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The Significance of Amphiregulin and Insulin-Like Growth Factor II in Follicular Fluid: Implications for Infertility and Oocyte Quality

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Abstract

The presented study explores the importance of amphiregulin and insulin-like growth factor II concentrations within a follicular fluid, and their relationship with gene expression in mural granulosa cells, particularly in the context of infertility. The investigation specifically targets polycystic ovary syndrome and considers patients' body mass index, as well as the impact of those proteins on oocyte quality.

A total of thirty-three women 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. In total 108 follicular fluid and mural granulosa cell samples were analyzed.

Amphiregulin and insulin-like grown factor II levels were determined via enzyme-linked immunosorbent assay. Expression of amphiregulin and insulin-like grown factor II was analyzed by StepOnePlus™ real-time PCR system using TaqMan fast advanced master mix assays. Results reveal that the concentration of amphiregulin and insulin-like grown factor II in follicular fluid statistically differs between healthy and infertile patients. Additionally, the results reveal that infertility diagnosis, especially polycystic ovary syndrome, affects amphiregulin concentration in follicles.

The concentration of amphiregulin in the follicular fluid significantly differs between patients based on body mass index. What is more, some findings such as the correlation between patients' body mass index and amphiregulin expression as well as between amphiregulin concentration and oocyte quality are entirely new.

In conclusion, by recognizing and highlighting the relationship between amphiregulin and infertility as well as oocyte quality, we contribute to the growing body of knowledge on reproductive biology and potentially pave the way for targeted interventions or treatments aimed at optimizing oocyte quality and enhancing fertility outcomes. This research underscores the importance of non-invasive biomarkers in optimizing assisted reproductive techniques.

Abbreviations

ACTB: Actine Beta; AREG: Amphiregulin; ART: Assisted Reproductive Technology; BMI: Body Mass Index; cDNA: Reverse-Transcribed Complementary DNA; COC: Cumulus-Oocyte Complex; EGF: Epidermal Growth Factor; EGFR: Epidermal Growth Factor Receptor; ESHRE: European Society of Human Reproduction and Embryology; GnRH: Gonadotropin-Releasing Hormone; GQO: Good Quality Oocytes; hCG: Human Chorionic Gonadotropin; ICSI: Intracytoplasmic Sperm Injection; IVF: *In-Vitro*-Fertilisation; LH: Luteinizing Hormone; MGC: Mural Granulosa Cells; mRNA: Messenger-RNA; NTC: No Template Control; PBS: Phosphate Buffer Saline; PCOS: Polycystic Ovary Syndrome; PQO: Poor Quality Oocytes; rFSH: Recombinant Follicle-Stimulating Hormone; rLH: Recombinant Luteinizing Hormone; RNA: Ribonucleic Acid; RT-qPCR: Real-Time Quantitative Polymerase Chain Reaction

Introduction

European Society of Human Reproduction and Embryology (ESHRE) reported that approximately 20 - 35% of infertility cases worldwide can be attributed to a female factor [1]. The most common disorders in females are polycystic ovary syndrome (PCOS) and endometriosis. PCOS is an endocrine disease that affects approximately 5% to 13% of females in reproductive age [2]. PCOS is the leading cause of hyperandrogenism and irregular menstrual cycles and is frequently linked with infertility and various metabolic and clinical issues. Moreover, women with PCOS are at a heightened risk of developing metabolic disorders like diabetes mellitus and dyslipidemia compared to healthy ones. Studies have reported prevalence rates of glucose intolerance ranging from 23% to 35% and diabetes mellitus from 4% to 10% in women with PCOS [3]. Besides the diabetes diagnosis, obesity plays a significant role in infertility associated with PCOS, accounting for 90% of cases [4]. Increased adiposity, defined by a body mass index (BMI) exceeding 25 kg/m^2, is correlated with anovulation, thereby increasing the risk of infertility due to elevated androgen levels [5]. Furthermore, folliculogenesis, the complex process of oocyte development and release, is disrupted in PCOS [6]. In this condition, there is excessive recruitment of primordial follicles for growth; subsequent development becomes arrested at the early preantral stage, leading to the formation of multiple cysts [4]. Increased gonadotropin-releasing hormone (GnRH) pulses in women with PCOS, promote heightened luteinizing hormone (LH) production which along with excess insulin, stimulates ovarian theca cells to produce more androgen, resulting in cessation of follicular growth and dominant follicle selection, thus affecting ovulation [7]. However, the precise molecular defects of follicular development in PCOS remain unknown.

Follicular fluid is produced within growing antral follicles and plays a crucial role in providing a microenvironment for developing oocytes. Changes in the composition of follicular fluid can reflect alterations in the secretory activities of ovarian cells and modifications in plasma constituents due to pathological conditions [6].

