Thieme

Impact of Amphiregulin on Oocyte Maturation and Embryo Quality: Insights from Clinical and Molecular Perspectives

Wirkung von Amphiregulin auf Eizellreifung und Embryoqualität: klinische und molekulare Erkenntnisse

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ABSTRACT

Introduction
Identifying non-invasive biomarkers which can predict the outcome of intracytoplasmic sperm injection (ICSI) is crucial, particularly in Germany where the challenges are intensified by the Embryo Protection Act. Recent research has highlighted biomarkers within the epidermal growth factor (EGF) family as central to follicular processes, although their predictive utility remains a subject of debate in the literature. Therefore, the primary objective of this study was to investigate the significance of amphiregulin concentrations in follicular fluid and gene expression in mural granulosa cells on oocyte maturation, fertilization, and embryo quality.

Patients and Methods
A total of 33 women were recruited at the University Clinic of Saarland Fertility Center (Homburg, Germany). Follicular fluid aspiration consisted of single/individual aspiration of follicles, enabling a 1:1 correlation with retrieved oocytes. Follicular fluid and mural granulosa cell samples from 108 oocytes were analyzed. Amphiregulin levels were determined with enzyme-linked immunosorbent assay, while gene expression was analyzed with the StepOnePlus Real-Time PCR System using TaqMan Fast Advanced Master Mix assays.

Results
Results showed that amphiregulin concentrations affect oocyte maturation, fertilization, and embryo quality, while luteinizing hormone concentrations influence oocyte maturation, with significant differences identified between fertilized/unfertilized and good/poor embryo groups. Amphiregulin expression significantly impacts oocyte maturation, with downregulation observed in immature oocytes, while luteinizing hormone/chorionic gonadotropin receptor expression showed no significant differences between groups and did not influence maturation, fertilization, or embryo quality.

Conclusion
These findings are very important for advancing infertility treatment, especially in Germany. The results for amphiregulin may provide prognostic insights which could be useful when selecting viable oocytes and embryos. This research underscores the importance of non-invasive biomarkers for optimizing ICSI outcomes and potentially enhancing the success rates of assisted reproductive technology.

ZUSAMMENFASSUNG

Einleitung
Die Identifikation von nicht invasiven Biomarkern, die das Outcome nach intrazytoplasmatischer Spermieninjektion (ICSI) prognostizieren könnten, ist sehr wichtig, besonders in Deutschland, wo die Herausforderung durch das Embryonenschutzgesetz noch intensiviert wird. Die jüngste Forschung hat die Bedeutung einiger Biomarker innerhalb der Familie der epidermalen Wachstumsfaktoren als zentral für follikuläre Prozesse hervorgehoben, aber ihr prädiktiver Nutzen ist in der Literatur noch umstritten. Das Hauptziel dieser Studie war es daher, die Wirkung von Amphiregulin-Konzentrationen in der Follikelflüssigkeit und der Genexpression in muralen Granulosazellen auf Eizellreifung, Fertilisation und Embryoqualität zu untersuchen.

Patientinnen und Methoden
Insgesamt wurden 33 Frauen in der Klinik für Reproduktionsmedizin des Universitätsklinikums des Saarlandes (Homburg, Deutschland) für die Studie rekrutiert. Die Aspiration der Follikelflüssigkeit bestand aus einer einzelnen/individuellen Aspiration von Follikeln, was eine 1 : 1 Korrelation mit den entnommenen Eizellen erlaubte. Die Follikelflüssigkeit- und muralen Granulosazell-Proben von 108 Eizellen wurden analysiert. Der jeweilige Amphiregulin-Spiegel wurde mit ELISA (Enzyme-Linked ImmunoSorbent Assay) bestimmt, und die Genexpression mithilfe des StepOnePlus Real-Time PCR Systems und TaqMan Fast Advanced Master Mix Assays analysiert.

Ergebnisse
Die Ergebnisse zeigten, dass Amphiregulin-Konzentrationen die Eizellreifung, Fertilisation und Embryoqualität beeinflussen, während die Konzentrationen von luteinisierendem Hormon die Eizellreifung beeinflussen. Es fanden sich signifikante Unterschiede in den Konzentrationen zwischen fertilisierten/nicht fertilisierten Gruppen und den Gruppen mit guter/schlechter Embryonenqualität. Die Expression von Amphiregulin hatte eine signifikante Wirkung auf die Eizellreifung. Eine Downregulation von Amphiregulin wurde bei unreifen Eizellen beobachtet, wohingegen die Expression von luteinisierendem Hormon/Choriongonadotropinrezeptor sich nicht signifikant zwischen den Gruppen unterschied und keinen Einfluss auf die Eizellreifung, Fertilisation oder Embryonenqualität hatte.

