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Impact of genetic polymorphisms on the degree of ovarian response to gonadotrophin stimulation in patients undergoing ICSI treatment

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1. Abstract:

Infertility is a common health problem associated with various medical, emotional, and social implications. It affects one in four couples in developing countries and one in six couples worldwide at least once during their reproductive lifetime. Intracytoplasmic sperm injection (ICSI) is the most common technique of assisted reproduction, accounting for approximately three-quarters of all infertility treatments worldwide.

Despite the availability of new ovarian reserve markers and improvements in the methodologies that support personalization of In vitro fertilization (IVF) treatment protocols, an accurate definition of the modalities for applying personalized therapy to optimize efficacy and daily clinical management is still required.

Genetic differences between patients are most likely the main factor responsible for different responses to the drugs. The gonadotrophin hormones, follicle-stimulating hormone (FSH) and luteinising hormone (LH), control folliculogenesis, and naturally occurring polymorphisms in genes encoding these hormones and their receptors may affect the ovarian response. However, a definite association between genetic polymorphisms and ovarian responses to gonadotrophins still needs to be determined.

The purpose of this study was to detect the association between five single nucleotide polymorphisms of the following four genes *follicle-stimulating hormone receptor (FSHR), anti-Mullerian hormone (AMH), luteinizing hormone/choriogonadotropin receptor (LHCGR), estrogen receptor (ESR1),* and the degree of the ovarian response to gonadotrophin in Egyptian Patients undergoing IVF/ICSI therapy.

The study population was Egyptian Women undergoing ICSI treatment. Two hundred and eighty women have participated in the study with mean aged 20 -35 years old. The clinical part of the study was performed in the IVF unit Sohag, Egypt starting with patient recruitment and selection. Preparatory phase and investigations before ICSI then Controlled ovarian stimulation (COS) by Long Gonadotrophin releasing hormone (GnRH) agonist protocol, patient follow-up, and samples collection. The patients were classified according to ovarian response into three groups: normal responders (retrieved oocytes=4-15) (n= 80), poor responders (retrieved oocyte <4) (n= 92), and high responders (retrieved oocytes= > 15) (n= 108). Approximately 5.0 ml of blood samples were collected from all participants in EDTA tubes and stored at -80°C until the genetic analysis to be performed in assisted reproduction

and Genetics Unit, Saarland University, Germany. Genomic DNA was extracted from the blood samples, and the PCR and DNA sequencing were performed to compare the variation in the DNA sequencing between the different study groups. The quantitative PCR (qPCR) was performed to evaluate the expression level of the following genes: *FSHR, AMH, LHCGR, ESR1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* as a reference gene among the study groups.

Data analysis was performed by SPSS software. The Kruskal–Wallis (H-test) and Mann-Whitney (U-test) were applied to compare the median quantitative variables between the study groups. The Spearman rank correlation was used to evaluate the association between genetic polymorphisms (rs4073366, rs6166, rs6165, rs2234693; rs17854573) and the different investigated parameters including clinical parameters e.g., age, Body mass index (BMI), hormonal parameters e.g., AMH level, FSH Level and ICSI cycle parameters e.g., dose of gonadotrophin, number of collected oocytes, number of fertilised oocytes and number of transferred embryos. Regarding the qPCR data, the comparative analysis was used to calculate the expression level of tested genes in the cases "poor/high responder" versus controls "normal responder". The results were considered statistically significant when P-value ≤ 0.05.

The data analysis of DNA sequencing showed a significant difference in the frequency of the following genotypes *FSHR (rs6166), AMH (rs17854573), and ESR1 (rs2234693)* in the poor responders compared to normal responders ($P \leq 0.001$, $P = 0.010$, and $P \leq 0.001$) respectively. No significant difference has been found in the frequency of *LHCGR* (rs4073366) and *FSHR* (rs6165) genotypes in patients with poor ovarian response compared to others with a normal ovarian response ($P = 0.312$ and $P = 0.192$).

Besides, no significant difference has been found in the frequency of the *FSHR* (rs6166), *FSHR* (rs6165), *ESR1* (rs2234693), *AMH* (rs17854573) or *LHCGR* (rs4073366) genotypes in high responders compared to normal responders ($P = 0.074$, $P = 0.353$, $P = 0.060$, $P = 0.060$ and $P = 0.091$ respectively).

Moreover, a significant difference has been found between the poor responders and the normal responders in the total dose of gonadotropin, the number of stimulation days, the number of collected oocytes, the number of injected oocytes, the number of fertilized oocytes, and the number of embryo transfer.

On the other hand, the analysis of qPCR results revealed a variation between the study groups (poor, normal, and high response) in the expression levels of *FSHR* (rs6166), FSHR (rs6165), AMH, *LHCGR*, and *ESR1* gene ($P \leq 0.0001$).

In conclusion, The results of this study suggest that polymorphisms in the genes for key reproductive hormones (AMH, FSHR, and ESR1) in combination with the patient's clinical characteristics and hormonal biomarkers could be used to predict the ovarian response to gonadotrophins, to personalize and adjust the dose of gonadotrophins before starting the stimulation protocol, to improve efficacy and to avoid possible complications such as cycle cancellation and OHSS; and, finally, to improve the pregnancy rate in patients undergoing ICSI treatment.

2. Zusammenfassung:

Infertilität ist ein häufiges Gesundheitsproblem, das mit verschiedenen medizinischen, emotionalen und sozialen Auswirkungen einhergeht. Es betrifft eines von vier Paaren in Entwicklungsländern und eines von sechs Paaren weltweit mindestens einmal im Laufe ihres reproduktiven Lebens. Die intrazytoplasmatische Spermieninjektion (ICSI) ist die häufigste Technik der assistierten Reproduktion und macht weltweit etwa drei Viertel aller Unfruchtbarkeitsbehandlungen aus.

Obwohl neue Marker für die ovarielle Reserve zur Verfügung stehen und die Methoden zur Personalisierung der Behandlungsprotokolle für die In-vitro-Fertilisation (IVF) verbessert wurden, ist eine genaue Definition der Modalitäten für die Anwendung der personalisierten Therapie zur Optimierung der Wirksamkeit und des täglichen klinischen Managements nach wie vor erforderlich.

Genetische Unterschiede zwischen den Patienten sind höchstwahrscheinlich der Hauptfaktor, der für die unterschiedlichen Reaktionen auf die Medikamente verantwortlich ist. Die Gonadotropin Hormone FSH und luteinisierendes Hormon (LH) steuern die Follikulogenese, und natürlich vorkommende Polymorphismen in Genen, die für diese Hormone und ihre Rezeptoren kodieren, können die Reaktion der Eierstöcke beeinflussen. Ein eindeutiger Zusammenhang zwischen genetischen Polymorphismen und der Reaktion der Eierstöcke auf Gonadotropine muss jedoch erst noch festgestellt werden.

Ziel dieser Studie war es, den Zusammenhang zwischen fünf Einzelnukleotid-Polymorphismen der folgenden vier Gene *Follikel-stimulierendes Hormon-Rezeptor (FSHR), Anti-Mullerian-Hormon (AMH), Luteinisierendes Hormon/Choriogonadotropin-Rezeptor (LHCGR), Östrogenrezeptor (ESR1)* und dem Grad der ovariellen Reaktion auf Gonadotropin bei ägyptischen Patientinnen, die sich einer IVF/ICSI-Therapie unterziehen, zu ermitteln.

Die Studienpopulation bestand aus ägyptischen Frauen, die sich einer ICSI-Behandlung unterzogen. Zweihundertachtzig Frauen, mit einem Mittelalter von 20 bis 35 Jahren, nahmen an dieser Studie teil. Der klinische Teil der Studie wurde in der IVF-Abteilung in Sohag, Ägypten durchgeführt. Zuerst erfolgten die Rekrutierung und Auswahl der Patientinnen, dann die Vorbereitungsphase und Untersuchungen vor ICSI danach die kontrollierte ovarielle Stimulation (COS) durch langwirkende GnRH-Agonisten-Protokoll.

Anschließend erfolgte das Follow-up. Nach der ovariellen Reaktion wurden die Patientinnen in drei Gruppen eingeteilt: normale Responder (entnommene Eizellen = 4-15) (n = 80), schlechte Responder (entnommene Eizellen < 4) (n = 92) und hohe Responder (entnommene Eizellen =>) 15) (n = 108). Ca. 5,0 ml Blutproben wurden von allen Teilnehmerinnen in EDTA-Röhrchen gesammelt und bei -80° C, bis die genetische Analyse in der Abteilung von der assistierten Reproduktion und Genetik an der Universität des Saarlandes in Deutschland, gelagert. Genomische DNA wurde aus den Blutproben extrahiert und die PCR und die DNA-Sequenzierung wurden durchgeführt, um die Variation in der DNA-Sequenzierung zwischen den verschiedenen Studiengruppen zu vergleichen. Die quantitative PCR (qPCR) wurde durchgeführt, um das Expressionsniveau der folgenden Gene zu bewerten: *FSHR, AMH, LHCGR, ESR1 und Glyceraldehyd-3-phosphat-Dehydrogenase (GAPDH)* als Referenzgen in den Studiengruppen.

Die Datenanalyse wurde mit der Software SPSS durchgeführt. Der Kruskal-Wallis-Test (H-Test) und der Mann-Whitney-Test (U-Test) wurden angewandt, um den Median der quantitativen Variablen zwischen den Studiengruppen zu vergleichen. Die Spearman-Rangkorrelation wurde verwendet, um den Zusammenhang zwischen dem genetischen Polymorphismus (rs4073366, rs6166, rs6165, rs2234693; rs17854573) und den verschiedenen untersuchten Parametern zu bewerten einschließlich klinischer Parameter wie Alter, Body-Mass-Index (BMI), hormoneller Parameter wie AMH-Spiegel, FSH-Spiegel und ICSI-Zyklusparameter wie Gonadotropin-Dosis, Anzahl der entnommenen Eizellen, Anzahl der befruchteten Eizellen und Anzahl der übertragenen Embryonen. In Bezug auf die qPCR-Daten wurde die vergleichende Analyse verwendet, um das Expressionsniveau der getesteten Gene in den Fällen "schlechte/hohe Responder" gegenüber den Kontrollen "normale Responder" zu berechnen. Die Ergebnisse wurden als statistisch signifikant angesehen, wenn der P-Wert $\leq 0{,}05$ war.

Die Datenanalyse der DNA-Sequenzierung zeigte einen signifikanten Unterschied bezüglich der Häufigkeit der folgenden Genotypen *FSHR (rs6166), AMH (rs17854573) und ESR1 (rs2234693)* bei den schlechten Respondern im Vergleich zu den normalen Respondern ($P \leq 0{,}001$, $P = 0{,}010$ und $P. \leq 0{,}001$). Es wurde kein signifikanter Unterschied in der Häufigkeit der Genotypen von *LHCGR (rs4073366) und FSHR (rs6165)* bei den schlecht ansprechenden Respondern im Vergleich zu den normalen Respondern beobachtet ($P \leq 0{,}312$ und $P = 0{,}192$).

Außerdem wurde kein signifikanter Unterschied in der Häufigkeit der Genotypen *FSHR (rs6166), FSHR (rs6165), ESR1 (rs2234693) , AMH (rs17854573) oder LHCGR (rs4073366)* bei den High-Respondern im Vergleich zu den normalen Respondern ($P \leq 0{,}074$) gefunden , $P \leq 0{,}35$ 3, $P \leq 0{,}060$, $P \leq 0{,}060$ bzw. $P \leq 0{,}091$).

Darüber hinaus wurde ein signifikanter Unterschied zwischen den schlechten Respondern und den normalen Respondern bezüglich der Gesamtdosis von Gonadotropin, der Anzahl der Stimulationstage, der Anzahl der gesammelten Eizellen, der Anzahl der injizierten Eizellen, der Anzahl der befruchteten Eizellen und der Anzahl der gefunden Embryotransfer sowie die Anzahl der kryokonservierten Embryonen. Andererseits zeigte die Analyse der qPCR-Ergebnisse einen Unterschied zwischen den Studiengruppen (schlechtes, normales und hohes Ansprechen) in den Expressionsniveaus der Gene *FSHR (rs6166), FSHR (rs6165), AMH, LHCGR und ESR1* ($P \leq 0{,}0001$).

Zusammenfassend zeigen die Ergebnisse dieser Studie, dass Polymorphismen in den Genen für die wichtige Fortpflanzungshormone (AMH, FSHR und ESR1) in Kombination mit den klinischen Merkmalen der Patientin und hormonellen Biomarkern verwendet werden könnten, um die ovarielle Reaktion auf Gonadotropen vorherzusagen, zu personalisieren und die Dosis von Gonadotropen vor Beginn des Stimulationsprotokolls anzupassen, um die Wirksamkeit zu verbessern und mögliche Komplikationen wie Zyklusabbruch und OHSS zu vermeiden; und schließlich zur Verbesserung der Schwangerschaftsrate bei Patientinnen, die sich einer ICSI-Behandlung unterziehen.

List of Abbreviations

A	Adenine
Ala	Alanine
AMH	Anti-Mullerian Hormone
AMHRII	*Anti-Müllerian Hormone (AMH) Receptor Type 2*
Asn	Asparagine
ART	Assisted Reproduction Techniques
BMP15	*Bone Morphogenetic Protein 15*
cDNA	Complementary DNA
COH	Controlled Ovarian Hyperstimulation
COS	Controlled Ovarian Stimulation
cAMP	Cyclic Adenosine Monophosphate
C	Cytosine
DNA	Deoxyribonucleic acid
EGF	Epidermal Growth Factor
E2	Estradiol
ESR	Estrogen Receptor
ESR-1	*Estrogen Receptor* 1
Erα	*Estrogen Receptors Alpha*
Erβ	*Estrogen Receptors Beta*
EDTA	Ethylenediaminetetraacetic acid
FSHR	*Follicle- Stimulating Hormone Receptor*
FSH	Follicle-Stimulating Hormone
SPSS	Statistical Package for the Social Sciences
GAPDH	*Glyceraldehyde 3-phosphate dehydrogenase*
GnRH agonist	Gonadotrophin-releasing hormone agonist
GDF9	Growth Differentiation Factor 9
G	Guanine
HCG	Human Chorionic Gonadotropin
HMG	Human Menopausal Gonadotrophins
IVF	In Vitro Fertilization
IGF-I	Insulin-Like Growth Factor 1
IL-1	Interleukin-1
ICSI	Intracytoplasmic Sperm Injection
Ile	Isoleucine

q-arm	Long arm
LH	Luteinizing Hormone
LHR	*Luteinizing Hormone Receptor*
LHCGR	*Luteinizing Hormone/Choriogonadotropin Receptor*
MIF	Mullerian-inhibiting factor
MIS	Mullerian-inhibiting substance
OHSS	Ovarian Hyperstimulation Syndrome
PCR	Polymerase Chain Reaction
PRL	Prolactin
qPCR	Quantitative Polymerase Chain Reaction
r-FSH	Recombinant FSH
RNA	Ribonucleic acid
Ser	Serine
p arm	Short arm
SNPs	Single-Nucleotide Polymorphisms
Thr	Threonine
T	Thymine
TSH	Thyroid-Stimulating Hormone
TGF-β	Transforming Growth Factor Beta
TGFa	Transforming Growth Factors
PvuII	Typr2 Restriction enzyme- Proteus hauseri
VEGF	*Vascular Endothelial Growth Factor*

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1. Introduction

1.1 Background

Infertility can be defined as the inability to conceive after 12 months or more of regular unprotected sexual intercourse **(WHO, 2018)**, which has many emotional, medical, and social consequences **(WHO, 2020).** It affects one in four couples in developing countries **(WHO, 2021)** and affects one in six couples worldwide at least once during their reproductive lifetime **(ESHRE, 2020).**

According to the European Society of Human Reproduction and Embryology (ESHRE), more than 9 million babies have been born worldwide since the first in vitro fertilization (IVF) baby was born in 1978. Intracytoplasmic sperm injection (ICSI) is the most common technique of assisted reproduction, accounting for approximately three-quarters of all infertility treatments worldwide **(ESHRE, 2020).**

The IVF protocol includes many measures, the cornerstone being controlled ovarian stimulation (COS), achieved by administering exogenous gonadotrophin, enabling the retrieval of a sufficient number of good quality oocytes, thus increasing the chances of success (**Boudjenah et al., 2012).**

The personalization of IVF protocols based on accurate prediction of ovarian response to gonadotrophins helps clinicians provide effective treatment regimens to IVF/ICSI patients and avoids complications, such as poor response associated with cycle cancellation and ovarian hyperstimulation syndrome (OHSS) **(La Marca and Sunkara, 2014).** Despite the availability of new ovarian reserve markers and improvements in the methodologies that support personalization of IVF treatment protocols, an accurate definition of the modalities for applying personalised therapy to optimise efficacy and daily clinical management is still required **(dos Reis Silva et al., 2016).** Furthermore, tailoring of the gonadotrophin dose to a particular population based only on the current ovarian reserve markers, such as basal follicle-stimulating hormone (FSH), antral follicle count, and anti-Mullerian hormone (AMH), does not improve the IVF/ICSI treatment outcome **(Lensen et al., 2018).**

Genetic differences between patients are most likely the main factor responsible for different responses to the drugs. The gonadotrophin hormones, FSH and luteinising hormone (LH), control folliculogenesis and naturally occurring polymorphisms in genes encoding these

hormones and their receptors may affect the ovarian response **(Greene et al., 2014)**. However, a definite association between genetic polymorphisms and ovarian responses to gonadotrophins still needs to be determined **(Riccetti et al., 2017).**

1.2 Physiology of the human ovary and folliculogenesis

Anatomically, the ovary is divided into three parts: an outer area, which is the cortex containing the germinal epithelium and follicles; a medullary area composed of connective tissue; and the hilum, containing blood vessels and nerves. The ovary has two important functions: gametogenesis for reproduction and synthesis of steroid hormones and other factors that are important for the body's endocrinological needs **(Hoffman et al., 2016).**

Folliculogenesis is a dynamic process that starts with the recruitment of a primordial follicle and ends with either ovulation or atresia (**Figure 1.1**).

