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Abstract

To date, approximately 15% of all couples worldwide suffer from infertility. The management include controlled ovarian hyper-stimulation of the women with follicle-stimulating hormone (FSH) and semen analysis of the men, prior to *in vitro* fertilization (IVF) and transfer of the fertilized embryo to the uterine cavity. Approximately 250 000 IVF cycles are performed worldwide each year, and as a result more than 5 million babies have been born since the technique was developed in 1978. It has been shown that common genetic variations, called single-nucleotide polymorphisms (SNPs), affect the reproductive ability of both men and women, and also the outcome of the IVF treatment. Especially the gene encoding the receptor to which FSH binds, the *FSHR* gene, has been investigated, since FSH is an essential hormone in both female and male reproductive function. However, since conflicting results have been produced from previous studies, no firm conclusions can be drawn. Therefore, the aims of this thesis were to investigate the impact of the *FSHR* SNPs T307A and N680S on male reproductive function in a cohort of homogenous Swedish men (n=313) from the general population, study the connection between the *FSHR* N680S and the *LHCGR* N312S SNPs in relation to outcomes after IVF in unselected women (n=617) attending an IVF clinic and explore the activity of the different *FSHR* and *LHCGR* variants *in vitro*. The *FSHR* genotypes of the men and *FSHR/LHCGR* genotypes of the women were associated with clinical parameters, and in the case of the women also associated with outcomes after IVF. The activity of the different receptor variants were investigated *in vitro* in granulosa cells from women undergoing IVF and in monkey kidney cells, by means of the level of the produced downstream signaling molecule cAMP, measured by ELISA, in response to FSH treatment. Swedish men from the general population that were homozygous for the *FSHR* T307/N680 genotype displayed a lower serum FSH concentration, as well as higher estradiol, sex hormone-binding globulin and testosterone concentrations, and also higher sperm counts and larger testicles. Women undergoing IVF got pregnant to a higher extent (four-fold in IVF cycle 1 and two-fold in IVF cycle 2 and 3) if they were homozygous for the combination of *FSHR* S680/*LHCGR* S312. *In vitro* results indicated that the *FSHR* S680/*LHCGR* N312 variant was superior in terms of cAMP production. In conclusion, the results from this thesis show favourable reproductive hormonal status and sperm parameters of men with the *FSHR* T307/N680 genotype, while women homozygous for the *FSHR* S680/*LHCGR* S312 combination more often got pregnant after IVF. *In vitro* results corroborated the observed higher pregnancy rate for women with the *FSHR* S680/*LHCGR* S312 genotype, in terms of a higher cAMP level in response to FSH treatment for this receptor combination. A gender difference may explain why the same pattern was not observed in men, since several differences in the hormonal regulation between the female and male reproductive system exist. In general, this knowledge may be utilized in the development of individualized treatments of infertile men and women.

Key words FSHR, LHCGR, single-nucleotide polymorphism (SNP), *in vitro* fertilization (IVF), reproduction, infertility, female fertility, male fertility

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The impact of gonadotropin receptor polymorphisms on human reproductive function

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Front cover picture "Min familj" by my nephew Alfred Anefur, 6 years old.

Back cover photo by Björn Martinsson.

Till Tor

PREFACE

This thesis comprises two parts. In the first part, an overview of the field is presented, starting with an introduction, followed by a materials and methods section and the overall results obtained. Subsequently, a general discussion of the scientific papers of which the thesis is built on is presented, and finally a general conclusion and some future perspectives are drawn. The second part of the thesis covers the three scientific papers (I-III) upon which the thesis is based.

CONTENT

LIST OF FIGURES

ABBREVIATIONS

7-AAD	7-amino actinomycin D
A	Alanine
APPLI1	Adaptor protein, phosphotyrosine interaction, PH domain and leucine zipper containing 1
ART	Assisted reproductive technologies
AS	Allele-specific
BCA	Bicinchinonic acid
BMI	Body mass index
C	Cysteine
CADMA	Competitive amplification of differentially melting amplicons
cAMP	3',5'-cyclic adenosine monophosphate
CREB	cAMP response element-binding
D	Aspartic acid
DMEM	Dulbecco's modified eagle's medium
E	Glutamic acid
E2	Estradiol
e.g.	*Exempli gratia*
ELISA	Enzyme-linked immunosorbent assay
ESHRE	European society of human reproduction and embryology
FACS	Fluorescent-activated cell sorting
FBS	Fetal bovine serum
FSH	Follicle-stimulating hormone
FSHR	Follicle-stimulating hormone receptor
G	Glycine
GC	Granulosa cell
GnRH	Gonadotrophin-releasing hormone
hCG	Human chorionic gonadotrophin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPG	Hypothalamic-pituitary-gonadal
I	Isoleucine
ICSI	Intracytoplasmic sperm injection
i.e.	*Id est*
IP$_3$	Inositol 1,4,5-triphosphate
IVF	*In vitro* fertilization
LH	Luteinizing hormone

LHCGR	Luteinizing hormone/human chorionic gonadotrophin receptor
N	Asparagine
NAFA	Nordic association for andrology
NCBI	National center for biotechnology information
PCOS	Polycystic ovarian syndrome
PCR	Polymerase chain reaction
PE	Phycoerythrin
PEI	Polyethylenimine
PerCP	Peridinin-chlorophyll-protein
R	Arginine
RFLP	Restriction fragment length polymorphism
RMC	Reproductive medicine centre
RPMI	Roswell park memorial institute
S	Serine
SHBG	Sex hormone-binding globulin
SNP	Single-nucleotide polymorphism
SSCP	Single-strand conformation polymorphism
T	Threonine
TSH	Thyroid-stimulating hormone
TSHR	Thyroid-stimulating hormone receptor
V-LHβ	Variant allele of the human LHβ subunit
vs.	*Versus*
W	Tryptophan
Y	Tyrosine

PAPERS INCLUDED IN THIS THESIS

This thesis is based on the following original publications, which hereafter are referred to in the text by their respective Roman numerals:

I.   Lindgren, I., Giwercman, A., Axelsson, J., Giwercman, Y.L. Association between follicle-stimulating hormone receptor polymorphisms and reproductive parameters in young men from the general population. *Pharmacogenetics and Genomics, 2012;22(9):667-672.*

II.   Lindgren, I., Bååth, M., Uvebrant, K., Dejmek, A., Kjaer, L., Henic, E., Bungum, M., Bungum, L., Cilio, C., Leijonhufvud, I., Skouby, S., Andersen, C.Y., Giwercman, Y.L. Combined assessment of polymorphisms in the *LHCGR* and *FSHR* genes predict chance of pregnancy after *in vitro* fertilization. *Human Reproduction, 2016;31(3):672-683.*

III. Lindgren, I., Henic, E., Bååth, M., Bungum, L., Bungum, M., Skouby, S., Bungum, L., Andersen, C.Y., Giwercman, Y.L. For women undergoing *in vitro* fertilization, chance of pregnancy is linked to gonadotropin receptor variants. *Manuscript.*

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Published papers not included in this thesis

Borgbo, T., Kristensen, L.S., **Lindgren, I.**, Andersen, C.Y., Hansen, L.L. Genotyping common FSHR polymorphisms based on competitive amplification of differentially melting amplicons (CADMA). *Journal of Assisted Reproduction and Genetics, 2014;31(11):1427-1436.*

Borgbo, T., Jeppesen, J.V., **Lindgren, I.**, Giwercman, Y.L., Hansen, L.L., Andersen, C.Y. Effect of the FSH receptor single nucleotide polymorphisms (FSHR 307/680) on the follicular fluid hormone profile and the granulosa cell expression in small human antral follicles. *Molecular Human Reproduction, 2015;21(3):255-261.*

INTRODUCTION

Gametogenesis

Gametogenesis is the process in which oocytes are formed in the female (oogenesis) and sperm cells are formed in the male (spermatogenesis). In humans, the female and male sex cells, called gametes, arise within the embryo when the embryo is only two weeks old (Larsen, *et al.*, 2001). These cells form the germ line and are called the primordial germ cells. The primordial germ cells populate the structure of the embryo that eventually will form the gonadal region, and with help from neighboring cell types, they develop strands of tissue called the primitive sex cords. The primitive sex cords proliferate and create the genital ridges, which represent the primordial gonads. The primordial gonads will subsequently give rise to the tissue that nourishes and regulates the developing gametes.

In the gonads, both female and male primordial germ cells continue to divide by mitosis, and start the process of gametogenesis (Larsen, *et al.*, 2001). However, the timing of the process of gametogenesis differs between the female and the male. In females, the primordial germ cells differentiate into oogonia in the genital ridges, and at the fifth month of fetal development, they all begin meiosis and the number of chromosomes will be split in two parts when the meiosis is complete, ending up in 23 chromosomes in each sex cell. The oogonium has at this stage become a primary oocyte and is surrounded by follicle cells. However, at an early stage the primary oocytes come to a meiotic arrest, and remain in this stage until puberty. When the sexual maturation starts at puberty, a limited amount of follicles mature and usually only one primary oocyte matures into a secondary oocyte and is ovulated. However, the secondary oocyte remains in a second phase of meiotic arrest until it is fertilized.

In males, by contrast, the primordial germ cells remain dormant from the sixth week of embryonic development until puberty, when they start to differentiate into spermatogonia (Larsen, *et al.*, 2001). At this stage the tissue arising from the primordial gonads, namely Sertoli cells and the surrounding tissue, starts to mature and provide a region suitable for the development of sperm cells to take place. A

17

certain amount of spermatogonia undergo meiosis and mature into spermatozoa, and this process will in most cases continue throughout a man's adult life.

Oogenesis

The development from primordial germ cells to large antral follicles that are ready for ovulation (called Graafian follicles) is called oogenesis and takes about six months in the female (Coward & Wells, 2013). As mentioned above, the primary oocyte is surrounded by follicular cells, and the complex of follicle cells enclosing a primary oocyte is called a primordial follicle (Larsen*, et al.*, 2001). The primordial follicles populate the ovaries from fetal life until puberty, when a limited amount of follicles resume development each month. The maximum number of follicles is reached during the fetal life, and when puberty starts, only about 400 000 follicles remain. During the period from early fetal life to the start of the menopause, approximately seven million primordial germ cells are generated within a women, whereas only approximately only 400 are ovulated (Coward & Wells, 2013).

The follicular cells surrounding the primary oocyte thicken and these developing follicles are now called primary follicles (Larsen*, et al.*, 2001). The follicular epithelium of approximately 5 to 12 of the primary follicles continue to develop and now become multi-layered, and at this stage the primary follicles are called growing follicles. Some of the growing follicles stop their development, and only a few remain growing in response to follicle-stimulating hormone (FSH). These growing follicles absorb fluid from the surroundings and develop a fluid-filled cavity called antrum, and the follicles are thereby called antral follicles. Only one (usually) of the antral follicles eventually gets dominant, and the other recruited antral follicles stop developing and instead undergo so called atresia and die. It is currently not known which properties the follicle that is selected for ovulation has, that the follicles that undergo atresia are missing (Coward & Wells, 2013). The dominant follicle subsequently increases further in size, and the oocyte that still is connected to the follicle cells that line the basement membrane of the follicle (granulosa cells), starts to project into the antrum of the follicle (Larsen*, et al.*, 2001).

Ovulation takes place as a result of a surge in FSH and luteinizing hormone (LH), usually around day 14 of the menstrual cycle (Larsen*, et al.*, 2001). The peak of FSH and LH during ovulation makes the primary oocyte of the mature Graafian follicle to resume meiosis, and the primary oocyte divides in order to form the secondary oocyte and the first polar body. As mentioned above, the secondary oocyte is arrested in the second meiotic division until it is fertilized. If the

secondary oocyte is fertilized by a sperm cell in the ampulla of the oviduct, the oocyte completes the second meiotic division and produces the second polar body. The fertilized oocyte is considered as a zygote, with a diploid set of chromosomes.

After ovulation, that may be likened to an inflammatory response with production of prostaglandins and histamines, the granulosa cells of the remaining ruptured follicle begin to proliferate in order to form the corpus luteum (Larsen, *et al.*, 2001). The corpus luteum is an endocrine structure which secretes estrogens and progesterone that maintain the uterine endometrium in a condition ready to receive an embryo. If fertilization does not occur and/or no embryo implants in the endometrium of the uterus, the corpus luteum degenerates and forms a structure called corpus albicans. The phases of the menstrual cycle are shown in Figure 1.
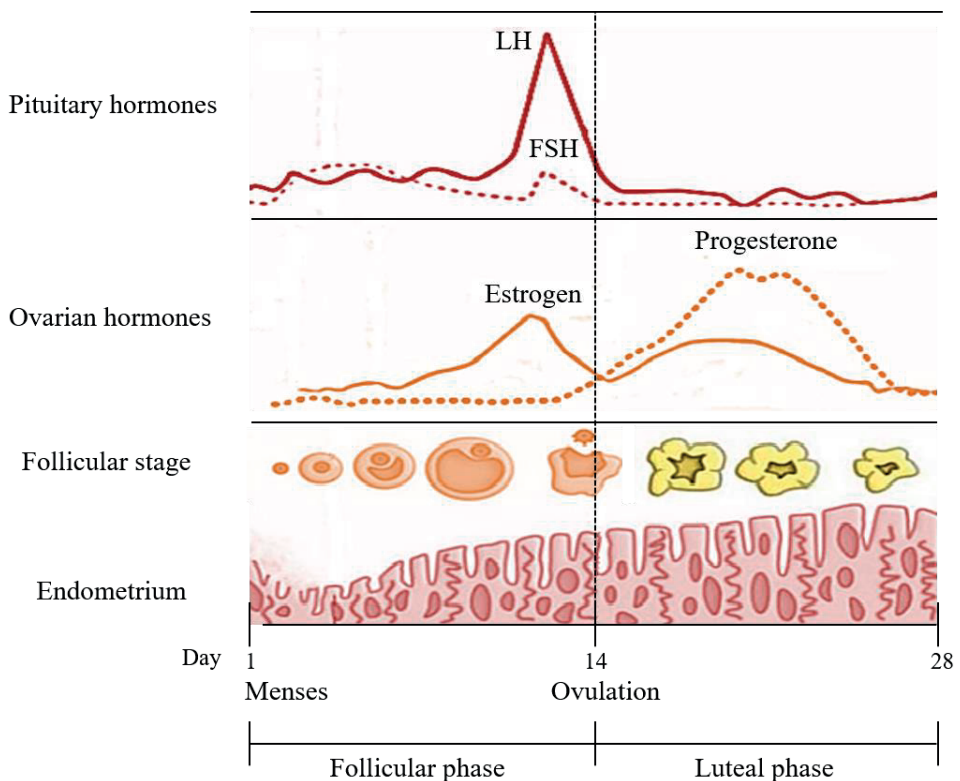
Figure 1. The phases of the menstrual cycle. FSH: follicle-stimulating hormone. LH: luteinizing hormone. The figure is modified from Saner-Amigh & Halvorson (Saner-Amigh & Halvorson, 2013).