Over the past years, many published reports about follicular fluid biomarkers' predictive value remain disputable. Besides regular reproductive hormones which have been the focus of research for over a decade, members of the Epidermal-like growth factor family (EGF) have increasingly become the focus as possible mediators of the crucial processes in the follicle [8,9]. It has been demonstrated that the Epidermal growth factor receptor (EGFR) and its ligands (epidermal growth factor (EGF), amphiregulin (AREG), betacellulin (BTC), epiregulin (EREG)), influence numerous essential reproductive functions such as follicular development and oocyte maturity processes [10,11].

Regarding *In-Vitro*-Fertilisation (IVF) patients, according to Zamah., *et al.* (2010), AREG is the most significant EGFR ligand in human follicular fluid and might be used as a predictor of follicle growth [12]. Furthermore, Inoue., *et al.* (2009) indicated that the AREG concentration in follicular fluid affected oocyte maturity and pregnancy outcome [13].

On the other side, only a few published data are available concerning AREG mRNA expression and infertility. Although, some of the research [14] indicated a significant correlation between AREG mRNA expression in human granulosa cells and following IVF parameters,

such as the number of retrieved oocytes and embryo quality, the signaling pathway involved in regulating AREG expression has not yet been completely characterized [15].

Therefore, we hypothesize that the protein expression profile of follicular fluid from women with PCOS can serve as an indicator of the abnormalities in the microenvironment of follicles. Based on the aforementioned facts and due to the limitations of the presented study of Zamah., *et al.* 2010 and Inoue., *et al.* 2009 on IVF patients where pooled follicular samples were used, the major aim of this study was to investigate the possible difference in protein concentration and gene expression of Amphiregulin and Insulin-Like Grown Factor II (IGF-II) between infertile and fertile patients as well between patients with normal BMI and those with increased values > 25 kg/m^2. In addition, a special focus of the research was to define whether there is a difference in the AREG and IGF-II protein concentration and mRNA expression in follicular fluid based on the oocyte quality.

Materials and Methods

Ethical approval

Ethical approval for the study was obtained from the local Ethics Committee of the Medical Association of Saarland (reference number: 146/19). Each study participant provided written informed consent.

Participants

Thirty-three women undergoing intracytoplasmic sperm injection (ICSI) were recruited at the Fertility Centre of the University Clinic of Saarland located in Homburg, Germany, between May 2021 and May 2022. Patients' ages ranged between 23 and 40 years. 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 after maximal stimulation; history or presence of tumors; and the presence of an ovarian cyst > 25 mm.

Stimulation protocol

Twenty patients underwent controlled ovarian stimulation antagonist Gonadotropin-Releasing-Hormone (GnRH) protocol treatment, where starting doses of recombinant Follicle-stimulating hormone (rFSH) (Gonal-F®; Merck Serono, Switzerland)) were based on serum Anti-Müllerian hormone (AMH) levels, antral follicle counts, or previous responses to ovarian stimulation. Subsequent doses were adjusted according to the monitoring of ovarian responses with serial ultrasound examination and serum estradiol measurement. On the other side for thirteen 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 of a combined 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. In each case, a human chorionic gonadotropin (hCG; Ovitrelle; Merck Europe, Darmstadt, Germany) injection was used to trigger final oocyte maturation, and ultrasound-guided ovum retrieval was performed approximately 36h later.

Follicular fluid aspiration and mural granulosa cell isolation

Oocyte retrieval was performed transvaginal, 34 to 36 hours after hCG administration. The follicular fluid contained in each follicle was collected independently. For each patient, from one up to 5 follicles were aspirated individually. For patients with more than 5 follicles due to the possible risk of bleeding and the prolonged time required for the procedure, individual aspiration was done maximally for up to the five follicles.

In the time following cumulus-oocyte complex (COC) harvesting, follicular fluid was replaced with a sterile spinal needle and syringe from the dish to the sterile tube (Vitrolife, Sweden) and centrifuged at 2000 rpm for 5 minutes to isolate Mural Granulosa Cells (MGC). The supernatant was stored at -80°C in CryoTube™ vials (Nunc, Denmark) in aliquots until assayed.

In the following steps, 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) density gradient and centrifuged at 2500 rpm for 30 minutes.

After centrifugation, the 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 200 µL of RNA later Stabilization Reagent (Qiagen, Germany) and cryostored at -80°C until RNA isolation.

Oocyte quality

Each COC was denuded separately and the oocyte quality was determined. Oocyte quality was determined based on the presence of granularity in the cytoplasm, the presence of inclusion in the perivitelline space, and fragmentation of the polar body. Therefore, mature oocytes with intact polar body and with no presence of inclusion in perivitelline space or granularity in the cytoplasm were characterized as Good Quality Oocytes (GQO) while oocytes with fragmented polar body and/or presence of inclusion in perivitelline space as well presence of the cytoplasm granularity were defined as Poor Quality Oocytes (PQO).

Enzyme-linked immunosorbent assay (ELISA)

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). All samples were analyzed in duplicate, and all reagents were prepared according to the manufacturer's instructions. Amphiregulin concentration (pg/ml) was converted and depicted as ng/ml. 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). IGF-II concentration in pg/mL was converted and depicted as ng/ml.