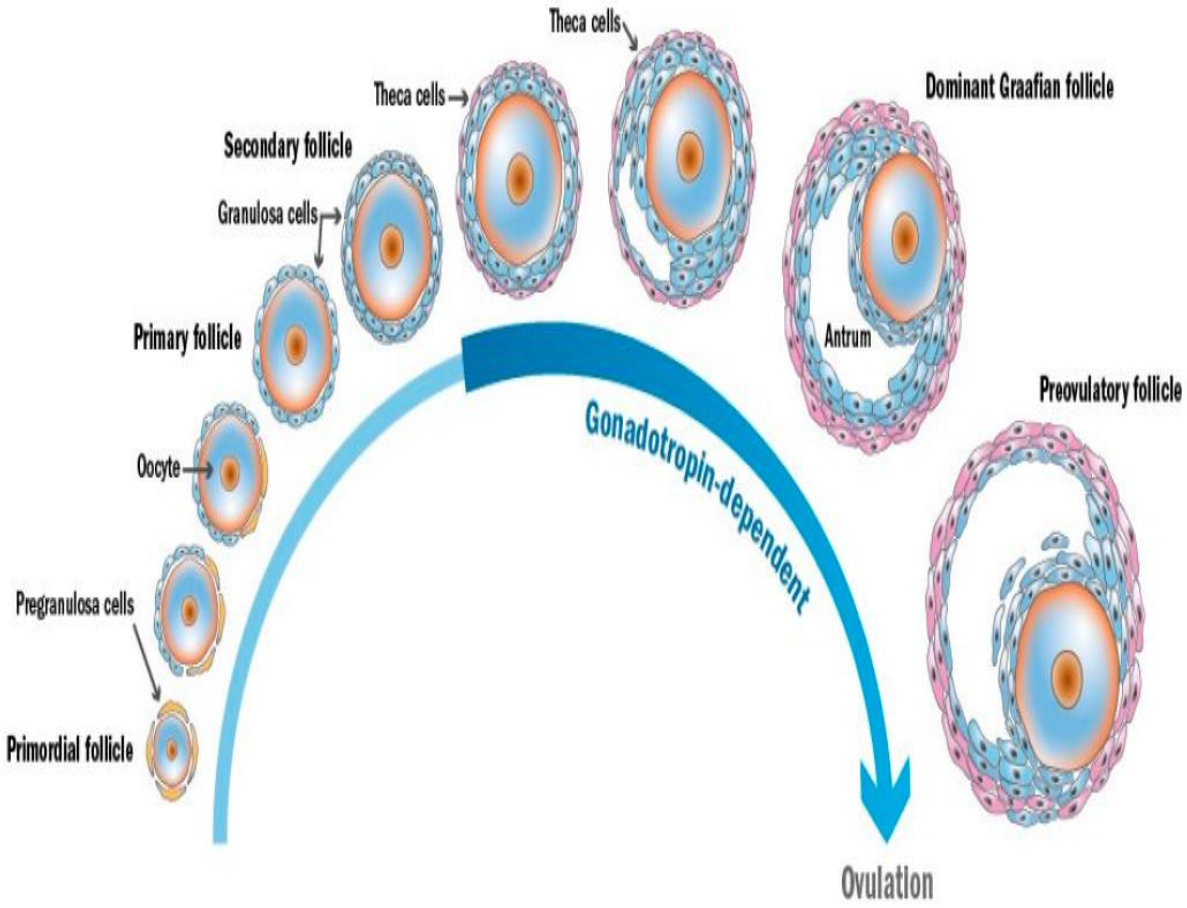

Figure 1.1: Stages of follicular development. [Adapted from **Hao et al., (2019)**].

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In women, folliculogenesis is a very long process, requiring approximately one year for a primordial follicle to grow and develop to the ovulation stage. The entry of an arrested primordial follicle into the growing follicle pool is known as primordial follicle recruitment or activation **(Williams and Erickson, 2000).** The recruitment process is relatively constant over the first three decades of a woman's life. However, the loss of non-growing follicles due to atresia continuously increases, resulting in a general loss of ovarian reserve and leading to a decrease in fertility from the age of 30 years and a marked decrease from 35 years of age **(Hansen et al., 2008).**

The oocyte and the surrounding granulosa and theca cells are controlled by FSH and LH, which are synthesised by the anterior pituitary gland under the control of gonadotrophin-releasing hormone (GnRH) from the hypothalamus. In addition, granulosa cells produce oestrogens, AMH, activins, and Inhibins **(Edson et al., 2009; Georges et al., 2014).**

Based on gonadotrophin requirements, follicular development can be divided into two phases: the gonadotrophin-independent phase (the preantral phase) that includes follicular growth from primordial to primary and secondary stages, and the gonadotrophin-dependent phase, (the antral phase) that includes the transition from the preantral to antral and preovulatory stages and to ovulation **(McGee and Hsueh, 2000).**

Gametogenesis begins during embryonic development through the migration of primordial germ cells to the genital ridge. The primordial germ cells grow to oogonia which are arrested at the diplotene stage of meiosis prophase I, where they are surrounded by a layer of flattened somatic cells to form primordial follicles **(Brevini and Georgia, 2012; Piprek, 2016)**. The primordial follicles represent the ovarian reserve, also known as the follicle pool. Although the follicle pool reaches a peak of 6–7 million primordial follicles at 20 weeks of gestation, this number decreases to 1–2 million at birth, and by puberty, only approximately 400,000 primordial follicles remain **(Boron and Boulpaep, 2012).**

After puberty, a large number of primordial follicles begin to be recruited into activation, a process which is irreversible. Under the control of locally produced growth factors, the oocytes grow and the surrounding pregranulosa cells differentiate into cuboidal granulosa cells, developing through primary follicles and secondary follicles before acquiring an antral cavity **(Dole et al., 2008; Paratcha and Ledda, 2008; Zhang et al., 2014).**

Only a few secondary follicles can develop to the preovulatory stage, with the majority undergoing atresia due to negative regulation by the dominant Graafian follicle. The growth of antral follicles is gonadotrophin-dependent, regulated by feedback mechanisms between GnRH, FSH, LH, and various growth factors **(Cossigny et al., 2012; Kishi et al., 2018)**.

The increasing levels of oestrogen in the preovulatory follicle results in a positive feedback loop, which causes an increase in LH levels. Consequently, the LH surge leads to a high concentration of LH, and 24 h later, to the release of the mature oocyte. This process is called ovulation. The LH surge stimulates the luteinisation of granulosa and theca cells and forms the corpus luteum, which is required for maintenance of the pregnancy (**Araujo et al., 2014; Georges et al., 2014; Orisaka et al., 2009).**

1.3 Controlled Ovarian Stimulation (COS)

Infertile patients can undergo IVF/ICSI therapy to conceive a healthy baby. Ovarian stimulation using exogenous gonadotrophins for multiple follicular development is a critical step in IVF/ICSI therapy. Various ovarian stimulation protocols in which FSH is the cornerstone hormone responsible for ovarian maturation have been established. To prevent endogenous LH surge and early ovulation, GnRH agonists or antagonists are administered simultaneously with gonadotrophins (**Macklon et al., 2006**).

Currently, highly purified and recombinant FSH (r-FSH) are the most common gonadotrophins used for stimulation of follicular development and retrieval of multiple mature oocytes **(Fatemi et al., 2012).** GnRH agonist, human chorionic gonadotrophin (HCG) or both are used to trigger oocyte ovulation (**Humaidan and Alsbjerg, 2014).**

1.4 Pharmacogenomics and Gene Polymorphisms

Single nucleotide polymorphisms (SNPs) are the mainstay in pharmacogenetics conceptualisation and represent approximately 90% of the genetic variability in the human genome **(International Human Genome Sequencing Consortium, 2004).**

A single nucleotide polymorphism (SNP) is a single DNA base substitution that is observed at a frequency of at least 1% in a given population. It accounts for 90% of the inter-individual variability (**Brookes, 1999**). SNPs are found in different genomic locations, including in the genome's coding regions known as exons (coding SNPs), in noncoding regions known as introns (intronic SNPs), or in regulatory regions between genes (intergenic SNPs). Most SNPs

are silent and do not affect the synthesis of the polypeptide chain; only a small percentage of SNPs affect gene function by changing or modifying the protein produced (missense SNPs) or causing premature termination (nonsense SNPs) **(Tennessen et al., 2012).**

Genetic polymorphisms can affect a drug's efficacy by altering its pharmacokinetics, pharmacodynamics, or both. Pharmacokinetics and pharmacodynamics are two major determinants of interindividual differences in drug responses. Pharmacokinetics deals with the amount of drug that needs to reach its target tissue, whereas pharmacodynamics deals with how well targets, such as receptors and enzymes, respond to various drugs **(Pirmohamed, 2014) (Figure 1.2).**

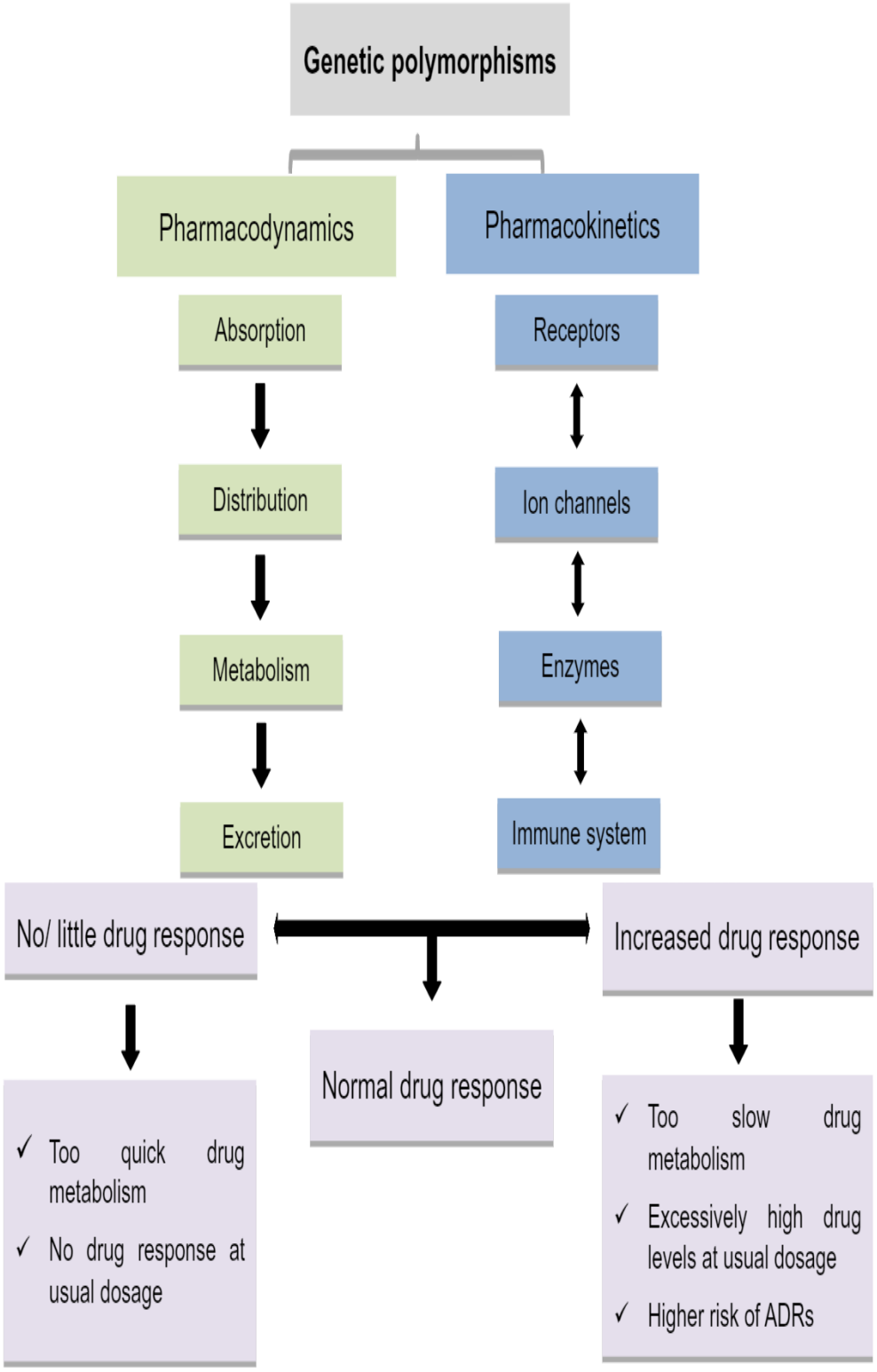

Figure 1.2: Effect of genetic polymorphisms on drug responses. [Adapted from **Ahmed et al., (2016)].**

1.5 Gene Polymorphisms and Ovarian Response to COS:

Certain Individuals with specific genotypes have the ability to rapidly metabolise drugs, such that increased doses are needed to reach optimal therapeutic levels, while others metabolise the same drug more slowly, meaning that smaller doses are necessary to avoid side effects. Accordingly, in assisted reproductive technology (ART), women respond to gonadotrophin stimulation in different ways, resulting in normal, poor, or high responders **(Nelson, 2017)**.

Several studies have discussed the common genetic variants in *follicle-stimulating hormone receptor (FSHR), oestrogen receptor (ESR)* and other genes that can be good predictors of COS outcome. These studies show the impact of individual variations on COS outcome and the ability to customise treatment according to the patient's genetic characteristics **(Huang et al., 2015)**.

1.5.1 *Anti-Mullerian hormone* (*AMH*) gene and its variants.

AMH gene is located at position 13.3 of the short (p) arm of chromosome 19 (19p13.3) (**Figure 1.3**). *AMH* is also known as: *Mullerian-inhibiting factor (MIF) and Mullerian-inhibiting substance (MIS).*

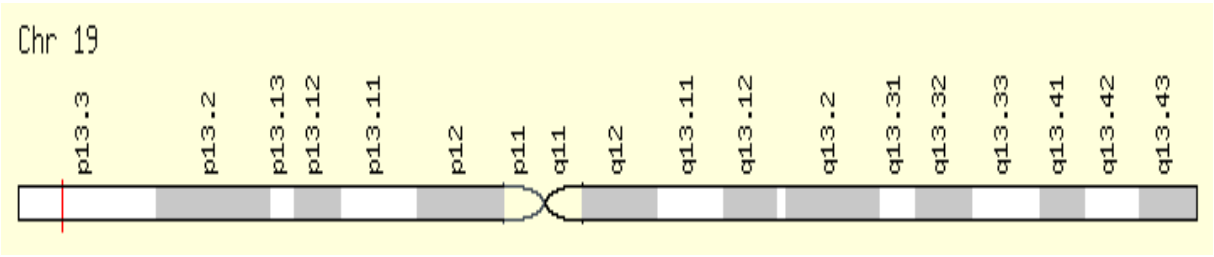

Figure 1.3: Cytogenetic location of the *AMH* gene [Adapted from **Gene Cards, (2021a)].**

Anti-Mullerian hormone (*AMH*) is one AMH is a transforming growth factor family that regulates follicular growth (**Di Clemente et al., 2003**). It is secreted by granulosa cells of preantral follicles (**Visser et al., 2006**). AMH regulates ovarian follicle recruitment and its sensitivity to FSH (**Durlinger et al., 2001**). It inhibits primordial follicle growth and decreases its sensitivity to FSH. Therefore, AMH plays a crucial role in regulating the recruitment of primordial follicles and in cyclic selection (**Durlinger et al., 2002**). It was recently shown that AMH serum level is an essential indicator of the primordial follicle pool and is positively correlated with the number of antral follicles. Moreover, it shows progressive reduction with advances in reproductive age (**van Rooij et al., 2005**). Therefore, AMH is an

important marker of ovarian reserve and is crucial in patients undergoing assisted reproduction (**Visser et al., 2006**).

Moreover, AMH is elevated in other pathological conditions where prenatal follicle numbers are elevated, such as polycystic ovary syndrome (PCOS) (**Laven et al., 2004**) and it can therefore be used as a diagnostic tool for PCOS (**Visser et al., 2006**).

Literature review: *AMH* gene Variants

Kevenaar et al., (2007) investigated the influence of the *AMH* Ile49Ser and *AMH type II receptor* 2482 A>G variants on the characteristics of the menstrual cycle. They found that the *AMH* Ser49 allele was associated with higher serum estradiol (E2) levels on menstrual cycle day 3 in a Dutch population ($P \leq 0.012$) and a combined Dutch and German cohort ($P \leq 0.03$). In addition, the *AMHR2* 2482G variant was associated with higher serum estradiol levels in the follicular phase in the Dutch population ($P \leq 0.028$), the German population ($P \leq 0.048$), and the combined cohort ($P \leq 0.012$). Carriers of both *AMH* Ser49 and *AMHR2* 2482G alleles had the highest follicular phase estradiol levels ($P \leq 0.001$).

Rigon et al., (2010) investigated the impact of *AMH* and *AMHRII* gene variants on treatment outcomes in women with idiopathic infertility. They found significant differences in the frequencies of –482 A>G and 146T>G polymorphisms in infertile patients compared with those in controls and concluded that *AMH* and *AMHRII* gene variants are associated with infertility.

Furthermore, **Zhao and Zhang (2013)** investigated the relationship between *AMH* and *AMHRII* gene polymorphisms and ovarian hyperstimulation syndrome (OHSS) in patients who underwent IVF/ICSI treatment. They found that SNPs G>T at position 146 of AMH exon 1 and G>A at position 134 of *AMH* exon 2 differed between the OHSS and control groups ($P<0.05$). However, SNP G>T at position 303 of *AMH* exon one did not show any significant difference between the OHSS and control groups ($P>0.05$).

Karagiorga et al., (2015) investigated the influence of *AMH* and *AMHRII* gene polymorphisms, Ile49Ser and -482A>G, respectively, on IVF treatment outcome. They found that patients who did not carry the *AMH* (Ile49Ser) polymorphism had significantly higher FSH levels and fertilisation rates.

Cerra et al., (2016) investigated the impact of *AMH* and *AMH type II receptor* (*AMHRII*) gene variants on the degree of ovarian response and IVF/ICSI therapy outcomes. They did not find any significant correlations between *AMH* c.146G>T and *AMHR2* -482A>G variants and either the number of retrieved oocytes (p=0.08 and p =0.64, respectively) nor the rate of live births (p=0.28 and p =0.52, respectively).

1.5.2 *Luteinizing hormone/choriogonadotropin receptor* (*LHCGR*) gene and its variants

Cytogenetic Location: 2p16.3, the short (p) arm of chromosome 2 at position 16.3 (**Figure 1.4**) This gene is also known as: *lutropin/choriogonadotropin receptor (LCGR) or luteinizing hormone receptor (LHR)*.

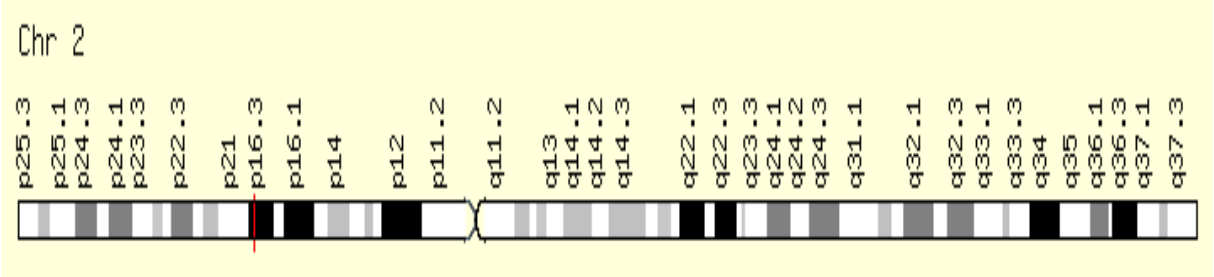

Figure 1.4: Cytogenetic location of the *Luteinizing hormone/choriogonadotropin receptor (LHCGR)* gene [Adapted from **Gene Cards, (2021b)].**

Gene Function

The *LHCGR* gene regulates the synthesis of the luteinizing hormone/chorionic gonadotrophin receptor, which is expressed in theca cells of the ovary and Leydig cells of the testicles and adipose tissue (**Capalbo et al., 2012**). It is the receptor of two hormones: luteinizing hormone and chorionic gonadotrophin hormone. In women, luteinizing hormone regulates the triggering of ovulation, while chorionic gonadotrophin hormone is produced by the placenta during pregnancy and controls the maintenance of the pregnancy (**Maman et al., 2012**).

