	12.85	0.586

Furthermore, the mean ΔCt values and Fold change levels indicated that expression of the *AREG* and *LHCGR* was downregulated while expression of *IGF-II*, was upregulated in the PQE group.

Table 20: The mean expression level of and fold change of examined genes based on the embryo quality.

Genes	Mean ΔCt GQE	Mean ΔCt PQE	ΔΔCt	Fold Change	Log2Fold Change	Regulation
AREG	3.15	3.43	0.28	0.82	-0.27	Down
IGF-II	6.83	6.71	-0.12	1.08	0.11	Up
LHCGR	10.11	10.16	0.05	0.96	-0.05	Down

3.8. Correlation between Protein Concentration in the Follicular Fluid and Hormonal Levels in the Blood

Obtained results indicated that studied proteins from follicular fluid had a significantly higher correlation with hormones measured on the day of follicle aspiration, than three days before aspiration. Due to the different stimulation protocols used between patients values of the FSH were not measured 3 days before follicular puncture (Table 21).

Table 21: Correlation between proteins in the follicular fluid and studied parameters.

Parameter		AREG	IGF-II	LH	FSH
AMH	r	-,345**	-0.166	0.064	-,281**
	p	**0.000**	**0.086**	0.517	**0.003**
LH*	r	-0.089	,235*	0.094	,209*
	p	0.361	**0.014**	0.338	**0.030**
Progesterone*	r	-0.120	-0.016	-0.143	0.108
	p	0.218	0.873	0.143	0.265
Oestradiol *	r	-,323**	0.150	0.017	-0.075
	p	**0.001**	0.120	0.864	0.439
LH**	r	-,274**	-0.028	,471**	-,192*
	p	**0.004**	0.770	**0.000**	**0.046**
FSH**	r	-0.044	0.046	-,212*	,750**
	p	0.652	0.633	**0.029**	**0.000**
Oestradiol**	r	-,394**	,243*	-0.061	-,223*
	p	**0.000**	**0.019**	0.563	**0.032**
Progesterone**	r	-0.175	-0.010	-,278*	-0.088
	p	0.157	0.938	**0.025**	0.480

*3 days before oocyte aspiration
** day of the oocyte aspiration

3.9. Correlation between the Studied Proteins in the Follicular Fluid

Results presented in Table 22 indicated no significant correlation between protein concentration in the follicular fluid.

Table 22: Correlation between proteins in the follicular fluid.

Parameter		AREG	IGF-II	LH	FSH
AREG	r	1.000	0.106	0.095	-.207
	p		0.275	0.332	0.051
IGF-II	r	0.106	1.000	-0.007	0.110
	p	0.275		0.942	0.258
LH	r	-0.095	-0.007	1.000	-0.164
	p	0.332	0.942		0.094
FSH	r	-.207	0.110	-0.164	1.000
	p	0.051	0.258	0.094	

3.10. Correlation between the Protein Concentration in the Follicular Fluid and Expression Levels of Studied Genes

Results presented in the Table 23 indicated no significant correlation between protein concentration and gene expression.

Table 23: Correlation between proteins in the follicular fluid and gene expression level.

Parameter		AREG	IGF-II	LH	FSH
AREG	r	-0.139	0.108	-0.021	0.049
	p	0.158	0.271	0.835	0.619
IGF-II	r	-0.042	0.146	0.098	0.185
	p	0.667	0.133	0.321	0.056
LHCGR	r	-0.011	-0.082	0.036	-0.145
	p	0.913	0.401	0.720	0.139

4. DISCUSSION

Infertility is a relatively common health condition, and, in the past decade has become one of the top priorities for the World Health Organization. Approximately 48 million couples and 186 million individuals struggle with infertility (WHO, 2020).

Due to the increasing number of people affected by infertility, research is required to improve infertility and subfertility diagnosis, regulations, and therapies. Infertility is a complicated condition influenced by several factors, including congenital malformations and hormonal, genetic, behavioural, environmental, and lifestyle factors (Sharma *et al*., 2013).

Additionally, recognizing the relationship between the possible causes of infertility, such as factors that regulate the expression of genes involved in fertility, inherited factors, hormonal production, and disordered epigenetic mechanisms may lead to a clear understanding of previously unknown causes of infertility. In the literature, different study designs have led to contradictory results. Consequently, the main focus of this study was to explore the correlation of AREG, LH, and IGF II with oocyte maturity, fertilization rate, and embryo quality.