Spermatogenesis

Spermatogenesis is the process in which male haploid germ cells are formed from diploid spermatogonial stem cells (Coward & Wells, 2013). The process of developing sperm takes place in tubular structures in the testes, called the seminiferous tubules. The testes are situated outside of the body in most mammals, in order to get a better regulation of the temperature so that a suitable environment for the temperature-sensitive sperm cells is created (Coward & Wells, 2013). The basement membrane of the seminiferous tubules is lined with peritubular myoid cells and Sertoli cells, which nourish the developing sperm cells and create an environment called the blood-testis barrier that protects the sperm from the body's immune system (Larsen, *et al.*, 2001). In men, the development of spermatogonia into mature sperm takes 64 days, and the spermatogenic process can be subdivided into four major steps:

1. Mitotic multiplication of the spermatogonium in order to maintain a pool of spermatogonia, but also differentiation into spermatocytes;
2. Meiotic division of spermatocytes into haploid spermatids;
3. Differentiation of spermatids into testicular sperm;
4. Release of testicular sperm into the lumen of the seminiferous tubules (Nieschlag, *et al.*, 2001).

The sperm cell continues its development after leaving the seminiferous tubules, and on its way to fertilize the oocyte in the ampulla of the oviduct, the sperm goes through a maturation process called capacitation (Larsen, *et al.*, 2001). Capacitation prepares the sperm so that it will be able to pass through the cell membrane of the oocyte; a process called acrosome reaction where enzymes break down the membrane surrounding the oocyte called zona pellucida. Approximately 1000 sperm cells are produced each second in the male (Coward & Wells, 2013); however, only a very low number of sperms actually reach the "right" oviduct for fertilization, and only one sperm cell eventually penetrates the egg.

Regulation of gametogenesis; the Hypothalamic-Pituitary-Gonadal axis

In humans, the endocrine regulation is governed from the hypothalamus, from where gonadotrophin-releasing hormone (GnRH) is secreted. Gonadotrophin-releasing hormone stimulates the anterior pituitary to release FSH and LH into the blood stream, and FSH and LH then exert their effect on the gonads. The so called hypothalamic-pituitary-gonadal (HPG) axis is assumed to be governed by kisspeptin, a protein involved in the onset of puberty (Hameed, *et al.*, 2011).

Kisspeptin receptors are present on GnRH neurons, and it is suggested that kisspeptin initiates the release of GnRH. The secretion of GnRH in the blood stream is pulsatile, and therefore also FSH and LH are secreted in a pulsatile fashion. However, this is most evident for LH, since the half-life in the circulation for LH is much shorter than the half-life for FSH (20 minutes versus 2 hours, respectively) (Nieschlag, *et al.*, 2001). The main hormones involved in the female and male HPG axis are displayed in Figure 2.
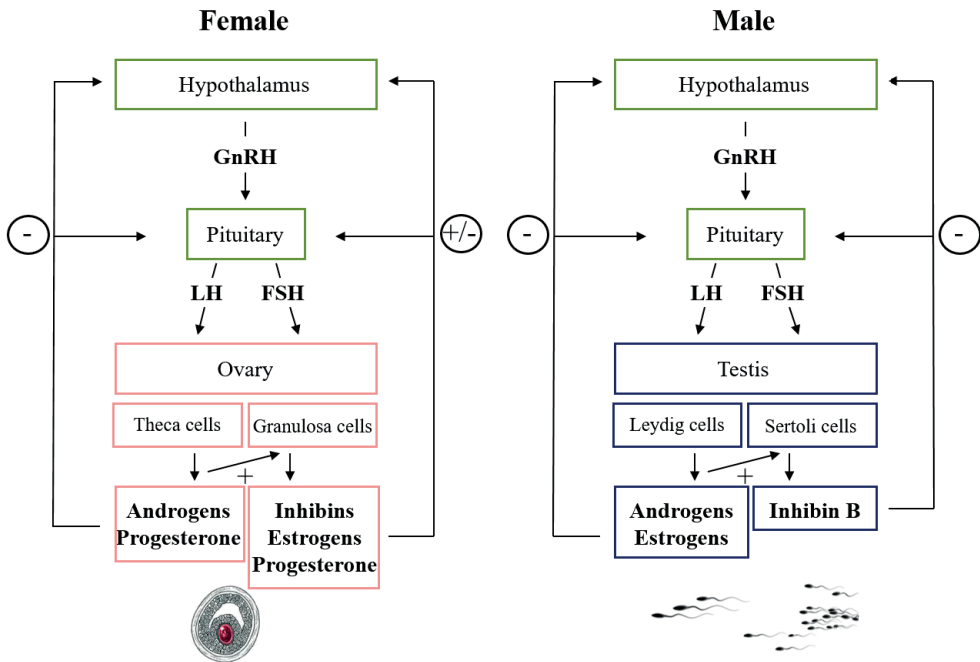
Figure 2. Endocrine regulation of the HPG axis. FSH: follicle-stimulating hormone. GnRH: gonadotrophin-releasing hormone. LH: luteinizing hormone. The Graafian follicle is modified from the 3rd edition of Human Embryology (Larsen, et al., 2001) and the swimming sperms are from www.babymed.com (160311).

In females, FSH acts on the granulosa cells surrounding the follicles in the ovaries through the FSH receptor (FSHR) located on the membrane of these cells (Camp, *et al.*, 1991). As mentioned above, FSH drives maturation and differentiation of the follicles (McNeilly, *et al.*, 1991), but also estrogen production (Nordhoff, *et al.*, 2011). Luteinizing hormone acts on the theca cells that surround the follicle in two layers, the theca interna and the theca externa (Larsen, *et al.*, 2001). Luteinizing hormone mediates its effects through the LH/human chorionic gonadotrophin (hCG) receptor (LHCGR) located on theca cells and make these

cells produce androgens, but also to induce ovulation, and to maintain the corpus luteum (Casarini, *et al.*, 2011). The sex steroids and inhibins regulate the secretion of GnRH and FSH and LH through a negative feedback on the hypothalamus and pituitary (Vadakkadath Meethal & Atwood, 2005).

In males, FSH regulates the spermatogenesis and maturation of the seminiferous tubules, through action on the FSHR located on the Sertoli cells. The Sertoli cells also secrete inhibin B as a consequence of FSH stimulation, and inhibin B controls FSH secretion from the pituitary by a negative feedback action on the hypothalamus and the pituitary (Meachem, *et al.*, 2001). Luteinizing hormone on the other hand, acts through the LHCGR in the plasma membrane of the Leydig cells (Herrera-Luna, *et al.*, 2015), located between the seminiferous tubules, and induce the production of testosterone in these cells (Casarini, *et al.*, 2011). Testosterone assists FSH in the development and maturation of the spermatogonia into mature sperms (Nieschlag, *et al.*, 2001), but also acts negatively, together with estrogens and progesterone produced by the testis, on GnRH and FSH and LH secretion through a feedback loop to the hypothalamus and the pituitary (Vadakkadath Meethal & Atwood, 2005).

FSH and LH –glycoprotein hormones

Both FSH and LH are glycoprotein hormones, i.e. they are built of a peptide backbone with covalently linked oligosaccharides, called glycans. The glycans are added to mainly arginine (R), asparagine (N), serine (S), threonine (T) and tyrosine (Y) residues in a post-translational process called glycosylation (Pierce & Parsons, 1981). The glycosylation status of a glycoprotein hormone may render the molecule more protected from e.g. proteases in the blood stream, and hence the half-life of a heavy glycosylated hormone may be longer (Alberts, *et al.*, 2015).

In humans, there are five glycoprotein hormones, which in addition to FSH and LH are hCG, thyroid-stimulating hormone (TSH) and inhibins. The glycoprotein hormones are heterodimers enclosing a common α subunit encoded by a gene located on chromosome 6 (6q12-q21) (Dias, *et al.*, 2002), expressing a protein that consists of 92 amino acids, and a hormone-specific β subunit consisting of 112-118 amino acids that accounts for the specificity of the hormone when it binds to its respective receptor (Saladin, 2010). The β subunit of FSH, LH, hCG and TSH shows structurally and functional similarities, with highly conserved amino acid residues (Dias, *et al.*, 2002).

Evolution of the glycoprotein hormones

The gene that encodes the β subunit of FSH is located on chromosome 11 (11p13), and its coding sequence is highly conserved among humans and other species, with homology values for mammalian species between 92% and 99% and an overall structural similarity of 77%. The transcriptional unit contains, apart from the translational part, two introns, of which the one located between amino acids 35-36 in the β subunit protein is strictly conserved in all human glycoprotein β subunits (Dias*, et al.*, 2002). The human LH and hCG genes encoding the β subunits are similar, and, as the FSHβ subunit, also has two introns (Pang*, et al.*, 1991). The placental hCG has in humans LH-like features; the two hormones even bind to the same receptor. However, hCG has, compared with LH, an additional carboxyterminal peptide consisting of 24 amino acids, and the two hormones also differ in their glycosylation status and in their half-life in the circulation (Casarini*, et al.*, 2011).

Because of the overall similarity of the α and β subunits of the glycoprotein hormones, it has been proposed that these arose from a common ancestor. Li and Ford (1998) proposed a model for the evolution of the α and β subunits, and according to this model the ancestor gene duplicated approximately 930 million years ago in order to produce an α subunit and a β subunit. If this model is accurate, the glycoprotein gene would be among the oldest genes in animal history (Li & Ford, 1998). The ancestor α subunit further evolved and formed species-specific genes, whereas the β subunit ancestor duplicated in order to give rise to the FSHβ, TSHβ and LHβ subunits. In primates, the LHβ subunits further developed to give rise to also an hCGβ subunit gene, which was expressed in the placenta, whereas most mammalian, fish and avian species kept only the LHβ subunit gene (Dias*, et al.*, 2002). The organization of the subunits in FSH, LH/hCG and TSH is presented in Figure 3.
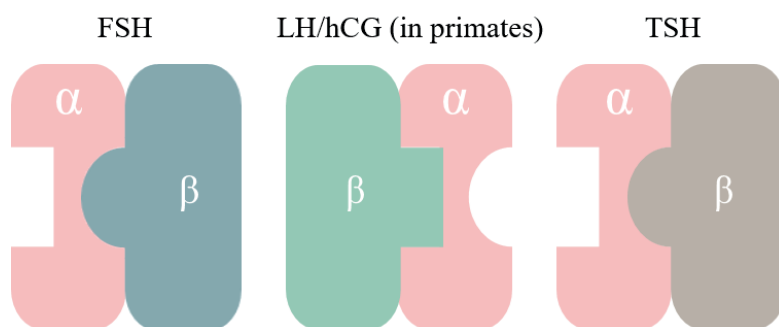
Figure 3. Organization of the subunits of FSH, LH/hCG and TSH. All hormones have a common α subunit (pink), but differ in the β subunits. FSH: follicle-stimulating hormone. hCG: human chorionic gonadotrophin. LH: luteinizing hormone. TSH: thyroid-stimulating hormone. Copyright© motifolio.com.

Single-nucleotide polymorphisms

The eventual response to glycoprotein hormones may vary with nucleotide variations in the glycoprotein hormone itself, but also with variations in the receptor gene (Themmen & Huhtaniemi, 2000). The variations in the nucleotide sequence may be single-base changes, where one nucleotide is substituted to another nucleotide, called single-nucleotide polymorphism (SNP), but there are also variations in genes where nucleotides are inserted, duplicated or deleted in the genome, called insertions, duplications and deletions, respectively. A SNP differs from a mutation in its frequency in the population; a one-nucleotide substitution is only called a SNP if it is present in more than 1% of the population. Single-nucleotide polymorphisms are often very common. They account for 90% of all variations that occur in the human genome in a population, and for some polymorphic sites in the genome, the substituting nucleotide is the norm (Brooker, 2009).