Literature review: *LHCGR* gene Variants

O'Brien et al., (2013) conducted a prospective study of 172 patients undergoing IVF to determine correlation between the insLQ and rs4073366 variants and the outcome of gonadotrophin stimulation. They found that the polymorphisms were in linkage

disequilibrium (LD). Furthermore, they found no significant association between the insLQ variant and any of the outcome measures of COS, including the number of oocytes, fertilisation rate, and pregnancy rate. However, the study found that patients carrying the rs4073366 C variant had an increased risk of developing OHSS (OR 2.95, 95% CI = 1.09-7.96, P = 0.033).

Thathapudi et al., (2015) conducted another study that included 204 patients with PCOS to investigate the relationship between the *LHCGR* polymorphism, rs2293275, and PCOS and found a positive association (p = 0.0026).

1.5.3 *Follicle-stimulating hormone receptor* (*FSHR*) gene and its variants:

Cytogenetic location: 2p16.3, the short (p) arm of chromosome 2 at position 16.3. (**Figure 1.5**).

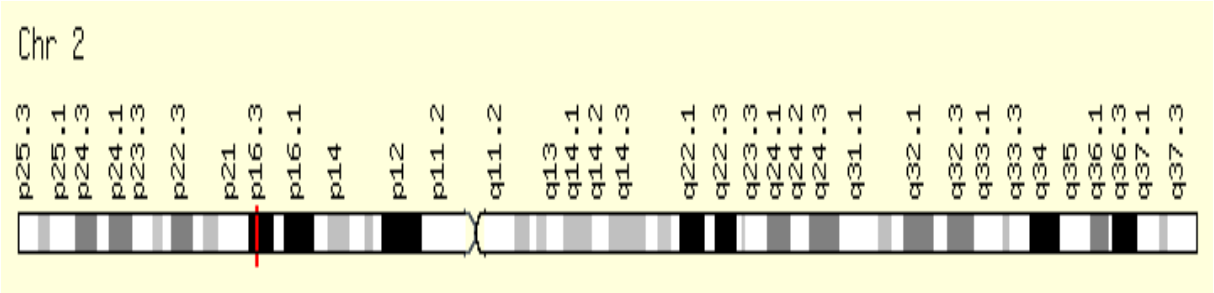

Figure 1.5: Cytogenetic location of the *follicle stimulating hormone receptor (FSHR) gene* [Adapted from **Gene Cards, (2021c)].**

Gene Function

FSHR gene regulates the synthesis of FSHR, which is a G-protein receptor responsible for the transduction of FSH through cyclic adenosine monophosphate (cAMP) (**Dufau and Tsai-Morris, 2007**).

FSH is presented on granulosa cells of ovarian follicles through activation of FSHR, where it regulates ovarian function mainly by stimulating the proliferation of the granulosa cells and controlling the maturation of the dominant oocytes. FSH also regulates the production of oestrogen by activating aromatase enzyme **(Tohlob et al., 2016)**.

Literature review: *FSHR* gene variants

The *FSHR* gene is the principal gene which controls the ovarian response and the outcome of ovarian stimulation. Approximately 1488 single nucleotide polymorphisms have been detected in the *FSHR* gene, of which the most widely investigated are Thr307Ala (rs6165) and Asn680Ser (rs6166), which are located on exon 10 **(La Marca et al., 2013)**. Many studies have analysed the impact of Thr307Ala (rs6165) and Asn680Ser (rs6166) on the degree of ovarian response and its outcome measures due to their reproductive importance. Due to discrepancies in the results of these studies, there is inadequate evidence that these variants have a significant and crucial role in assisted reproduction therapy **(Tohlob et al., 2016).**

The rs6166 polymorphism is an extensively studied SNP in the *FSHR* gene that causes a p. Asn680Ser missense variation. Many studies have demonstrated an association between rs6166 and the number of retrieved oocytes in different populations, including Chinese women **(Huang et al., 2015)**, Iranian Women **(Sheikhha et al., 2011)**, Greek Women **(Anagnostou et al., 2012; Anagnostou et al., 2021)** and Arab women **(Sindiani et al., 2021)** undergoing IVF/ICSI treatment. However, other studies have found no association between the rs6166 polymorphism and ovarian response to gonadotrophins **(Mohiyiddeen et al, 2013; Sun et al., 2018; Trevisan et al., 2014).**

Several meta-analyses have also investigated the association between the rs6166 polymorphism and ovarian response to gonadotrophins during IVF/ICSI treatment. **Morón and Ruiz (2010)** conducted a meta-analysis which included various European populations and evaluated the impact of Asn680Ser on the reproductive outcomes of IVF/ICSI. They concluded that the Asn680Ser polymorphism is a potential genetic marker for poor responders. In agreement with these results, a separate meta-analysis by **Yao et al., (2011)** found that the Asn680Ser polymorphism is associated with poor ovarian response to gonadotrophins and that carriers of the Asn680Ser polymorphism needed higher gonadotrophins.

Additionally, another meta-analysis investigated the role of the Asn680Ser polymorphism in identifying poor responders during controlled ovarian hyperstimulation. It recommended the use of the Asn680Ser polymorphism in combination with other biomarkers to predict poor ovarian response before IVF/ICSI therapy **(Pabalan et al., 2014).**

A recent meta-analysis evaluated the role of the Asn680Ser polymorphism in predicting ovarian response in Asian women and found that the polymorphism is a good genetic marker of poor ovarian response (**Tang et al., 2015).** Furthermore, a more recent meta-analysis conducted by **Alviggi et al., (2018)**, which included 21 studies and 4,425 women, investigated the correlation between the number of retrieved oocytes and the *FSHR* rs6166 genotype and found that the rs6166 polymorphism is associated with poor ovarian response to gonadotrophins.

The rs6165 A>G SNP is a missense variant that causes a p.Thr307Ala amino acid substitution in the FSHR protein. Many studies have demonstrated an association between the rs6165 polymorphism and ovarian response. A systematic review and meta-analysis conducted by **Alviggi et al., (2018)** found an association between the rs6165 variant and poor responders. Moreover, many other studies found a significant association between the rs6165 polymorphism and poor ovarian response in patients undergoing IVF/ICSI treatment (**Livshyts et al., 2009; Motawi et al., 2017; Yan et al., 2013).** However, other studies found no significant association between the rs6165 variant and ovarian response to gonadotrophins (**Meng et al., 2018; Sindiani et al., 2021; Trevisan et al., 2014).**

1.5.4 *Estrogen receptor-1* gene (*ESR-1*) and its variants

Cytogenetic Location: It is located on 6q25.1-q25.2, the long (q) arm of chromosome 6, between positions 25.1 and 25.2. (**Figure 1.6**).

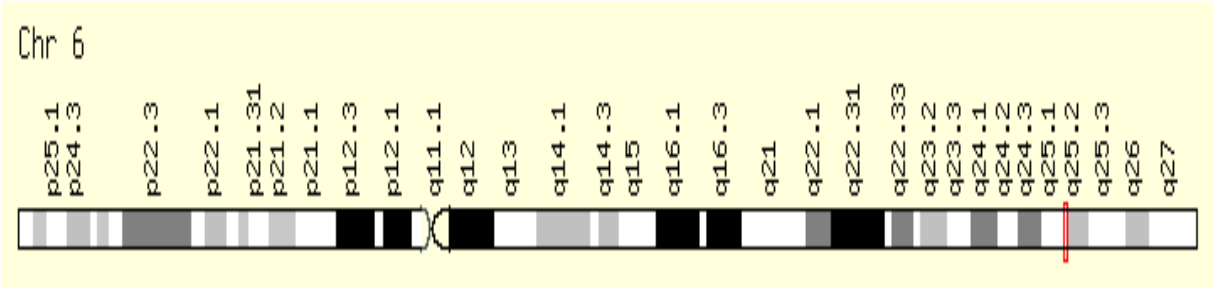

Figure 1.6: Cytogenetic location of the *Estrogen receptor 1* (*ESR*-1) gene [adapted from **Gene Cards, (2021d)].**

Gene Function

Oestrogen is a sex hormone that plays an essential role in folliculogenesis, ovulation, implantation, and maturation of reproductive organs **(Saunders, 2005).** It is produced by the

granulosa cells of the ovarian follicles and functions by binding to its receptor, oestrogen receptor (ER), in the target organs, and polymorphisms in the ER lead to disorders in oestrogen function and infertility **(Ayvaz et al., 2009; Swaminathan et al., 2016).**

There are two types of oestrogen receptors: ERα, encoded by the *ESR1* gene, and ERβ, encoded by the *ESR2* gene. ERα moderates the proliferative action of oestrogen on theca cells, while ERβ mediates the antiproliferative action of oestrogen on granulosa cells **(Britt and Findlay, 2002)**.

Literature review: *ESR-1* gene variants.

ESR genes were the first to be studied and investigated for their role in ovarian response. *ESR-1* is a highly polymorphic gene, with the polymorphisms correlating with different disorders in the female reproductive system, including endometriosis, infertility, and recurrent pregnancy loss **(Altmäe et al., 2007; Bahia et al., 2020; De Mattos et al., 2014; Lamp et al., 2011; Molvarec et al., 2007)**.

Several studies have investigated the relationship between the *ESR-1* gene polymorphism, rs2234693, and female infertility and outcomes of IVF/ICSI treatment in different populations. **Ayvaz et al., (2009)** found a significant association between rs2234693 and infertility, with a negative impact on IVF/ICSI outcomes, including oocyte maturation, fertilisation, pregnancy rates, and embryo quality. In Brazilian women, rs2234693 was associated with poor ovarian response and a larger amount of gonadotrophins in IVF cycles (**De Mattos et al., 2014)**. Moreover, **Liaqat et al., (2015)** demonstrated a significant association between the rs2234693 polymorphism and the risk of infertility in Pakistani women.

The rs2234693 polymorphism was also associated with implantation failure in Iranian women **(Pagard et al., 2020)**. However, no significant correlation was found between the rs2234693 polymorphism and the risk of infertility in Indian women **(Swaminathan et al., 2016)**. Furthermore, In Indian women, the rs2234693 polymorphism in combination with other polymorphisms had a protective effect and was associated with better IVF outcomes in terms of implantation and clinical pregnancy rates **(Ganesh et al., 2018)**. Recently**,** rs2234693 has been associated with recurrent pregnancy loss in Tunisian women **(Bahia et al., 2020)**.

Although multiple studies have attempted to define the genetic predictors of gonadotrophin stimulation, there is inadequate evidence to support the routine use of genetic biomarkers in clinical practice to personalize the stimulation protocol to improve the outcome of IVF/ICSI therapy. Further research is needed to enable the development of this attractive personalized stimulation protocol, taking into account the population type, geographic distribution, number of patients, and type of stimulation protocol. Considering the influence of different stimulation protocols on the degree of patient response to gonadotrophins, we included only patients on a long GnRH agonist stimulation protocol.

1.6. The purpose of the study:

1.6.1 General objective

The overall aim of this study was to investigate the relationship between the following five SNPs of four genes: *LHCGR* (rs4073366), *FSHR* (rs6166 and rs6165), *ESR-1* (rs2234693), and *AMH* (rs17854573) and ovarian response to gonadotrophin stimulation in Egyptian women undergoing ICSI treatment.

1.6.2. Specific objectives

- To estimate the association between the following genetic polymorphisms: rs4073366, rs6166, rs6165, rs2234693, and rs17854573, and different ovarian responses during gonadotrophin stimulation.
- To evaluate the association between the following genetic polymorphisms: rs4073366, rs6166, rs6165, rs2234693, and rs17854573, and the clinical and Hormonal parameters e.g., age, BMI, AMH, basal FSH and basal LH.
- To determine the association between the following genetic polymorphisms: rs4073366, rs6166, rs6165, rs2234693, and rs17854573, and ICSI cycle parameters e.g., starting dose of gonadotrophin, total dose of gonadotrophin, duration of stimulation, number of mature oocytes and number of fertilized oocytes.

1.6.3. Significance

- This study is one of a small number of studies using samples from an Egyptian population.
- Identifying women who are at a high risk of either poor or high response during ovarian stimulation is important as it may assist in preventing this type of response and the associated complications, as well as in selecting the proper protocol and dosage that can increase the success rate of IVF/ICSI therapy.

2. Materials and Methods

2.1. Study design

This case-control study assessed the relationship between five SNPs of four genes and ovarian response in women who underwent assisted reproductive technology (ART).

2.2. Ethical Considerations

This study was approved by the Institutional Ethics Committee of Sohag University, Sohag City, Egypt (Reference number. 25/2016), where the specimens were collected (**Appendix 1**). All patients signed written informed consent forms prior to enrolment into the study. All samples were processed and analysed at the Department of Gynaecology and Obstetrics, Assisted Reproduction Laboratory, University of Saarland, Homburg, Germany. All samples were analysed according to standard operating procedures and all possible measures were taken to prevent sample contamination.

2.3. Study population

From January 2017 to May 2018, a total of 280 blood samples were collected from women aged 25–35 years who underwent ART. All women included in the study were of the same ethnicity (Egyptian). In addition, the personal, medical, and family histories, body mass index, clinical and serological status, hysterosalpingography, and day-three hormone profiles (E2, FSH, LH, Prolactin (PRL), Thyroid stimulating hormone (TSH), and AMH) of all the participants were extensively evaluated. All samples were collected based on the following exclusion criteria.

- **Inclusion criteria**:
    - 25–35 years of age
    - Women undergoing to ART due to tubal factor and unexplained infertility
    - Normal BMI
    - Normal ovulation and regular cycles
    - Normal hormone levels
    - Women from the same population.

- **Exclusion criteria**:
  - ≥ 36 years of age
  - Exposure to chemotherapy or radiotherapy
  - Having undergone unilateral ovariectomy or any identified genetic abnormalities
  - Women with endometriosis
  - Women with PCOS
  - A history of OHSS.

2.4. Materials

2.4.1. Chemicals and reagents

Chemicals and reagents used in this study were shown in **Table 2.1.** Besides that, all the kit used in the present study was illustrated in **Table 2.2.**

Table 2.1 Chemicals and reagents used in the present study in alphabetical order

Chemicals or reagents	Manufacturer/Distributor
Absolute Ethanol Alcohol	Sigma-Aldrich, Germany
Absolute Isopropanol Alcohol	Sigma-Aldrich, Germany
Absolute Methanol Alcohol	Sigma-Aldrich, Germany
Buffer(10X) with EDTA	Applied Biosystems, USA
Ethidium Bromide	Sigma-Aldrich, Germany
Nuclease-Free Water	Sigma-Aldrich, Germany
TAE Buffer	Sigma-Aldrich, Germany

Table 2.2 List of kit that used in the present study in alphabetical order

Kit	Manufacturer/Distributor
Agarose Tablets	Bioline, Germany
Cyclic Sequencing Kit	Applied Biosystems, USA
HotStarTaq Master Mix Kit (250 U)	Bioline, UK.
ISOLATE II RNA/DNA/Protein Kit	Bioline, UK.
miScript II Reverse Transcription Kit	Qiagen, Germany
PCR Purification Kit	Invitrogen, Germany
POP 6 Electrophoresis Polymer	Applied Biosystems, USA
Primers for Routine PCR	Microsynth AG, Schweiz

QuantiTect Primer Assay (200 reactions), AMH	Qiagen, Germany
QuantiTect Primer Assay (200 reaction), ESR1	Qiagen, Germany
QuantiTect Primer Assay (200 reactions), FSHR(rs6166)	Qiagen, Germany
QuantiTect Primer Assay (200 reactions), FSHR(rs6165)	Qiagen, Germany
QuantiTect Primer Assay (200 reactions), GAPDH	Qiagen, Germany
QuantiTect Primer Assay (200 reactions), LHCGR	Qiagen, Germany
QuantiTect SYBR Green PCR Kit (1000 reaction)	Qiagen, Germany
Quick-Load® 2-Log DNA Ladder	BioLabs, New England

2.4.2. Disposable materials

All the disposable materials used in the present study were shown in **Table 2.3**. Disposable materials were certified to be free from RNase, DNase, human DNA, PCR inhibitors, and pyrogens.

Table 2.3 List of disposables materials in alphabetical order

Disposables Materials	Manufacturer/Distributor
Aerosol-Resistant Pipette Filter Tips	Labcon, USA
Disposables Gloves Free Powder	Sigma-Aldrich, Germany
EDTA Tubes	Sigma-Aldrich, Germany
Eppendorf PCR Tubes, 0.2 ml	Eppendorf GmbH, Germany
Eppendorf PCR Tubes, 1.5 ml	Eppendorf GmbH, Germany
Fast Optical 96-Well Reaction Plate for qPCR	Thermo Fisher, Germany
MicroAmp® Optical 96-Well Plate and Septa for the Genetic Analyzer	Applied Biosystems, Foster City, USA
Microfuge Tubes for PCR - thin wall 0.2 ml	Labcon, USA
Optical Adhesive Covers	Thermo Fisher, Germany
Plate Septa, Retainer and Base	Applied Biosystems, Foster City, USA
Sterile Filter Tips, 10 µl	Sorenson BioScience, Inc., USA
Sterile Filter Tips, 1000 µl	Sorenson BioScience, Inc., USA
Sterile Filter Tips, 20 µl	Sorenson BioScience, Inc., USA
Sterile Filter Tips, 200 µl	Sorenson BioScience, Inc., USA

2.4.3. Instrument and Equipment

The instrument and equipment used in this study were shown in **Table 2.4.**

Table 2.4 The principal instrument and equipment used in this study

Instrument and Equipment	Manufacturer/Distributor
Applied Biosystems Genetic Analyser	Applied Biosystems, Foster City, USA
Automatic Pipette (10-50 µl)	Eppendorf GmbH, Germany
Automatic Pipette (5-20 µl)	Eppendorf GmbH, Germany
Automatic Pipette (1000 µl)	Eppendorf GmbH, Germany
Automatic Pipette (100-200 µl)	Eppendorf GmbH, Germany
Bench-top Centrifuge	Eppendorf GmbH, Germany
Bench-top Cold Centrifuge	Eppendorf GmbH, Germany
C1000™ Thermal Cycler	Bio-Rad, USA
Digital Balance	AE adam, USA
Electrophoresis Chambers and Tanks	BioRad, USA
Electrophoresis Power Supply	BioRad, USA
Freezer, -20ºC	Sanyo, Japan
Freezer, -70ºC	Sanyo, Japan
Gel Documentation System	Bio-Rad, USA
Incubator	Memmert GmbH, Germany
Microwave	L.G, Korea
NanoDrop ND-2000c Spectrophotometer	Thermo Fisher, Germany
Personal Computer	DELL, USA
Safety Cabinet	Heraeus, Germany
Shaker	Heidolph Instruments GmbH, Germany
StepOnePlus™ real-time PCR system	Thermo Fisher, Germany
Vortex Mixer	Uniequip, Germany

2.5. Methodology

2.5.1. Controlled Ovarian Stimulation protocol

The clinical portion of the study was performed at the IVF/ICSI unit, Sohag, Egypt, starting with patient recruitment and selection based on the inclusion and exclusion criteria. This was followed by the preparatory phase and investigations prior to ICSI, controlled ovarian stimulation (COS), patient follow-up, and sample collection. A long GnRH agonist protocol was used for ovarian stimulation. GnRH agonist (leuprolide acetate; TAP Pharmaceuticals) treatment was initiated on day 21 of the previous cycle, and recombinant FSH (Gonal-F, Merk-Serono, Darmstadt, Germany) was initiated on day 2 of the next cycle, with dosing depending on AMH levels as follows: patients with < 1 ng/mL AMH received a recombinant FSH dose of 450 IU/day, patients with 1–2 ng/mL AMH received a recombinant FSH dose of 375 IU/day, patients with 2–3 ng/mL AMH received a recombinant FSH dose of 225 IU/day and patients with > 3 ng/mL AMH received a recombinant FSH dose of 150 IU/day.