Schlussfolgerung
Diese Ergebnisse liefern wichtige Hinweise für eine weitere Verbesserung von Fertilitätsbehandlungen, besonders in Deutschland. Die Ergebnisse könnten prognostische Erkenntnisse liefern, die bei der Selektion von lebensfähigen Eizellen und Embryonen nützlich wären. Diese Studie unterstreicht die Bedeutung von nicht invasiven Biomarkern bei der Optimierung von ICSI-Outcomes und könnte potenziell die Erfolgsraten der assistierten Reproduktionstechnologie verbessern.

Introduction

In the rapidly advancing field of reproductive medicine, identifying and transferring the most viable embryo is a key goal, but the challenge of finding an optimal selection method remains. Although there are well-designed and published algorithms for the selection of embryos based on morphologic parameters [1, 2], their use is not possible in countries such as Germany due to the German Embryo Protection Act (EPA). Additionally, according to the EPA, in Germany the selection of embryos with the highest potential for successful implantation must be made at the pronucleus stage [3]. Defining possible parameters that might usefully serve as non-invasive biomarkers for intracytoplasmic sperm injection (ICSI) outcomes is therefore crucial.

Many reports published over the past years on the predictive value of follicular fluid (FF) biomarkers are still disputed. In addition to regular reproductive hormones which have been the focus of research for over a decade, members of the epidermal-like growth factor (EGF) family have increasingly become an object of interest as possible mediators of crucial follicular processes [4, 5]. 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 maturation processes [6, 7].

The production of AREG, EREG, and BTC is an essential component of the paracrine function of luteinizing hormone (LH) ovulatory signaling. These three proteins mediate a large network of gene activation in mural granulosa cells (MGC) and cumulus oocyte complex (COC), with AREG serving as a critical intermediary between those cells following the mid-cycle LH surge [5]. Therefore, AREG, EREG, and BTC are not observable in FF before the LH surge but rapidly increase to maximum levels before oocyte germinal vesicle breakdown, and decrease immediately thereafter [8]. Following human chorionic gonadotropin (hCG) administra-

tion, AREG can be found in FF whereas, even though BTC and EREG can potentially be up-regulated by hCG, their protein levels in human FF are minimal. LH can also stimulate MGCs to secrete AREG, which activates EGFR signaling in cumulus cells and regulates processes such as oocyte maturation, cumulus development, and ovulation [9].

According to Zamah et al., AREG is the most significant EGFR ligand in human FF and might be used as a predictor of follicle growth, which could be important for in vitro fertilization (IVF) patients [10]. Furthermore, Inoue et al. (2009) indicated that the amphiregulin concentrations in FF affected oocyte quality and pregnancy outcomes [11].

The published data on AREG mRNA expression and infertility is still limited. Although some studies have pointed to a significant correlation between LH-induced AREG mRNA expression in human granulosa cells and various IVF parameters, such as the number of retrieved oocytes and embryo quality [12], the signaling pathway of the LH receptor involved in regulating amphiregulin expression has not yet been completely described [13].

The use of specific biomarkers and carrying out necessary changes to the EPA has been a topic of constant discussion since the number of ICSI/IVF cycles in Germany increased significantly [14]. Based on the aforementioned facts and due to the limitations of studies on IVF patients [10, 11] in which pooled follicular samples were used, the main aim of this study was to define the possible impact of amphiregulin on oocyte maturation, fertilization rate, and embryo quality. In addition, a special focus of the research was to define whether there is a difference in amphiregulin mRNA expression based on oocyte maturity.

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 ICSI were recruited at the Fertility Center of the University Clinic of Saarland in Homburg, Germany, between May 2021 and May 2022. Patient ages ranged between 23 and 40 years. Patients were recruited according to the following criteria: age between 18 and 40; inability to achieve natural pregnancy over 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 maximum stimulation; history or presence of tumors; the presence of an ovarian cyst > 25 mm; use of testicular or epididymal sperm; cryptozoospermia or strict teratospermia (< 2 % of normal sperm morphology).