Genetic variation of FSH and LH

The common α subunit
So far, only one genetic alteration in the α subunit of the glycoprotein hormones, secreted by a human carcinoma, has been found (Nishimura, *et al.*, 1986). The substitution of glutamic acid (E) in amino acid position 56 to alanine (A) in this isoform of the α subunit resulted in a protein that failed to associate with the β subunits, and thus the formation of the different glycoproteins failed. Furthermore, the mutated α subunit had a higher molecular weight than the non-mutated protein, indicating that the mutation resulted in a different tertiary structure, altered glycosylation and self-dimerization (Themmen & Huhtaniemi, 2000). The lack of genetic variation of the α subunit of the glycoprotein hormones indicates that such changes may not be compatible with life.

The FSHβ subunit
There are some genetic alterations described in the FSHβ subunit gene. The first concerned a women suffering from primary amenorrhea and infertility, with a two base pair-deletion in codon 61 of the gene, resulting in lack of translation of amino acids 87-111, due to a premature stop codon in the altered amino acid chain 61-86 (Matthews, *et al.*, 1993). A truncated form of the FSHβ protein was produced, and association with the α subunit in order to form the FSHαβ dimer could therefore not occur (Themmen & Huhtaniemi, 2000).

Also in men, FSHβ mutations have been described, leading to variations in the reproductive function. The first activating mutation described concerned a hypophysectomized man with normal semen parameters that fathered three children under testosterone substitution (Gromoll*, et al.*, 1996). Analysis revealed a heterozygous aspartic acid (D) to glycine (G) substitution in amino acid position 567 that resulted in receptor activation independent of ligand-binding. In one man from Sweden presenting with azoospermia, puberty was normal (Lindstedt*, et al.*, 1998), and a cysteine (C) to R substitution in amino acid position 82 was found as the factor causing the phenotype. Based on the hCG biosynthesis (Bedows*, et al.*, 1992), it was thought that the C82R substitution caused a defective tertiary structure of the FSHβ protein, resulting in intracellular degradation of the protein. Subsequently, no association with the α subunit would occur, and therefore no biologically active FSH hormone would be produced (Themmen & Huhtaniemi, 2000). In another man from Israel, presenting with delayed puberty, small testes, azoospermia and hypogonadism (Phillip*, et al.*, 1998), the same two base pair-deletion as previously described for the woman (Matthews*, et al.*, 1993) was found, where a truncated form of the FSHβ protein was produced.

The LHβ subunit

Only one inactivating human mutation of the LHβ subunit has been described so far (Weiss*, et al.*, 1992). The phenotype of the 17 year-old male harboring the mutation was delayed puberty with low testosterone and high LH. *In vitro* experiments showed that his LH was lacking bioactivity. He had a family history of consanguinity, with three infertile maternal uncles, indicating that he had inherited a defect form of the LHβ subunit. Yet, his mother and sister had normal reproductive function. Furthermore, a genetic variant allele of the human LHβ subunit (V-LHβ) has been found (Pettersson*, et al.*, 1992). The V-LHβ allele presented with two missense alterations in the amino acid chain that are in complete linkage disequilibrium: tryptophan (W) that was substituted with R in amino acid position 8, and isoleucine (I) in amino acid position 15 that was substituted with T (Nilsson*, et al.*, 1998). The I15T substitution adds an extra glycosylation site to the V-LHβ, which is similar to the hCG N13 that also is glycosylated (Talmadge*, et al.*, 1984). The frequency of the V-LHβ is highest in Australian aboriginals with an allelic frequency of 28%, and in Northern European populations (>10%) and lowest (2.5-5%) in Asian populations and American Indians (Nilsson*, et al.*, 1997; Themmen & Huhtaniemi, 2000). The V-LHβ allele has been associated both as a protector of LH-related pathologies, but also as a predisposing factor, but since it is present in such a high frequency in certain populations, it cannot be deleterious to human reproduction function (Themmen & Huhtaniemi, 2000). However, a polymorphism encoding amino acid 102 of the LHβ gene, changing S to G, has been associated with female infertility (Liao*, et al.*, 1998), indicating that polymorphisms in the LHβ gene indeed can alter the human reproductive function.

Follicle-stimulating hormone receptor

The FSHR belongs to the superfamily of G protein-coupled receptors, together with the LHCGR and the thyroid-stimulating hormone receptor (TSHR) (Casarini, *et al.*, 2011). As mentioned above, the FSHR is present in the cell membrane of granulosa cells in women (Camp, *et al.*, 1991) and in the cell membrane on Sertoli cells in men (Herrera-Luna, *et al.*, 2015). G protein-coupled receptors span the cell membranes of the cells of which they are present with seven transmembrane hydrophobic alpha helices. The G protein-coupled receptors have also one extracellular and one intracellular part, and structurally they are similar although they are encoded by different genes. The location and overall structure of G protein-coupled receptors are presented in Figure 4. The extracellular part is the hormone-binding part, and this part differs in structure between different G protein-coupled receptors, so that it is shaped to fit only the hormone that is destined for it. The intracellular part is the signal transduction part, which is responsible for passing forward the message from the hormone that has formed a complex with the extracellular part of the receptor, into the cell nucleus, through specific second messengers. In the nucleus, cell and receptor specific nuclear events happen, as for example transcription of certain genes (La Marca, *et al.*, 2013).
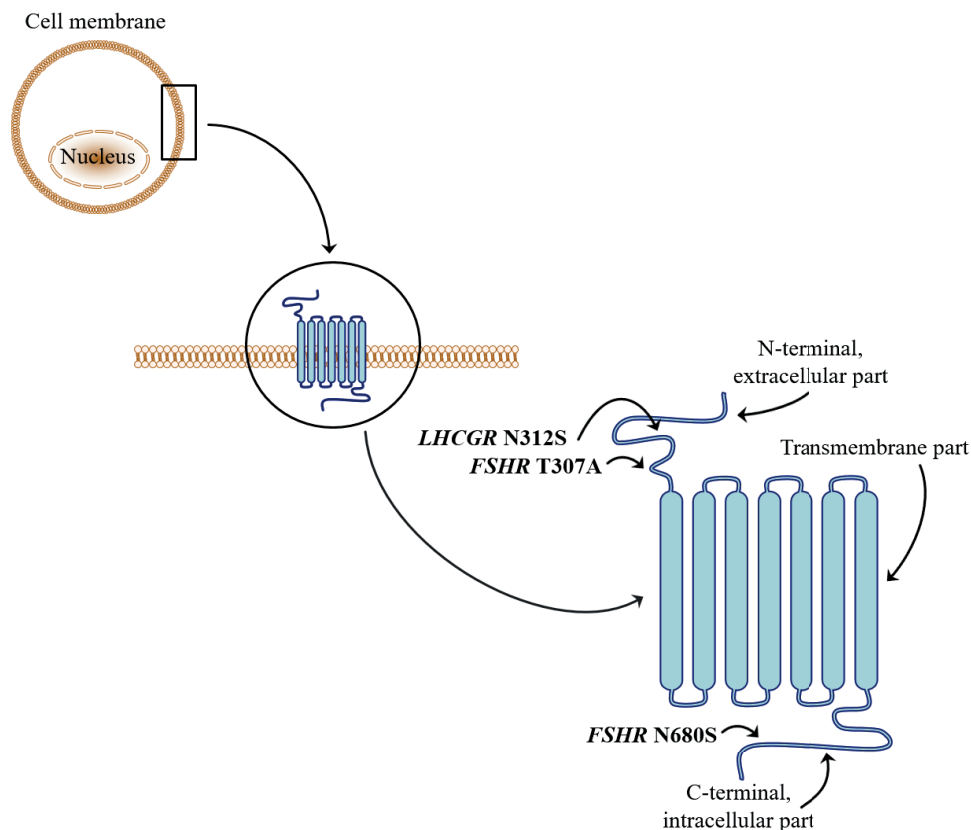
Figure 4. *Cellular location and structure of the FSHR and the LHCGR. FSHR: follicle-stimulating hormone receptor. LHCGR: luteinizing hormone/human chorionic gonadotrophin receptor. N: asparagine. S: serine. Copyright© motifolio.com.*

The FSHR signals through the classical $G\alpha_s$/3',5'-cyclic adenosine monophosphate (cAMP)/protein kinase A pathway (Dattatreyamurty*, et al.*, 1987; Dattatreyamurty*, et al.*, 1986; Means*, et al.*, 1974), but also through for example β-arrestins (Kara*, et al.*, 2006; Krishnamurthy*, et al.*, 2003; Marion*, et al.*, 2006; Marion*, et al.*, 2002), the adaptor protein Adaptor protein, phosphotyrosine interaction, PH domain and leucine zipper containing 1 (APPLI1)/inositol 1,4,5-triphosphate (IP_3) signaling pathway (Thomas*, et al.*, 2011), $G\alpha_q$/IP_3 signaling pathway (Escamilla-Hernandez*, et al.*, 2008; Quintana*, et al.*, 1994) and through epidermal growth factor receptor transactivation (Wayne*, et al.*, 2007). The nuclear events involved in FSH stimulation include activation of cAMP response element-binding (CREB) protein, which in turn controls the expression of aromatase, which is responsible for the aromatization of androgens produced by

the theca cells to estrogens; FSH stimulation thereby increases estrogen production. Nuclear protein kinase A also leads to histone H3 phosphorylation, which gives increased expression of FSH induced genes (Salvador*, et al.*, 2001). The classical cAMP/protein kinas A pathway is presented in Figure 5.
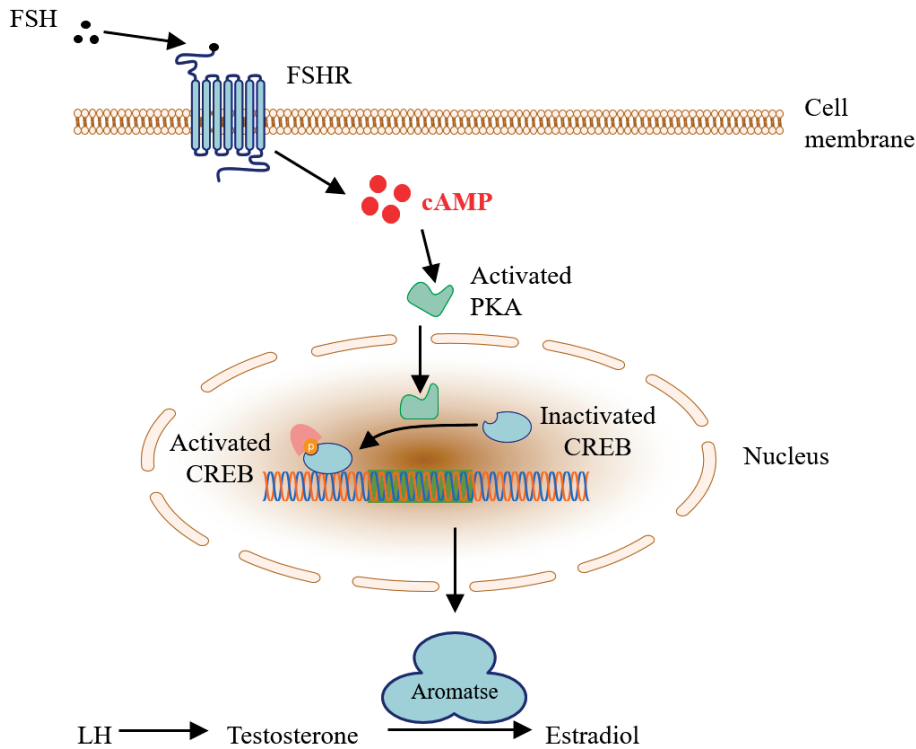
Figure 5. *The classical cAMP/protein kinase A FSHR signaling pathway. CREB: 3',5'-cyclic adenosine monophosphate (cAMP) response element-binding (protein). FSH: follicle-stimulating hormone. FSHR: follicle-stimulating hormone receptor. LH: luteinizing hormone. PKA: protein kinase A. Copyright © motifolio.com.*

The FSHR is encoded by the *FSHR* gene located on the short arm of chromosome 2, p21-16. The *FSHR* gene consists of 10 exons, of which exons 1-9 encode the extracellular part of the FSHR, whereas exon 10 encodes for the C-terminal end of the extracellular part, the transmembrane part and the intracellular part of the receptor. The *FSHR* gene is transcribed and translated to form the FSHR protein consisting of 695 amino acids and that has a molecular mass of 76 kDa (Gromoll*, et al.*, 1992; Gromoll & Simoni, 2005; Simoni*, et al.*, 1997; Ulloa-Aguirre*, et al.*, 2014).

FSHR mutations

In 1995, Aittomäki et al. described the first inactivating mutation of the *FSHR* gene (Aittomaki*, et al.*, 1995). The mutation was fairly common in some Finnish families, and was mapped to amino acid residue 189 in the FSHR protein, changing A to valine (V). Women with this mutation displayed a phenotype of premature ovarian failure with elevated FSH and LH concentrations in the circulation. Homozygous men in the affected families displayed various degrees of spermatogenic failure; however, they were not completely infertile since two out of three had fathered children. Expression of the mutated *FSHR* gene *in vitro* demonstrated a protein with normal hormone-binding ability, but with reduced signal transduction capacity. In 1999, a heterozygous V241A transition in the FSHR protein was found in one infertile man (Simoni*, et al.*, 1999). However, since the effect of the mutation was not seen *in vitro*, the mutation was said not to be responsible for the infertile condition. A few other mutations in the *FSHR* gene have been found since then (Beau*, et al.*, 1998; Doherty*, et al.*, 2002; Touraine*, et al.*, 1999), but since they are so infrequent it is believed that mutations in the *FSHR* gene rarely are the cause of an infertile phenotype.