Follicular growth was followed by serum E2 and transvaginal sonography until at least three follicles reached a diameter of 18 mm, after which patients received 10000 IU HCG. Transvaginal ultrasound-guided follicular aspiration was performed 36 hours after administration of HCG, and then the ICSI procedure was performed, followed by embryo transfer. The luteal phase was supported by daily progesterone for 14 days using either Cyclogest vaginal 400 mg twice daily (L.D. Collin, Barnstaple, UK) or Prontogest 100 mg IM once Daily (IBSA Pharmaceutical, Lugano, Switzerland), based on the patient's preference.

Chemical pregnancy was evaluated by measuring serum β-hCG level 14 days after embryo transfer. At the end of the stimulation phase, the study population was divided into three groups based on the number of collected oocytes as the following:

1) **Poor response:** retrieved oocytes < 4 (n= 92),
2) **Normal response (control):** retrieved oocytes 4-15 (n=80),
3) **High response:** retrieved oocytes > 15 (n=108).

2.5.2. Collection of Blood Samples

Approximately 5.0 mL blood samples were collected from each participant in EDTA tubes and stored at -80 °C until the genetic analysis which was performed in the assisted reproduction and Genetics Unit, Saarland University, Germany. Each blood sample was separated into two: one part was used for clinical chemistry (FSH, LH, PRL, E2, TSH, and AMH) and the other part was used for genetic analysis.

2.5.3. Genomic DNA and RNA Isolation

Genomic DNA and RNA were isolated from blood samples using a standard isolation kit (ISOLATE II RNA/DNA/Protein, Bioline, UK) according to the manufacturer's protocol.

2.5.3.1. Buffer preparation

- **Preparing Lysis Buffer TX:** 10 µL of ß-ME was combined with 1 mL of buffer X.
- **Combining Wash Buffer (W1) with ethanol:** 90 mL of 96 %–100% ethanol was combined with 38 mL of wash buffer W1 concentrate, giving a volume of 128 mL.
- **Combining Wash Buffer (W2) with ethanol:** 42 mL of 96 %–100% ethanol was added to 18 mL of wash buffer W2 concentrate, giving a final volume of 60 mL.
- **Preparing DNase I (RNase-free):** 15 µL of DNase I was added to 100 µL of DNase Reaction Buffer DRB and mixed gently.

2.5.3.2. Genomic DNA and total RNA purification:

Before genomic DNA purification, a lysate was prepared for each blood sample as follows:

- 100 µL of blood was collected in a 1.5 mL (RNase-free) microcentrifuge tube.
- 300 µL of Lysis Buffer TX was added to the sample and the mixture vortexed for 15 s.

Then, DNA was purified as follows:

1. An ISOLATE II DNA column (white ring) with a collection tube was assembled.
2. 600 µL of the lysate was added to the column and centrifuged for 1 min at 14,000 x g.
3. The flow-through, containing RNA and proteins, was retained for total RNA purification. The flow-through was stored on ice or at -20 °C until the total RNA purification protocol was carried out.
4. The spin-column with the collection tube was reassembled.

5. 500 µL of Wash Buffer W1 was added to the column and centrifuged for 1 min at 14,000 x g and the flow-through discarded.
6. 500 µL of Wash Buffer W2 was added to the column and centrifuged for 1 min at 14,000 x g and the flow-through discarded.
7. After discarding the flow-through, the column was dried by spinning for 2 min at 14,000 x g.
8. The column was inserted into a fresh 1.5 mL Elution Tube.
9. 100 µL of DNA Elution Buffer was added to the column and centrifuged for 2 min at 200 x g, and then at 14,000 x g for 1 min. The isolated DNA was stored at -80 °C until use.

Total RNA was purified as follows:

The flow-through previously stored for total RNA purification was processed as follows:

1. 60 µL of 100% ethanol was added to each 100 µL of flow-through and mixed by vortexing.
2. The provided RNA column was assembled with the collection tube and 600 µL of lysate was applied on it and centrifuged at 3,500 x g for 1 min.
3. Total RNA in the column was washed three times by applying 400 µL of wash buffer W1, centrifuging the column at 14,000 x g for 1 min and discarding the flow-through.
4. The column was dried by spinning at 14,000 x g for 2 min.
5. 50 µL of RNA elution buffer was applied to the column placed in a fresh elution tube and centrifuged for 2 min at 300 x g, and then at 14,000 x g for 1 min.
6. The extracted RNA was stored at -80 °C for later use.

2.5.4. Assessment of DNA and RNA concentration and purity

The quantity and purity of the isolated DNA and RNA were determined using a NanoDrop 2000C spectrophotometer (Thermo Fisher, Germany). Purity was assessed based on the 260/280 ratio. The acceptable value of DNA purity was 1.8 and that of RNA purity was 2.0. Elution buffer was used as a blank solution to calibrate the equipment.

2.5.5. Single Nucleotide Polymorphism screening in *AMH, ESR-1, FSHR,* and *LHCGR* genes

Information on the *LHCGR* (rs4073366), *FSHR* (rs6166 & rs6165), *ESR-1* (rs2234693), and *AMH* (rs17854573) SNPs were obtained from the public SNP database (National Institute of Health, USA; http://www.ncbi.nlm.nih.gov/SNP) **(Sherry et al., 2001).**

2.5.5.1 Allele-specific Polymerase chain reaction

An allele-specific PCR technique was used to detect these SNPs. The primer design was based on previous studies of *LHCGR* rs4073366 **(Schmitz *et al.*, 2015)**, *FSHR* rs6166 and rs6165 **(Rod *et al.*, 2014)**, *ESR*-1 rs2234693 **(Altmäe *et al.*, 2007)**, and *AMH* rs17854573 **(Rigon *et al.*, 2010)**. The sequences of the primers are listed in **Table 2.5.**

Table 2.5 Sequences of primers used in the study**.**

SNP	Primer sequence	
LHCGR (rs4073366)	**F**	5'-CAC TCA GAG GCC GTC CAA G-3'
	R	5'-GGA GGG AAG GTG GCA TAG AG-3'
FSHR (rs6166)	**F**	5'-TTT GTG GTC ATC TGT GGC TGC-3'
	R	5'-CAA AGG CAA GGA CTG AAT TAT CAT T-3'
FSHR (rs6165)	**F**	5'-CAA ATC TAT TTT AAG GCA AGA AGT TGA TTA TAT GCC TCA G-3'
	R	5'-GTA GAT TCC AAT GCA GAG ATC A-3'
ESR-1(rs2234693)	**F**	5'-CTG CCA CCC TAT CTG TAT C-3'
	R	5'-ACC CTG GCG TCG ATT ATC TG-3'
AMH (rs17854573)	**F**	5'-ACC AGT GGC CTC ATC TTC C-3'
	R	5'-AGG AAG GCC TGC TCA TAG G-3'

F: Forward, R: Reverse

Polymerase chain reaction was performed in a total volume of 30 µL. The PCR components in each tube are listed in **Table 2.6.** PCR tubes were placed in a thermal cycler (Bio Rad-Germany) and PCR amplification was performed following the program shown in **Table 2.7**. The PCR annealing temperatures and PCR products for the various genes are listed in were given in **Table 2.8.**

Table 2.6 PCR components for each sample

Reagent	Volume (µl)	Final concentration
HotStarTaq Master Mix	15 µL	1X
Foreword primer	0.6 µL	20 µM
Revers primer	0.6 µL	20 µM
Nuclease free water	11.3 µL	
DNA sample	2.5 µL	≈ 100 ng
Total volume	30 µl	

Table 2.7 Thermal cycling program for PCR amplification

Cycle	T (ºC)	Duration	No. of cycles
Initial Denaturation	95°C	5 min	1
Denaturation	95°C	30 s	**40**
Annealing	**See (Table 2.8)**	30 s	
Extension	72°C	30 s	
Final extension	72 ºC	5 min	1
Forever	4 ºC		

Table 2.8 Annealing temperatures and PCR products for each gene

Gene	Annealing temperature	Product Size (bp)
LHCGR (rs4073366)	59 ºC	295 bp
FSHR (rs6166)	61 ºC	520 bp
FSHR (rs6165)	57 ºC	364 bp
ESR-1 (rs2234693)	60 ºC	1000 bp
AMH (rs17854573)	57 ºC	316 bp

2.5.5.2. Verification of the quality and sizes of PCR products

To verify the amplification of PCR products, the following steps were followed:

1. Dried agarose gel (1.5 g) was melted by heating in 100 mL 1x Tris-Acetate-EDTA buffer (TAE) (2 M Tris base 1 M Glacial Acetic acid, 0.05 M EDTA).
2. Then, 3 µL ethidium bromide was added and mixed, and the gel was cast into a mould fitted with a well-forming comb.
3. The agarose gel was submerged in TAE buffer inside a horizontal electrophoresis apparatus.
4. 5 µL of PCR products and a DNA ladder marker (Biolabs, New England) were loaded into the wells.
5. The PCR products were identified based on their sizes on the agarose gel (**Table 2.8**). Electrophoresis was performed using an electrophoresis apparatus (Bio Rad, USA) at 80 V for 60 min at room temperature and the DNA bands were visualised and documented using a Molecular Imager Gel Doc TM system (BioRad, USA) (**Figures 2.1, 2.2, 2.3, 2.4, and 2.5**).

Finally, PCR products were purified using a PCR purification kit (Invitrogen, Germany), according to the manufacturer's protocol.

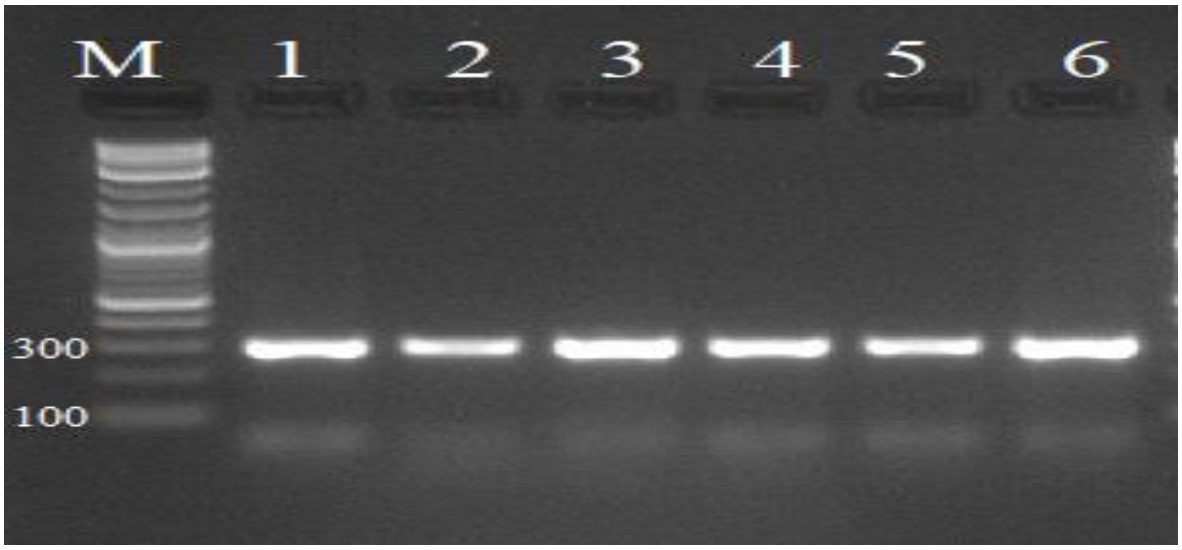

Figure 2.1 Amplification products of the *LHCGR* (rs4073366) gene on 1.5% agarose gel. Product size: 295 bp; lane M: DNA ladder, Lanes 1–6: PCR products.

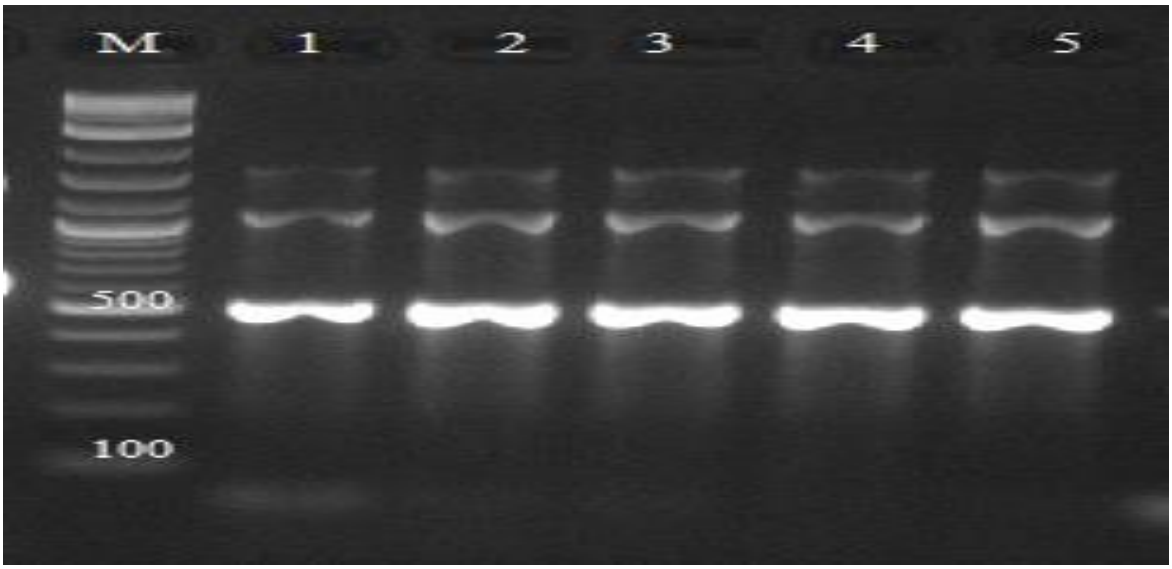

Figure 2.2 Amplification products of the *FSHR* (rs6166) gene on 1.5% agarose gel. Product size: 520 bp; lane M: DNA ladder, Lanes 1–5: PCR products.

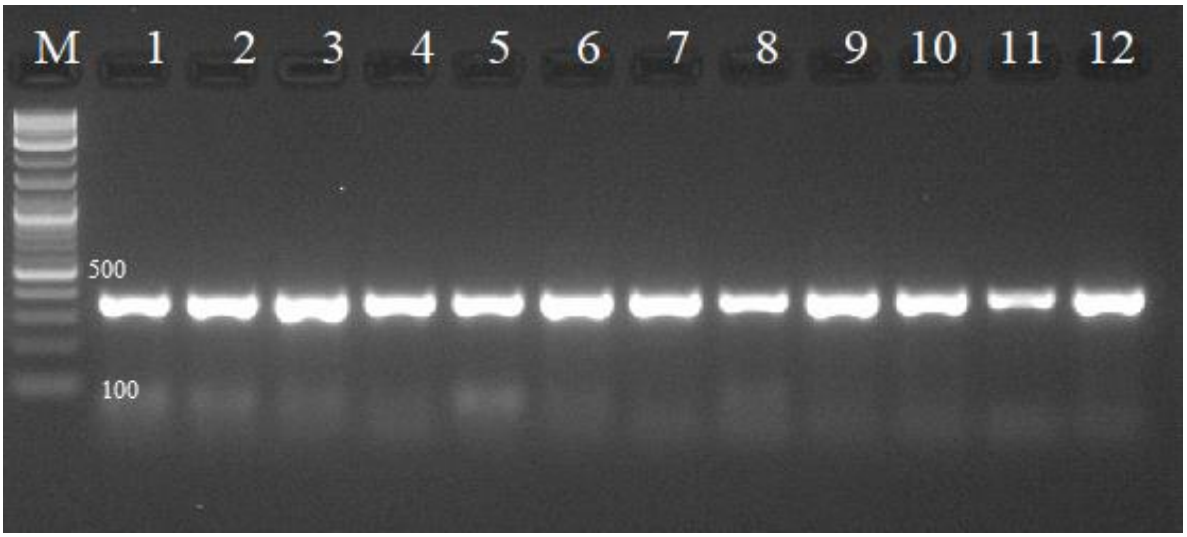

Figure 2.3 Amplification products of the *FSHR* (rs6165) gene on 1.5% agarose gel. Product size: 364 bp; lane M: DNA ladder; Lanes 1–12: PCR products

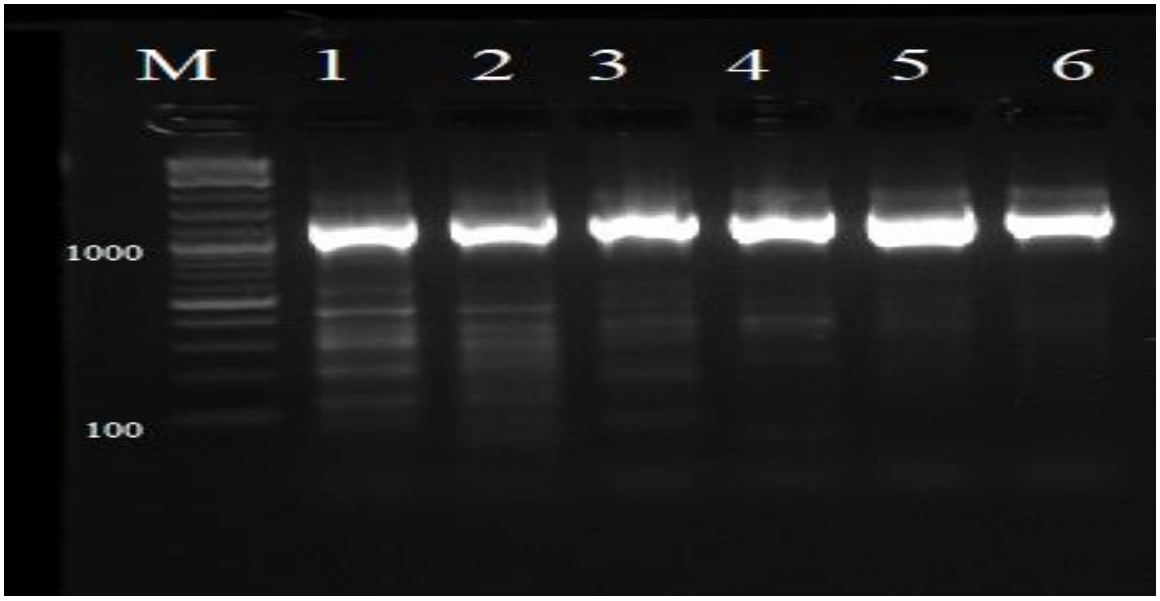

Figure 2.4 Amplification products of the *ESR-1* (rs2234693) gene on 1.5% agarose gel. Product size: 1000 bp; lane M: DNA ladder; Lanes 1–6: PCR products.