Because the main role of MGC is the production and secretion of steroidal hormones and growth factors into the follicular fluid as well as the regulation of oocyte maturation through gap junctions, the special aim of this study was to determine gene expression in the MGCs of *LHCGR*, *AREG*, and *IGF-II* and their correlation with protein concentration and oocyte maturity, fertilization rate, and development of embryos.

Unlike many published manuscripts, the present study involved single/individual aspiration of follicles, enabling a 1:1 correlation with retrieved oocytes. Moreover, the oocytes retrieved after fertilization were followed and embryo quality was evaluated. Based on the aforementioned study design, the results obtained are especially useful and applicable in Germany due to EPA.

4.1 Age and Stimulation Protocol impact on Protein Concentration

The age of a woman is one factor that can affect fertility. In a study conducted by the National Survey of Family Growth that covered 12,000 women in the United States, the prevalence of infertility increased with a woman's age: In women aged 15 to 34 years, infertility rates ranged from 7.3 to 9.1% while in women aged 35 to 39 years old, the infertility rates increased to 25% (Chandra *et al*., 2013; Walker & Tobler, 2022).

In line with numerous published studies (Armstrong, 2001; Lee *et al*., 2012; Scheffer *et al*., 2017), our findings indicate that women below 35 years produce more MII oocytes, have higher fertilization rates, and more frequently develop good-quality embryos than older women.

Among the researched proteins in the follicular fluid, only FSH concentrations were significantly different between patients based on age, which is in line with published data (Huang *et al*., 2022). Additionally, our results were almost the same as those of the aforementioned study. One of the possible reasons for a higher value of FSH in older patients might be that elevated FSH is associated with a shorter follicular phase and cycle length in aging women (in their late 30s and mid-40s) before the menopause transition (Van Zonneveld *et al*., 2003), coinciding with a marked decline in fecundity (Baird *et al*., 2005).

In our research, the concentration of LH in follicular fluid did not significantly differ between young and aging women, consistent with findings from multiple published studies (De los Santos *et al*., 2013; Pacella *et al*., 2012). Additionally, neither the concentration of AREG nor IGF II statistically differed between groups, in line with the findings by Zamah *et al*. (2010) but in contrast with the findings of other researchers (Choi *et al*., 2006; Liu *et al*., 2012; Wang *et al*., 2006). However, those studies had different designs and aims for the researched parameters, which were based on the patients' ages. Furthermore, one key fact was that there was no difference for AREG, LH, and IGF-II based on age and that allowed

further evaluation on the basis of different conditions/groups because the obtained results were not biased by age.

The age of the patients in this study was similar to that of patients in the stimulation protocol group, and the results in the present investigation did not show any significant differences between protein concentration in FF based on the stimulation protocol, which is in contrast with some published data. Liu *et al*. (2012) reported that the concentration of AREG in FF significantly differed based on the stimulation protocol. One of the possible reasons for the differing findings from those of our study might be the fact that the concentration of administered rFSH varied from 150 IU to 300 IU and was individually adjusted for every patient. Additionally, Liu *et al.* (2012) aspirated only one dominated follicle per patient while in our study the number of aspirated follicles was up to six. However, for the present study, the most important aspect was that due to the possible mixing up in other groups, neither age nor stimulation protocol affected the results obtained.

4.1.1 Age/Stimulation Protocol and Expression of Studied Genes

The relative amounts of the investigated genes' mRNA indicated that only the values of *LHCGR* were different between groups based on age. One possible reason might be the fact that out of the 9 immature oocytes obtained in the study, 7 were from patients older than 35 years. A correlation between oocyte maturity and *LHCGR* expression was found by Maman *et al*. (2012) who reported low levels of *LHCGR* expression by immature cells. Additionally, Regan *et al*. (2017) studied *LHCGR* mRNA expression density in 327 ovarian follicles from young and old IVF patients and reported that *LHCGR* density was increased higher in young women than it was among older women.

On the other hand, as far as we know, this is the first research where the expression of *AREG* and *IGF-II* from MGC was studied based on patients' ages. However, the difference

between younger and older patients was not statistically significant. Due to the lack of similar research in the literature, further studies are necessary.

4.2 BMI and Protein Concentration

The increasing incidence of obesity has been identified as one of the greatest worldwide health challenges of the current century. Vahratian *et al*. (2009) reported that the risk of infertility is significantly higher in obese women than in non-obese women. Moreover, Supramaniam *et al*. (2018) reported that obese women responded poorly to ovulation stimulation and their pregnancy and live birth rates were low following IVF treatment.