Gene expression of AREG and IGF-II in mural granulosa cells

RNA isolation

Total RNA extraction of individual MGCs was carried out using the High Pure RNA Isolation Kit (Roche Applied Science, Mannheim, Germany) and all reagents were prepared according to the manufacturer's instructions. RNA samples were stored at -80°C until the reverse-transcribed complementary DNA (cDNA) process.

Reverse transcription and quantitative real-time PCR

From each sample, cDNA was synthesized using the High Capacity RNA-to-cDNA kit (4387406; Applied Biosystems, CA, USA). Reverse transcription reactions were performed in Bio-Rad S1000 (Bio-Rad, USA). Samples were stored at -20°C until a Real-time quantitative polymerase chain reaction (RT-qPCR).

Expression of AREG and IGF-II was analyzed by StepOnePlus™ Real-Time PCR System (Applied Biosystems, CA, United States) using TaqMan Fast Advanced Master Mix (Applied Biosystems) and the TaqMan gene expression assays for *AREG* (Hs00950669_m1) and *IGF-II* (Hs00538954_g1).

All listed components were pipetted by the Liquid Handling Robot QIAgility™ (Qiagen, Germany) into the 96-well plate (MicroAmp®, Applied Biosystems) and subsequently loaded into the StepOnePlus™ Each sample was analyzed in triplicate, with no template control (NTC) included in each run.

The samples were normalized concerning the reference gene Actine Beta [*ACTB*] (Hs99999903_m1) using the relative quantification $2^{-\Delta\Delta Ct}$ method (Livak method) where the mean value of mRNA transcripts from each probe was set as one for each gene [16].

Statistic

All variables were analyzed using IBM SPSS version 27 (IBM Corp., Armonk, NY, USA). Mann-Whitney U test was used to compare the medians of the two group variables. Univariate logistic regression analyses were used to detect a correlation between the concentration of proteins, and gene expression levels, according to, patients' BMI, and infertility diagnoses. Furthermore, receiver operating characteristic (ROC) analyses were used to investigate the diagnostic performance of the parameters considering the area under the curve AUC. The fold change was calculated using the equation $2^{-\Delta\Delta C}$. Differences with $p \leq 0.05$ were considered statistically significant.

Results

Descriptive statistics for evaluated parameters

From the thirty-three patients, a total of 108 follicular fluids and MGC samples were included in the study. For the study purpose patients were divided into groups based on age, BMI, and infertility diagnosis. While oocytes were divided based on the quality.

Impact of patients' age and stimulation protocol on AREG and IGF-II concentration

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.

The descriptive statistics for the studied parameters did not show a statistically significant difference between the groups, as illustrated in table 1. Those findings allowed further evaluation based on other different groups because the obtained results were not biased by age or stimulation protocol.

Parameter	Group I (N = 20)			Group II (N = 13)			p-value
	Median	Minimum	Maximum	Median	Minimum	Maximum	
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

Table 1: *Comparison of the AREG and IGF-II concentration between the groups based on the patients' age.*

Correlation between patients' age and gene expression

The relative amounts of the investigated genes mRNA (mean ΔCt) (*AREG, IGF-II*) neither statistically differed between the groups (Table 2).

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

Table 2: *Comparison of the studied gene expression level (ΔCt) between groups based on the patients' age.*

Impact of BMI on AREG and IGF-II concentration

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 25.0 kg/m^2.

Out of the thirty-three patients, 54.5% (N = 18) had normal BMI, while 45.6% (N = 15) had BMI over 25.0 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.

The obtained results showed that the concentration of AREG was significantly different between groups, where patients with normal BMI had increased values of AREG (Table 3).

Since the difference between groups was statistically significant, logistic regression analysis was performed to confirm the impact of BMI on AREG concentration (r = 0.257; p = 0.008).

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

Table 3: *Comparison of the AREG and IGF-II concentration in the follicular fluid based on the patients' BMI.*

Correlation between BMI and gene expression

The mean of the relative expression levels of *AREG* and *IGF-II* were significantly different between groups (Figure 1). Additional analysis indicates that the relative expression level of both studied genes was lower in the overweight group (Table 4).

Due to the aforementioned difference in the relative expression level of *AREG* and *IGF-II* between the groups, linear regression analysis was performed, and the 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).

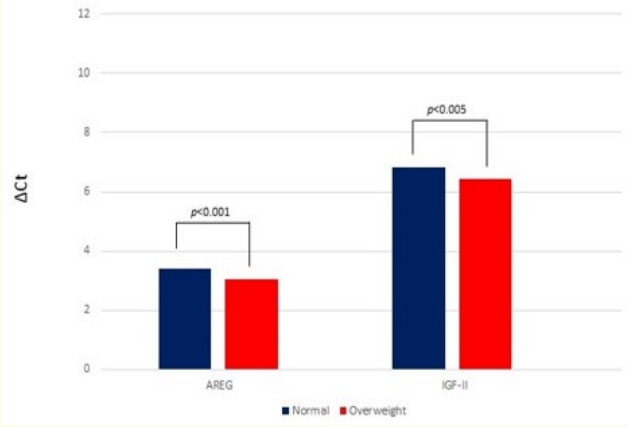

Figure 1: *Differences in AREG, and IGF-II expression levels between groups based on BMI.*

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

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

Since the correlation between ΔCt and the level of gene expression is inverse, lower ΔCt values indicate an increase in gene expression. Therefore, table 5 illustrates that gene expression of *AREG* and *IGF-II* was upregulated in the group with increased BMI values.