Stimulation protocol

Twenty patients of the study group underwent controlled ovarian stimulation with the antagonist GnRH protocol, where the starting doses of recombinant follicle-stimulating hormone (rFSH) (Gonal-F; Merck Europe, Darmstadt, Germany) were based on serum AMH levels, antral follicle counts, or previous responses to ovarian stimulation. Subsequent doses were adjusted based on monitoring of ovarian responses using serial ultrasound and serum estradiol measurement. A different type of stimulation protocol was used for 13 patients aged more than 35 years or with AMH levels lower than 1.0 ng/ml. Ovarian stimulation was initiated on the second day of the menstrual cycle by administering combined recombinant follicle-stimulating hormone (rFSH) and recombinant luteinizing hormone (rLH) (Pergoveris, Merck Europe, Darmstadt, Germany). From the fifth day of stimulation therapy, a daily dose of 0.25 mg GnRH antagonist (Cetrotide; Merck Europe, Darmstadt, Germany) was administered. In each case, a human chorionic gonadotropin (Ovitrelle; Merck Europe, Darmstadt, Germany) injection was used to trigger final oocyte maturation, and ultrasound-guided ovum retrieval was performed approximately 36 h later.

Follicular fluid aspiration and mural granulosa cell isolation

Oocyte retrieval was performed transvaginally at 34 to 36 hours after hCG administration. The FF contained in each follicle was collected independently. For each patient, between one and five follicles were aspirated individually. Due to the possible risk of bleeding and the prolonged time required for the procedure, a maximum of 5 follicles was retrieved by individual aspiration, even in patients with more than 5 follicles.

In the time following COC harvesting, FF was replaced using 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. The supernatant was stored at − 80 °C in CryoTube vials (Nunc, Denmark) in aliquots until assayed.

Subsequently, 2 ml of phosphate buffer saline (PBS) were added to the pellet, and the diluted solution was slowly layered using a 40 : 80 % PureSperm (Sigma Aldrich) density gradient and centrifuged at 2500 rpm for 30 min. 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 RNALater Stabilization Reagent (Qiagen, Germany) and cryostored at − 80 °C until RNA isolation.

ICSI, embryo culture, and embryo assessment

Each COC was denuded separately and the maturation status was determined as mature oocyte (MII), immature oocyte (MI), germinal vesicle (GV), or empty zona pellucida. Oocytes (M II and MI) were fertilized using conventional ICSI procedures and placed immediately after injection in the sequential culture medium G-1 Plus (Vitrolife, Goteborg, Sweden) and incubated at 37 °C, with 18 % O_2, and 6 % CO_2. The oocytes were examined for fertilization on the following day within 18 hours after the injection, and only normally fertilized oocytes (those with two pronuclei) were cultivated further.

On day 2 (44–48 h after injection) and day 3 (68–72 h 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) [15].

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

Enzyme-linked immunosorbent assay (ELISA)

Quantitative determination of AREG concentrations in FF was done with ELISA according to the instructions for Human Amphiregulin Quantikine Kit (R&D Systems Inc., Minneapolis, MN, USA). All samples were analyzed in duplicate, and all reagents were prepared according to the manufacturer's instructions. Amphiregulin concentrations (pg/ml) were converted and presented as ng/ml.

Since production of AREG is an essential component of the paracrine function of the LH ovulatory signal, LH concentrations were also determined. The automated Cobas 8000 analyzer (Module e801; Roche Diagnostics, Germany) and Elecsys Kits (Roche Diagnostics) were used to measure the concentration of LH in FF.

Gene expression of amphiregulin (*AREG*) and luteinizing hormone/chorionic gonadotropin receptor (*LHCGR*) 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 reverse transcription of complementary DNA (cDNA).

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 with the Bio-Rad S1000 (Bio-Rad, USA). Samples were stored at − 20 °C until a real-time quantitative polymerase chain reaction (RT-qPCR) was carried out.

Expression of *AREG* and *LHCGR* was analyzed with the StepOnePlus Real-Time PCR System (Applied Biosystems, CA, USA) using TaqMan Fast Advanced Master Mix (Applied Biosystems) and the TaqMan gene expression assays for *AREG* (Hs00950669_m1) and *LHCGR* (Hs00174885_m1).

All listed components were pipetted by the liquid handling robot QIAgility (Qiagen, Germany) into a 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 with the actin-beta reference gene [*ACTB*] (Hs99999903_m1) using the relative quantification $2^{-\Delta\Delta Ct}$ method where the mean value of mRNA transcripts from each probe is set as one for each gene [16].