FSHR polymorphisms

More than 19 000 SNPs are present in the *FSHR* gene, according to the National Center for Biotechnology Information (NCBI) (NCBI, 2016). Of those, only a minor portion is in the coding region and are fairly common in the population (Casarini*, et al.*, 2011). A frequently studied SNP in the *FSHR* gene is the adenine-to-guanine substitution in nucleotide position 2039 (rs6166 in the NCBI SNP database [dbSNP]). This nucleotide change results in an amino acid substitution from N to S in amino acid position 680 (N680S), and is because of the amino acid substitution classified as a non-synonymous SNP (Casarini*, et al.*, 2011). The *FSHR* N680S SNP is located in the intracellular part of the FSHR, which is indicated in Figure 4. The SNP in amino acid position 680 is in linkage disequilibrium with a SNP in amino acid position 307 (rs6165 in dbSNP), and those two SNPs hence are inherited together and form two haplotypes. The SNP in amino acid position 307 causes an amino acid change from T to A. Threonine in amino acid position 307, co-inherited with N680, is present in approximately 60% of white populations, while A307, co-inherited with S680, is present in approximately 40% of white populations (Conway*, et al.*, 1999; Simoni*, et al.*, 1999).

Single-nucleotide polymorphisms where the nucleotide shift not results in an amino acid substitution is called synonymous, and may instead of altering the amino acid, alter the messenger RNA splicing, stability and structure, and thereby also the folding of the protein (Hunt*, et al.*, 2009). Non-synonymous SNPs on the other hand, can alter the amino acid chain, and they may thereby influence promotor activity and hence gene expression, messenger RNA stability and translation efficiency (Hunt*, et al.*, 2009). The N680S SNP in the *FSHR* gene is located in the intracellular part of the receptor, and could possibly alter the glycosylation status of this intracellular part, and thereby affect the downstream signaling response and receptor activity when FSH binds to its receptor, since N is an amino acid that often is glycosylated (Pierce & Parsons, 1981; Weerapana & Imperiali, 2006).

In men, conflicting results have been produced regarding the impact of the *FSHR* N680S polymorphism on the reproductive function. A similar distribution of the *FSHR* N680S SNP in fertile controls and infertile men have been reported (Gharesi-Fard*, et al.*, 2015; Lend*, et al.*, 2010; Song*, et al.*, 2001; Zalata*, et al.*, 2008), as well as the opposite where S680 was more frequent in infertile men than in controls and N680 was more prevalent in controls than in patients (Ahda*, et al.*, 2005; Balkan*, et al.*, 2010). Regarding the impact of *FSHR* N680S on reproductive hormone concentrations and sperm and testicular parameters, virtually nothing was known at the start of this project. However, in women it had previously been shown that normally menstruating women with the *FSHR* S680 genotype had longer menstrual cycles and a higher sensitivity threshold to FSH compared to women with the *FSHR* N680 genotype (Greb*, et al.*, 2005). The *FSHR* N680S polymorphism had also been linked to controlled ovarian hyperstimulation prior to *in vitro* fertilization (IVF) in different ways. A previous study indicated that the *FSHR* N680S polymorphism may affect the ovarian threshold to exogenous FSH, since women with the *FSHR* S680 genotype demanded a higher dose of FSH to produce an equal amount of follicles compared to women with other genotypes (Perez Mayorga*, et al.*, 2000; Sudo*, et al.*, 2002). Additionally, women homozygous for S680 more often developed ovarian hyperstimulation syndrome compared to those with other genotypes (Daelemans*, et al.*, 2004).

The mechanism behind the different biological responses between women with different *FSHR* genotypes is not fully elucidated. In COS-7 cells, no difference in cAMP production was noted between the two receptor forms, when cells were treated with increasing concentrations of recombinant FSH, from 0.03 IU/L to 1 IU/L (Simoni*, et al.*, 1999).

Luteinizing hormone/human chorionic gonadotrophin receptor

Also the LHCGR belongs to the G protein-coupled receptors, with the same overall structure as the FSHR. Luteinizing hormone and hCG mediate their effects through the same receptor, the LHCGR, located on cell membranes of granulosa and theca cells in women (Camp*, et al.*, 1991), and on Leydig cells in men (Herrera-Luna*, et al.*, 2015). Luteinizing hormone is released from the pituitary in a pulsatile fashion, which results in progesterone production, ovulation, luteinization and corpus luteum formation when bound to LHCGR on granulosa cells (Segaloff & Ascoli, 1993). When LH binds to LHCGR on theca cells, androstenedione production is triggered; androstenedione is subsequently aromatized to estradiol (E2) by the enzyme aromatase produced by granulosa cells. The *LHCGR* gene is located on chromosome 2, close to the *FSHR* gene, and contains 11 exons that are translated to a protein consisting of 675 amino acids (Gromoll*, et al.*, 1992; Ulloa-Aguirre*, et al.*, 2014).

LHCGR mutations

In 1993, activating mutations in the *LHCHR* causing early puberty in boys were identified (Kremer*, et al.*, 1993; Shenker*, et al.*, 1993). The mutations resulted in single amino acid changes in the LHCGR protein, causing partial activity of the receptor in the absence of LH-binding to the extracellular domain. The single amino acid changes resided in the transmembrane domain, which were assumed to be important for coupling to the G protein and subsequent signal transduction, since elevated cAMP levels were measured in *in vitro* experiments (Shenker*, et al.*, 1993). In females, activating *LHCGR* mutations seem to have no clinical effect, since no (or very little) amount of *LHCGR* is expressed before puberty (Themmen & Huhtaniemi, 2000). Since the timing of ovulation in adult women is of great importance, an activating mutation of the *LHCGR* may have deleterious effects on the reproductive function. However, for the mother of two boys with an activating mutation in the *LHCGR* gene showing signs of early onset of puberty, no clinical effect of the mutation on the reproductive function was shown (Rosenthal*, et al.*, 1996).

Inactivating mutations of the *LHCGR* gene cause various symptoms of Leydig cell hypoplasia, of which sex differentiation may be the most pronounced (Themmen & Huhtaniemi, 2000). Several clinical cases have been described (Kremer*, et al.*, 1995; Latronico*, et al.*, 1996; Laue*, et al.*, 1995); however, the molecular mechanisms are still not fully elucidated (Themmen & Huhtaniemi, 2000). In

some cases G protein-coupling is thought to be affected, whereas in other cases the transport to the Golgi apparatus may be affected, causing e.g. an incorrect glycosylation status. The activating mutations of the *LHCGR* gene cause a more severe phenotype in men than in women, which may be explained by a more pronounced effect on sex differentiation and puberty of LH in males than in females, due to its effect in inducing testosterone production.

LHCGR polymorphisms

There are a few known polymorphisms in the *LHCGR* gene, of which the N312S polymorphism (rs2293275 in dbSNP) in exon 10 is one of the most studied. Approximately 18% of Caucasians are homozygous for the A allele of the *LHCGR* N312S polymorphism, 49% are heterozygous and 33% are homozygous for the G allele (Valkenburg*, et al.*, 2009). The *LHCGR* N312S polymorphism in the extracellular part of the LHCGR (Figure 4) is located near a glycosylation site which indicates that variations in the sequence could affect receptor sensibility.

The *LHCGR* N312S has been linked to polycystic ovarian syndrome (PCOS), where the presence of one A allele generated a 2-fold increased risk of PCOS and the presence of two A alleles generated a 2.7-fold increased risk in a Sardinian population of 198 cases and 187 controls (Capalbo*, et al.*, 2012). The A allele of the *LHCGR* N312S polymorphism was also considered a weak breast cancer risk allele in two breast cancer cohorts (Piersma*, et al.*, 2007). However, transfection studies in HEK293 cells did not reveal an altered activity for variants of the *LHCGR* N312S polymorphism.

In men, the *LHCGR* N312 has been reported less frequent in men with maldescended testes than in healthy controls (Simoni*, et al.*, 2008). In this study it was also found that men seeking help for couple infertility, with or without maldecended testes, had a lower frequency of the A allele encoding N than healthy controls. It was therefore hypothesized that the G allele encoding S could be linked to impaired spermatogenesis. The *LHCGR* N132S polymorphism has also been associated to testicular cancer, with the heterozygous genotype being less frequent in cancer patients than in healthy controls (Kristiansen*, et al.*, 2012). However, it was not associated with testicular germ cell cancer (Brokken*, et al.*, 2012). Altogether, these findings may indicate an increased LH sensitivity in carriers of the A allele of the *LHCGR* N312S polymorphism. However this effect has not yet been fully investigated *in vitro*.

The interplay between FSHR and LHCGR

Evolution of *FSHR* and *LHCGR*

As mentioned above, the FSHR is a protein consisting of 695 amino acids in its mature form, while the mature LHCGR consists of 675 amino acids. *In situ* hybridization and linkage analysis showed that the human *FSHR* and *LHCGR* genes map to the same chromosomal location; however, a considerable distance was evident between the two genes (Gromoll*, et al.*, 1992). The *FSHR* and *LHCGR* genes display great similarity regarding structure and organization, and therefore it has been suggested that they evolved from a common ancestral gene (Gromoll*, et al.*, 1994; Gromoll*, et al.*, 1996). The *FSHR* gene from human, monkey, mouse, rat, ovine, bovine, equine, porcine, chicken and reptile has been isolated and revealed that each species has a single *FSHR* gene (Akazome*, et al.*, 1996; Gromoll*, et al.*, 1993; Houde*, et al.*, 1994; Kelton*, et al.*, 1992; Minegishi*, et al.*, 1991; Remy*, et al.*, 1995; Sprengel*, et al.*, 1990; Tena-Sempere*, et al.*, 1999; Yarney*, et al.*, 1993; You*, et al.*, 1996). Regarding the *LHCGR* gene, a presumed evolutionary drive resulted in different receptor subtypes. An insertion of an extra exon between exon 6 and 7 (exon 6A) is present in human and primates only. Exon 10 was also deleted in the new world monkey linage, and generated two receptor subtypes based on the presence or absence of exon 10 (Troppmann*, et al.*, 2013). The structure of the exons of the human *FSHR* and *LHCGR* genes are displayed in Figure 6.

Human *FSHR*

Human *LHCGR*

Figure 6. The structure of the exons of the human FSHR and LHCGR genes. FSHR: follicle-stimulating hormone receptor. LHCGR: luteinizing hormone/human chorionic gonadotrophin receptor.

The extracellular domains of the human *FSHR* and *LHCGR* show approximately 46% homology when their sequences were compared, whereas the transmembrane parts of the receptors show nearly 72% homology, and the intracellular parts show approximately 27% of homologous sequences (Dias*, et al.*, 2002; Kleinau & Krause, 2009).

Molecular interactions between FSHR and LHCGR

As mentioned above, when LH binds to LHCGR on theca cells, androstenedione production is triggered; androstenedione is subsequently aromatized to E2 by aromatase produced by granulosa cells on induction of FSH stimulation. A fine-tuned interplay between FSH and LH thus comprise the so called two-cell-two gonadotropin concept and govern ovarian estrogen production (La Marca*, et al.*, 2013).

Since both FSH and LH are required for a normal oocyte maturation and development (Segaloff & Ascoli, 1993), it is not surprising that variants of the *LHCGR* may play a role in the outcome of IVF treatments, even though most commonly FSH preparations are used for ovarian hyperstimulation. The mechanism behind the FSHR/LHCGR interplay is unknown, but it has been proposed that G protein-coupled receptors may form homo- and heterodimers (Angers*, et al.*, 2002), so that stimulation by one of the hormones could be mediated in part through the other hormone's receptor. The heterodimerization hypothesis is presented in Figure 7. Heterodimerization among closely related receptors such as the SST3 and SST2a somatostatin receptors has been observed (Pfeiffer*, et al.*, 2001), as well as between more distantly related receptors such as angiotensin AT1 and bradykinin B2 (AbdAlla*, et al.*, 2000). It could therefore be of reason to believe that FSHR and LHCGR may form heterodimers, and if they do, that some isoforms of the different receptors may fit better than other isoforms. Heterodimers with a more suitable fit may in that case have a higher receptor activity, and may also favour specific signaling pathways, so that a heterodimer constructed of two specific isoforms may give a slightly different gene expression of target genes than a heterodimer constructed of two different receptor isoforms.