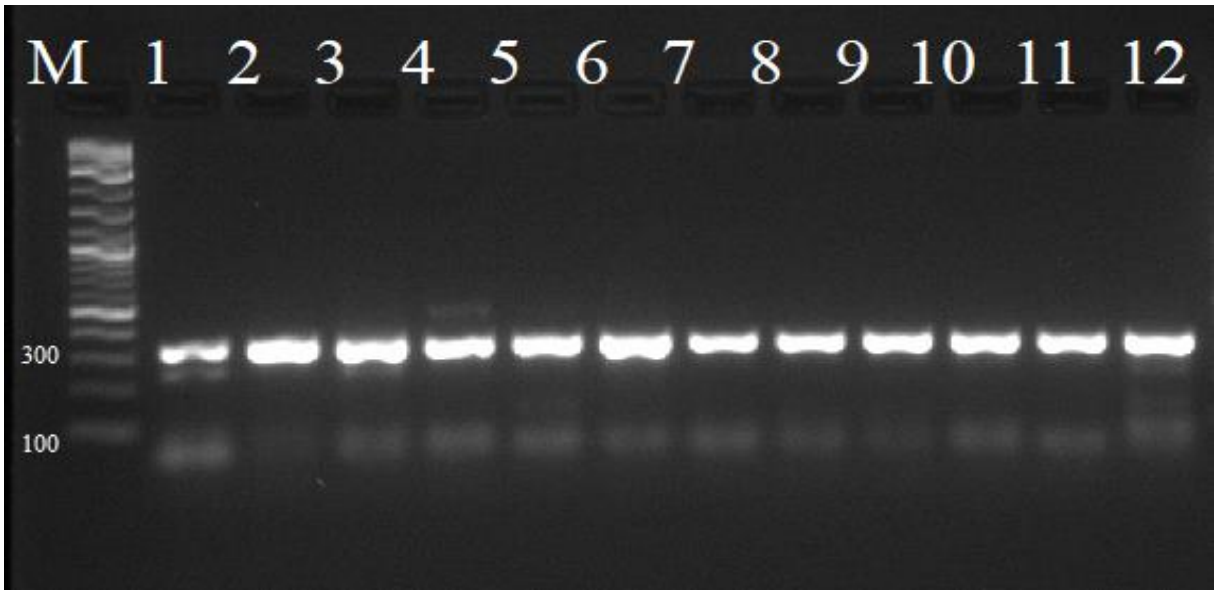

Figure 2.5 Amplification products of the *AMH* (rs17854573) gene on 1.5% agarose gel. Product size: 316 bp; lane M: DNA ladder; Lanes 1–12: PCR products.

2.6. DNA sequencing

2.6.1. Automated Sanger sequencing

Automated Sanger sequencing technique was used to sequence the nucleotides of all genes in this study. The quantity of DNA template from the amplified and purified gene fragments was 10–20 ng. Sequencing was performed at Seqlab Sequence Laboratories GmbH, Göttingen, Germany.

2.7. Reverse transcription and quantitative PCR (qPCR)

2.7.1. Reverse transcription of RNA

Reverse transcription (RT) is the synthesis of complementary DNA (cDNA) from an RNA template. This process was performed using the miScript reverse transcription kit (Qiagen, Germany), according to the manufacturer's instructions, as follows:

1. Total RNA (5 µL of 40 ng/µL) was mixed with 4 µL of 5X miScript HiFlex buffer, 2 µL nucleic acid mix (10X), and 2 µL miScript reverse transcriptase mix, and the reaction volume topped up to 20 µL using RNase-free water.
2. The samples were incubated at 37 °C for 1 hr to generate the first-strand cDNA.
3. The reaction was inactivated by heating at 95 °C for 15 min and the cDNA stored at -20 °C.

4. This reaction was performed twice in two separate tubes to generate sufficient cDNA for all qPCR reactions.

2.7.2. Quantitative PCR

Quantitative PCR (qPCR) was performed to determine the expression levels of genes analysed in the present study, namely *follicle-stimulating hormone receptor (FSHR), anti-Mullerian hormone (AMH), luteinizing hormone/choriogonadotropin receptor (LHCGR), and oestrogen receptor 1 (ESR1)*, using a StepOnePlus™ System (Applied Biosystems, USA). The housekeeping gene, G*lyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), was used as a reference.

SYBR green binding to the amplified cDNA was estimated to detect increased fluorescence. cDNA was used as a template for qPCR analysis.

qPCR was performed using the QuantiTect primer assay (Qiagen, Germany) according to the manufacturer's instructions, as follows:

1. The PCR reaction involved the use of 2 µL cDNA, 10 µL of 2× miScript SYBR Green PCR master mix, 2 µL QuantiTect primer assay, and RNase-free water was added to give a total reaction volume of 20 µL. The reaction mixture was added to the wells of a MicroAmp Fast 96-tube strip (0.1 mL).
2. Reactions were performed using the following thermal cycling parameters:
   50 °C for 2 min, initial activation at 95 °C for 15 min, 40 cycles of 94 °C for 15 s (denaturation), 55 °C for 30 s (annealing), and 70 °C for 30 s (extension).
3. A final dissociation curve (melting curve) was generated, and the PCR plates were maintained at 4 °C.
4. The *GAPDH* QuantiTect primer assay was used as the housekeeping gene for normalisation. In addition, no template control (NTC) and no reverse transcriptase control (NRT) were included in each run.

- **Note**: All qPCR reactions were performed in triplicate, and the resulting C_t values were normalised to that of *GAPDH*.

2.8. Statistical analysis

IBM SPSS software (version 24.0; SPSS Inc., Chicago, IL, USA) was used for data analysis. The skewness, Shapiro, and Kurtosis tests were used to evaluate the non-parametric data distribution. The Kruskal–Wallis (H) and Mann-Whitney (U) tests were applied to compare the median quantitative variables between the study groups. Spearman rank correlation was used to evaluate the association between the genetic polymorphisms (rs4073366, rs6166, rs6165, rs2234693, and rs17854573) and different investigated parameters. The results were considered statistically significant when the p-value was less than or equal to 5% ($P \leq 0.05$).

For DNA sequence analysis, the Tracy tool (https://github.com/gear-genomics/tracy) was used to obtain the allelic sequences of each gene. The aligner BWA (**Li & Durbin, 2009**), samtools mpileup (**Li *et al*., 2009**), and WhatsHap (**Ebler *et al*., 2018; Patterson *et al*., 2015**) were used for variant calling. From the set of all identified SNPs, all positions with an allele frequency above 5% across all studied individuals were selected and tested for Hardy-Weinberg equilibrium using Fisher's exact test. To test the association between alleles and each of the studied groups, Fisher's exact test was performed to identify significant differences in allele distributions among the study groups and Benjamini Hochberg correction was used for multiple testing (alpha=0.05).

Comparative qPCR data analysis was used to calculate the expression level of the tested genes in the cases “poor/high responder” versus controls “normal responder” The threshold cycle (C_t) indicates the cycle number at which the fluorescence curve generated within the reaction crossed the threshold for qPCR and ΔC_t = (C_t of the target gene) - (C_t *GAPDH*). The $2^{-\Delta\Delta Ct}$ equation was used to calculate the fold-change of the following target genes (*FSHR, AMH, LHCGR,* and *ESR1*), where comparative $\Delta\Delta C_t$ = (ΔC_t Controls “normal responder”- ΔC_t Cases “poor/high responder”).

3. Results

Table 3.1 illustrates the general and clinical characteristics of the study population. The study consists of 280 blood samples collected from women who underwent assisted reproductive technology. The samples were divided into three groups, depending on the degree of ovarian response during the ovarian stimulation program: poor responders (n=92), normal responders (n=80), and high responders (n=108). The women were 25–35 years of age.

Table 3.1 General and clinical characteristics of the study population (n = 280)

General and Clinical characteristics	Mean ± SD
Age (Year)	29.37±3.04
BMI (kg/m2)	22.31±2.29
AMH Level (ng/ml)	1.99±0.58
Basal FSH Level (mIU/ml)	6.74±1.81
Basal LH Level (mIU/ml)	4.58±1.40
Basal PRL Level (ng/mL)	11.21±4.95
Starting FSH Dose (IU)	297.75±77.38
Total Dose of Gonadotropin (IU)	2,854.45±1,107.53
Number of Stimulation Days	10.03±1.23
Number of Collected Oocytes	10.84±7.52
Number of Injected Oocytes	8.74±6.37
Number of Fertilized Oocytes	6.77±5.34
Number of Transferred embryos	1.90±1.17
Number of Embryo Freezing	3.60±4.10

All data illustrated as Mean ± Standard deviation; SD: Standard deviation

3.1. Descriptive characteristics of the different study groups

As shown in **Table 3.2**, the study found significant variations in AMH levels among the study groups (P = 0.039). Additionally, significant variations in the total dose of gonadotrophin, number of stimulation days, number of collected oocytes, number of injected oocytes, number of fertilised oocytes, number of transferred embryos, and number of frozen embryos was found among the study groups ($P \leq 0.001$). In contrast, no significant difference was observed among the study groups in the basal FSH level (P = 0.197), basal LH level (P = 0.285), basal PRL level (P = 0.080), starting dose of FSH (P = 0.118), body mass index (P = 0.496), and age (P = 0.906). The results of this study showed no significant variations in pregnancy rates among the study groups (poor responders, 36.96%; normal responders, 63.75%; and highresponders,46.30%).

Table 3.2 General and the ICSI parameters of high responders, poor responders compared to normal responders (n = 280)

General and ICSI Parameters	Poor responders (n=92)	Normal responders (n=80)	High responders (n= 108)	P-Value
	Mean± SD	Mean± SD	Mean± SD	
Age (Year)	29.29±3.00	29.48±2.86	29.34±3.29	0.906
BMI (kg/m2)	22.02±2.39	22.47±2.19	22.45±2.28	0.496
AMH Level (ng/ml)	2.13±0.57	1.91± 0.45	1.92±0.61	0.039
Basal FSH Level (mIU/ml)	6.55±1.76	7.05±1.81	6.65±1.85	0.197
Basal LH Level (mIU/ml)	4.44±1.40	4.84±1.42	4.48±1.36	0.285
Basal PRL Level (ng/mL)	11.13±4.65	10.21±4.99	12.24±5.09	0.080
Starting FSH Dose (IU)	283.70±73.74	298.83±77.34	311.19±79.69	0.118
Total Dose of Gonadotropin (IU)	4,205.36±495.20	2,471.02±530.11	1,829.48±246.14	≤ 0.001
Number of Stimulation Days	11.28±0.78	9.83±0.70	8.94±0.78	≤ 0.001
Number of Collected Oocytes	2.32±1.25	10.55±2.32	19.90±2.10	≤ 0.001
Number of Injected Oocytes	1.65±1.01	8.30±2.02	16.45±2.07	≤ 0.001
Number of Fertilized Oocytes	1.09±0.89	6.14±1.82	13.21±2.41	≤ 0.001
Number of Transfered embryos	0.96±0.74	2.50±0.69	2.28±1.29	≤ 0.001
Number of Embryo Freezing	0.03±0.24	2.33±1.24	8.48±3.19	≤ 0.001

Kruskal–Wallis (H-test), data illustrated as Mean ± SD; SD: Standard deviation; SE: Standard of error; BMI: Body Mass Index; P ≤ 0.05: Significant.

3.2. Investigating targeted single nucleotide polymorphisms (SNPs) in *FSHR*, *LHCGR*, *ESR1*, and *AMH* genes.

3.2.1 Variant calling

Primary and secondary sequences of each sample were extracted from chromatogram (.ab1) files using the Tracy tool (https://github.com/gear-genomics/tracy) to obtain the allelic sequences for each gene. The aligner BWA (**Li &Durbin, 2009**) was used to map the resulting FASTA reads to the hg19 reference genome. A BAM file containing two reads was produced for each individual. Next, known SNP positions were genotyped from these reads using the genotyping module in WhatsHap (**Ebler *et al.*, 2018; Patterson *et al.*, 2015**). This set of known SNPs includes the provided variants and additional SNPs reported by the 1000 Genomes Project (**1000 Genomes Project Consortium, 2012**) **Figure 3.1**.

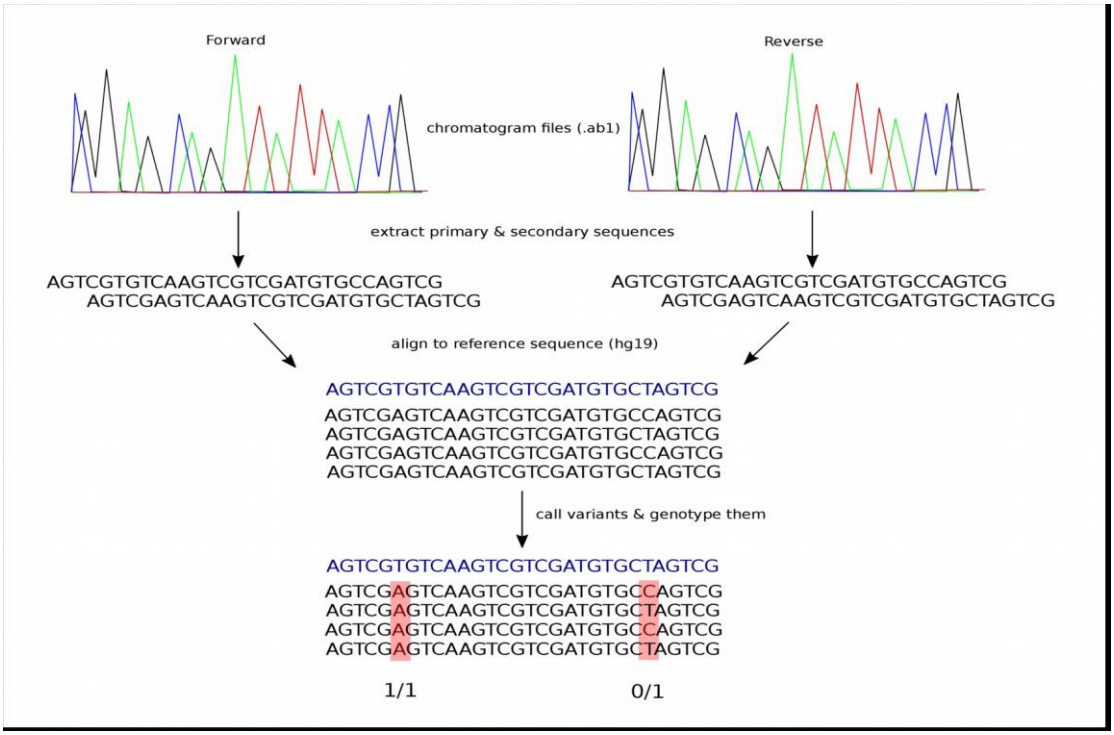

Figure 3.1 Variant Calling

Allelic sequences were extracted from the chromatogram files and aligned to a reference genome. The variants were then genotyped in all samples.

3.2.2. Quality Control

In addition to the SNPs that were successfully genotyped, we selected all 1000 Genomes SNPs for which allele frequencies (of the alternative alleles) were above 5% across all genotyped individuals and tested them for Hardy-Weinberg equilibrium (HWE) using Fisher's exact test. The SNPs in Table 3.3 below did not show significant deviations from HWE.

Table 3.3 Considered SNPs. All SNPs (given + 1000 Genomes) that were considered for statistical analysis

ID	Gene	Genomic Position (hg19)	Reference Allele	Alternative allele	Allele Frequency (across all samples)
rs4073366	***LHCGR***	Chromosome 2 48982622	C	G	0.11
rs6166	***FSRH***	Chromosome 2 49189921	C	T	0.48
rs6165	***FSRH***	Chromosome 2 49191041	C	T	0.47
rs17854573	***AMH***	Chromosome 19 2250469	G	A	0.06
rs2234693	***ESR1***	Chromosome 6 152163335	T	C	0.46

Fisher's exact test.

As an additional quality control, we compared the allele frequencies that we obtained for these SNPs to those reported by the 1000 Genomes project and found that they matched (**Figure 3.2**). The blue dots correspond to the allele frequencies observed across our samples, and the boxplots show the distribution of allele frequencies for these variants observed across several populations studied in the 1000 Genomes project.

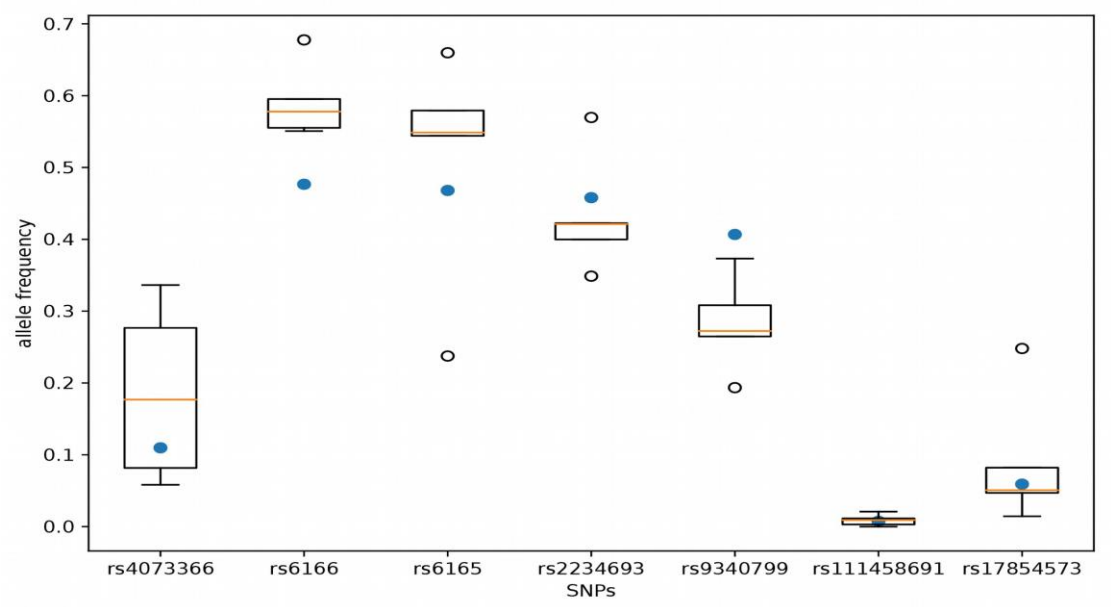

Figure 3.2 Comparison of allele frequencies
We compared the allele frequencies of the considered SNPs (blue) with those previously reported by the 1000 Genomes Project for the indicated variants across several populations.

3.3. Genotype distribution and allele frequencies between the study groups

Table 3.4 illustrates the position of the polymorphisms and their effect on the protein sequence. Genetic information showed that two polymorphisms (rs6166 and rs6165) led to a change in the sequence and the type of amino acid (Ser/Asn and Ala/Thr, respectively).

DNA sequence analysis showed significant differences in the frequencies of *FSHR* (rs6166) and ESR1 (rs2234693) in the poor responders compared with normal responders ($P \leq 0.001$ and $P \leq 0.001$, respectively). In contrast, no significant differences were found in the frequencies of *FSHR* (rs6166), *FSHR* (rs6165), or *ESR1* (rs2234693) genotypes in high responders compared with normal responders ($P = 0.074$, $P = 0.353$, and $P = 0.060$, respectively).

Moreover, DNA sequence data analysis showed a significant difference in the frequency of *AMH* (rs17854573) genotype in poor responders compared with normal responders ($P = 0.010$), whereas no significant differences were found in the frequencies of the *AMH* (rs17854573) and *LHCGR* (rs4073366) genotypes in high responders compared with normal responders ($P = 0.060$ and $P = 0.091$, respectively). No significant difference was found in the frequency of the *LHCGR* (rs4073366) genotype in poor responders compared with normal responders ($P = 0.312$).