The results obtained in our research indicate that fertilization rates were significantly high in the group with normal BMI, in line with published data (Gonzalez *et al*., 2022; Kudesia *et al*., 2018; Pandey *et al*., 2010). Additionally, the concentration of AREG significantly differs between groups; but as far as we know, this is the first research that compared AREG concentrations in FF based on BMI. Several studies have analysed FF in obese women undergoing ART in an attempt to understand how the FF environment may affect oocyte quality. Obese women have a modified follicular environment, which has been assessed mainly through the analysis of FF compounds (Gonzalez *et al*., 2018; Ruebel *et al*., 2017; Valckx *et al*., 2012).

Broughton *et al*. (2017) reported that obesity has a negative effect on reproductive potential, primarily thought to be due to functional alteration of the hypothalamic–pituitary–ovarian axis. Many researchers have investigated differences in serum basal based on BMI, but there is still no data about LH concentration in FF after controlled ovarian stimulation (COS) from obese patients undergoing ICSI.

Cruickshank *et al.* (2001), in a study involving three ethnic groups, did not find an association between IGF-II levels and BMI, which is similar to data obtained in our study where IGF-II did not differ between groups based on the patients' BMI.

Our research was done on only 15 obese patients. Out of this number, seven had an abnormal BMI and PCOS. We assume that differences in the concentration of AREG might be partly because of the PCOS diagnosis; however, a Spearman correlation test indicated a trend between BMI and AREG. Due to the aforementioned facts and with the aim of clearly understanding processes in the FF of obese women, large, well-defined prospective studies are necessary.

4.2.1 BMI and Expression of Studied Genes

Although in the last decade obesity has become one of the predominant health problems, there are very few studies which correlate BMI to gene expression in the field of reproductive medicine. Many molecular tools and techniques, such as single-gene mutations, quantitative trait loci mapping, association mapping, and gene expression signatures, have been used in genetic studies to investigate the biological causes of obesity (Akiyama *et al*., 2017; Xia *et al*., 2013). Butler *et al*. (2015) reported that out of the 370 genes which play a role in obesity, 21 genes are also associated with infertility. Exposure to environmental factors or specific nutritional influences during critical developmental periods can impact epigenetic modifications at the genomic level. These alterations may lead to disruptions in genomic imprinting or the regulation of gene activity. In cases where genetic predispositions contribute to severe obesity the failures in imprinting can also affect fertility. As far as we know, our study is the first one to research the correlation between patients' BMI and *AREG* expression.

Differences in *IGF-II* expression have also been observed between obese and normal weight groups. Some research has confirmed a correlation between *IGF-II* gene expression and obesity (O'Dell *et al*., 1997; Roth *et al*., 2002). On the other hand, no study has correlated BMI and *IGF-II* and *LHCGR* expression in MGC after controlled ovary stimulation.

We believe that in order to provide an accurate genetic diagnosis and counselling for patients with non-syndromic and syndromic obesity and patients presenting with infertility for medical care, current updated lists of clinically relevant known and candidate genes for obesity and infertility in humans are required.

4.3 Infertility and Protein Concentration

ESHRE (2014) reported that approximately 20–35% of infertility cases worldwide can be attributed to a female factor. The most common disorders in females are PCOS and endometriosis. PCOS is an endocrine disease that affects approximately 5–10% of females of reproductive age (Carson *et al*., 2022). In the current study, 45.5% of patients were diagnosed with infertility. The results obtained indicate that LH and progesterone in the serum were significantly different between infertile and fertile patients. Some previous studies reported that endometriosis and PCOS both involve altered functioning of the female HPG axis and cause differences in the secretion of androgens and LH, which was also confirmed in our study (Dinsdale *et al*., 2021).

A comparison of protein concentration in the FF between infertile and healthy patients indicated that AREG and IGF-II were significantly different. The concentration of AREG was lower in the infertile group. Additionally, further analysis confirmed that infertility generally—but PCOS especially—affects the concentration of AREG in FF. Our results are in line with previously published studies (Ambekar *et al*., 2015; Liu *et al*., 2022). Therefore, one possible reason for the difference in the AREG concentration between fertile and infertile patients might be the fact that patients diagnosed with PCOS have an altered biochemical status in the FF (Zhang *et al*., 2017; Chen *et al*., 2020).

Giudice (2001) reported that the IGF system plays a significant role in the autocrine and paracrine regulation of follicular and embryonal development. Consequently, modifications to any part of the IGF system have the ability to alter the process of follicular

development as well as the maturation of oocytes. Van Dessel *et al*. (1999) suggested that disruption of the insulin/IGF system contributes to the pathogenesis of follicular maturation arrest in PCOS.