Genes	Mean ΔCt Normal BMI	Mean ΔCt Decreased BMI	ΔΔCt	Fold Change	Log2Fold Change	Regulation
AREG	3.48	3.03	-0.45	1.37	0.45	UP
IGF-II	7.04	6.37	-0.67	1.84	0.67	UP

Table 5: *The mean expression level of and fold change of examined genes based on the patients' BMI.*

Impact of infertility on AREG and IGF-II concentration

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.

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.

Regarding the concentration of AREG and IGF-II from the follicular fluid, a significant difference between the groups was observed for the concentration of both parameters (Table 6). 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 2).

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

Table 6: *Comparison of the protein concentration in the follicular fluid between healthy (Group I) and infertile patients (Group II).*

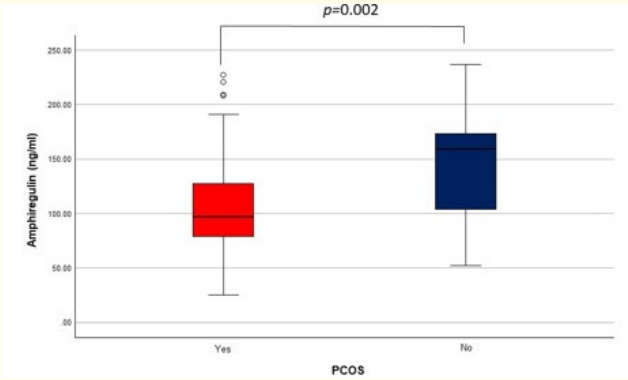

Figure 2: *Difference in the amphiregulin concentration between healthy patients and patients diagnosed with PCOS.*

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. 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 3). On the other side, infertility diagnosis based on the logistic regression analysis results did not significantly affect IGF-II concentration.

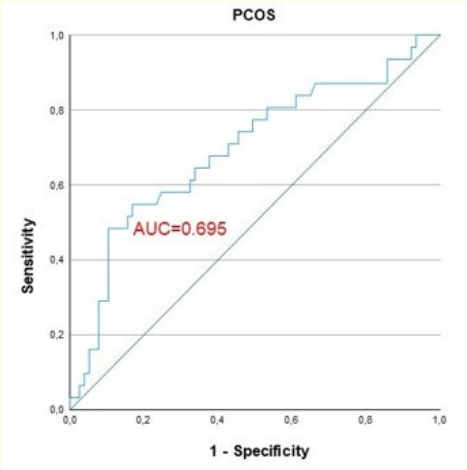

Figure 3: *The ROC curve for AREG concentration by the patients diagnosed with PCOS*

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 7).

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

Table 7: *Multiple regression analysis-the impact of BMI, Infertility and PCOS on the AREG.*

a. Dependent Variable: Amphiregulin (AREG).

Correlation between infertility diagnosis and gene expression

Despite the fact that protein concentrations in the follicular fluid were significantly different, obtained results indicated that the difference between healthy and infertile patients in the relative expression level of studied genes was not statistically significant (Table 8). Additionally, the relative expression of the *AREG* and *IGF-II* compared between healthy and PCOS patients neither significantly differed.

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

Table 8: *Comparison of the studied gene expression level (ΔCt) between groups based on the patient infertility diagnosis.*

Impact of the AREG and IGF-II concentration on oocyte quality

ICSI was performed on a total number of 84 oocytes, where 54 (65.47%) had good quality (GQO) while 29 (34.52%) had poor quality (PQO). Out of these 29 PQO, 18 (62.06%) had fragmented polar body, inclusion in perivitelline space and/or cytoplasm granularity, while 4 (13.89%) oocytes had only inclusion in perivitelline space and 7 (24.13%) had only fragmented polar body. By conducting a comparative analysis of protein concentrations within the follicular fluid, the obtained results indicated that the concentration of AREG was significantly different between the GQO and PQO groups (Table 9). Moreover, the concentration of AREG (Figure 4) was elevated in the GQO group.