It has been shown in animal studies that transgenic mice that are lacking the wild-type LHCGR, but are expressing two isoforms of the LHCGR, one that has a mutated hormone-binding region and one that has a mutated signal transduction region, actually can re-establish normal hormonal actions by functional complementation (Rivero-Muller*, et al.*, 2010). In that way, the receptor with the mutated hormone-binding region but with a normal signal transduction region can compensate for the mutated signal transduction region of the other LHCGR isoform and vice versa. The same has also been shown for the FSHR. A heavily mutated FSHR variant, where the extracellular domain of the receptor has been linked to a phospholipid, was able to dimerize with a FSHR variant lacking the hormone-binding region and the dimer was able to activate signaling cascades into the cell (Ji*, et al.*, 2004). It may be of reason to believe that the same would be true also for interaction between different isoforms of the FSHR and the LHCGR, so that e.g. a FSHR with an altered signal transduction region would dimerize with a

LHCGR that has signal transduction region with better fit for the G protein. It is also plausible that this reasoning is correct not only for mutated variants of FSHR and LHCGR, but also for other receptors. It is e.g. a well-known problem that pregnant women may develop hyperthyroidism, as a result of the elevated hCG that may interact with the TSHR, and maybe TSHR/LHCGR complexes, leading to a higher activity of the TSHR signaling pathway, which may result in the hyperthyroidism seen in pregnant women.
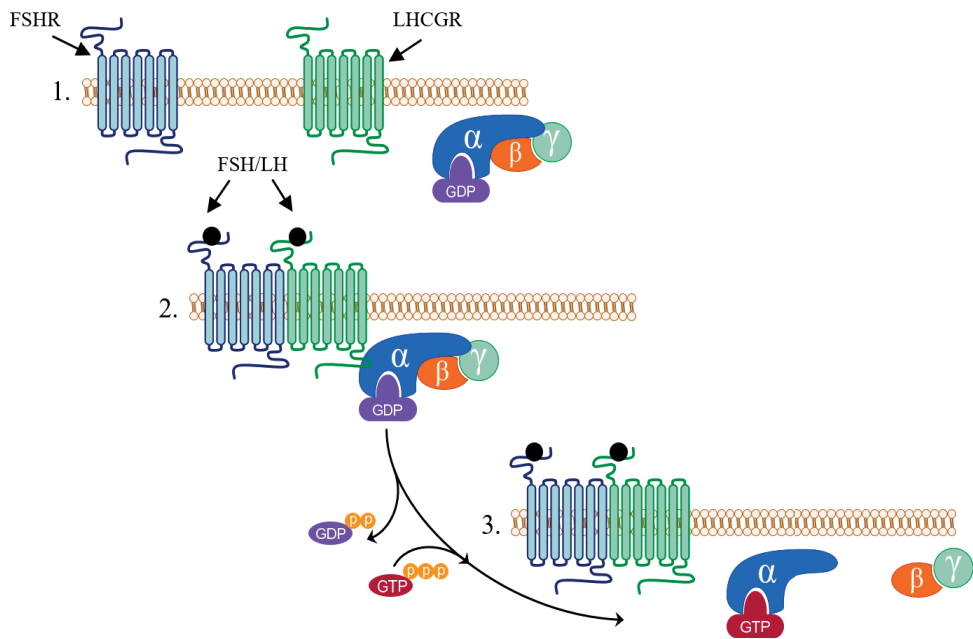
Figure 7. *The theory behind heterodimerization between FSHR and LHCGR. 1: FSHR and LHCGR are situated in the plasma membrane of a granulosa cell. 2: Dimerization of the receptors when binding of FSH occurs and docking to the G protein. 3: Activation of the α subunit of the G protein and initiation of the signaling cascade. FSH: follicle-stimulating hormone. FSHR: follicle-stimulating hormone receptor. GDP: guanosine diphosphate. GTP: guanosine triphosphate. LH: luteinizing hormone. LHCGR: luteinizing hormone/human chorionic gonadotrophin receptor. Copyright © motifolio.com.*

Infertility

Infertility, defined as the inability to obtain a pregnancy after at least one year of unprotected intercourse, affects approximately 15% of all couples (Kelton*, et al.*, 1992). However, this number is expected to increase as a result of the current postponing of the time to start a family to an age where especially female fecundity is declining, and also as a result of the access to assisted reproductive techniques worldwide. The age at which a couple starts to conceive will affect the chance to actually get a child or a family of the intended size. Also, where the use of contraceptive medications and devices has been available, a trend of especially higher educated women postponing their pregnancy has been noted. Altogether, these factors all affect the population size since total fertility rate will fall below the population replacement level of 2.1 births per couple (Espenshade*, et al.*, 2003).

About 20-35% of infertility cases can be explained by a female factor and 20-30% can be explained by a male factor, whereas 25-40% of cases can be assigned a problem in both partners. In 10-20% of cases, no known factor can be found to explain the infertility (ESHRE, 2014). Age alone, especially the age of the female, is one of the major risk factors for infertility, since the ovarian reserve diminishes with increasing age (Ginsburg & Racowsky, 2012). According to Ginsburg and Racowsky (2012), reduced ovarian reserve is present in 11.5% of infertile women; multiple female factors in 10.6%, tubal factor in 7.7%, ovulatory dysfunction in 6.8%, endometriosis in 4.2% and uterine factor in 1.4%. Male factor is present in 18.8% of infertility cases, female and male factors in 17.8%, unexplained infertility in 13.5% and other causes in 7.7%. As female infertility, male infertility is a heterogeneous disease that may be dependent on congenital and genetic factors in 15-30% of cases, acquired factors in approximately 20% and idiopathic infertility in 50% of cases (Krausz, 2011; O'Flynn O'Brien*, et al.*, 2010).

Assisted reproductive technologies

Assisted reproductive technologies (ART) involve all sorts of handling with gametes outside of the body (Ginsburg & Racowsky, 2012). In intrauterine insemination, sperm cells are prepared outside of the body and subsequently inseminated into the uterus of the women. In IVF, approximately 100 000 sperm cells are co-incubated with the egg in the laboratory for 1.5 hour or longer, where as in intracytoplasmic sperm injection (ICSI), one single sperm cell is injected into the oocyte. If fertilization and satisfying embryo development occurs after IVF or ICSI, one or in some cases two embryos are transferred to the uterus of the

women. Embryo transfer often takes place at cleavage stage (day 2-3 after oocyte retrieval) or at blastocyst stage (day 5 after oocyte retrieval).

Prior to IVF and ICSI, a hormonal stimulation of the women is performed in order to grow multiple pre-ovulatory follicles that can be aspirated from the ovaries and subsequently fertilized *in vitro*, at the laboratory (Ginsburg & Racowsky, 2012). The hormonal stimulation is achieved with exogenous FSH or a mixture of FSH/LH, with flexible set doses that can be changed during follicle development (monitored by ultrasound) in order to achieve an adequate number of mature oocytes. Premature ovulation of the oocytes is avoided with a GnRH agonist that inhibits the effect of the rising estrogen levels that would trigger LH surges and ovulation. This type of hormonal stimulation is called a "long protocol" because of the pre-treatment period that is necessary in order to control pituitary release of FSH and LH. A "short protocol" may also be applied, and in this treatment, a GnRH antagonist instead of the GnRH agonist is used. The stimulation with FSH or FSH/LH then starts in connection to a menstrual period, and after a couple of days, the GnRH antagonist is introduced, preventing endogenous LH surges.

In 1978, the first baby was born after the use of IVF (Steptoe & Edwards, 1978), and since then ART has been applied worldwide, and as a result, more than five million babies have been born (ESHRE, 2014). In Sweden, approximately 18 000 IVF treatments are performed annually, and as a result 4000 babies (3.6% of all babies) are born each year (Q-IVF, 2015).

In 2011, at the time of starting this thesis project, it was known that polymorphisms in the gonadotropin receptors are important in normal female reproductive hormonal regulation. However, it was not known what their roles were in normal male reproductive hormonal regulation.

Moreover, only some small experimental studies had been conducted, but no firm proof for the functional properties of polymorphisms in gonadotropin receptors existed.

AIMS

The overall aim of this thesis was to investigate the impact of *FSHR* and *LHCGR* gene polymorphisms on human reproductive function.

The specific aims of the thesis were to:

- Investigate the impact of *FSHR* T307A and N680S polymorphisms on the reproductive function in young men from the general population.

- Study the impact of a combination of *FSHR* N680S and *LHCGR* N312S receptor variants on the pregnancy outcome and clinical parameters in women undergoing IVF.

- Explore the *FSHR* N680S and the *LHCGR* N312S polymorphisms *in vitro*.

MATERIALS AND METHODS

Study subjects

Swedish young men from the general population (Study I)

The study population used in study I consisted of young Swedish men (n=313) from the general population. Since we wanted to associate male reproductive parameters with the *FSHR* T307A and N680S polymorphisms, it was of interest to use a study population of men of the same age. The study population consisted of 241 military conscripts and 73 men recruited as friends of participants or through advertisements in schools. All men were recruited from 1st of December 2008 to 27th of May 2010. The 73 men that were recruited as friends of participants or through advertisements in schools were recruited to the study in order to increase the number of participants. Due to savings in the military budget, only approximately 25% of all men living in Sweden during the years 2008-2010 underwent medical health examination prior to military service, and even though all 1681 men that lived <60 km from Malmö, Sweden, that were born and raised in Sweden, were asked to participate in the study, only 241 men accepted to participate (participation rate: 14%). The men were between 17-20 years of age (mean age: 18.0 ± 0.41) on the day of inclusion. All men were of White origin. Of the 314 men, 1 man was excluded due to genotyping difficulties. The men were paid 500 SEK for their participation in the study and they signed a written informed consent form. The study was approved by the local ethical committee board of Lund University, Sweden.

All study participants underwent a medical examination, in which testicular size was measured using ultrasound. The men were also asked to fill in a questionnaire prior to the medical examination. Abstinence time and details on their height and length were recorded. They also provided a semen sample that was analyzed according to the guidelines provided by the World Health Organization from 1999 (WHO, 1999), and a blood sample for subsequent hormonal analysis and DNA extraction for *FSHR* genotyping. The study population has been described in detail previously (Axelsson*, et al.*, 2011).

Women undergoing IVF (Studies II & III)

In studies II and III, the same study population was used, consisting of women undergoing IVF at Reproductive medicine centre (RMC), Skåne University Hospital, Malmö, Sweden. The women (n=384) were consecutively enrolled in the study from September 2010 to February 2015, and the inclusion criteria were younger than 40 years of age, a regular menstruation cycle of 21-35 days, bilateral ovaries, a body mass index (BMI) below 30 kg/m^2 and nonsmokers. On the day of follicular fluid aspiration, the women were between 22 and 39 years of age (mean age: 32.0 ± 3.82). Since BMI values were missing for n=11 women, these women were excluded when associating genotype with clinical parameters in study II, which in such cases resulted in a total of n=373 women. Additionally, FSH values were missing for n=30 women and data on cycle length was not recorded for n=37 women (although the cycle length was in the normal range for these women, since this was an inclusion criteria), and these women were therefore excluded when associating genotype with FSH concentration in serum and with cycle length, respectively.

An independent cohort of n=233 women was enrolled *a posteriori* as a validation cohort in study II, and in study III the two cohorts were used as one single cohort of n=617 women. The 233 women in this cohort underwent IVF at the same medical unit as the women in the first cohort, between the start of year 2007 until June 2015. The women were between 20-39 years of age (mean age: 32.5 ± 3.93) on the day of follicular fluid aspiration. Data regarding BMI was not recorded for this population (although BMI was below 30 kg/m^2, since this was an inclusion criteria). Clinical data was retrieved from medical records for both cohorts.

A venous blood sample was drawn for DNA extraction and subsequent *FSHR* and *LHCGR* genotyping, and for hormonal analysis. From n=135 women in the first cohort, follicular fluid was collected in order to isolate granulosa cells for functional *in vitro* studies. All women signed an informed consent form, and the study was approved by the local ethical committee board of Lund University, Sweden.

Granulosa cells from women undergoing IVF (Study II)

For n=135 women in the study population of n=384 women used in study II, granulosa cells were isolated from follicular fluid. The women were between 22 and 39 years of age (mean age: 31.9 ± 3.78). The granulosa cells were used as a functional *in vitro* model regarding the ability of different isoforms of the FSHR and LHCGR to produce cAMP and IP$_3$. While performing oocyte retrieval through follicular aspiration, the follicular fluid is collected for oocyte isolation, and when all visible oocytes are isolated, the follicular fluid is discarded. In the case of the

n=135 women from whom granulosa cells were isolated, the follicular fluid was analyzed also for visible clusters of granulosa cells before it was discarded.

Molecular methods

Genotyping (Studies I-III)

The DNA used in the genotyping procedure in study I-III was extracted from peripheral leukocytes using standard procedures.

There are several methods for analyzing DNA polymorphisms, e.g. DNA sequencing, several polymerase chain reaction (PCR)-based techniques, the use of restriction enzymes and hybridization to DNA microarrays, among others. Regarding the *FSHR* T307A polymorphism, e.g. the single-strand conformation polymorphism (SSCP) analysis has previously been used among others, whereas regarding the *FSHR* N680S polymorphism e.g. the restriction fragment length polymorphism (RFLP) method has been used. In SSCP, PCR is used in order to amplify the DNA region of interest, and subsequently a polyacrylamide gel electrophoresis is used in order to separate the DNA as single-stranded molecules. If two single strands of DNA molecules differ from each other by one single nucleotide base, the folding of the DNA strands differs, and can be detected as new bands by radioactive detection, silver staining or by the use of fluorescent PCR primers that are visualized by DNA sequencing (Biogene, 2016). In RFLP, the DNA region of interest is amplified by PCR, followed by treatment of the amplified fragment with a suitable restriction enzyme. Depending on the presence or absence of a restriction site in the amplified DNA region (which differs according to the presence or absence of a polymorphic site in the DNA), the amplified DNA is cleaved, and the different cleavage patterns can be visualized on an agarose gel electrophoresis (Rasmussen, 2012).

For genotyping of the *FSHR* T307A (Study I) and N680S (Studies I-III) polymorphisms, allele-specific PCR was used. In allele-specific PCR, allele-specific primers that match the two variants of the polymorphic site in the DNA are used. One primer matches the wild-type allele and the other primer matches the mutant allele, creating a short allele-specific band, together with two flanking primers, generating a longer control band. In this way, amplification of two different fragments depending on which allele that is present is carried out (Gaudet*, et al.*, 2009). As mentioned above, for the allele-specific PCR in our laboratory, a common forward control primer was also used, for visualization of a control fragment for each PCR reaction, independent of the presence or absence of

the allele-specific fragment. In order to confirm the results from the allele-specific PCR, purified samples representative for each genotype were directly sequenced on an eight-capillary Applied Biosystems sequencing gear (Applied Biosystems, Stockholm, Sweden).