Statistical analysis

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 analysis was used to detect a correlation between the concentrations of proteins and gene expression levels, based on the number of mature oocytes, fertilization rate, and the percentage of good-quality embryos. Receiver operating characteristic (ROC) analysis was used to investigate the diagnostic performance of the parameters using 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

Clinical characteristics of participants

A total of 108 oocytes from the 33 patients were included in the study. Details on the case series are given in ▶ **Fig. 1**. For the study, proposed oocytes were divided into groups based on maturity and fertilization, while embryos were divided according to quality.

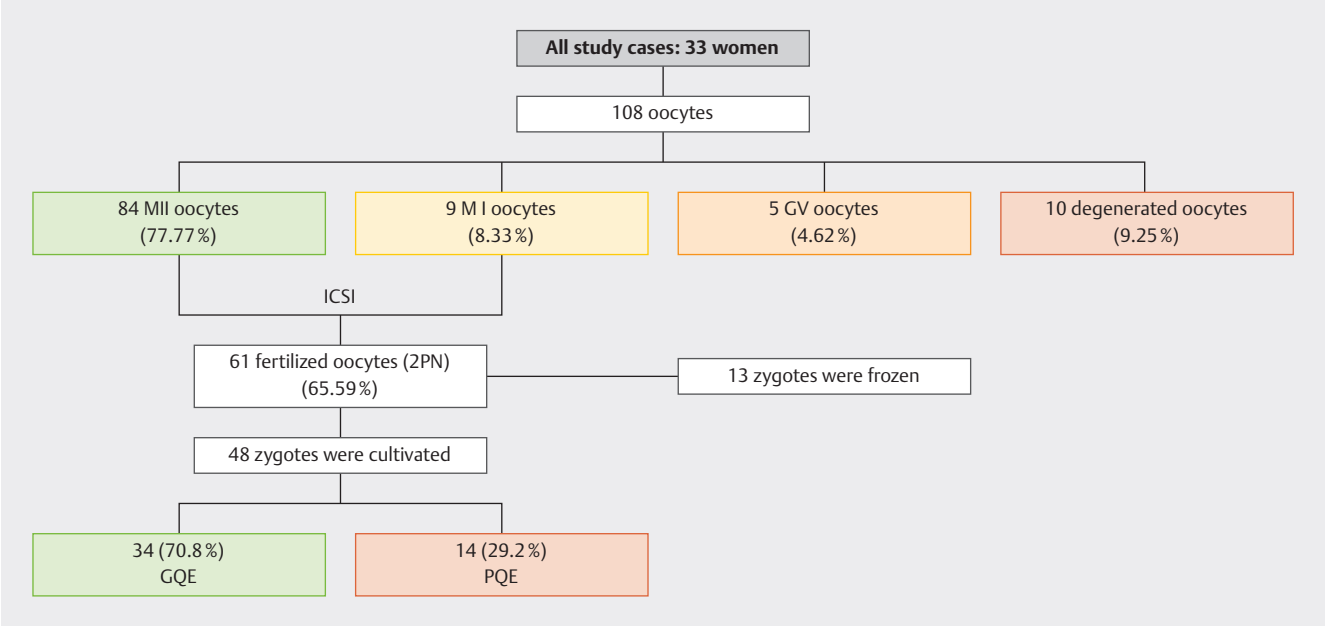
Patient age and stimulation protocol

The results obtained in the present study did not show any significant differences between AREG and LH concentrations in FF based on the stimulation protocol or patient age. Further evaluation based on different groups could be carried out because the obtained results were not biased by age or stimulation protocol.