Parameter	GQO (N = 55)			PQO (N = 29)			p-value
	Median	Minimum	Maximum	Median	Minimum	Maximum	
AREG (ng/ml)	140.60	71.63	227.54	79.75	27.69	135.07	0.0001
IGF-II (ng/ml)	376.64	274.87	467.190	369.75	264.63	467.92	0.587

Table 9: *Comparison of the protein concentration in the follicular fluid between GQO and PQO groups.*

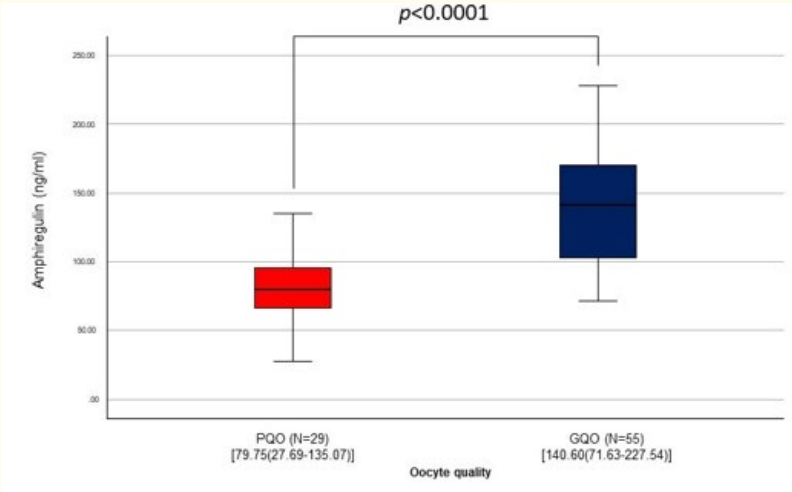

Figure 4: *Difference in the AREG concentration between follicles of the GQO and PQO. Follicles that produce PQO oocytes had lower concentrations of amphiregulin in the follicular fluid. Results are present as median (range).*

In addition, binary logistic regression analysis was performed to evaluate the impact of AREG, on oocyte quality. The obtained results demonstrated that AREG concentrations in follicular fluid could significantly affect oocyte quality (OR: 1.05 Cl: 1.029 - 1.084 p < 0.0001). Predictive strength was quantified using the area under the curve (AUC) of the receiver operating characteristic (ROC), where the area under the ROC curve for AREG was AUC = 0.889 (Figure 5).

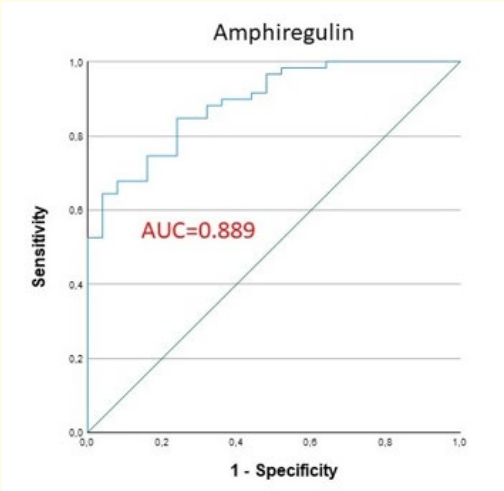

Figure 5: *The ROC curve for oocyte quality by the AREG concentration.*

Correlation between gene expression and oocyte quality

Although AREG concentrations in the follicular fluid affect oocyte quality, gene expression was not significantly different between groups (Table 10).

Parameter	GQO (N = 545)			PQO (N = 29)			p-value
	Median	Minimum	Maximum	Median	Minimum	Maximum	
ΔCt *AREG*	3.14	0.33	5.87	3.04	1.24	4.36	0.469
ΔCt *IGF-II*	6.56	2.74	14.00	6.58	3.63	10.22	0.743

Table 10: *Comparison of the studied gene expression level (ΔCt) between GQO and PQO groups.*

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 [17].

Due to the increasing number of people affected by infertility, research is required to improve infertility and subfertility diagnosis, regulations and therapies. 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 our study was to explore the correlation of AREG, and IGF II with infertility especially with PCOS diagnosis, as well as with BMI and oocyte quality.

Since 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 [9], the special aim of this study was to determine gene expression in the MGCs of *AREG*, and *IGF-II* and their correlation with oocyte quality as well to investigate does patients with infertility diagnoses have different gene expression compare to healthy ones. Unlike many published manuscripts, the present study involved single/individual aspiration of follicles, enabling a 1:1 correlation with retrieved oocytes.

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% [18,19].

The concentration of AREG and IGF II did not statistically differ between groups, which is in line with the findings by Zamah., *et al*. (2010) [12]. The age of the patients in this study was similar to the patients in the stimulation protocol group, and the results in the present investigation did not show any significant differences between protein concentration in follicular fluid based on the stimulation protocol, which is in contrast with some published data. Liu., *et al*. (2012) reported that the concentration of AREG in follicular fluid significantly differed based on the stimulation protocol [20]. 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) [20] 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 infertility or BMI groups, neither age nor stimulation protocol affected the results obtained.

Age/stimulation protocol and expression of studied genes

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.

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 [21]. Moreover, Supramaniam., *et al*. (2018) reported that obese women responded poorly to stimulation, and their pregnancy and live birth rates were low following IVF treatment [22].