In our research, infertile patients had higher concentrations of IGF-II compared to healthy ones, in line with published data (Zhong *et al*., 2011). Due to the small number of available papers concerning IGF-II and infertility, the comparison of our findings with the others was highly constrained. We assume that based on the published data one of the possible reasons for the differences might be that increased IGF-II levels may pathologically increase androgen production (Cara, 1994). Additionally, insulin resistance, which is very often present in infertile patients, likely contributes to androgen production since the treatments that improve insulin sensitivity also improve hyperandrogenism (Iurno *et al*., 2001). Moreover, it is well known that hyperinsulinemia present in insulin-resistant PCOS subjects suppresses hepatic IGFBP-1 production, resulting in an increase in bioavailable IGF-II (Homburg *et al*., 1992).

Differences observed in LH concentration in FF between healthy patients and patients diagnosed with PCOS indicated that patients diagnosed with PCOS had a higher concentration of LH in the FF, which is in line with published data (Brinca *et al.,* 2022; Cordeiro *et al*., 2018; Fauser *et al*., 1995). Furthermore, although Liu *et al*. (2022), in a very well-designed study, indicated that elevated LH concentrations could stimulate the secretion of AREG in MGCs, our results do not support their findings. One reason for this disparity might be the difference in the study design. Compared to our research, Liu *et al.'s* study involved pooled samples which were not individually punctured, and the cells were cultivated and treated with LH.

4.3.1 Infertility and Expression of Studied Genes

According to published studies, infertility—especially PCOS—is caused by a combination of genetic predisposition and environmental factors (Khan *et al*., 2019). However, the data obtained in our research did not reveal a difference in gene expression between infertile and healthy patients although we found a trend when we compared gene expression of *AREG* between healthy and PCOS patients.

Our results indicate that *AREG* expression was downregulated in the PCOS group vis-à-vis the control, which is in line with previously published data (Ambekar *et al*., 2015; Haouzi *et al*., 2012; Schmidt *et al*., 2014). However, compared to the findings where the expression of *AREG* between patients' groups was highly significant, in our study, the number of patients diagnosed with PCOS was relatively small (N = 9). Furthermore, our results are in line with the findings by Liu *et al*. (2022) where a decrease in the expression of *AREG* in GCs from individuals with PCOS when compared to those from individuals with normal ovarian function was observed. Moreover, the expression of *LHCGR* was comparable in the PCOS and normal groups, regardless of LH stimulation, indicating that the downregulation of *AREG* in PCOS ovarian cells may not be caused by the decreased expression of *LHCGR*, which mediates the effect of LH.

According to the results of earlier studies, the IGF systems of women with PCOS differ from those of women with normal ovaries (Kwon *et al*., 2010). In our study, the expression of *IGF-II* was not significantly different between the groups, which contradicts some previously published data (Haouzi *et al*., 2012; Kaur *et al*., 2012; Kwon *et al*., 2010). A possible reason for these variations might be due to differences in study design as well as the number of study participants.

An interesting finding is that according to Kaur *et al*. (2012), *IGF-II* expression differed between patients based on insulin resistance. As we previously discussed, insulin resistance is one of the main characteristics of obese patients. Therefore, compared to our study where

we found differences in *IGF-II* expression based on BMI and confirmed a correlation between BMI and *IGF-II* expression, we believe that a detailed and very well-designed study is warranted to investigate variations in *IGF-II* expression between fertile and infertile patients with normal BMI.

In addition to its function in gonads, *LHCGR* has been shown to be widely expressed in reproductive organs such as the uterus and oviduct (Fields & Shemesh, 2004; Zhang *et al*., 2001). It has been proposed that *LHCGR* plays a role in early pregnancy survival, endometrial receptivity modulation, and implantation (Shemesh, 2001).

To date, only a few reports of therapies for infertility in women with inactive *LHCGR* mutations have been published (Mitri *et al*., 2014; Yariz *et al*., 2011; Yuan *et al*., 2017).

LHCGR expression in the present study did not differ between healthy and infertile patients, which is in line with results obtained by Foong *et al*. (2006). On the other hand, Kanamarlapudi *et al.* (2016) reported that the expression and activation of *LHCGR* were up-regulated in MGC from PCOS women but the mechanism of agonist-induced *LHCGR* internalization was unaltered. Nevertheless, our study had a different stimulation protocol and study design from that of Kanamarlapudi *et al.* (2016).