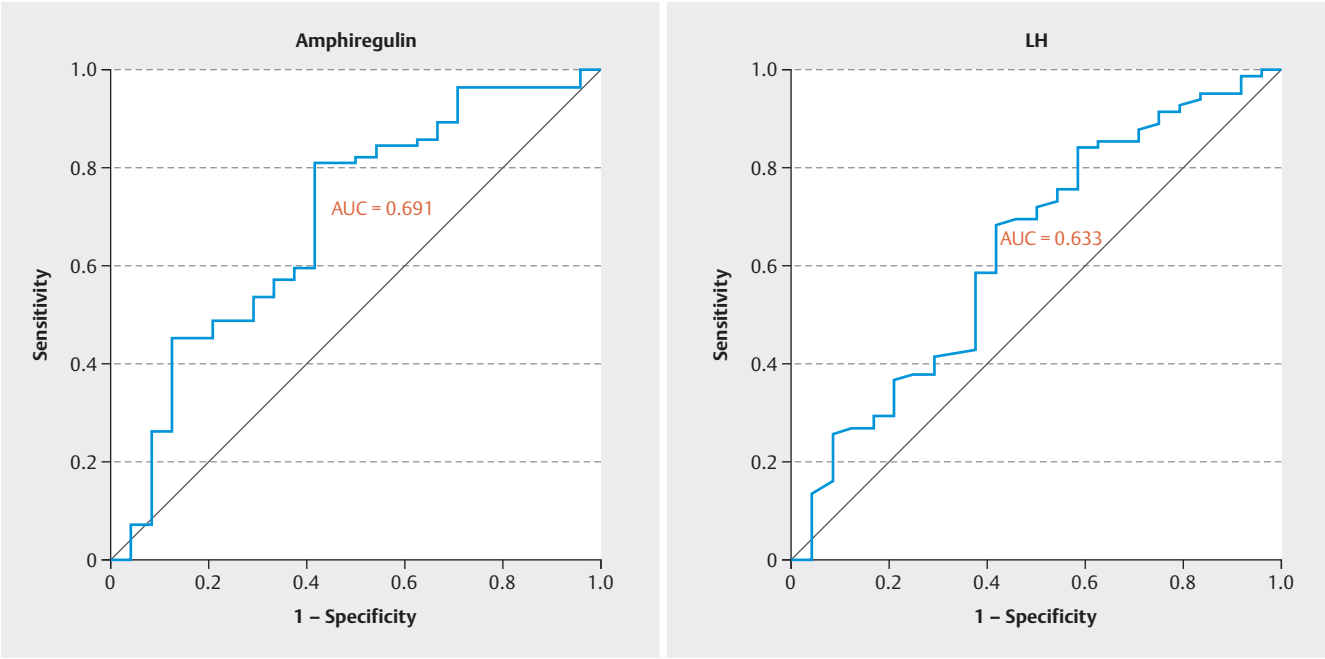
Impact of AREG and LH concentrations on oocyte maturation

Out of the total number of retrieved oocytes (n = 108), 84 (77.77 %) were mature MII oocytes, nine (8.33 %) were immature MI, five (4.62 %) cells were at the GV stage, and 10 (9.25 %) cells had degenerated. A comparative analysis of protein concentrations within the FF was done and the results indicated that the concentrations of AREG and LH differed significantly between the mature and immature oocyte groups (▶ **Table 1**). Moreover, the concentrations of both AREG and LH were elevated in the mature oocyte group.

Univariate logistic regression analysis was performed to evaluate the impact of AREG and LH on oocyte maturation. The results demonstrated that AREG (OR: 1.01; CI: 1.002–1.025; p = 0.020) as well as LH (OR: 0.762; CI: 0.600–0.967; p = 0.026) concentrations in FF could significantly affect oocyte maturation. Predictive



▶ **Fig. 1** Details of the case series (fertilization rate and embryo quality). 2 PN = fertilized oocyte; GQE = good quality embryo; GV = germinal vesicle; MI = immature oocytes; MII = mature oocytes; PQE = poor quality embryo.



▶ **Fig. 2** ROC curves of the predictive performance of AREG and LH concentrations for oocyte maturation. The AUC indicates moderate predictive values for both factors.

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 = 0.691 and for LH was AUC = 0.633 (▶ **Fig. 2**).

Since the univariate logistic regression analysis confirmed statistical significance, AREG and LH were included in a multiple regression analysis. The data confirmed the significant impact of both studied proteins on oocyte maturation (▶ **Table 2**).

▶**Table 1** Comparison of protein concentrations in FF between mature and immature oocyte groups (n = oocyte number; p ≤ 0.05 was considered statistically significant).

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**
LH (mIU/ml)	1.21	0.10	9.97	0.88	0.10	7.72	**0.046**

▶**Table 2** Multiple regression analysis: the impact of AREG and LH concentrations in FF on oocyte maturation.

Model	B	Std. Error	Beta	T	p value
(Constant)	0.612	0.108		5.645	0.000
AREG (ng/ml)	0.002	0.001	0.246	2.652	**0.009**
LH (mIU/ml)	0.058	0.022	0.243	2.622	**0.010**

▶**Table 3** Comparison of AREG and LHCGR gene expression levels (ΔCt) in 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 *LHCGR*	9.94	5.15	13.01	10.21	8.01	14.41	0.39

▶**Table 4** Comparative analysis of AREG expression levels and fold changes in mature and immature oocyte 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