The results obtained in our research indicate the concentration of AREG significantly differs between groups; but as far as we know, this is the first research that compared AREG concentrations in follicular fluid based on BMI. Several studies have analyzed follicular fluid in obese women undergoing assisted reproductive technology (ART) in an attempt to understand how the follicular fluid environment may affect oocyte quality. Obese women have a modified follicular environment, which has been assessed mainly through the analysis of follicular fluid compounds [23-25].

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 [26].

The obtained results about IGF-II concentration in the follicular did not differ significantly between groups based on the patient's BMI. Cruickshank., *et al.* (2001) study, involved three ethnic groups and did not find an association between IGF-II levels and BMI, which is similar to data obtained in our study [27].

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 PCOS ($p = 0.056$). Due to the aforementioned facts and with the aim of clearly understanding processes in the follicular fluid of obese women, large, well-defined prospective studies are necessary.

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 that 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 [28,29]. Butler., *et al*. (2015) reported that out of the 370 genes that play a role in obesity, 21 genes are also associated with infertility. As far as we know, our study is the first one to research the correlation between patients' BMI and *AREG* expression [30].

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 [31,32]. On the other hand, no study has correlated BMI and *IGF-II* 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.

Infertility and protein concentration

It is very well known that endometriosis and PCOS involve altered functioning of the female hypothalamic-pituitary-gonadal (HPG) axis and cause differences in the secretion of androgens and LH [33].

A comparison of protein concentration in the follicular fluid 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 follicular fluid. Our results are in line with previously published studies [6,8]. 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 follicular fluid [34,35].

Giudice (2001) reported that the IGF system plays a significant role in the autocrine and paracrine regulation of follicular and embryonal development [36]. 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 [37].

In our research, infertile patients had higher concentrations of IGF-II compared to healthy ones, in line with published data [38]. 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 [39]. Moreover, it is well known that hyperinsulinemia present in insulin-resistant PCOS subjects suppresses hepatic insulin like growth factor binding protein 1 production, resulting in an increase in bioavailable IGF-II [40].

Infertility and expression of studied genes

According to published studies, infertility-especially PCOS-is caused by a combination of genetic predisposition and environmental factors [41]. However, the data obtained in our research did not reveal a difference in gene expression between infertile and healthy patients. According to the results of earlier studies, the IGF systems of women with PCOS differ from those of women with normal ovaries [42]. In our study, the expression of *IGF-II* was not significantly different between the groups, which contradicts some previously published data [42-44]. 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 [43]. 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.

Oocyte quality and protein concentration

Female fertility is highly dependent on normal oocyte development, and oocyte quality is a significant rate-limiting factor in ART. Considering the EGF signalling network's essential function in the ovulatory cascade, it can also be expected to be crucial for oocyte developmental competence [9].

The results obtained in our study indicate that AREG concentrations in follicular fluid significantly differ between good and poor-quality oocytes, and were higher in the good-quality group. As far as we know this is the first study, which correlates AREG concentration and oocyte quality. Additionally, several studies confirmed the impact of AREG on the oocyte maturation process [12,45]. Some of the research indicated that oocytes obtained from the follicles with a high concentration of AREG have an elevated fertilization rate [14].

Due to the limited number of publications available, making a comparison with our results was difficult. However, based on the existing research findings that confirm the impact of AREG on oocyte maturity and fertilization rate, our assertion regarding the influence of AREG on oocyte quality holds substantial value. The established impact of AREG on oocyte maturation and subsequent fertilization underscores the importance of AREG as a biomarker or regulator of oocyte quality. By recognizing and highlighting this relationship, we contribute to the growing body of knowledge on reproductive biology and potentially pave the way for targeted interventions or treatments aimed at optimizing oocyte quality and enhancing fertility outcomes.

In summary, our statement regarding the impact of AREG on oocyte quality is supported by robust scientific evidence, and further research in this area could lead to valuable insights and advancements in assisted reproductive technologies and fertility management.

Oocyte quality and expression of studied genes

When we speak about gene expression and oocyte quality obtained results did not reveal a difference between groups. As far as we know this is the first study, that correlates AREG expression and the quality of oocytes. On the other side, Inoue., *et al*. (2009) reported a non-significant correlation between AREG levels and fertilization rate [13]. Due to the limited number of publications available, making a comparison with our results was difficult. However, based on the existing research findings we believe that our assertion regarding the influence of AREG expression on oocyte quality might be correct since oocyte quality is recognized as one of the most critical factors for successful fertilization. What is more, according to Feuerstein., *et al*. (2007), AREG does not impact either embryo quality [46].

When we speak about IGF-II obtained results did not confirm the difference in the gene expression between groups based on oocyte quality. In literature, Kaya., *et al*. (2012) reported that expression of IGF-II did not differ between groups based on embryo quality [47]. Additionally, 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.

Conclusion

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

The concentration of AREG in the follicular fluid significantly differs between patients based on BMI. What is more, some findings such as the correlation between patients' BMI and *AREG* expression as well as between AREG concentration and oocyte quality are entirely new.

In summary, the obtained results together with previous studies are of great importance to the future development of infertility treatment. AREG may offer prognostic information aiding the selection of the most viable oocyte. 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.