Table 3.4 Genotype distribution and allelic frequency of the single nucleotide polymorphisms in the cases compared to the control group (n = 280)

Genotype	Chromosome localization	Amino acid	SNP	Normal Responders		Poor Responders			High Responders		
				(n=80)	(%)	(n=92)	(%)	P-Value	(n=108)	(%)	P-Value
***FSHR* (rs6166)**	Chromosome 2:49189921 (**Position:** 48962782)	Ser/ Asn	C/C	63	78.75	46	50.00		56	51.85	
			C/T	17	21.25	46	50.00	≤ 0.001	52	48.15	0.074
***FSHR* (rs6165)**	Chromosome 2:49191041 (**Position:** 48963902)	Ala/Thr	C/C	37	46.25	28	30.43		35	32.41	
			C/T	43	53.75	64	69.57	0.192	73	67.59	0.353
***ESR1* (rs2234693)**	Chromosome 6:152163335 (**Position:** 151842200)	N/A	T/T	62	77.50	41	44.57		51	47.22	
			T/C	18	22.50	51	55.43	≤ 0.001	57	52.77	0.060
***AMH* (rs17854573)**	Chromosome 19:2250469 (**Position**: 2250470)	Pro	G/G	74	92.50	72	78.26		106	98.15	
			G/A	6	7.50	20	21.74	0.010	2	1.85	0.060
***LHCGR* (rs4073366)**	Chromosome 2:48755483 (**Position**: 48755483)	N/A	C/C	69	86.25	84	91.30		101	93.52	
			C/G	11	13.75	8	8.70	0.312	7	6.48	0.091

Mann-Whitney (U-test), P ≤ 0.05: Significant.

3.4. Association between the *FSHR* (rs6166) genotype and female general and ICSI parameters.

Analysis of the association between *FSHR* (rs6166) and female general characteristics showed no significant differences in the general characteristics between individuals with the CT and CC genotypes in the poor, normal, and high response groups (**Table 3.5**). Analysis of the association between the ICSI cycle parameters and *FSHR* (rs6166) gene polymorphism showed a significant reduction in the number of injected oocytes in individuals with the CT genotype compared with those with the CC genotype among the normal responders (P = 0.03). A significant decline was observed in the number of injected oocytes between individuals with the CT and CC genotypes among the poor responders (P = 0.013), whereas a significant increase was found in the number of stimulation days in individuals with the CT genotype compared with those with the CC genotype among the high responders (P ≤ 0.001) (**Table 3.6**).

3.5. Association between the *FSHR* (rs6165) genotype and female general and ICSI parameters.

The results associated with the *FSHR* (rs6165) genotype showed significant elevation in the AMH levels on day 3 in individuals with the CT genotype compared with individuals with the CC genotype among the poor responders (P = 0.03). There were significant reductions in PRL levels on day 3 in individuals with the CT genotype compared with individuals with the CC genotype among the high responders (P = 0.02). In contrast, no significant variations were found based on age, body mass index, AMH levels on day 3, FSH levels on day 3, LH levels on day 3, and PRL levels on day 3 between individuals with CT and CC genotypes among the normal responders (P = 0.62, P = 0.38, P = 0.82, P = 0.79, P = 0.61, and P = 0.70, respectively) (**Table 3.7**). The results of the association between ICSI cycle parameters and *FSHR* (rs6165) gene polymorphism showed a significant reduction in the starting FSH dose in individuals with the CT genotype compared with individuals with the CC genotype among the poor responders (P = 0.01). Among the high responders, a significant decrease was observed in the number of embryo transfers in individuals with the CT genotype compared with individuals with the CC genotype (P = 0.01). In addition, a significant increase in the number of stimulation days (P ≤

0.001) and significant reductions in the number of fertilised oocytes (P = 0.03) and the number of embryos frozen (P = 0.02) were observed in individuals with the CT genotype compared with individuals with the CC genotype (**Table 3.8**).

3.6. Association between the *ESR1* (rs2234693) genotypes and female general and ICSI parameters.

Table 3.9 illustrates the association between the *ESR1* (rs2234693) genotypes and general female characteristics. The results showed a significant decrease in the body mass index of normal responders with the TC genotype compared with those with the TT genotype (P = 0.05). When poor and high responders were analysed, there were no significant variations in the general characteristics between individuals with TC and TT genotypes.

Table 3.10 illustrates the association between *ESR1* (rs2234693) genotypes and ICSI cycle parameters. No significant differences were found in the clinical characteristics of normal, high, and poor responders with TC and TT genotypes.

Table 3.5 Association between the *FSHR* (rs6166) genotype and the female general and ICSI parameters (n = 280)

FSHR-rs6166 / General Characteristics	Normal responders (n=80)		P-Value	Poor responders (n=92)		P-Value	High responders (n=108)		P-Value
	C/C (n= 63)	C/T (n=17)		C/C (n=46)	C/T (n=46)		C/C (n=56)	C/T (n=52)	
	Mean±SD	Mean±SD		Mean±SD	Mean±SD		Mean±SD	Mean±SD	
Female Age (Year)	29.51±2.83	29.06±3.07	0.51	28.67±2.81	29.87±3.04	0.060	29.71±3.25	28.90±3.18	0.21
BMI (kg/m2)	22.52±1.98	22.62±2.61	0.60	21.86±2.37	22.17±2.64	0.542	22.14±2.15	22.78±2.36	0.10
AMH Level (ng/ml)	1.90±0.52	1.95±0.52	0.57	2.18±0.71	2.13±0.50	0.851	1.81±0.55	1.99±0.65	0.11
Basal FSH Level (mIU/ml)	7.40±1.83	6.57±2.28	0.09	6.37±1.80	6.55±1.61	0.603	6.47±1.62	6.71±1.97	0.49
Basal LH Level (mIU/ml)	5.05±1.51	4.54±1.43	0.21	4.25±1.36	4.53±1.39	0.306	4.21±1.10	4.60±1.46	0.17
Basal PRL Level (ng/mL)	10.46±4.94	11.31±5.61	0.63	11.27±4.57	10.43±4.44	0.373	13.20±4.85	11.45±5.11	0.10

Mann-Whitney (U-test), data are illustrated as Mean ± Standard deviation; SD: Standard deviation; BMI: Body Mass Index; $P \leq 0.05$: Significant.

Table 3.6 Association between the *FSHR* (rs6166) genotype and the ICSI parameters (n = 280)

FSHR rs6166 / ICSI Parameters	Normal responders (n=80)		P-Value	Poor responders (n=92)		P-Value	High responders (n=108)		P-Value
	C/C (n= 63)	C/T (n=17)		C/C (n=46)	C/T (n=46)		C/C (n=56)	C/T (n=52)	
	Mean±SD	Mean±SD		Mean±SD	Mean±SD		Mean±SD	Mean±SD	
Starting FSH Dose (IU)	298.81±75.59	291.18±83.36	0.71	286.96±74.67	277.17±72.23	0.522	321.43±72.52	304.33±85.85	0.29
Total Dose of Gonadotropin IU)	2486.03±537.24	2442.65±528.76	0.54	4245.11±471.25	4188.48±488.04	0.401	1798.21±244.57	1861.06±236.47	0.17
Number of Stimulation Days	9.79±0.68	9.82±0.73	0.90	11.24±0.77	11.37±0.88	0.392	8.59±0.73	9.23±0.65	≤ 0.001
Number of Collected Oocytes	10.87±2.41	10.00±1.41	0.06	2.22±0.76	2.39±1.44	0.929	20.13±2.09	19.58±2.03	0.19
Number of Injected Oocytes	8.60±2.04	7.76±1.39	0.03	1.85±0.61	1.39±1.13	0.013	16.71±2.17	16.27±1.95	0.31
Number of Fertilized Oocytes	6.32±1.84	5.76±1.35	0.14	0.91±0.72	1.17±0.95	0.219	13.61±2.30	13.08±2.30	0.53
Number of Embryo Transfer	2.59±0.69	2.41±0.51	0.09	0.89±0.71	0.98±0.75	0.624	2.25±1.31	2.54±1.09	0.22
Number of Embryo Freezing	2.44±1.27	2.12±0.99	0.33	0.00±0.00	0.04±0.29	0.317	8.95±3.68	8.19±2.69	0.45

Mann-Whitney (U-test), data are illustrated as Mean ± Standard deviation; SD: Standard deviation; P ≤ 0.05: Significant.

Table 3.7 Association between the *FSHR* (rs6165) genotype and the general female characteristics (n = 280)

General Characteristics \ *FSHR- rs*6165	Normal responders (n=80)		P-Value	Poor responders (n=92)		P-Value	High responders (n=108)		P-Value
	C/C (n=37)	C/T (n= 43)		C/C (n=28)	C/T (n=64)		C/C (n=35)	C/T (n= 73)	
	Mean±SD	Mean±SD		Mean±SD	Mean±SD		Mean±SD	Mean±SD	
Female Age (Year)	29.24±2.74	29.56±3.00	0.62	28.39±2.77	29.66±3.00	0.06	29.71±3.38	29.14±3.16	0.44
BMI (kg/m2)	22.87±1.62	22.25±2.44	0.38	21.88±2.38	22.08±2.57	0.85	21.89±2.33	22.71±2.20	0.08
AMH Level (ng/ml)	1.93±0.49	1.89±0.54	0.82	2.06±0.85	2.19±0.47	0.03	2.04±0.63	1.83±0.58	0.16
Basal FSH Level (mIU/ml)	7.24±1.78	7.22±2.10	0.79	6.44±1.86	6.47±1.64	0.98	6.76±1.70	6.50±1.84	0.47
Basal LH Level (mIU/ml)	5.01±1.47	4.89±1.54	0.61	4.36±1.51	4.40±1.33	0.86	4.48±1.20	4.35±1.34	0.42
Basal PRL Level (ng/mL)	10.35±4.66	10.90±5.43	0.70	11.39±3.81	10.61±4.78	0.31	14.02±4.86	11.56±4.95	0.02

Mann-Whitney (U-test), data are illustrated as Mean ± Standard deviation; SD: Standard deviation; BMI: Body Mass Index; $P \leq 0.05$: Significant.

Table 3.8 Association between the *FSHR* (rs6165) genotype and the ICSI parameters (n = 280)

ICSI Parameters \ *FSHR*-rs6165	Normal responders (n=80)		P-Value	Poor responders (n=92)		P-Value	High responders (n=108)		P-Value
	C/C (n=37)	C/T (n= 43)		C/C (n=28)	C/T (n=64)		C/C (n=35)	C/T (n= 73)	
	Mean±SD	Mean±SD		Mean±SD	Mean±SD		Mean±SD	Mean±SD	
Starting FSH Dose (IU)	297.97±76.01	296.51±78.41	0.39	310.71±75.59	269.53±69.07	0.01	297.86±82.33	320.55±77.32	0.16
Total Dose of Gonadotropin(IU)	2446.62±486.85	2502.79±573.17	0.42	4095.54±479.25	4269.84±471.27	0.12	1746.43±219.97	1867.81±243.08	0.08
Number of Stimulation Days	9.76±0.64	9.84±0.72	0.66	11.21±0.74	11.34±0.86	0.37	8.51±0.89	9.08±0.62	≤ 0.001
Number of Collected Oocytes	10.57±1.82	10.79±2.60	0.35	2.18±0.72	2.36±1.29	0.71	20.37±2.22	19.62±1.96	0.14
Number of Injected Oocytes	8.32±1.67	8.51±2.16	0.46	1.39±0.50	1.72±1.06	0.13	17.06±2.30	16.23±1.91	0.09
Number of Fertilized Oocytes	6.05±1.70	6.33±1.81	0.36	1.00±0.67	1.06±0.92	0.96	14.17±2.33	12.96±2.20	0.03
Number of Embryo Transfer	2.51±0.77	2.58±0.54	0.98	0.93±0.60	0.94±0.77	0.93	2.97±1.44	1.59±1.04	0.01
Number of Embryo Freezing	2.32±1.16	2.42±1.28	0.78	0.00±0.00	0.03±0.25	0.51	9.54±3.42	8.12±3.08	0.02

Mann-Whitney (U-test), data illustrated as Mean ± Standard deviation; SD: Standard deviation; P ≤ 0.05: Significant.

Table 3.9 Association between the *ESR1* (rs2234693) genotype and the female general characteristics (n = 280)

General characteristics \ *ESR1-rs*2234693	Normal responders (n=80)		P-Value	Poor responders (n=92)		P-Value	High responders (n=108)		P-Value
	T/T (n= 62)	T/C (n=18)		T/T (n=41)	T/C (n=51)		T/T (n=51)	T/C (n=57)	
	Mean±SD	Mean±SD		Mean±SD	Mean±SD		Mean±SD	Mean±SD	
Female Age (Year)	29.31±2.96	29.78±2.60	0.57	28.90±3.10	29.57±2.87	0.341	29.02±3.18	29.61±3.27	0.36
BMI (kg/m2)	22.76±2.15	21.78±1.80	0.05	22.43±2.28	21.68±2.64	0.171	22.38±2.31	22.50±2.25	0.81
AMH Level (ng/ml)	1.92±0.52	1.85±0.52	0.56	2.14±0.52	2.16±0.68	0.724	1.80±0.57	1.99±0.62	0.13
Basal FSH Level (mIU/ml)	7.39±1.93	6.67±1.96	0.29	6.52±1.82	6.41±1.61	0.994	6.65±1.73	6.52±1.86	0.59
Basal LH Level (mIU/ml)	5.02±1.48	4.69±1.60	0.41	4.40±1.33	4.38±1.43	0.826	4.47±1.20	4.32±1.38	0.49
Basal PRL Level (ng/mL)	10.41±5.15	11.44±4.81	0.43	11.52±4.94	10.31±4.08	0.289	12.87±5.54	11.88±4.51	0.59

Mann-Whitney (U-test), data are illustrated as Mean ± Standard deviation; SD: Standard deviation; BMI: Body Mass Index; P ≤ 0.05: Significant.

Table 3.10 Association between the *ESR1* (rs2234693) genotype and the ICSI parameters (n = 280)

ESR1-rs2234693 / ICSI parameters	Normal responders (n=80)		P-Value	Poor responders (n=92)		P-Value	High responders (n=108)		P-Value
	T/T (n= 62)	T/C (n=18)		T/T (n=41)	T/C (n=51)		T/T (n=51)	T/C (n=57)	
	Mean±SD	Mean±SD		Mean±SD	Mean±SD		Mean±SD	Mean±SD	
Starting FSH Dose (IU)	298.79±77.41	291.67±76.70	0.73	279.88±73.15	283.82±73.96	0.797	333.17±70.31	294.64±83.22	0.01
Total Dose of Gonadotropin(IU)	2434.60±516.61	2622.22±575.09	0.22	4187.20±466.17	4240.59±490.47	0.572	1803.85±246.46	1851.34±237.00	0.29
Number of Stimulation Days	9.820.69	9.72±0.67	0.60	11.32±0.72	11.29±0.90	0.983	8.69±0.78	9.09±0.69	0.01
Number of Collected Oocytes	10.65±2.34	10.83±2.01	0.97	2.27±0.59	2.33±1.45	0.719	19.75±1.84	19.96±2.28	0.50
Number of Injected Oocytes	8.44± 2.01	8.39±1.72	0.77	1.46±0.64	1.75±1.11	0.240	16.37±1.86	16.63±2.26	0.66
Number of Fertilized Oocytes	6.21±1.80	6.17±1.62	0.66	1.00±0.74	1.08±0.93	0.790	13.21±2.34	13.48±2.29	0.97
Number of Embryo Transfer	2.56±0.69	2.50±0.51	0.36	0.90±0.66	0.96±0.77	0.671	2.54±1.09	2.25±1.31	0.22
Number of Embryo Freezing	2.39±1.25	2.33±1.14	0.77	0.00±0.00	0.04±0.28	0.370	8.31±2.97	8.84±3.50	0.81

Mann-Whitney (U-test), data are illustrated as Mean ± Standard deviation; SD: Standard deviation; $P \leq 0.05$: Significant.

3.7. Association between the *LHCGR* (rs4073366) genotypes and female general and ICSI parameters

Analysis of the association between *LHCGR* (rs4073366) genotypes and the patients' general characteristics showed a significant reduction in the body mass index, FSH level on day 3, and LH level on day 3 in individuals with the CG genotype compared with individuals with the CC genotype among poor responders (P = 0.01, P = 0.03, and P = 0.01, respectively) (**Table 3.11**).

In contrast, a significant elevation in AMH levels was observed on day 3 in individuals with the CG genotype compared with those with the CC genotype (P = 0.01) among the high responders. There were no significant variations in the general characteristics of normal responders with CG and CC genotypes. Analysis of the association between ICSI cycle parameters and *LHCGR* (rs4073366) genotypes showed a significant decrease in the starting FSH dose and the total dose of gonadotrophin in individuals with the CG genotype compared with those with the CC genotype among the normal responders (P = 0.02 and P = 0.01, respectively). Additionally, a significant decrease was found in the starting dose of FSH in individuals with the CG genotype compared with those with the CC genotype among the high responders (P = 0.02). In contrast, the results showed a significant increase in the number of injected oocytes, the number of fertilised oocytes, and the number of embryos transferred (P = 0.001, P = 0.05, and P = 0.02, respectively) in individuals with the CG genotype compared with those with the CC genotype among the poor responders (**Table 3.12**).

Table 3.11 Association between the *LHCGR* (rs4073366) genotype and the female general characteristics (n = 280)

General Characteristics \ *LHCGR*- rs4073366	Normal responders (n=80)			Poor responders (n=92)			High responders (n=108)		
	C/C (n=69)	C/G (n=11)	P-Value	C/C (n= 84)	C/G (n=8)	P-Value	C/C (n= 101)	C/G (n=7)	P-Value
	Mean±SD	Mean±SD		Mean±SD	Mean±SD		Mean±SD	Mean±SD	
Female Age (Year)	29.32±2.98	30.00±2.10	0.41	29.33±3.03	28.63±2.39	0.46	29.36±3.25	28.86±3.18	0.60
BMI (kg/m2)	22.58±2.15	22.28±1.92	0.57	22.21±2.39	19.93±2.83	0.01	22.50±2.28	21.69±2.11	0.34
AMH Level (ng/ml)	1.87±0.52	2.17±0.45	0.13	2.16±0.62	2.06±0.50	0.69	1.86±0.60	2.43±0.42	0.01
Basal FSH Level (mIU/ml)	7.30±1.82	6.78±2.67	0.25	6.58±1.69	5.20±1.26	0.03	6.62±1.78	6.05±1.96	0.36
Basal LH Level (mIU/ml)	5.04±1.48	4.36±1.57	0.08	4.50±1.37	3.26±0.83	0.01	4.42±1.27	3.98±1.62	0.25
Basal PRL Level (ng/mL)	10.76±5.14	9.94±4.73	0.52	11.10±4.44	8.20±4.59	0.09	12.10±4.98	16.06±4.65	0.06

Mann-Whitney (U-test), data are illustrated as Mean ± Standard deviation; SD: Standard deviation; BMI: Body Mass Index; $P \leq 0.05$: Significant.