4.4 Oocyte Maturity and Protein Concentration

Ovulation and the subsequent last stage of oocyte maturation are complex biological processes that provide the basis for the development of a healthy embryo and foetus (Lane *et al*., 2014). Female fertility is highly dependent on normal oocyte development, and oocyte quality is a significant rate-limiting factor in ART techniques such as ICSI. It is very well known that the complex and interconnected processes of oocyte growth, maturation, and ovulation are triggered by endocrine cues and controlled by the ovarian follicular environment, where the oocyte is housed (Richani *et al*., 2018).

Ovulation is triggered by an increase in pituitary gonadotrophin LH, which acts directly on the theca and MGCs of a follicle to promote the last phases of oocyte cytoplasmic and nuclear maturation, followed by follicle rupture and corpus luteum formation (Russell & Robker, 2007). The preovulatory oocyte does not express the LH receptor despite being profoundly affected by LH. Furthermore, although the cumulus cells surrounding the preovulatory oocyte have been reported to contain LH receptors, functional LH signalling in the preovulatory oocyte is noticeably lower than that on GCs (Peng *et al*., 1991). Therefore, the LH signal must pass the follicle using paracrine signals and a gap junction-mediated exchange of small nucleotides in order to reach the oocyte (Gilchrist *et al*., 2016). Considering the EGF signalling network's essential function in the ovulatory cascade, it can also be expected to be crucial for oocyte developmental competence (Richani *et al*., 2018).

The results obtained in our study indicate that AREG and LH concentrations in FF significantly differ between immature and mature oocytes. Additional statistical analysis also confirmed the strong impact of these proteins on oocyte nuclear maturity. Differences in AREG concentration align with previously published data (Sasaki *et al*., 2010; Zamah *et al*., 2010). Furthermore, the impact of AREG on the oocyte maturation process was confirmed by Ben Ami *et al*. (2011), whose data indicated that the incubation of human GV-stage oocytes in a standard medium supplemented with AREG resulted in a significantly heightened rate of MII oocyte development. Akin *et al*. (2022) reported that the addition of 100 ng/ml of AREG to the maturation culture step significantly improved the number of MII in patients diagnosed with PCOS.

Zemah *et al*. (2010) gave a detailed explanation of the process and indicated that gonadotropin stimulation of the human ovulatory follicle produces a great accumulation of AREG in FF. Furthermore, no immunoreactive AREG is detected in FF prior to the LH surge, and LH/hCG stimulates AREG secretion *in vitro*. Moreover, a positive correlation was established between AREG concentration in FF and the mature size of the follicle. Although

our study design was slightly different from that of the aforementioned study, we also confirmed a strong correlation between serum LH concentration on the day of oocyte aspiration (34 h after the hCG surge) and AREG concentration as well as FF size.

It is well known that the oocyte maturation process is initiated when an LH signal is generated in the ovarian follicle. LH binds the mural granulosa cell LH receptor (LHCGR), activating a G protein that activates the cAMP system (Arroyo *et al*., 2020). The results in the present study confirm that the concentration of LH is lower in the follicles where immature oocytes are developed, which is consistent with many published papers (Cha *et al*., 1986; Mendoza *et al*., 2002, Sarhan *et al*., 2017). The impact of LH on oocyte maturity was confirmed in our study as well in many previous studies: the presence of LH in FF is crucial for oocyte development (Filicori, 1999; Mendoza *et al*., 2002; Zemah *et al*., 2010).

There was no statistical difference in the concentration of IGF-II between mature and immature oocyte groups. Nevertheless, Wang et al. (2006) suggested that elevated levels of IGF-II could potentially have a direct role in promoting oocyte maturation by interacting with IGF receptors. Additionally, they proposed that this interaction might trigger the subsequent *de novo* production of IGF-II within preimplantation embryos through autocrine regulation. However, due to the limited number of studies in the literature that investigated possible differences in IGF-II concentration after COS and based on oocyte maturity, a detailed comparison would be unreliable.