Bibliography

1. ESHRE. "ESHRE-European Society of Human Reproduction and Embryology. ART Fact Sheet" (2014).
2. Carson Sandra Ann and Amanda N Kallen. "Diagnosis and Management of Infertility: A Review". *JAMA* 326.1 (2021): 65-76.
3. Macut Djuro., *et al*. "Insulin and the polycystic ovary syndrome". *Diabetes Research and Clinical Practice* 130 (2017): 163-170.

4. Nandi Anindita., *et al*. "Polycystic ovary syndrome". *Endocrinology and Metabolism Clinics of North America* 43.1 (2014): 123-147.

5. Bloom Michael S., *et al*. "Adiposity is associated with anovulation independent of serum free testosterone: A prospective cohort study". *Paediatric and Perinatal Epidemiology* 35.2 (2021): 174-183.

6. Ambekar Aditi S., *et al*. "Proteomics of follicular fluid from women with polycystic ovary syndrome suggests molecular defects in follicular development". *The Journal of Clinical Endocrinology and Metabolism* 100.2 (2015): 744-753.

7. Gorry Anwen., *et al*. "Infertility in polycystic ovary syndrome: focus on low-dose gonadotropin treatment". *Endocrine* 30.1 (2006): 27-33.

8. Liu Yang., *et al*. "Luteinizing hormone stimulates the expression of amphiregulin in human theca cells". *Journal of Ovarian Research* 15.1 (2022): 129.

9. Richani Dulama and Robert B Gilchrist. "The epidermal growth factor network: role in oocyte growth, maturation and developmental competence". *Human Reproduction Update* 24.1 (2018): 1-14.

10. Harris RC., *et al*. "EGF Receptor Ligands". *Experimental Cell Research* 284.1 (2003): 2-13.

11. Schneider Marlon R and Eckhard Wolf. "The epidermal growth factor receptor and its ligands in female reproduction: insights from rodent models". *Cytokine and Growth Factor Reviews* 19.2 (2008): 173-181.

12. Zamah AM., *et al*. "Human oocyte maturation is dependent on LH-stimulated accumulation of the epidermal growth factor-like growth factor, amphiregulin". *Human Reproduction (Oxford, England)* 25.10 (2010): 2569-2578.

13. Inoue Yoshihito., *et al*. "Amphiregulin is much more abundantly expressed than transforming growth factor-alpha and epidermal growth factor in human follicular fluid obtained from patients undergoing in vitro fertilization-embryo transfer". *Fertility and Sterility* 91.4 (2009): 1035-1041.

14. Huang Ying., *et al*. "Altered amphiregulin expression induced by diverse luteinizing hormone receptor reactivity in granulosa cells affects IVF outcomes". *Reproductive Biomedicine Online* 30.6 (2015): 593-601.

15. Wang Huarong., *et al*. "HDAC3 maintains oocyte meiosis arrest by repressing amphiregulin expression before the LH surge". *Nature Communications* 10.1 (2019): 5719.

16. Livak KJ and Schmittgen TD. "Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method". *Methods* 25.4 (2001): 402-408.

17. World Health Organization. "WHO Fact Sheet" (2020).

18. Chandra Anjani., *et al*. "Infertility and impaired fecundity in the United States, 1982-2010: data from the National Survey of Family Growth". *National Health Statistics Reports* 67 (2013): 1-18, 1 p following 19.

19. Walker Matthew H and Kyle J Tobler. "Female Infertility". StatPearls, StatPearls Publishing (2022).

20. Liu Nana., *et al*. "Comparison of follicular fluid amphiregulin and EGF concentrations in patients undergoing IVF with different stimulation protocols". *Endocrine* 42.3 (2012): 708-716.

21. Vahratian Anjel and Yolanda R Smith. "Should access to fertility-related services be conditional on body mass index?". *Human Reproduction (Oxford, England)* 24.7 (2009): 1532-1537.

22. Supramaniam Prasanna Raj., *et al*. "The correlation between raised body mass index and assisted reproductive treatment outcomes: a systematic review and meta-analysis of the evidence". *Reproductive Health* 15.1 (2018): 34.

23. Valckx S D M., *et al*. "BMI-related metabolic composition of the follicular fluid of women undergoing assisted reproductive treatment and the consequences for oocyte and embryo quality". *Human Reproduction (Oxford, England)* 27.12 (2012): 3531-3539.

24. Ruebel Meghan L., *et al*. "Obesity modulates inflammation and lipid metabolism oocyte gene expression: a single-cell transcriptome perspective". *The Journal of Clinical Endocrinology and Metabolism* 102.6 (2017): 2029-2038.

25. Gonzalez Macarena B., *et al*. "Inflammatory markers in human follicular fluid correlate with lipid levels and Body Mass Index". *Journal of Reproductive Immunology* 130 (2018): 25-29.

26. Broughton Darcy E and Kelle H Moley. "Obesity and female infertility: potential mediators of obesity's impact". *Fertility and Sterility* 107.4 (2017): 840-847.