Correlation between gene expression and oocyte maturation

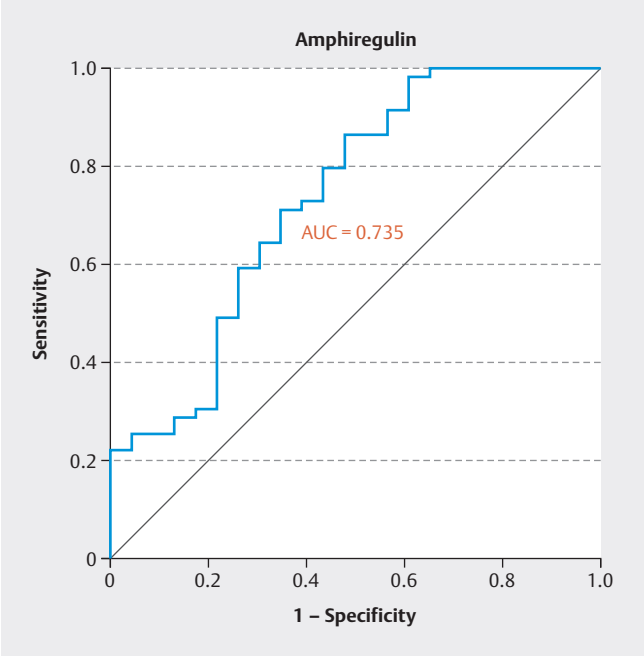
The relative concentrations of mRNA (mean ΔCt values) for the *AREG* differed significantly differently between the mature and immature oocyte groups (p = 0.031) (▶ **Table 3**).

In addition, univariate logistic regression analysis confirmed that *AREG* expression correlated with oocyte maturity (OR: 0.49; CI: 0.26–0.91; p = 0.024).

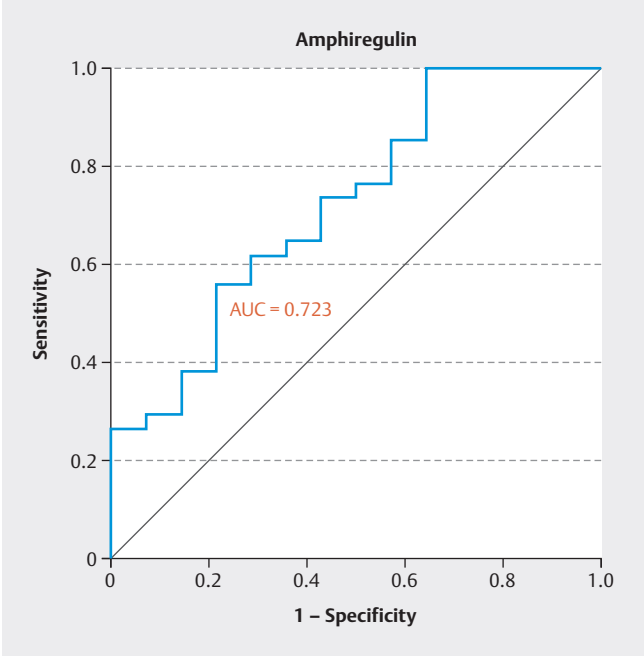
Since the correlation between ΔCt and the level of gene expression is inverse, higher ΔCt values indicate a decrease in gene expression. Therefore, ▶ **Table 4** shows that gene expression of *AREG* was downregulated in the immature oocyte group.

Impact of AREG and LH concentrations on fertilization rate

Out of the 108 oocytes included in the study, ICSI was carried out with 93 oocytes (84 MII and 9 MI). The fertilization rate was 67.74 % (n = 63), and 61 oocytes were correctly fertilized (2 PN). Of the nine MI oocytes, two were correctly fertilized. However, due to possible bias in the result, MI oocytes were excluded from further statistical analysis. When protein concentrations in FF between fertilized and unfertilized oocytes were compared, the results indicated that AREG and LH differed significantly between the fertilized and unfertilized oocyte groups (▶ **Table 5**). Moreover, the AREG and LH concentrations were significantly elevated in the fertilized oocyte group.



▶ **Fig. 3** The ROC curve of the predictive performance of AREG concentrations for oocyte fertilization. The AUC indicates a fair level of accuracy in distinguishing between fertilized and unfertilized oocytes based on AREG concentration.



▶ **Fig. 4** The ROC curve of the predictive performance of AREG concentrations for embryo quality. The AUC indicates a good level of accuracy in distinguishing between good and low-quality embryos based on AREG concentrations.