4.4.1 Oocyte Maturation and Expression of Studied Genes

Some studies indicate the expression of *AREG* may play the most important role in oocyte maturation (Ben Ami *et al*., 2006; Freimann *et al*., 2004; Freimann *et al*., 2005). A non-redundant contribution of *AREG* to mouse oocyte maturation *in vivo* was demonstrated by a null mutation of *AREG* resulting in delayed meiotic development (Hsieh *et al*., 2007) and a significant 40–50% decrease in the proportion of oocytes that could be

fertilized (Chen *et al*., 2013). *AREG* demonstrates significant activities for GV induction as well as cumulus expansion (Conti *et al*., 2006). Additionally, according to Huang *et al*. (2015), *AREG* mRNA induction in human GCs or COCs is connected to oocyte meiotic development, the number of retrieved oocytes, as well as general ICSI outcomes. Moreover, Shimada *et al*., (2006) reported that *AREG* expression is upregulated after hCG treatment at the protein level.

The results of the present study are in accordance with the findings of other studies (Feuerstein *et al*., 2007; Sasaki *et al*., 2010) that demonstrated a significant correlation between the expression of *AREG* and oocyte maturation. Ben-Ami *et al*. (2006) reported that human primary GCs display increased expression of *AREG* 2–8 h after LH stimulation.

We have found scant information on mural granulosa gene expression and oocyte maturation after controlled ovary stimulation. Therefore, in order to confirm our results, further analyses are necessary, especially at molecular levels, to clearly understand the role of EGF – particularly *AREG* – in oocyte maturation after the gonadotropin stimulation process.

IGF-II is the only IGF expressed in human GCs, and FSH stimulates *IGF-II* expression (Baumgarten *et al*., 2014). The importance of *IGF-II* in the oocyte maturation process was confirmed in a recent study, which demonstrated that the expression of *IGF-II* in human GCs undergoes a substantial approximately 64-fold increase as follicles advance from the small antral stage to the preovulatory stage. Importantly, this process is entirely regulated by FSH (Bøtkjær *et al*., 2019). The results obtained in our study did not confirm a significant difference in the expression of *IGF-II* based on oocyte maturity. As far as we know, this is the first study that compares the expression of *IGF-II* in MGCs from patients who undergo ICSI treatment.

LHCGR is exclusively expressed in the MGCs of the ovarian follicle. Similar to the case of animals, LH receptor expression in humans is highest in MGCs in preovulatory follicles (Assou *et al*., 2006). Moreover, *LHCGR* mRNA expression is 10-fold higher in preovulatory follicles compared with expression in small antral follicles (Yung *et al*., 2014).

On the other hand, *LHCGR* expression is suppressed by an LH surge, and the LH surge downregulates *LHCGR* expression in preovulatory follicles in women (Regan *et al*., 2016; Regan *et al*., 2017).

In the present study, the expression of *LHCGR* was higher in the group of mature oocytes, compared to immature oocytes which is in line with the findings made by Maman *et al*., (2012). However, the difference between the groups was not significant. Additionally, Huang *et al*., (2015) reported that *LHCGR* expression in MGCs did not differ or correlate with IVF outcomes.

4.5 Fertilization Rate and Protein Concentration

A combination of male and female factors influence fertilization rates in assisted reproduction techniques. ICSI is mostly preferred over standard IVF in cases of poor sperm quality, where even spermatozoa from low-quality samples yield high fertilization and pregnancy rates (Nagy *et al*., 1995). Therefore, fertilization failure after ICSI is likely to be mainly due to abnormalities of the oocyte. Javed *et al*. (2012) reported that two main factors behind successful fertilization after ICSI are oocyte quality and oocyte maturity. Additionally, numerous studies have confirmed the impact of FF biomarkers on fertilization rate and ICSI outcome (Barroso-Villa *et al*., 2023; Mendoza *et al*., 2002).

The results of our study indicate that oocytes obtained from the follicles with a high concentration of AREG have an elevated fertilization rate, which is in accordance with the literature (Huang *et al*., 2015). Contrary to our results, Inoue *et al*. (2009) reported a non-significant correlation between AREG levels and fertilization rate. However, FF samples in the aforementioned study by Inoue *et al.,* were pooled whereas our study allowed correlations with individual oocyte developmental outcomes. Due to the limited number of publications available, making a comparison with our results was difficult. Firstly, in our study, when we speak about oocyte maturity, we mean nuclear maturity (i.e. PB extrusion and clear visibility).

Nevertheless, cytoplasm maturity remains an open question in research. However, since many papers as well as our results confirm the impact of AREG on oocyte nuclear maturity, we hypothesize that AREG affects oocyte quality, which may be the main reason for the correlation between AREG and fertilization rate.