Table 3.12 Association between the *LHCGR* (rs4073366) genotype and the ICSI parameters (n = 280)

LHCGR-rs4073366 / ICSI parameters	Normal responders (n=80)			Poor responders (n=92)			High responders (n=108)		
	C/C (n=69)	C/G (n=11)	P-Value	C/C (n= 84)	C/G (n=8)	P-Value	C/C (n= 101)	C/G (n=7)	P-Value
	Mean ± SD	Mean ± SD		Mean ± SD	Mean ± SD		Mean ± SD	Mean ± SD	
Starting FSH Dose (IU)	305.43±75.35	245.45±67.84	0.02	283.93±73.70	262.50±69.44	0.43	317.82±78.77	246.43±56.69	0.02
Total Dose of Gonadotropin(IU)	2522.03±554.39	2193.18±216.24	0.01	4208.57±486.36	4303.13±394.03	0.74	1831.44±246.31	1785.71±166.99	0.88
Number of Stimulation Days	9.83±0.66	9.64±0.81	0.33	11.30±0.83	11.38±0.74	0.83	8.89±0.79	9.00±0.00	0.65
Number of Collected Oocytes	10.71±2.35	10.55±1.63	0.53	2.27±1.19	2.63±0.52	0.09	19.85±2.04	20.00±2.65	0.89
Number of Injected Oocytes	8.41±1.99	8.55±1.63	0.84	1.56±0.95	2.25±0.46	0.001	16.53±2.07	16.00±2.16	0.39
Number of Fertilized Oocytes	6.17±1.78	6.36±1.63	0.98	1.00±0.86	1.50±0.53	0.05	13.35±2.30	13.43±2.64	0.82
Number of Embryo Transfer	2.58±0.67	2.36±0.50	0.10	0.88±0.72	1.50±0.53	0.02	2.44±1.18	1.71±1.60	0.13
Number of Embryo Freezing	2.36±1.25	2.45±1.04	0.83	0.02±0.22	0.00±0.00	0.76	8.60±3.28	8.29±3.04	0.89

Mann-Whitney (U-test), data are illustrated as Mean ± Standard deviation; SD: Standard deviation; $P \leq 0.05$: Significant.

3.8. Association between the *AMH* (rs17854573) genotypes and female general and ICSI cycle parameters

Analysis of the association between *AMH* (rs17854573) genotypes and the patients' general characteristics showed that basal FSH levels and patient age were significantly higher in individuals with the GA genotype than in individuals with the GG genotype among the normal responders (P = 048 and P = 0.036, respectively). BMI, AMH levels, and age were significantly lower in individuals with the GA genotype compared with individuals with the GG genotype among the high responders (P = 0.023, P = 0.040, and P =0.039, respectively), but PRL levels were significantly higher in individuals with the GA genotype than in individuals with the GG genotype among the high responders (P = 0.036). On the other hand, the study did not find any significant variations in the general characteristics of poor responders with the GA genotype compared with those with the GG genotype (**Table 3.13**).

When ICSI cycle parameters and *AMH* (rs17854573) gene polymorphisms were measured, the results showed a significant reduction in the number of stimulation days, the number of collected oocytes, the number of injected oocytes, the number of fertilised oocytes, and the number of embryo transfers in individuals with the GA genotype compared with individuals with the GG genotype among the poor responders ($P \leq 0.001$). In contrast, the results showed significant increase in the total dose of gonadotrophins ($P \leq 0.001$) in individuals with the GA genotype compared with those with the GG genotype among the normal responders. The results of the *AMH* (rs17854573) gene polymorphism showed no significant difference in the ICSI cycle parameters in the high response group (**Table 3.14**).

Table 3.13 Association between the *AMH* (rs17854573) genotypes and the female general characteristics (n = 280)

AMH-rs17854573 / General Characteristics	Normal responders (n=80)			Poor responders (n=92)			High responders (n= 108)		
	G/G (n=74)	G/A (n=6)	P-value	G/G (n=72)	G/A (n= 20)	P-value	G/G (n= 106)	G/A (n= 2)	P-value
	Mean± SD	Mean± SD		Mean± SD	Mean± SD		Mean± SD	Mean± SD	
Female Age (Year)	29.20±2.80	32.00±2.61	0.036	29.13±2.87	29.80±3.33	0.36	29.41±3.20	25.00±0.00	0.039
BMI (kg/m2)	22.56±2.17	22.26±1.12	0.559	21.81±2.54	22.76±2.24	0.10	22.52±2.23	18.58±0.00	0.023
AMH Level (ng/ml)	1.93±0.52	1.71±0.46	0.385	2.09±0.50	2.37±0.89	0.31	1.91±0.60	1.12±0.00	0.040
Basal FSH Level (mIU/ml)	7.13±1.96	8.46±1.46	0.048	6.51±1.61	6.26±2.03	0.57	6.59±1.81	6.32±0.00	0.927
Basal LH Level (mIU/ml)	4.90±1.48	5.45±1.81	0.385	4.39±1.29	4.38±1.70	0.91	4.40±1.31	4.19±0.00	0.784
Basal PRL Level (ng/mL)	10.68±5.16	10.23±4.07	1.000	10.48±4.56	12.18±4.12	0.13	12.22±4.99	19.36±0.00	0.036

Mann-Whitney (U-test), data are illustrated as Mean ± Standard deviation; SD: Standard deviation; BMI: Body Mass Index; $P \leq 0.05$: Significant.

Table 3.14 Association between the *AMH* (rs17854573) genotypes and the ICSI parameters (n = 280)

AMH - rs17854573 / ICSI parameters	Normal responders (n=80)			Poor responders (n= 92)			High responders (n=108)		
	G/G (n=74)	G/A (n=6)	P-Value	G/G (n=72)	G/A (n= 20)	P-Value	G/G (n= 106)	G/A (n= 2)	P-Value
	Mean±SD	Mean±SD		Mean±SD	Mean±SD		Mean±SD	Mean±SD	
Starting FSH Dose (IU)	292.91±76.70	350.00±61.24	0.080	283.33±73.64	277.50±73.40	0.75	312.03±79.60	375.00±0.00	0.263
Total Dose of Gonadotropin(IU)	2393.85±413.43	3500.00±790.57	≤ 0.001	4243.68±483.21	4120.00±457.01	0.16	1824.76±242.40	2025.00±0.00	0.222
Number of Stimulation Days	9.82 ±0.67	9.50±0.84	0.214	11.42±0.83	10.90±0.64	≤ 0.001	8.90±0.77	9.00±0.00	0.813
Number of Collected Oocytes	10.69±1.94	10.67±5.09	0.260	2.44±1.20	1.80±0.77	≤ 0.001	19.84±2.08	21.00±0.00	0.368
Number of Injected Oocytes	8.47±1.75	7.83±3.82	0.882	1.75±1.00	1.15±0.37	≤ 0.001	16.49±2.09	17.00±0.00	0.563
Number of Fertilized Oocytes	6.23±1.63	5.83±3.06	0.867	1.18±0.86	0.55±0.60	≤ 0.001	13.34±2.33	14.00±0.00	0.548
Number of Embryo Transfer	2.55±0.64	2.50±0.84	0.974	1.06±0.71	0.50±0.61	≤ 0.001	2.38±1.22	3.00±0.00	0.472
Number of Embryo Freezing	2.39±1.19	2.17±1.60	0.699	0.03±0.24	0.00±0.00	0.60	8.63±3.26	6.00±0.00	0.146

Mann-Whitney (U-test), data illustrated as Mean ± Standard deviation; SD: Standard deviation; P ≤ 0.05: Significant.

3.9. Analysis of gene expression levels

3.9.1 Descriptive characteristics of samples included in the gene expression study

In this section of the study, 140 blood samples were randomly selected to assess the expression levels of *FSHR*, *AMH*, *LHCGR*, *ESR1*, and *GAPDH* (housekeeping gene). The samples were divided into three groups based on ovarian response to the stimulation program: poor response (n= 66), normal response (n= 20), and high response (n= 54). **Table 3.15** illustrates the descriptive characteristics of the study population. The women included in the study were 25–35 years old (29.69 ± 3.17 years). The results showed significant differences between the study groups in terms of the total dose of gonadotrophin, the number of stimulation days, the number of collected oocytes, the number of injected oocytes, the number of fertilised oocytes, and the number of embryo transfers ($P \leq 0.0001$) (**Table 3.16**). In contrast, no significant variations among the study groups were found in terms of age ($P = 0.172$), body mass index ($P = 0.091$), AMH hormone levels ($P = 0.066$), FSH hormone levels ($P = 0.119$), LH hormone levels ($P = 0.060$), and PRL hormone levels ($P = 0.489$).

3.9.2 Comparison of gene expression among the study groups

The analysis of qPCR results revealed variations in the expression levels of *FSHR (rs616)*, *FSHR (rs6165)*, *AMH*, *LHCGR*, and *ESR1* ($P \leq 0.0001$) genes among the study groups (poor, normal, and high response) (**Table 3.17*).*** *FSHR (rs616)*, *FSHR (rs6165)*, *AMH*, *LHCGR*, and *ESR1* expression was upregulated in high responders compared with normal responders (13.83, 13.64, 16.80, 2.91, and 53.45, respectively). In addition, the expression of *LHCGR* (3.84) was upregulated in poor responders compared with normal responders. In contrast, the expression of *FSHR (rs616)*, *FSHR (rs6165)*, *AMH*, and *ESR1* (0.07, 0.07, 0.01, and 0.01, respectively) was downregulated in poor responders compared with normal responders (**Table 3.18**).

3.9.3 Correlation between gene expression levels and other parameters in the high response group

Finally, this study evaluated the correlation between the level of gene expression (*FSHR [rs6166], FSHR [rs6165], AMH, LHCGR,* and *ESR1*) and the different parameters in the high response group. The results revealed significant positive correlations between the expression levels of *FSHR (rs6166), FSHR (rs6165),* and *LHCGR* genes and PRL hormone levels ($P \leq 0.0001$). In contrast, negative associations were found between PRL hormone levels, body mass index, and *AMH* expression levels ($P \leq 0.018$ and $P \leq 0.001$, respectively). In addition, a significant negative correlation was found between the levels of FSH, LH, and starting doses of FSH, and *LHCGR* expression levels ($P = 0.006$, $P \leq 0.001$, and $P = 0.008$, respectively). A positive correlation was found between age and level of FSH and the expression level of *FSHR (rs616)* ($P = 0.024$). However, a negative correlation was found between LH level, starting dose of FSH, and the expression level of *FSHR (rs6166)* ($P \leq 0.001$ and $P = 0.008$, respectively) (**Table 3.19).** When the ICSI outcomes were measured in the high response group, significant associations were found between the expression levels of *FSHR (rs6166), FSHR (rs6165), LHCGR*, and the number of frozen embryos ($P = 0.002$, $P = 0.002$, $P \leq 0.001$, respectively). Additionally, significant positive associations were observed between the expression levels of *AMH* and *LHCGR* and the number of fertilised oocytes ($P \leq 0.001$ and $P = 0.007$, respectively). Positive correlations were found between the number of collected oocytes and number of injected oocytes and the expression level of *AMH* ($P \leq 0.001$ and $P = 0.003$, respectively). In contrast, negative associations were observed between the expression levels of *FSHR (rs6166), FSHR (rs6165),* and *LHCGR* and the number of stimulation days ($P = 0.044$, $P = 0.044$, and $P = 0.045$, respectively) (**Table 3.20**).

3.9.4 Correlation between gene expression levels and other parameters in the poor response group

The study assessed the association between gene expression levels and other parameters in the poor response group, and found significant negative correlations between FSH, LH, and PRL levels and *FSHR* (rs6166) expression ($P = 0.02$, $P \leq$

0.001, and P ≤ 0.001, respectively). A significant negative correlation was observed between *FSHR (6165)* expression level, *AMH* expression level, and PRL hormone level (P ≤ 0.001). In contrast, significant positive correlations were found between *LHCGR* and *ESR1* expression levels and PRL hormone levels (P = 0.050 and P ≤ 0.001, respectively). A significant negative association was found between *FSHR* (rs6165) and *LHCGR* expression levels and LH hormone levels (P ≤ 0.001), and similar correlations were found between *FSHR (rs6165)* and *LHCGR* expression levels and FSH hormone levels (P = 0.02 and P ≤ 0.001, respectively). A significant relationship was found between *LHCGR* and *ESR1* expression levels and body mass index (P ≤ 0.001 and P ≤ 0.001, respectively) (**Table 3.21**).

Analysis of the ICSI outcomes in the poor responder group showed significant negative associations between *FSHR* (rs6166) and *LHCGR* expression levels and the total dose of gonadotrophins (P ≤ 0.001 and P ≤ 0.001, respectively). Additionally, significant negative associations were found between *FSHR* (rs6166), *FSHR (rs6165)* and *AMH* expression levels and the number of stimulation days (P ≤ 0.001, P ≤ 0.001, and P = 0.01, respectively). The results showed significant positive correlations between the number of oocytes, the number of injected oocytes in fertilised oocytes, and the number of embryo transfers, and *FSHR (rs6166)* expression levels (P ≤ 0.001, P = 0.04, P ≤ 0.001, and P ≤ 0.001, respectively), and the same trend was observed between the same parameters and *FSHR (rs6165)* expression levels (**Table 3.22**).

Table 3.15 Descriptive characteristics of the study population according to gene expression analysis (n = 140)

Parameters - (n=140)	Mean±SD
Age (Year)	29.69±3.17
BMI (kg/m2)	22.27±2.38
AMH Level (ng/ml)	2.05±0.76
Basal FSH Level (mIU/ml)	7.22±2.10
Basal LH Level (mIU/ml)	4.65±1.55
Basal PRL Level (ng/mL)	11.51±4.88
Starting FSH Dose (IU)	216.84±24.14
Total Dose of Gonadotropin (IU)	2897.11±858.50
Number of Stimulation Days	9.77±0.98
Number of Collected Oocytes	10.67±8.48
Number of Injected Oocytes	8.59±7.22
Number of Fertilized Oocytes	6.65±5.80
Number of Transfered embryos	2.07±1.10
Number of Embryo Freezing	3.40±3.80

Data are presented as mean ±SD, SD: Standard deviation.

Table 3.16 Descriptive characteristics of the different groups according to gene expression analysis (n = 140)

Parameters	Normal Response group (n=20)	High Response group (n=54)	Poor Response group (n=66)	P-Value
	Mean±SD	Mean±SD	Mean±SD	
Age (Year)	29.550±3.052	30.333±2.775	29.212±3.453	0.172
BMI (kg/m2)	21.387±2.122	22.735±2.514	22.164±2.280	0.091
AMH Level (ng/ml)	1.990±0.504	1.860±0.611	2.219±0.896	0.066
Basal FSH Level (mIU/ml)	7.809±1.825	6.908±1.995	7.301±2.240	0.119
Basal LH Level (mIU/ml)	5.308±1.423	4.446±1.402	4.621±1.665	0.060
Basal PRL Level (ng/mL)	10.283±4.310	11.599±5.558	11.809±4.450	0.489
Starting FSH Dose (IU)	211.70±47.51	219.020±14.52	216.62±20.06	0.283
Total Dose of Gonadotropin(IU)	2674.750±558.347	2025.000±264.664	3678.030±401.955	≤ 0.0001
Number of Stimulation Days	9.800±0.834	8.889±0.317	10.485±0.789	≤ 0.0001
Number of Collected Oocytes	11.300±2.179	20.556±1.269	2.394±0.492	≤ 0.0001
Number of Injected Oocytes	9.000±1.777	17.000±1.346	1.576±0.609	≤ 0.0001
Number of Fertilized Oocytes	6.650±1.531	13.444±1.076	1.091±0.836	≤ 0.0001
Number of Transfered embryos	2.800±0.410	3.000±0.000	1.091±0.836	≤ 0.0001
Number of Embryo Freezing	2.500±1.235	7.889±1.462	0.000±0.000	≤ 0.0001

Kruskal–Wallis (H-test); SD: Standard deviation; P ≤ 0.05: Significant.

Table 3.17 Gene expression (C_t) levels among the different groups (n = 140)

Gene's expression	Normal response (n=20)	High response (n=54)	Poor response (n=66)	P-Value
	Mean±SD	Mean±SD	Mean±SD	
***FSHR* (rs6166) expression level**	38.133±2.460	35.738±2.019	41.718±1.372	< 0.0001
***FSHR* (rs6165) expression level**	38.347±2.253	35.692±2.157	41.246±1.194	< 0.0001
***AMH* expression level**	36.265±1.236	33.596±0.770	42.327±1.773	< 0.0001
***LHCGR* expression level**	37.272±2.360	37.124±2.654	34.975±1.292	< 0.0001
***ESR1* expression level**	37.542±1.840	33.189±1.425	44.086±2.267	< 0.0001

Kruskal–Wallis (H-test); SD: Standard deviation; P ≤ 0.05: Significant.

Table 3.18 Regulation of relative gene expression in cases (high and poor response) compared to the control group (normal response) (n= 140)

Gene	Normal Response – Mean ΔC_t	High Response vs. Normal Response			Poor Response vs. Normal Response		
		High Response- Mean ΔC_t	Fold change	Regulation	Poor Response- Mean ΔC_t	Fold change	Regulation
***FSHR* (rs6166)**	3.03	-0.76	13.83	UP	6.96	0.07	down
***FSHR* (rs6165)**	2.96	-0.81	13.64	UP	6.85	0.07	down
AMH	1.16	-2.91	16.80	UP	7.57	0.01	down
LHCGR	2.16	0.62	2.91	UP	0.22	3.84	up
ESR1	2.43	-3.31	53.45	UP	9.33	0.01	down

Table 3.19 Correlation coefficient between the gene expression level "C_t" and different parameters at high response group (n = 54)

Gene Expression \ Parameters		Female age	BMI	AMH Level	FSH Level	LH Level	PRL Level	Starting FSH Dose
***FSHR* (rs6166) expression level**	R	0.308	0.156	-0.234	-.307	-.504	.745	-.357
	P-Value	0.024	0.259	0.088	0.024	≤ 0.001	≤ 0.001	0.008
***FSHR* (rs6165) expression level**	R	.308*	0.156	-0.234	-.307	-.504	.745	-.357
	P-Value	0.024	0.259	0.088	0.024	≤ 0.001	≤ 0.001	0.008
***AMH* expression level**	R	-0.197	-.320	0.067	-0.112	0.059	-.544	0.134
	P-Value	0.154	0.018	0.631	0.422	0.673	≤ 0.001	0.333
***LHCGR* expression level**	R	0.153	-0.026	-0.150	-.367	-.577	.633	-.358
	P-Value	0.269	0.852	0.279	0.006	≤ 0.001	≤ 0.001	0.008
***ESR1* expression level**	R	-0.256	-0.005	-0.050	0.008	0.160	0.042	-0.127
	P-Value	0.061	0.973	0.718	0.952	0.249	0.764	0.362

Spearman rank correlation, r: Correlation coefficient, P-value>0.05: Not significant, P-value ≤ 0.05: Significant.