For genotyping of the *LHCGR* N312S SNP (Studies II-III), PCR amplification of DNA followed by direct sequencing on an eight-capillary Applied Biosystems sequencing gear (Applied Biosystems) was used. All primer sequences used in study I-III are presented in Table 1.

Table 1. Primer sequences.

SNP	Assay	Primer direction	Primer sequence
FSHR T307A	AS PCR	Forward (flanking)	5'-TAGCCTCAAGGGCAGGTATG-3'
FSHR T307A	AS PCR	Reverse (flanking)	5'-GATGCAATGAGCAGCAGGTA-3'
FSHR T307A	AS PCR	Reverse (AS T)	5'-GAGGATCTCTGACCCCTAGT-3'
FSHR T307A	AS PCR	Reverse (AS A)	5'-AGGATCTCTGACCCCTAGC-3'
FSHR N680S	AS PCR	Forward (flanking)	5'-TTCACCCCATCAACTCCTGT-3'
FSHR N680S	AS PCR	Reverse (flanking)	5'-TCCTGGCTCTGCCTCTTACA-3'
FSHR N680S	AS PCR	Reverse (AS N)	5'-GACAAGTATGTAAGTGGAACCAT-3'
FSHR N680S	AS PCR	Reverse (AS S)	5'-GACAAGTATGTAAGTGGAACCAC-3'
LHCGR N312S	PCR	Forward	5'-TGTTGACCATGTGACTAGGGA-3'
LHCGR N312S	PCR	Reverse	5'-ACTCTCTCCTCAGGAAGCAT-3'

A: alanine. AS: allele-specific. FSHR: follicle-stimulating hormone receptor. LHCGR: luteinizing hormone/human chorionic gonadotrophin receptor. N: asparagine. S: serine. T: threonine.

Hormonal analysis (Studies I & II)

In study I E2, FSH, LH, sex hormone-binding globulin (SHBG) and total testosterone concentrations in serum were analyzed. In study II E2, FSH and LH concentrations in serum were analyzed.

For several decades, immunoassay methods involving the use of monoclonal antibodies for endocrine serum analysis has been the first choice. However, there is an ongoing shift towards the use of mass spectrometry for analysis of steroids in serum, because of its superior specificity, small samples sizes and fast analysis needed (Keevil, 2013; Taylor*, et al.*, 2015). The mass spectrometry technique is based on the principle that a compound can be ionized and the different excited ions in the sample then can be sorted based on their mass-to-charge ratio. Since the mass spectrometry method is expensive and demands advanced equipment, and since it is not a standard method in many clinical and research settings, the

immunoassay technique is still used because of its sensitivity, simplicity and low cost. A debate whether it is right or wrong to use the immunoassay method is ongoing (Handelsman & Wartofsky, 2013; Taylor, *et al.*, 2015), and a conclusion has not yet been drawn. However, studies comparing the two methods showed no difference in results (Huhtaniemi, *et al.*, 2012). In the studies comprising this thesis, the immunoassay technique was used.

In study I and II, blood samples for endocrine serum analysis were drawn between 8 and 10 a.m. In study I E2 was measured using an immunofluorometric assay (Autodelfia; Wallac Oy, Turku, Finland) at the routine clinical chemistry laboratory at Skåne University Hospital (Malmö, Sweden), whereas FSH, LH, SHBG and total testosterone concentrations were measured using an electrochemiluminiscence immunoassay (Cobas-Roche, Mannheim, Germany) at the routine clinical chemistry laboratory at Skåne University Hospital (Lund, Sweden). In the immunofluorometric assay, the immunocomplex is labeled with a fluorescent probe for detection, whereas in the electrochemiluminiscence immunoassay a kind of luminescence is produced during an electrochemical reaction (Hemmila, 1985). The sensitivities of the assays were 8 pmol/L (E2), 0.10 IU/L (FSH), 0.10 IU/L (LH), 0.35 nmol/L (SHBG), and 0.087 nmol/L (total testosterone). The intra-assay and interassay coefficients of variations (CV) were 20% (intra-assay CV) for E2, 3.9% (intra-assay CV) and 3.9% (interassay CV) for FSH, 2.2% (intra-assay CV) and 2.3% (interassay CV) for LH, 0.9% (intra-assay CV) and 0.8% (interassay CV) for SHBG, and 0.6% (intra-assay CV) for total testosterone. In study II E2, FSH and LH concentration in serum were measured using an electrochemiluminescence immunoassay (Cobas-Roche, Mannheim, Germany) at the routine clinical chemistry laboratory at Skåne University Hospital (Lund, Sweden). The sensitivities of the assays were 18.4 pmol/L for E2 and 0.10 IU/L for FSH and LH. The coefficients of variances for E2 were 7% at 289 pmol/L and 4% at 2011 pmol/L, for FSH 3% at 5 IU/L and 3% at 41 IU/L, and for LH 3% at 5 IU/L and 2% at 37 IU/L. The use of different methods for E2 measurements in study I and II is explained by that in study I, E2 was measured in men, and since men usually have lower E2, a method with a higher sensitivity had to be applied (8 pmol/L for the immunofluorometric assay used in study I and 18.4 pmol/L for the electrochemiluminescence immunoassay used in study II).

Semen analysis (Study I)

Prior to semen sample delivery, all men were asked to keep an abstinence time of 48-72 hours. However, in each individual case, the actual abstinence time was recorded. Semen samples were collected in a wide-mouthed plastic container by masturbation in a room at the laboratory. Prior to liquefying, all semen samples

were weighed in order to obtain the total volume of each ejaculate in mL, and when liquefied, all samples were analyzed according to the guidelines provided by the World health organization from 1999 (WHO, 1999) and the European society of human reproduction and embryology (ESHRE) manual on basic semen analysis (ESHRE, 2002) in duplicates with comparisons of concentration and motility. Sperm concentration was measured by use of a haemocytometer with improved Neubauer ruling. All semen analyses were performed by laboratory assistants at the laboratory at RMC, which is a reference laboratory for ESHRE-Nordic association for andrology (NAFA) quality control.

Isolation of granulosa cells from follicular fluid (Study II)

Granulosa cells were isolated from n=135 of the 384 women recruited to the study cohort in study II. A common method to isolate granulosa cells from follicular fluid is by the use of a centrifuge gradient where the enriched granulosa cell layer is removed after centrifugation and then washed in order to get rid of contaminating cells (Nordhoff, *et al.*, 2011). However, since we wanted to get hold of a granulosa cell population that was basically free of contaminating cells and where we could get the exact cell number of each sample, we invented a new fluorescent-activated cell sorting (FACS)-based method for this purpose.

Follicular fluid aspirates from the left and right ovary were pooled and granulosa cell aggregates were manually identified when oocytes were removed. The granulosa cell aggregates were placed in phenol-red free Roswell park memorial institute (RPMI) 1640 medium (Gibco Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (FBS; Biological Industries, Beit HaEmek, Israel) and 1% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Mediatech, Inc., Manassas, VA, USA), and were subsequently washed and filtered through a 70 μm cell strainer (Becton Dickinson Biosciences, San José, CA, USA), in order to get rid of contaminating cells as red blood cells, leukocytes and debris. The concentrated granulosa cells were then centrifuged for 10 min at 300×*g*, and after discarding the supernatant the cells were resuspended in supplemented RPMI.

Cell sorting
In order to avoid non-specific binding of antibodies to contaminating cells, blocking of the Fc receptor on blood and immune cells was carried out with TrueStain (BioLegend, London, UK) for 5 min. Granulosa cells were subsequently stained with anti-CD45 peridinin-chlorophyll-protein (PerCP) (BioLegend) and anti-CD99 phycoerythrin (PE) (eBioscience, SanDiego, CA, USA) for 15 min in darkness at room temperature, in order to sort out the granulosa cells from the

mixed cell population. After staining, cells were filtered through a 50 µm filcon with syringe fitting (Biogenetics, Padova, Italy), washed in supplemented RPMI, centrifuged at $430 \times g$ for 5 min and then resuspended in 200 µl supplemented RPMI. Five min prior to flow cytometry acquisition and cell sorting, 7-amino actinomycin D (7-AAD) (BioLegend) was added to the cells, and the samples were then acquired and sorted in a FACSAria (Becton Dickinson Biosciences). An acquisition gate based on side scatter and forward scatter, including cells with high granularity and excluding dead cells, debris and leukocytes (7-AAD and CD45 positive cells) was used as gating strategy for the definition of viable granulosa cells, and remaining cells were then gated on the most granular and CD99 positive cells in order to sort out viable cells consisting of only granulosa cells. Cells were sorted into supplemented RPMI containing 55% FBS in order to prevent cell death. For analysis of flow cytometry data, FlowJo (TreeStar, Inc., Ashland, OR, USA) was used.

Identification of granulosa cells

Unsorted and sorted samples from 3 randomly selected patients were morphologically evaluated at the Department of pathology and cytology, Skåne University Hospital, Malmö, Sweden. Cells were kept in R10 medium, centrifuged at $760 \times g$ for 10 min, the supernatant was discarded and BectonDickinson Cytorich™ (red) was added. In order to obtain optimal fixation of cells, the samples were left for at least 30 min at 4°C, and were then centrifuged at $760 \times g$ for 10 min. The supernatant was discarded and the cells were resuspended in deionized water. The cells were then added to pre-prepared cell chambers (Settling chamber 240, TriPath Imaging, Becton Dickinsion Diagnostics, Sparks, NV, USA) placed on pre-coted slides (SurePath precoat slides, TriPath Imaging, Becton Dickinson Diagnostics), left for 15 min in order to ensure optimal adherence, and afterwards fixed in 95% ethanol for a minimum of 30 min. Cells were subsequently stained with hematoxylin-eosin (Histolab Products AB, Gothenburg, Sweden) according to routine cyto-staining procedures. Cells were examined in an Olympus BH-2 microscope (Olympus Corporation, Center Valley, PA, USA) and morphologically evaluated. Approximately 300 cells were counted in randomly chosen high power microscope fields (objective lens×40, ocular lens×10).

Culture and hormonal stimulation of granulosa cells *in vitro* (Study II)

Sorted and isolated human granulosa cells were seeded and cultured in 24-well plates at a density of approximately 75 000 cells in supplemented RPMI with 0.02 mg/mL gentamicin (PAA Laboratories, Pasching, Austria) for 2-3 days prior to stimulation with 150 mIU/mL Follitropin alpha (GONAL-f [Merck-Serono], Darmstadt, Germany) or 150 mIU/mL Menotropin (Menopur [Ferring GmbH],

Kiel, Germany) in serum-free RPMI containing HEPES and gentamicin for 1 h at 37°C, 5%CO_2. Prior to hormonal stimulation, the cells were incubated in serum-free RPMI, containing HEPES and gentamicin for 1 h at 37°C, 5% CO_2. After stimulation, cell culture medium was aspirated and centrifuged for 1000×g, at room temperature. Inactivation of endogenous phosphodiesterases was carried out by incubation of cell supernatants at 95°C for 5 min. Cells were lysed with Passive lysis buffer (Promega, Stockholm, Sweden). Whenever a sufficient number of granulosa cells were obtained from the women, experiments were carried out in duplicates. Activity of the FSHR and LHCGR through the Gαs/cAMP/protein kinase A pathway was analyzed in duplicates by quantification of cAMP amount with enzyme-linked immunosorbent assay (ELISA) (cAMP ELISA, ENZO Life Sciences, Lausen, Switzerland). The activity through the IP_3 pathway was analyzed in duplicates in 55 randomly selected samples by quantification of IP_3 amount with ELISA (IP_3 ELISA, Cusabio, Wuhan, China). All results obtained by ELISA were adjusted for total protein concentrations in the cell lysates, with use of Pierce BCA (bicinchoninic acid) protein assay reagent (Thermo Fisher Scientific, Inc.). Samples were also adjusted for basal activity in unstimulated cells from each patient.

Transfection and hormonal stimulation of eukaryotic cells *in vitro* (Study III)

Transfection is a method where foreign genetic material is introduced to a cell without the use of viruses. When the DNA of interest is transfected into the cell, the cell's own machinery is used to express the protein encoded by the introduced genetic material. In that way, it is possible to study a protein of interest, e.g. a mutated receptor, in a controlled environment. The transfection can be stable or transient, i.e. incorporated into the cell's own genome or degraded after a short time of expression of the gene. There are several methods for transfection of cells, e.g. electroporation, where an external electric field is applied that transiently breaks down parts of the cell membrane and subsequently the cell can be loaded with foreign genetic material, through calcium channels, or by the use of liposomes packed with foreign genetic material, and after fusing of the liposome and the cell membrane, the DNA is release into the cytoplasm.

In study III, the African green monkey kidney cell line (COS-1) was transfected with different variants of pCMV6-FSHR and pCMV6-LHCGR constructs. The COS-1 cell line was used since it does not have any endogenous steroid production, nor does it have any steroid receptors; in fact, the COS-1 cell line is suitable for transfection with the pCMV6 vector since it has a SV40 genome that expresses viral T antigen, and since the pCMV6 vector has the SV40 origin of

replication that will be recognized by the viral T antigen, it will as a result be replicated in overload by the COS-1 cells.