An oocyte that is developmentally competent can mature, fertilize, cleave, form a blastocyst, implant, and grow into a healthy foetus. After the ICSI procedure, the most important step is fertilization since the fertilization of even an MI oocyte can lead to a good quality embryo and pregnancy (Arroyo *et al*., 2022). Our results indicate that LH concentration in FF significantly differs between fertilized and unfertilized oocytes. The impact of LH on oocytes and fertilization has been researched for decades. Our results are in accordance with many published papers (Mendoza *et al.*, 1999; Mendoza *et al.*, 2002; Willem et al., 2000).

Concerning IGF-II, as we mentioned before, there are a limited number of studies in the literature. However, the results obtained in the present study indicate that the concentration of IGF-II in the FF of fertilized oocytes was higher compared to its concentration in unfertilized oocytes, but the difference was not statistically significant, which is in line with some papers (Mendoza *et al*., 2002; Wang *et al*., 2006). Nevertheless, compared to those studies, the number of samples used in our study was significantly lower.

4.5.1 Fertilization and Gene Expression

To date, numerous studies have been performed to identify gene markers, profile granulosa or cumulus gene expression, and predict oocyte or embryo competence (Cillo *et al*., 2007; McKenzie *et al*., 2004; van Montfoort *et al*., 2008). In the present study, the expression of all three studied genes did not statistically differ between fertilized and unfertilized oocytes. The results obtained for the expression of *AREG* and *LHCGR* contrasted with the results obtained by Huang *et al*. (2015), who reported that *AREG* expression levels positively correlated with the number of 2PN while *LHCGR* negatively correlated with

fertilization. One of the possible reasons for the discrepancy might be differing patient characteristics as well as ovarian responses. Moreover, the time-dependent change of *AREG* expression after HCG stimulation in MGCs was confirmed in previous studies (Fan *et al*., 2009; Fru *et al.*, 2007). Therefore, the variance in detection times might also account for the discrepancy.

There is no current research that is comparable to our study, which includes the expression of IGF-II in the MGCs of patients under controlled ovary stimulation. However, gene expression in MGCs is still a wide area for research aiming to clarify and categorize the role and impact of genes on oocyte competence and successful ICSI outcomes.

4.6 Embryo Quality and Protein Concentration

The selection of high-quality embryos continues to be a major challenge in assisted reproductive technology for humans. Globally, embryo selection is based on morphological evaluations although embryo morphology is not always an absolute indicator of implantation potential, especially due to intra- and inter-observer variability (Sundvall *et al*., 2013). Nevertheless, there is a lack of a unique evidence-based and globally accepted standard for evaluating embryos and identifying an embryo with the highest implantation potential.

In the last two decades, FF has attracted researchers' attention because it is non-invasive and easily available. In some countries such as Germany, due to EPA, there is a restriction on the number of embryos that can be cultured up to the blastocyst stage; furthermore, the utilization of FF biomarkers for predicting embryo quality faces challenges due to limitations in clinical effectiveness and cost-efficiency (Beier *et al*., 1991; Chen *et al*., 2016; Kljajic *et al*., 2022).

In this study, AREG significantly differed between good and poor-quality embryo groups: the oocyte which created a good quality embryo came from a follicle with a high AREG concentration. Our results are in contrast with data published by Inoue *et al*. (2009)

who reported that AREG concentration did not statistically differ between good and poor embryos. However, due to the limited number of studies, we can only compare our results with that of Inoue *et al*. (2009). Nevertheless, the study design used by *Inoue et al.* (2009) involved pooled FF samples, which prevents a direct correlation to embryo quality outcomes.

Besides AREG, the concentration of LH statistically differed between good and poor-quality embryo groups, which is in line with published data (Mendoza *et al*., 2002; Verpoest *et al*., 2000; Yu *et al*., 2022). Although the biological role of LH has been confirmed in many studies, there are few papers about IVF patients under COS based on the concentration of LH in FF, while the published data based on LH serum levels and embryo quality exhibit discrepancies (Depalo *et al.*, 2018; Eftekhar *et al.*, 2021). Moreover, some recent studies reveal that recombinant LH supplementation during GnRH antagonist cycles may improve embryo quality as well as live birth rate (Wang *et al*., 2022; Wiser *et al*., 2011).

On the other hand, the concentration of IGF-II did not differ between groups based on embryo quality, in accordance with the findings of Kaya *et al*. (2012). Additionally, Wang *et al*. (2006) claimed that IGF-II concentrations affected embryo development potential; however, their study design did not consider embryo quality.

Most investigations on the physiological and pathological function of IGF-II on embryo quality are based on animal research, and some roles of IGF-II in regulating function are species-specific. Despite the evident physiological and clinical implications, the underlying molecular processes that mediate the physiological and pathological activity of IGF-II on embryo development and quality are poorly understood and require additional research (Duarte *et al*., 2013; Neira *et al*., 2010).