▶**Table 5** Comparison of protein concentrations in FF between the fertilized and unfertilized oocyte groups (n = oocyte number; $p \leq 0.05$ was considered statistically significant).

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**
LH (mIU/ml)	0.93	0.15	5.77	0.64	0.16	3.45	**0.025**

Univariate logistic regression analysis was additionally performed to evaluate the impact of AREG and LH on the fertilization rate. The results demonstrated that AREG 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 (▶ **Fig. 3**). In contrast, according to the results of the univariate logistic regression analysis, LH concentrations did not significantly affect oocyte fertilization ($p = 0.123$).

Correlation between gene expression and oocyte fertilization

Despite the fact that protein concentrations in FF differed between fertilized and unfertilized oocyte groups, gene expression did not differ significantly between the groups (▶ **Table 6**).

Impact of AREG and LH concentrations on embryo quality

Because of the Embryo Protection Act, 13 zygotes of the 61 fertilized oocytes were frozen, while 48 were cultivated. Thirty-four embryos (70.8 %) were good quality (GQE) and 14 (29.2 %) were poor quality (PQE).

A comparison of protein concentrations in FF between GQE and PQE showed that AREG and LH concentrations were elevated in the good-quality embryo group, and the difference was statistically significant (▶ **Table 7**).

Univariate logistic regression analysis was performed to determine the impact of AREG and LH concentrations on embryo quality. The results demonstrated that AREG concentrations could significantly affect embryo quality (OR: 1.02, CI: 1.00–1.04; $p = 0.003$). In this case, the area under the ROC curve was determined to be AUC = 0.723 (▶ **Fig. 4**). In contrast, univariate logistic

▶**Table 6** Comparison of AREG and LHCGR gene expression levels (ΔCt) in 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 *LHCGR*	10.23	5.15	13.01	9.71	7.27	12.02	0.278

▶**Table 7** Comparison of protein concentrations in FF between Good Quality Embryo and Poor Quality Embryo groups (n = oocyte number; p ≤ 0.05 was considered statistically significant).

Parameter	Good Quality Embryo group (n = 34)			Poor Quality Embryo group (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
LH (mIU/ml)	1.15	0.37	5.77	0.64	0.46	1.39	0.017

▶**Table 8** Comparison of AREG and LHCGR gene expression levels (ΔCt) in Good Quality Embryo and Poor Quality Embryo groups.

Parameter	Good Embryo Quality group (n = 34)			Poor Embryo Quality group (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 *LHCGR*	10.27	5.15	11.75	9.81	8.57	12.85	0.586

regression analysis showed that LH concentrations did not significantly affect embryo quality (p = 0.072).

Correlation between gene expression and embryo quality

Differences in the relative expression level of studied genes (*AREG*, *LHCGR*) did not reach statistical significance when GQE and PQE were compared (▶ **Table 8**).

Discussion

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 unknown causes of infertility. As the most important outcomes of ICSI are the fertilization rate and embryo quality, identifying possible biomarkers that affect those parameters is still one of the main challenges in reproductive medicine.

Unlike many other publications, single/individual aspiration of follicles was done in the present study, which enabled a 1 : 1 correlation with retrieved oocytes. Moreover, the oocytes retrieved after fertilization were followed and embryo quality was evaluated. Because of the above-described study design, the results obtained are especially useful and applicable in Germany due to the Embryo Protection Act.

Oocyte maturation and protein concentrations

Female fertility is highly dependent on normal oocyte development, and oocyte quality is a significant rate-limiting factor in ART techniques such as ICSI. Considering the EGF signaling network's essential function in the ovulatory cascade, it can also be expected to be crucial for oocyte developmental competence [5].

The results in our study indicate that AREG and LH concentrations in FF differ significantly between immature and mature oocytes, and were higher in the mature group. Differences in AREG concentration were in accordance with previously published data [10, 17]. 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 higher rate of MII oocyte development [18].

It is well known that the oocyte maturation process is initiated when an LH signal is generated in the ovarian follicle. The results of our study confirm that the LH concentration is lower in the follicles where immature oocytes are developed, which is consistent

with many published papers [19, 20, 21]. The impact of LH on oocyte maturation was confirmed in our study as well as in many previous studies: the presence of LH in FF is crucial for oocyte development [10, 20, 22].

Oocyte maturation and expression of studied genes

Some studies have indicated that *AREG* expression may play the most important role in oocyte maturation [23, 24, 25]. According to Huang et al., *AREG* mRNA induction in human granulosa cells (GCs) or COCs is connected to oocyte meiotic development, the number of retrieved oocytes, as well as overall ICSI outcomes [12].