Site-directed mutagenesis of FSHR and LHCGR
The *FSHR* cDNA (OriGene Technologies Inc., Rockville, MD, USA) was cloned into the pCMV6-XL5 vector (OriGene Technologies Inc.), by *EcoR I* restriction in the 5' end and *Sal I* restriction in the 3' end. The amino acid position 680 of the *FSHR* was mutated from N (AAT) to S (AGT) by site-directed mutagenesis with the QuickChange II-E Site-Directed Mutagenesis Kit (Strategene, La Jolla, CA, USA) according to the instructions from the manufacturer. For mutagenesis, the following primers were used: forward 5'-CAGCTCCCAGAGTCACCAGTGGTT CCACTTACATACTTG-3' and reverse 5'-CAAGTATGTAAGTGGAACCACTG GTGACTCTGGGAGCTG-3'. Confirmation of the mutagenesis was performed by direct sequencing on a 16-capillary Applied Biosystems 3130 sequencing gear (Applied biosystems). The *LHCGR* cDNA (OriGene Technologies Inc.) was cloned into the pCMV6-AC-GFP vector (OriGene Technologies Inc.) by *Sgf I* restriction in the 5' end and *Mlu I* restriction in the 3' end. The amino acid in position 312 was mutated from N (AAT) to S (AGT) also by the use of site-directed mutagenesis, but since this vector was slightly larger than the *FSHR* vector, the QuickChange II XL Site-Directed Mutagenesis Kit (Strategene) was used according to the instructions from the manufacturer. The mutagenesis primers used were: forward 5'-GAAAGCACAGTAAGGAAAGTGAGTAACAAAACAC TTTATTCTTCC-3' and reverse 5'-GGAAGAATAAAGTGTTTTGTTACTCACT TTCCTTACTGTGCTTTC-3'. The mutagenesis was confirmed by direct sequencing.

Receptor activation and hormonal stimulation
The plasmids containing the genetic variants were transiently transfected into the COS-1 cells using the polymeric transfection reagent polyethylenimine (PEI; Warrington, PA, USA) according to the instructions from the manufacturer. Polyethylenimine makes the DNA positively charged in order to attract anionic residues on the cell surface so that the DNA can be endocytosed into the cell. Approximately 100 000 COS-1 cells were seeded into 12-well plates in Dulbecco's modified eagle's medium (DMEM; Gibco Invitrogen) supplemented with 10% FBS (Biological Industries) and 1% penicillin-streptomycin (5000 units penicillin and 5 mg/mL streptomycin; Sigma-Aldrich Sweden AB), and 0.8 µg of the plasmids were used for transfection in each well. Twenty-four hours after transfection cells were washed twice in phosphate-buffered saline, and subsequently incubated for 1 h in serum-free DMEM at 37°C, 5% CO_2. Stimulation of cells with either FSHR or LHCGR were then carried out with 150 mIU/mL of Follitropin alpha (Merck-Serono), Menotropin (Ferring GmbH) or Lutropin alpha (Luveris; Merck-Serono). A transfection control (an empty pCMV4 vector) was stimulated with 150 mIU/mL Menotropin only. Combination

experiments with FSHR/LHCGR were also performed, and in these experiments a dose-response relationship was elucidated with 150 mIU/mL, 300 mIU/mL and 450 mIU/mL of Follitropin alpha (Merck-Serono), Menotropin (Ferring GmbH) and Lutropin alpha (Merck-Serono), respectively. Cells were stimulated for 1 h at 37°C, 5% CO_2, and subsequently, cell culture medium was aspirated and centrifuged at room temperature for 20 min, $1000 \times g$. Endogenous phosphodiesterases present in the cell culture medium were inactivated at 95°C for 5 min, and cells were lysed with Passive lysis buffer (Promega). All experiments were repeated 4 times.

The activity of the FSH and LHCGR through the Gαs/cAMP/protein kinase A pathway was measured in duplicates by cAMP quantification (cAMP ELISA, ENZO Life Sciences), and all results were adjusted for total protein concentration in corresponding cell lysates by use of BCA protein assay reagent (Thermo Fisher Scientific, Inc.). All results were also adjusted for basal activity of each experiment (i.e. transfected but unstimulated cells), and expressed as proportion (%) of a reference value set as the value obtained from cells transfected with the wild-type form of the receptor stimulated with 150 mIU/mL Follitropin alpha.

Enzyme-linked immunosorbent assay (ELISA) (Studies II & III)

Enzyme-linked immunosorbent assay is a technique that uses designed antibodies that bind to antigens on the substance of interest, and a color change of the sample is used for identification and quantification. There are different types of ELISA methods, e.g. sandwich ELISA. The principle of the sandwich ELISA is that standards of known concentrations and samples are added to wells pre-coated with an antibody directed towards the substance of interest. After incubation and washing, a biotinylated antibody is added, and after a second incubation and washing step, incubation with a streptavidin-horseradish peroxidase conjugate is carried out. After washing, a final incubation step is performed where a substrate is added, and the antibody-antigen-biotin conjugate in complex with the streptavidin-horseradish peroxidase bound to the well is detected by the enzyme-substrate reaction. The absorbance is read at 450 nm, and is directly proportional to the amount of the substance of interest in the standards and samples. However, in studies II and III a competitive ELISA specific for cAMP was used. The ELISA plates come pre-coated with a G×R IgG antibody. cAMP standards of known concentrations and samples are added to the wells. Subsequently, a solution of cAMP conjugated to alkaline phosphatase is added, followed by a solution of a rabbit antibody that is polyclonal to cAMP. During incubation, the polyclonal antibody binds cAMP in the sample or the cAMP that is conjugated to alkaline phosphatase, and when the plate is washed after incubation, only cAMP that is

bound to the well via the G×R IgG antibody-polyclonal cAMP antibody complex is left. A substrate solution is then added, and when the substrate is catalyzed by the alkaline phosphatase on the cAMP conjugate a yellow color is generated, which can be read at 405 nm, and which is indirectly proportional to the cAMP amount in the sample.

BCA protein assay (Studies II & III)

There are several ways of measuring total protein concentration in a cell lysate, e.g. the Bradford protein assay and the BCA protein assay. The Bradford protein assay is a spectrophotometric assay, where an absorbance shift of the dye Coomassie brilliant blue G-250 can be measured and related to the protein content of the sample (Bradford, 1976). The red form of the dye is under acidic conditions converted to the blue form that binds the protein being assayed through two forms of bond interactions, both hydrophobic and ionic. Those bindings stabilizes the anionic (blue) form of the dye and an absorbance shift from 465 nm to 595 nm occurs and can be measured. The BCA protein assay that is used in studies II and III is also a spectrophotometric assay, but the principle of this method is different from the Bradford assay since it is based on the reduction of the copper ion Cu^{2+} to Cu^+ by proteins in an alkaline medium, and subsequently, two BCA molecules chelate with each of the Cu^+ ions, and a purple instead of a green solution is formed and can be measured at 562 nm (Smith, *et al.*, 1985). The standard curve used for determination of the protein concentration in the samples was produced with dilution series of bovine serum albumin with known concentrations. The samples were run in duplicates.

Statistical methods

All statistical calculations were performed using SPSS software version 18-23 (SPSS, Inc., Chicago, IL, USA). A p-value of less than 0.05 was considered as statistically significant. All variables were tested for Gaussian distribution in the Kolmogorov-Smirnov test, where appropriate. The variables that did not approximate Gaussian distribution were log transformed. No correction for mass significance was performed, since the analyses were performed on candidate genes (Cordell & Clayton, 2005).

In study I, the calculations concerning clinical parameters were performed with all possible genetic models: T307/N680 vs. T307A/N680S and A307/S680, A307/S680 vs. T307/N680 and T307A/N680S and at last T307/N680 vs.

T307A/N680S vs. A307/S680. An additive genetic model was also investigated, where a trend of the increased number of G alleles (i.e. A307 and S680) was associated with clinical parameters. All parameters were provided as mean ± SD, and all statistical tests, except the additive model, were performed using a univariate analysis of variance. For the additive genetic effect of increasing number of G alleles a linear regression model was used. When analyzing differences in hormonal concentrations and in testicular volumes, BMI as a continuous variable was considered as a confounder, whereas when analyzing differences in sperm parameters also abstinence time was considered as a confounder.

The allele frequencies for the *FSHR* N680S and *LHCGR* N312S in the cohort of women used in studies II and III were analyzed in comparison to two control populations using a q^2 test. For the *FSHR* N680S polymorphism a normal population of 1431 Caucasians (Kuijper*, et al.*, 2010) was used, and for *LHCGR* N312S a normal population of 2996 Caucasians (Piersma*, et al.*, 2007) was used. For analyzing linkage between the two polymorphisms, data from the Phase I data from the 1000 genomes project (ENSEMBL) was used for the calculation of linkage disequilibrium.

In studies II and III, all genotype groups were tested separately when analyzing differences in clinical parameters (i.e. N680 vs. N680S vs. S680 for the *FSHR* and N312 vs. N312S vs. S312 for the *LHCGR*). An additive genetic model was also investigated in association to pregnancy rate, in which the number of G alleles (i.e. *FSHR* S680 and *LHCGR* S312; 0-4 G alleles) was evaluated. When investigating differences in total doses among genotype groups for the combined *FSHR/LHCGR* characteristics for the cohort used in studies II and III (age, BMI, cycle length, serum baseline hormones, hormonal doses for ovarian hyperstimulation and follicle and oocyte counts) were presented as mean ± SD, and comparisons among genotype groups were carried out using a univariate analysis of variance. Incidence of endometriosis, PCOS, occurrence of embryo transfer or pregnancy among genotype groups for the study cohort was evaluated with a q^2 test. Incidence of pregnancy among genotype groups was also evaluated with logistic regression (Study II) and cox regression for incidence of pregnancy in 7 IVF cycles (Study III). Age as a continuous variable and BMI divided in two groups ($\leq$ 25 or > 25) was considered as confounders when analyzing differences in background characteristics, and age, BMI, use of ICSI, type of stimulation protocol and type of hormonal agent used for ovarian hyperstimulation were set as confounding factors when analyzing differences in incidence of pregnancy in study II, whereas only age was considered as confounder in study III, since data on the other confounders were missing in cycles 2-7. In study II, all calculations regarding pregnancy outcomes were also performed after exclusion of the 13 women that were diagnosed with PCOS.

Regarding the *in vitro* experiments in study II, the combined effect of the *FSHR* and *LHCGR* polymorphism (i.e. number of G alleles), as well as each polymorphism investigated separately, regarding cAMP and IP_3 concentrations in cell culture supernatants from cultured human granulosa cells, a linear regression analysis was performed. Also, a univariate analysis of variance was used in order to analyze concentrations from separate groups of the combined genotypes. Age of the women was considered as a confounder. For the *in vitro* experiments in study III, independent samples t-test was used when associating the homozygous variants of the *FSHR* and *LHCGR*, respectively, with means of cAMP concentrations in cell culture supernatants from COS-1 cells. When calculating differences in cAMP concentrations from pooled hormone concentrations for each hormone, in relation to receptor variants, an independent samples Mann-Whitney U test was performed.

RESULTS AND DISCUSSION

FSHR polymorphisms and male reproductive function (Study I)

Since conflicting results regarding the impact of *FSHR* polymorphisms on male reproductive function previously have been generated (Ahda*, et al.*, 2005; Balkan*, et al.*, 2010; Gharesi-Fard*, et al.*, 2015; Lend*, et al.*, 2010; Song*, et al.*, 2001; Zalata*, et al.*, 2008), the objective was to investigate the *FSHR* T307A and N680S effect in a well-defined and homogenous cohort of men when the current project begun, in order to minimize the risks of not finding a relationship where there actually is one, or the other way around (Study I).

In study I, we found that in a homogenous cohort of men from the general population, of approximately the same age, those that were homozygous for *FSHR* T307/N680 had a lower mean serum FSH concentration (3.07 vs. 3.65 IU/L, p=0.009), and higher mean serum E2 (94.0 vs. 86.1 pmol/L, p=0.001), SHBG (33.6 vs. 31.3 nmol/L, p<0.0001) and total testosterone (19.1 vs. 17.9 nmol/L, p<0.0001) concentrations, compared to men carrying other *FSHR* genotypes. They also had higher sperm concentrations (71.9×10^6 vs. 70.8×10^6/ml, p=0.040) and total sperm counts (212×10^6 vs. 206×10^6, p=<0.0001), as well as larger testicles (left: 11.5 vs. 11.0 mL, p<0.0001; right: 12.4 vs. 11.6 mL, p=0.002). However, a trend for an additive genetic effect was only valid for E2 (p=0.001), FSH (p=0.036) and testis volume for the left testicle (left: p=0.021; right: p=0.055). The results from study I regarding the additive genetic effect are presented in Figure 8.