4.6.1 Embryo Quality and Gene Expression

Although the concentration of proteins differed in good and poor embryo quality groups, gene expression did not significantly vary between these groups. To the best of our knowledge, little information is available on *AREG*, *IGF-II*, and *LHCGR* gene expression and *in vitro* embryo development. The results obtained in the present study for *AREG* expression are in line with previously published data (Feuerstein *et al*., 2007). Moreover, our results align with data reported by Inoue *et al*. (2009) although this study was performed using pooled samples. Conversely, our findings contrast with data published by Huang *et al*. (2015), who reported that the expression of *AREG* was positively correlated with embryo quality. Additionally, the same group of authors reported that *LHCGR* expression did not differ based on embryo quality which is consistent with our results. Furthermore, we hypothesise that this difference might be caused by the diversity in the patients' age and stimulation protocols.

Concerning IGF-II gene expression and embryo quality, only a few studies performed using human cells are available, and, by design, these studies differ from the present investigation. For example, Liu *et al*. (1997) reported that *IGF-II* gene expression correlates well with morphological assessment and that upregulated gene expressions are associated with embryos of high growth potential. However, their study was performed on donated human embryos, so the medical or therapeutically utility of their findings is restricted.

5. CONCLUSION

The results of the current study indicate that AREG concentration in FF affects oocyte maturation, fertilisation rate, and embryo quality. Additionally, the concentration of AREG in the FF significantly differs between patients based on BMI. Besides, some findings such as the correlation between patients' BMI and *AREG* expression are entirely new.

The concentration of LH in FF affects oocyte maturation and significantly differs between fertilized and unfertilized oocyte groups as well as between good and poor embryo quality groups.

The results obtained for IGF-II indicate that the concentration of this protein in FF does not affect oocyte maturity, fertilization rate, or embryo quality. Additionally, as far as we know, this is the first study that compared the expression of *IGF-II* in MGCs from patients undergoing ICSI treatment.

The gene expression of the studied genes (*AREG, IGF-II*, and *LHCGR*) indicates that only the expression of *AREG* significantly affects oocyte maturity, with *AREG* expression being downregulated in the immature oocyte group. *LHCGR* and *IGF-II* did not differ between groups and neither did it affect maturation, fertilization, or embryo quality.

The results on infertility indicate that the concentration of AREG and IGF-II in FF statistically differ between healthy and infertile patients. Additionally, the results reveal that infertility diagnosis, especially PCOS, affects AREG concentration in FF.

In summary, given the complexity of the numerous independent processes involved in oocyte development, a single biomarker is unlikely to predict the outcome of IVF. However, these findings together with previous studies are of great importance to the future development of infertility treatment, especially in Germany due to its EPA. AREG may offer prognostic information aiding the selection of the most viable oocytes and hence embryos because concentration affects oocyte maturity, fertilisation rate, and embryo quality.

Moreover, the availability and ease of analysis allow for the results obtained in the present study to be easily implemented in everyday IVF procedures and improve ICSI outcomes.

6. ACKNOWLEDGMENT

First of all, I would like to express my deepest gratitude to my family for their unwavering support throughout this journey. Especially to my uncle Kile to whom I dedicated this thesis. Their encouragement and understanding have been the pillars that sustained me during the highs and lows of these 4 years.

A heartfelt thank you goes to my supervisors, Prof. Dr med. Solomayer Erich-Franz and Dr.rer.nat. Kasoha Mariz for their invaluable guidance, mentorship, and dedication as well as for precious financial and academic support. Dr. Kasõha insights and constructive feedback have been instrumental in shaping the course of my work**.**

I am indebted to all of my colleagues from Reproductive Medicine Department, who provided amazing understanding and selfless support. Especially gratitude to Jasmin Ney who in crucial moment saved my work. Cheers to that Frau Dr. Ney. The shared experiences made the challenges more manageable and the successes more meaningful.

I am also very grateful to Dipl.-Stat. Gudrun Wagenpfeil for her advices and guidance in the statistical analysis of the present work.

Additionally, I am deeply grateful to my friends whose unwavering support provided the strength and motivation needed to overcome challenges. Your belief in me has been a constant source of inspiration, and I cherish the friendship we share.

In conclusion, this accomplishment is the result of a collective effort. To everyone who has played a role, your support has been invaluable, and I am thankful for the solidarity that has marked this chapter of my life.