27. Cruickshank J K., *et al*. "Epidemiology of the insulin-like growth factor system in three ethnic groups". *American Journal of Epidemiology* 154.6 (2001): 504-513.

28. Akiyama Masato., *et al*. "Genome-wide association study identifies 112 new loci for body mass index in the Japanese population". *Nature Genetics* 49.10 (2017): 1458-1467.

29. Xia Qianghua and Struan F A Grant. "The genetics of human obesity". *Annals of the New York Academy of Sciences* 1281.1 (2013): 178-190.

30. Butler Merlin G., *et al*. "Clinically relevant known and candidate genes for obesity and their overlap with human infertility and reproduction". *Journal of Assisted Reproduction and Genetics* 32.4 (2015): 495-508.

31. O'Dell S D., *et al*. "Apal polymorphism in insulin-like growth factor II (IGF2) gene and weight in middle-aged males". *International Journal of Obesity and Related Metabolic Disorders: Journal of the International Association for the Study of Obesity* 21.9 (1997): 822-825.

32. Roth S M., *et al*. "IGF2 genotype and obesity in men and women across the adult age span". *International Journal of Obesity and Related Metabolic Disorders Journal of the International Association for the Study of Obesity* 26.4 (2002): 585-587.

33. Dinsdale Natalie L and Bernard J Crespi. "Endometriosis and polycystic ovary syndrome are diametric disorders". *Evolutionary Applications* 14.7 (2021): 1693-1715.

34. Zhang, Yan., *et al*. "Follicular metabolic changes and effects on oocyte quality in polycystic ovary syndrome patients". *Oncotarget* 8.46 (2017): 80472-80480.

35. Chen Xiaojiao., *et al*. "Metabolic alterations associated with polycystic ovary syndrome: A UPLC Q-Exactive based metabolomic study". *Clinica Chimica Acta; International Journal of Clinical Chemistry* 502 (2020): 280-286.

36. Giudice LC. "Insulin-like growth factor family in Graafian follicle development and function". *Journal of the Society for Gynecologic Investigation* 8.1 (2001): S26-S29.

37. Thierry van Dessel H J., *et al*. "Elevated serum levels of free insulin-like growth factor I in polycystic ovary syndrome". *The Journal of Clinical Endocrinology and Metabolism* 84.9 (1999): 3030-3035.

38. Zhong Gang and Biheng Chen. "Serum and follicular fluid levels of IGF-II, IGF-binding protein-4 and pregnancy-associated plasma protein-A in controlled ovarian hyperstimulation cycle between polycystic ovarian syndrome (PCOS) and non-PCOS women". *Gynecological Endocrinology: The Official Journal of the International Society of Gynecological Endocrinology* 27.2 (2011): 86-90.

39. Cara J F. "Insulin-like growth factors, insulin-like growth factor binding proteins and ovarian androgen production". *Hormone Research* 42.1-2 (1994): 49-54.

40. Homburg R., *et al*. "The role of insulin-like growth factor-1 (IGF-1) and IGF binding protein-1 (IGFBP-1) in the pathogenesis of polycystic ovary syndrome". *Human Reproduction (Oxford, England)* 7.10 (1992): 1379-1383.

41. Khan Muhammad Jaseem., *et al*. "Genetic basis of polycystic ovary syndrome (PCOS): Current perspectives". *The Application of Clinical Genetics* 12 (2019): 249-260.

42. Kwon Hwang., *et al*. "mRNA expression pattern of insulin-like growth factor components of granulosa cells and cumulus cells in women with and without polycystic ovary syndrome according to oocyte maturity". *Fertility and Sterility* 94.6 (2010): 2417-2420.

43. Kaur Surleen., *et al*. "Differential gene expression in granulosa cells from polycystic ovary syndrome patients with and without insulin resistance: identification of susceptibility gene sets through network analysis". *The Journal of Clinical Endocrinology and Metabolism* 97.10 (2012): E2016-E2021.

44. Haouzi D., *et al*. "Altered gene expression profile in cumulus cells of mature MII oocytes from patients with polycystic ovary syndrome". *Human Reproduction (Oxford, England)* 27.12 (2012): 3523-3530.

45. Ben-Ami I., *et al*. "*In vitro* maturation of human germinal vesicle-stage oocytes: role of epidermal growth factor-like growth factors in the culture medium". *Human Reproduction (Oxford, England)* 26.1 (2011): 76-81.

46. Feuerstein P., *et al*. "Gene expression in human cumulus cells: one approach to oocyte competence". *Human Reproduction (Oxford, England)* 22.12 (2007): 3069-3077.

47. Kaya Aysenur., *et al*. "Follicular fluid concentrations of IGF-I, IGF-II, IGFBP-3, VEGF, AMH, and inhibin-B in women undergoing controlled ovarian hyperstimulation using GnRH agonist or GnRH antagonist". *European Journal of Obstetrics, Gynecology, and Reproductive Biology* 164.2 (2012): 167-171.

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