Table 3.20 Correlation coefficient between the gene expression level "C_t" and ICSI outcomes at high response group after stimulation (n = 54)

Gene Expression \ ICSI outcomes		Total Dose of Gonadotropin	Stimulation Days	No. of oocytes	No. Injected oocytes	No. Fertilized oocytes	No. of Embryo Freezing
***FSHR* (rs6166) expression level**	r	0.085	-.275	0.030	0.159	0.203	.413
	P-Value	0.543	0.044	0.829	0.251	0.142	0.002
***FSHR* (rs6165) expression level**	r	0.085	-.275	0.030	0.159	0.203	.413
	P-Value	0.543	0.044	0.829	0.251	0.142	0.002
***AMH* expression level**	r	-.361	0.000	.451	.399	.504	-0.028
	P-Value	0.007	1.000	≤ 0.001	0.003	≤ 0.001	0.844
***LHCGR* expression level**	r	-0.142	-.274	0.051	.308	.361	.529
	P-Value	0.306	0.045	0.713	0.024	0.007	≤ 0.001
***ESR1* expression level**	r	0.231	0.206	-0.017	0.094	0.039	-0.257
	P-Value	0.092	0.135	0.902	0.497	0.781	0.061

Spearman rank correlation, *r*: Correlation coefficient, *p*-value>0.05: Not significant, *p*-value≤0.05: Significant.

Table 3.21 Correlation coefficient between the gene expression level "C_t" and different Parameters at poor response group (n= 66)

Gene Expression \ Parameters		age	BMI	AMH Level	FSH Level	LH Level	PRL Level	Starting FSH Dose
***FSHR* (rs6166) expression level**	r	0.05	-0.15	-0.12	-.278	-.626	-.526	-0.15
	P-Value	0.68	0.22	0.34	0.02	≤ 0.001	≤ 0.001	0.24
***FSHR* (rs6165) expression level**	R	0.05	-0.15	-0.12	-.278	-.626	-.526	-0.15
	P-Value	0.68	0.22	0.34	0.02	≤ 0.001	≤ 0.001	0.24
***AMH* expression level**	R	0.20	.435	-0.15	.286	-0.07	-.369	0.04
	P-Value	0.11	≤ 0.001	0.22	0.02	0.59	≤ 0.001	0.76
***LHCGR* expression level**	R	-0.08	-.433	0.22	-.508	-.713	.247	0.12
	P-Value	0.50	≤ 0.001	0.07	≤ 0.001	≤ 0.001	0.06	0.35
***ESR1* expression level**	R	0.08	-.577	.542	-0.07	0.03	.691	0.09
	P-Value	0.50	≤ 0.001	c 0.001	0.58	0.81	≤ 0.001	0.47

Spearman rank correlation, *r*: Correlation coefficient, *p*-value>0.05: Not significant, *p*-value≤0.05: Significant.

Table 3.22 Correlation coefficient between the gene expression level "C_t" and ICSI outcomes at poor response group after stimulation (n= 66)

Gene Expression \ ICSI outcomes		Total Dose of Gonadotropin	Stimulation Days	No. of oocytes	No. Injected oocytes	No. Fertilized oocytes	No. of Embryo transfer
***FSHR* (rs6166) expression level**	R	-.598	-.491	.654	.251	.371	.371
	P-Value	≤ 0.001	≤ 0.001	≤ 0.001	≤ 0.04	≤ 0.001	≤ 0.001
***FSHR* (rs6165) expression level**	R	-.598	-.491	.654	.251	.371	0.371
	P-Value	≤ 0.001	≤ 0.001	≤ 0.001	0.04	≤ 0.001	≤ 0.001
***AMH* expression level**	R	-0.11	-.302	0.21	0.02	0.14	0.14
	P-Value	0.39	≤ 0.01	0.09	0.88	0.26	0.26
***LHCGR* expression level**	R	-.798	0.09	.385	-0.13	0.14	0.14
	P-Value	≤ 0.001	0.46	≤ 0.001	0.30	0.28	0.28
***ESR1* expression level**	R	0.14	.718	-0.09	-0.22	-0.22	-0.22
	P-Value	0.28	≤ 0.001	0.46	0.08	0.08	0.08

Spearman rank correlation, *r*: Correlation coefficient, *p*-value>0.05: Not significant, *p*-value≤0.05: Significant.

4. Discussion

One of the most critical challenges in assisted reproduction is the variable ovarian response to gonadotrophins. This variability affects the success rate and safety of IVF/ICSI therapy. The embryologist classified the responses to gonadotrophin stimulation by different degrees, from poor responses, resulting in few ovarian follicles and treatment cancellation, to high responses, resulting in potentially life-threatening development of OHSS (**Mohiyiddeen *et al*., 2013**). Therefore, personalization and optimisation of different ovarian stimulation protocols are necessary to avoid complications and improve the success rate of assisted reproduction techniques.

Many modalities have been suggested to help predict the ovarian response to gonadotrophin stimulation and avoid the possible complications of gonadotrophin treatment. However, all current modalities have limited accuracy in predicting the degree of response (**Broekmans *et al*., 2006).**

One strategy that can be used to improve and control ovarian response rates is pharmacogenetics, where genetic polymorphisms can affect drug responses. Many studies have investigated the association between polymorphisms in *LHCGR, FSHR, ESR-1,* and *AMH* genes and ovarian responses in women undergoing IVF treatment, with different results. Therefore, the present study was designed to investigate the relationship between *LHCGR* (rs4073366), *FSHR* (rs6166 and rs6165), *ESR-1* (rs2234693), and *AMH* (rs17854573) polymorphisms and the degree of ovarian response to controlled ovarian stimulation in an Egyptian population.

4.1 The *FSHR* (rs6166) genotype among the study groups

The results of the current study showed significant variations in the frequencies of the *FSHR* rs6166 variant between poor and normal responders. These results are in agreement with those of other studies which demonstrated an association between rs6166 and ovarian response to gonadotrophins **(Anagnostou et al., 2012; Anagnostou et al., 2021; Huang et al., 2015; König et al., 2019; Meng et al., 2018; Sheikhha et al., 2011; Sindiani et al., 2021).**

The results also agree with meta-analyses which showed that the rs6166 polymorphism can be used as a predictor of poor ovarian response to gonadotrophins in patients undergoing IVF/ICSI therapy **(La Marca et al., 2013; Morón and Ruiz, 2010; Pabalan et al., 2014)**.

Furthermore, a recent meta-analysis conducted by **Alviggi et al., (2018)** which included 21 studies and 4,425 women investigated the correlation between the number of retrieved oocytes and the *FSHR* rs6166 polymorphism and concluded that the rs6166 polymorphism is associated with poor ovarian response to gonadotrophins.

However, the current study results are not in agreement with other studies which found no significant association between rs6166 polymorphism and ovarian response to gonadotrophin stimulation **(Klinkert et al, 2006; Mohiyiddeen et al, 2013; Trevisan et al., 2014)**. Furthermore, another study conducted in a Chinese population did not demonstrate the significance of the rs6166 polymorphism in predicting ovarian response to gonadotrophin stimulation in ART therapy **(Sun et al., 2018)**. Recently, **Paschalidou et al., (2020)** found no significant association between FSHR rs6166 and ovarian response to gonadotrophins.

When the association between the *FSHR* rs6166 variant and clinical and ICSI cycle parameters was assessed, no significant differences were observed in the general characteristics and basal hormone levels between patients with the CT and CC genotypes These results are in agreement with those of a study which also found no significant difference in clinical parameters in the three groups (**Sun et al., 2018).** Moreover, these results are in agreement with those of the study by **Mohiyiddeen et al., (2013)** which did not find any significant difference in clinical parameters between different genotypes.

However, these results differ from those of previous studies which reported a significant difference in basal FSH levels in rs6166 variant AA genotype carriers compared with GG genotype carriers **(Huang et al., 2015; Jun et al., 2006; Čuš et al., 2019)**. In addition, there is no concordance between these results and those previously reported, showing that women with the Ser/Ser genotype have increased basal gonadotrophin levels (**Sudo et al., 2002)**. Furthermore, previous studies also found that AA genotype carriers had higher day 3 FSH levels than AG and GG genotype carriers **(Jun et al., 2006; Wunsch et al., 2005; Sheikhha et al., 2011**).

Among poor responders, the results showed a significant reduction in the number of injected oocytes in the CT genotype carriers compared with the CC genotype carriers, which is consistent with the results by **Greb et al., (2005)**, who found a significant variation in the number of follicles and injected oocytes in patients with the AA genotype compared with those with the GG genotype**.** In addition, previous studies have shown that women with homozygous (p.680Ser) have higher levels of FSH (**Kuijper et al., 2011; Loutradis et al., 2006; Mayorga et al., 2000).** On the other hand, the current results are discordant with those of other studies which found no significant differences in the outcomes of *in vitro* fertilisation treatment between individuals with the CT and CC genotypes **(Mohiyiddeen et al., 2013; Yao et al., 2011**)**.**

4.2 The *FSHR* (rs6165) genotypes among the study groups

The current study did not show any significant variation in the frequency of the *FSHR* rs6165 variant in poor and high responders compared with normal responders, which is in agreement with studies which found no statistically significant association between the rs6165 variant and ovarian response in assisted reproduction treatment **(Meng et al., 2018; Trevisan et al., 2014).** Moreover, a recent study found no association between the rs6165 variant and the number of retrieved oocytes, particularly after adjusting for age and BMI **(Song et al., 2019).** More recently, **Sindiani et al., (2021)** found no significant association between *FSHR* rs6165 polymorphism and poor ovarian response in Jordanian Arabian infertile women undergoing IVF treatment.

However, the current results are not concordant with those of other studies which found an association between the rs6165 variant and poor ovarian response to gonadotrophin stimulation **(Livshyts et al., 2009; Motawi et al., 2017; Yan et al., 2013).** Furthermore, a systematic review and meta-analysis conducted by **Alviggi et al., (2018)** found an association between the rs6165 variant and poor ovarian response to gonadotrophin stimulation in patients undergoing IVF/ICSI therapy.

4.3 The *ESR1 (*rs2234693*)* genotypes among the study groups

The results showed a significant variation in the frequency of *ESR1* rs2234693 between poor and normal responders, and the CT genotype was more frequent in poor responders than the TT genotype. These results are in agreement with those of **Anagnostou *et al*., (2012)** which showed that the C allele was more frequent in poor responders. Moreover, **Lledó *et al.,***

(2019) found that significantly fewer oocytes were retrieved from patients with the C allele than from those with the T allele. In contrast, the current results are contrary to the results of other studies which found no significant variation in the frequency of *ESR1* rs2234693 between poor and normal responders **(Ayvaz *et al*., 2009; de Mattos *et al*., 2014; Čuš *et al*., 2019).**

The current study found no significant differences in the dose of gonadotrophins or the number of stimulation days between individuals with the TT and TC genotypes when analysing the association between the *ESR1* rs2234693 variant and the ICSI cycle parameters. These results are in line with those of previous studies **(Lledó *et al*., 2019; Čuš *et al*., 2019)**. However, a separate study found that carriers of the C allele had a significantly poorer outcome in ovarian stimulation **(Anagnostou et al., 2012).** Controversially, two other studies found that patients carrying the TT genotype required higher doses of gonadotrophins and a longer induction period **(de Castro et al., 2004; de Mattos et al., 2014).**

4.4 The *AMH* (rs17854573) genotypes among the study groups

AMH plays a crucial role in regulating the sensitivity of ovarian follicles to follicle-stimulating hormone, follicular recruitment, and final selection. Various polymorphisms in the *AMH* gene have been studied, particularly rs10407022 and rs2002555. Some studies found a significant association between the rs10407022 and rs2002555 polymorphisms with the degree of ovarian response **(Behre et al., 2005; Mayorga et al., 2000),** and the number of embryos **(Peluso *et al*., 2015).** However, other studies have not found any association **(Boudjenah et al., 2012; Cerra et al., 2016; Hanevik et al., 2010; Čuš et al., 2019).**

The current results showed a significant variation in the frequency of *AMH* rs17854573 variant between the poor and normal responders. After analysing the subgroups, the present study showed significant elevations in the basal FSH levels in individuals with the GA genotype compared with individuals with the GG genotype among the normal responders. The results showed a significant elevation in the total dose of gonadotrophin in individuals with the GA genotype compared with those with the GG genotype. In the poor response group, the results showed a significant decline in the number of stimulation days, the number of collected oocytes, the number of injected oocytes, the number of fertilised oocytes, and the number of embryos transferred in individuals with the GA genotype compared with individuals with the GG genotype. Consequently, the present study results suggest the role of the *AMH* gene variant (rs17854573) in the prediction of ovarian response.

However, the results of the current study did not confirm the results of another study which did not find a significant association between different *AMH* polymorphisms, including rs17854573, and the degree of ovarian response to gonadotrophins or the risk of OHSS **(Wang et al., 2015)**.

4.5. The *LHCGR* (rs4073366) genotypes among the study groups

Several studies have been conducted to find associations between *LHCGR* gene polymorphisms and ovarian response to gonadotrophins. **Lledó *et al.* (2019**) investigated the association between the *LHCGR* gene variant (rs2293275) and the degree of ovarian response to gonadotrophins. They found no significant variations in the number of stimulation days, the total dose of gonadotrophins, or the number of retrieved oocytes between genotypes.

Moreover, in their meta-analysis, **Alviggi *et al.* (2018**) tested for associations between *LHCGR* gene variants and ovarian response. They found only one study conducted by **Lindgren *et al.*, (2016)** and investigated the total dose of FSH and number of retrieved oocytes in relation to the *LHCGR* (rs2293275) genotype, and no significant differences were reported among genotypes. Additionally, they found only one study conducted by **Yin *et al.*, (2015)** that investigated gonadotrophin consumption and the number of retrieved MII oocytes in relation to the *LHCGR* (rs13405728) genotype, and no significant differences were reported among genotypes.

The current study investigated the association between *LHCGR* (rs4073366 +28G>C) and the degree of ovarian response to gonadotrophins. *LHCGR* (rs4073366 +28G>C) is a newly discovered polymorphism that has a potential influence on *LHCGR* mRNA processing (**Haasl *et al.*, 2008**). The current study did not find any significant differences in the frequencies of *LHCGR* (rs4073366) genotypes in the study groups (poor responders and high responders) compared with normal responders.

However, these results are inconsistent with the results of the study by **O'Brien *et al.*, (2013)** which investigated the correlation between the *LHCGR* (rs4073366) polymorphism and the outcome of COH in 172 patients undergoing IVF/ICSI therapy, and found no association between age, basal FSH, and CC or CG genotypes of the *LHCGR* (rs4073366) variant. Moreover, they found that rs4073366 carriers have approximately 3-fold increased risk of developing OHSS.

4.6. Expression levels of genes analysed in the present study

qPCR analysis showed significant variations in the expression levels of *FSHR*, *AMH*, *LHCGR*, and *ESR1* genes among the study groups (poor, normal, and high response). The expressions of *FSHR* (rs6166), *FSHR* (rs6165), *AMH*, *LHCGR*, and *ESR1* gene variants were upregulated between normal and high responding females. In addition, *AMH* gene expression was upregulated in poor versus normal response groups. In contrast, the expression of *FSHR* (rs6166), *FSHR* (rs6165), *LHCGR*, and *ESR1* genes was downregulated in poor versus normal response groups.

In a study conducted by **Cai et al., (2007)**, One hundred infertile women were included and divided into three groups: poor, normal, and high responders. They found that FSHR mRNA and protein expression levels were significantly different between poor, normal, and high responders. They concluded that different levels of *FSHR* gene expression in granulosa cells led to different degrees of ovarian responses, and that low expression of the *FSHR* gene may have a negative effect on ovarian response to gonadotrophin stimulation, which shows the critical role of *FSHR* in ovarian response to gonadotrophin stimulation.

Moreover, **Desai et al., (2013)** investigated the association between *FSHR* polymorphism at position _29 with FSHR expression level in 100 women undergoing IVF treatment. They found that carriers of AA genotype at _29 of the *FSHR* gene had a lower level of *FSHR* gene expression, which led to poor ovarian response. Furthermore, the same study investigated the impact of two *FSHR* gene polymorphisms (at two different positions: -29 and 680) on the degree of ovarian response to gonadotrophin stimulation and the level of *FSHR* gene expression. The study found that the polymorphism at position 680 had a negative effect on ovarian response, with a lower level of FSHR mRNA expression, supporting their insensitivity to exogenous FSH treatment**.**

Finally, we observed discrepancies in the results of different studies which investigated the impact of the same polymorphism on the degree of ovarian responses to gonadotrophin stimulation. Factors that may explain the lack of consensus among the different studies include: (I) Variations in study populations analysed by different studies; (II) Variations in the stimulation protocols used, for example some studies used a long agonist protocol while others used antagonist protocols; (III) variations in the types and amounts of gonadotrophins administered, leading to different stimulation responses; and (IV) Different study designs and outcome measures.

5. Conclusion

The findings from this study indicate that:

1. Significant variations were found in the frequencies of *AMH* (rs17854573), *FSHR* (rs6166), and *ESR1* (rs2234693) genotypes between poor and normal responders.
2. No significant variations were found in the frequencies of *FSHR* (rs6165) and *LHCGR* (rs4073366) genotypes in the poor and high responders compared with the normal responders.
3. Significant differences between poor and normal responders were found in the total gonadotrophin dose, the number of stimulation days, the number of collected oocytes, the number of injected oocytes, the number of fertilised oocytes, the number of embryo transfers, and the number of embryos frozen.

To our knowledge, this is the first study to examine the role of *LHCGR* (rs4073366) and *AMH* (rs17854573) in predicting the degree of ovarian response to gonadotrophins in an Egyptian population. However, considering the conflicting results between studies, more studies are needed to estimate the role of *LHCGR* (rs4073366 +28G>C) and *AMH* (rs17854573) in predicting the degree of ovarian response to gonadotrophins.

The results of this study suggest that polymorphisms in the genes of key reproductive hormones (*AMH*, *FSHR*, and *ESR1*) together with the patient's clinical characteristics and hormonal biomarkers can be used to predict ovarian response to gonadotrophins, to personalise and adjust the dose of gonadotrophins before starting the stimulation protocol, to improve efficacy, and to prevent possible complications such as cycle cancellation and OHSS, and, finally, to improve the pregnancy rate in patients undergoing ICSI treatment.

6. References

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Gene Cards (2021c) the human gene database, *FSHR* Gene, https://www.genecards.org/cgi-bin/carddisp.pl?gene=FSHR&keywords=FSHR

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Curriculum Vitae

The curriculum vitae was removed from the electronic version of the doctoral thesis for reasons of data protection.

Research and publications:

- *Evaluation of different therapeutic modalities of polycystic ovary syndrome; an evidence- based approach. [Master thesis), 2010, Sohag University, Egypt.*

- ***Ahmed, I.,*** *Abdelateef, S., Laqqan, M., Amor, H., Abdel-Lah, M. A., & Hammadeh, M. E. (2018). Influence of extended incubation time on Human sperm chromatin condensation, sperm DNA strand breaks and their effect on fertilization rate. Andrologia, 50(4), e12960.*

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Appendix (1)

Medical research ethics committee.

Sohag Faculty Of Medicine.

MEDICAL RESEARCH ETHICS COMMITTEE APPROVAL

Researcher Name: Islam Ahmed Abdelsalam Ahmed.

We would like to inform that the ethical committee discussed the research proposal about:

تأثير تعدد الاشكال الجيني على درجة الاستجابة لتنشيط المبيضين في المرضي الذين يخضعون للعلاج بالحقن المجهري

"Impact of genetic polymorphism on degree of response to ovarianStimulation in patients undergoing ICSI treatment"

And, the committee has decided to approve the above mentioned research with approval number 25 in its meeting on 17.09.2016.

Much obliged

Head of the ethical committee

Hamdy Ali mohamed

Dean of faculty of medicine