The results of our study are in accordance with the findings of other studies [17, 26] 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 [23].

LHCGR is exclusively expressed in the MGCs of the ovarian follicle. Additionally, *LHCGR* expression in humans is highest in MGCs in preovulatory follicles [27]. However, *LHCGR* expression is suppressed by an LH surge, and the LH surge downregulates *LHCGR* expression in preovulatory follicles in women [28, 29].

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 of Maman et al. [30]. However, the difference between the groups was not significant. Huang et al. reported that *LHCGR* expression in MGCs did not differ or correlate with IVF outcomes [12].

Fertilization rate and protein concentrations

The main factor behind oocyte quality and oocyte maturation after ICSI is successful fertilization [31]. The results of our study indicate that oocytes obtained from follicles with a high concentration of AREG have a higher fertilization rate, which is in accordance with the literature [12]. Contrary to our results, Inoue et al. reported a non-significant correlation between AREG levels and fertilization rate [10]. However, in the study by Inoue et al., FF samples 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. However, since many studies 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.

The results indicate that LH concentrations in FF differ significantly 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 studies [20, 32].

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 [33, 34, 35]. In the present study, the expression of the 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., who reported that *AREG* expression levels correlated positively with the number of 2 PN while *LHCGR* correlated negatively with fertilization [12]. 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 has been confirmed in previous studies [36, 37]. Therefore, the variance in detection times might also account for the discrepancy.

Embryo quality and protein concentrations

The selection of high-quality embryos continues to be a major challenge for assisted reproductive technology. Globally, there is a lack of a unique evidence-based and globally accepted standards for evaluating embryos and identifying an embryo with the highest implantation potential.

In this study, AREG differed significantly between good and poor-quality embryo groups: the oocyte that 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., who reported that AREG concentrations did not differ statistically between good and poor embryos [11]. Due to the limited number of studies, we can only compare our results with those of Inoue et al. However, the study design used by Inoue et al. involved pooled FF samples which prevents a direct correlation with embryo quality outcomes.

In addition to AREG, the concentrations of LH differed statistically between good and poor-quality embryo groups, which is in line with published data [32, 38, 39]. Although the biological role of LH has been confirmed in many studies, there are only a few studies on IVF patients and the concentration of LH in FF [40, 41]. Moreover, some recent studies have reported that recombinant LH supplementation during GnRH antagonist cycles may improve embryo quality as well as the live birth rate [42, 43]. The obtained results are very promising and could easily be implemented in everyday clinical practice as well as being very useful for embryo selection in specific countries.

Embryo quality and gene expression

Although the concentration of proteins differed between good and poor embryo quality groups, gene expression did not differ significantly between these groups. To the best of our knowledge, little information is available on *AREG* 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. [26]). Conversely, our findings contrast with data published by Huang et al., who reported that *AREG* expression was positively correlated with embryo quality [12]. Additionally, the same group of authors reported that *LHCGR* expression did not differ according to embryo quality, which is consistent with our results.

Study limitations

This study is limited by its exclusive focus on ICSI couples and its exclusion of IVF patients, which may affect the applicability of the findings. The study design also prevented correlation analysis

between total oocyte yield and amphiregulin concentration, potentially limiting insights into oocyte quality across the entire yield. Furthermore, the study did not examine the relationship between amphiregulin concentration and blastocyst quality or transfer outcomes, which could be explored in future studies.

Conclusion

The results of the current study indicate that AREG concentrations in FF affect oocyte maturation, the fertilization rate, and, most importantly, embryo quality.

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

The gene expression of the studied genes *AREG* and *LHCGR* indicates that only the *AREG* expression significantly affects oocyte maturation, with *AREG* expression being downregulated in the immature oocyte group. *LHCGR* did not differ between groups and did not affect maturation, fertilization, or embryo quality.

In summary, given the complexity of the numerous independent processes involved in oocyte development, a single biomarker is unlikely to predict the outcome of ICSI. However, the obtained results, together with previous studies, are very important for the future development of infertility treatment, especially in Germany due to its EPA. AREG may offer prognostic information which could aid the selection of the most viable embryos. 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.

Clinical Trial

Registration number (trial ID): 146/19 | Ethics Committee of the Medical Association of Saarland | Type of Study: Prospective

Funding information

| University Hospital of Saarland, Department of Gynecology, Obstetrics and Reproductive Medicine |

Conflict of Interest

The authors declare that they have no conflict of interest.

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