The allele frequencies and the genotype distributions for the group of Swedish men used in study I were of expected sizes and are presented in Table 2. Of the 313 men, all except three men (1%) had alleles that were co-inherited, i.e. if they had the A allele (T) in position 307, they also had the A allele (N) in position 680, if they were heterozygous in one of the positions, they also were heterozygous in the other position and if they were homozygous for the G allele (A) in position 307, they also were homozygous for the G allele (S) in position 680. For the three men with recombinant alleles, two had *FSHR* T307T/N680S and one had *FSHR* T307A/S680S. One man of each of these two allelic combinations had

spermatocele on the left testicle, but all other reproductive parameters were within the normal range for these three men.
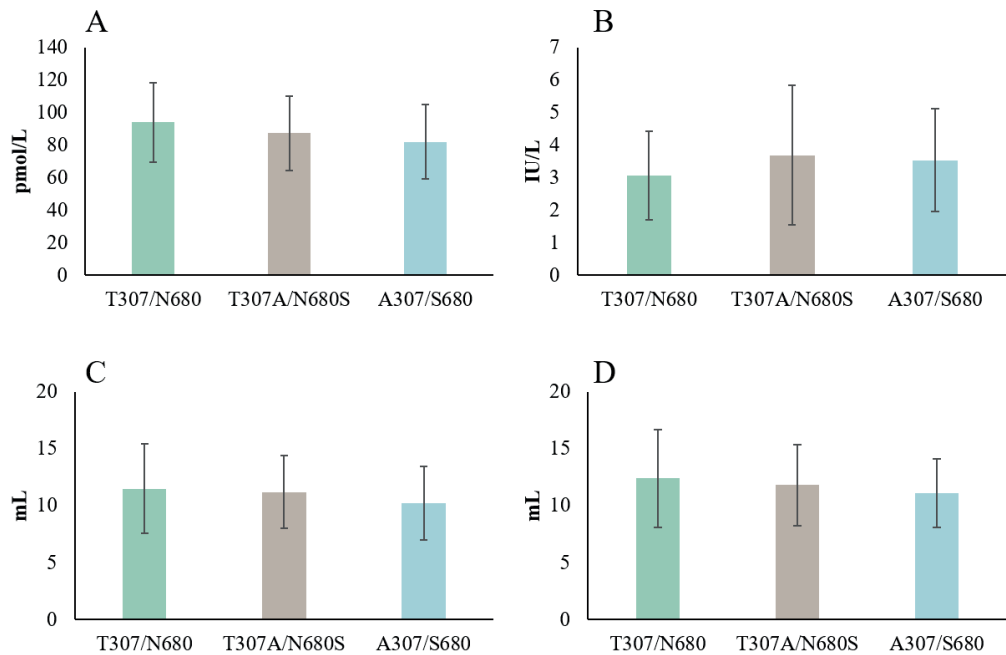
Figure 8. *Additive genetic effect of FSHR T307A/N680S genotypes on A: E2, B: FSH, C: Testis volume, left, and D: Testis volume, right. A: alanine. N: asparagine. S: serine. T: threonine.*

Table 2. Allele frequencies and genotype distributions.

			FSHR T307A*	*FSHR* N680S	*LHCGR* N312S
Swedish Men (Study I)	Allele frequency (%)	A	55	55	
		G	45	45	
	Genotype distribution % (n)	AA	29 (90)	28 (88)	
		AG	53 (167)	54 (168)	
		GG	18 (56)	18 (57)	
Swedish Women (Studies II & III)	Allele frequency (%)	A		57	39
		G		43	61
	Genotype distribution % (n)	AA		30 (186)	16 (101)
		AG		53 (327)	46 (281)
		GG		17 (104)	38 (235)

*alanine. A: adenine. *FSHR*: follicle-stimulating hormone receptor. G: guanine. *LHCGR*: luteinizing hormone/human chorionic gonadotrophin receptor. N: asparagine. S: serine. T: threonine.

The findings in study I are in accordance with some previous findings on infertile men or on a mix of infertile men and healthy controls (Song, *et al.*, 2001; Zalata, *et al.*, 2008). The findings are also in agreement with some previous findings on women (Perez Mayorga, *et al.*, 2000; Sudo, *et al.*, 2002). In 2013 a study from Estonia confirmed the results from study I (Grigorova, *et al.*, 2013).

The finding in the *FSHR* T307/N680 group of men that presented with lower FSH, higher E2, SHBG and total testosterone concentrations, could be explained by a more active FSHR in this group of men, since a FSHR variant harboring a higher activity demands a lower amount of FSH to produce a downstream signaling response that is similar to the response produced by an individual with a higher serum FSH concentration for compensation of a FSHR variant capable of a lower activity. As a result, an individual with a high activity FSHR, consequently also could present with a higher E2, since aromatase is expressed in Sertoli cells as a result of FSHR signaling (Schteingart, *et al.*, 1995), and a higher level of aromatase would result in a larger amount of testosterone converted to E2. Since FSH also regulates SHBG production in the testis (Hansson, *et al.*, 1975), a more active FSHR could also explain the higher serum SHBG concentration observed in this group of men, as well as the higher total testosterone, since a higher amount of circulating SHBG would result in lower testosterone that through the negative feedback loop to the hypothalamus would signal an induced testosterone secretion through higher LH. However not statistically significant, a higher LH concentration was observed in the group of men harboring T307/N680, which fits in this argumentation.

It seems plausible that the fact that an association between *FSHR* polymorphisms and reproductive parameters in men was found in study I, but not in many previous papers, could depend on the study population. In study I, a homogenous group of men regarding age was studied, which could affect the results since FSH in known to increase with age (Morley*, et al.*, 1997; Nieschlag*, et al.*, 1982). Also in the Estonian paper, a similar study population was enrolled, and the same results obtained (Grigorova*, et al.*, 2013).

The mechanism underlying the higher activity of the *FSHR* seen in the men with the T307/N680 genotype was not known at the time when study I was conducted, but an altered glycosylation status might play a role, since N introduces a potential glycosylation site in amino acid position 680 of the FSHR (Weerapana & Imperiali, 2006).

It remains to be elucidated if the results obtained in study I could be used in the management of the infertile male in terms of who would benefit best from treatment with exogenous FSH, or if the reference values used for the evaluation of infertile men need to be adjusted according to the genotype of the man.

In conclusion, in a large group of Swedish men of the same age from the general population, lower FSH, higher E2, SHBG and total testosterone concentrations were found for men with the *FSHR* T307/N680. These men also presented with higher sperm concentrations, total sperm counts and larger testicles. The underlying mechanism was not known when study I was conducted, but could be explained by a higher activity of this isoform of the *FSHR*.

FSHR/LHCGR polymorphisms and female reproductive function (Studies II & III)

The allele frequencies and the genotype distributions for the cohort of women used in studies II and III were of expected sizes and are presented in Table 2. There were no differences in allele frequencies between the first cohort used in study II and study III and the previously reported cohorts for the *FSHR* N680S polymorphism (p=1) (Kuijper*, et al.*, 2010), or for the *LHCGR* N312S polymorphism (p=0.554) (Piersma*, et al.*, 2007). Additionally, the allele frequencies for the two polymorphisms were in Hardy-Weinberg equilibrium (χ^2=1.29, p>0.05 for *FSHR* N680S and χ^2=0.06, p>0.05 for *LHCGR* N312S) and in linkage equilibrium (D´=0.042, r^2=0.0015). There were no differences in allele frequencies between the first cohort and the replication cohort (p=0.191 for *FSHR* N680S and p=0.080 for *LHCGR* N312S), and when merging the two cohorts, the

allele frequencies were in Hardy-Weinberg equilibrium (χ^2=0.11, p>0.05 for *FSHR* N680S and χ^2=0.52, p>0.05 for *LHCGR* N312S).

It has previously been shown that the *FSHR* N680S polymorphism affects female reproductive function, in terms of less sensitivity for FSH for the *FSHR* S680 variant (Perez Mayorga, *et al.*, 2000). However, there have been some discrepancies in the studies conducted since then, with some studies confirming the original result (Boudjenah, *et al.*, 2012; Falconer, *et al.*, 2005; Jun, *et al.*, 2006; Laven, *et al.*, 2003; Loutradis, *et al.*, 2006; Sudo, *et al.*, 2002), and other studies not (Achrekar, *et al.*, 2009; Anagnostou, *et al.*, 2012; Genro, *et al.*, 2012; Klinkert, *et al.*, 2006). An explanation could be that the studies not confirming the initial result were conducted on older women. However, since the hormonal regulation of the female gametogenesis is dependent on both FSH and LH, and since the production and development of a competent oocyte is such a complex action involving a complex interplay of the reproductive hormones and their receptors, we wanted to investigate a combination *FSHR* and *LHCGR*, and the effect on the female reproductive function (Studies II and III).

FSHR/LHCGR polymorphisms and the first IVF cycle (Study II)

In study II, we found a relationship in women with the combined genotypes *FSHR* S680 and *LHCGR* S312 (n=35) and a four-fold increased chance of achieving a pregnancy after the first IVF cycle, compared to women homozygous for *FSHR* N680/*LHCGR* N312 (n=30) (unadjusted: OR=4.70, 95% CI: [1.18, 18.7], p=0.028; adjusted: OR=11.5, 95% CI: [1.86, 71.0], p=0.009). The same pattern was found in women who received embryo transfer (unadjusted: OR=5.26, 95% CI: [1.30, 21.3], p=0.020; adjusted: OR=11.5, 95% CI: [1.89, 69.9], p=0.008). Also, a linear relationship with increased number of G alleles (*FSHR* S680/*LHCGR* S312) and increasing pregnancy rate was noted (unadjusted: OR=1.30, 95% CI: [1.08, 1.58], p=0.007; adjusted: OR=1.34, 95% CI: [1.10, 1.64], p=0.004). The same was also true for women receiving embryo transfer (unadjusted: OR=1.30, 95% CI: [1.07, 1.58], p=0.009; adjusted: OR=1.33, 95% CI: [1.09, 1.63], p=0.005). The association between pregnancy rate and the combination of *FSHR* N680S and *LHCGR* N312S is shown in Figure 9.
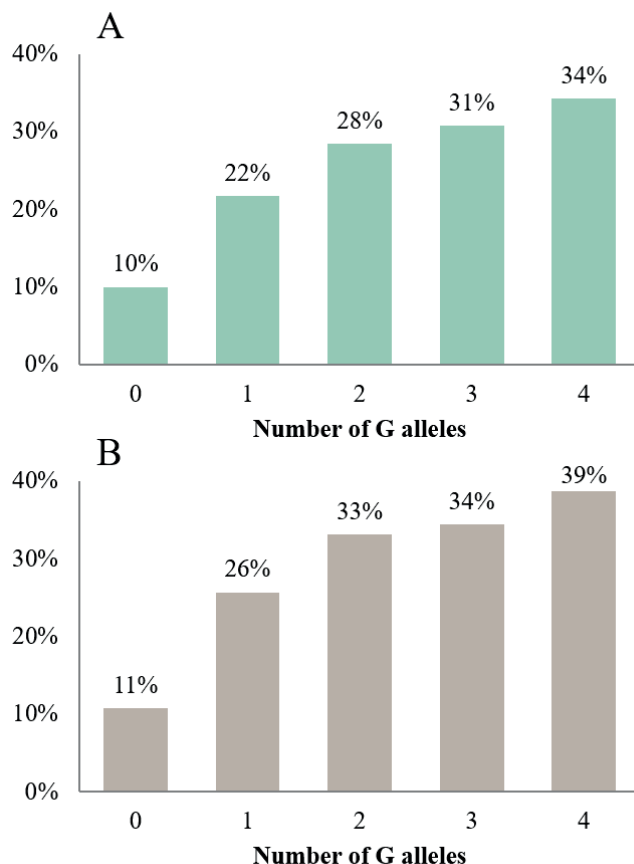
Figure 9. Pregnancy frequencies for the combination of FSHR N680S and LHCGR N312S. A: All women. 0: n=30 women, 1: n=129 women, 2: n=243 women, 3: n=169 women, 4: n=35 women; B: Only women receiving embryo transfer. 0: n=28 women, 1: n=109 women, 2: n=208 women, 3: n=151 women, 4: n=31 women.

When evaluating only the *FSHR* N680S polymorphism in the group of n=606 women, no association with pregnancy was noted (unadjusted: OR=1.18, 95% CI: [0.906, 1.54], p=0.216; adjusted: OR=1.17, 95% CI: [0.889, 1.55], p=0.260). The same was observed among *FSHR* S680 carriers receiving embryo transfer (unadjusted: OR=1.19, 95% CI: [0.903, 1.56], p=0.218; adjusted: OR=1.17, 95% CI: [0.878, 1.55], p=0.288): However, when analyzing *LHCGR* N312S with pregnancy, it was evident that heterozygous women with one S had a 56% higher pregnancy rate and women homozygous for S312 had an 83% higher pregnancy rate, compared to women homozygous for *LHCGR* N312 (unadjusted: OR=1.40, 95% CI: [1.07, 1.81], p=0.013; adjusted: OR=1.49, 95% CI: [1.14, 1.96], p=0.004). A higher pregnancy rate was also found among those receiving embryo

transfer (unadjusted: OR=1.39, 95% CI: [1.07, 1.83], p=0.016; adjusted: OR=1.50, 95% CI: [1.13, 1.99], p=0.005). Women homozygous for *LHCGR* N312 received a lower daily, as well as a lower total dose of FSH for successful ovarian hyperstimulation, compared to women with other genotypes in the first cohort of n=373 women (daily dose: 148 ± 47 IU for *LHCGR* N312 vs. 165 ± 53 IU for *LHCGR* N312S vs. 161 ± 50 IU for *LHCGR* S312, unadjusted: p=0.030, adjusted: p=0.031; total dose: 1546 ± 827 IU for *LHCGR* N312 vs. 1762 ± 781 IU for *LHCGR* N312S vs. 1694 ± 753 IU for *LHCGR* S312, unadjusted: p=0.0064, adjusted: p=0.037). The associations between pregnancy rate and *FSHR* N680S and *LHCGR* N312S are shown in Figure 10.
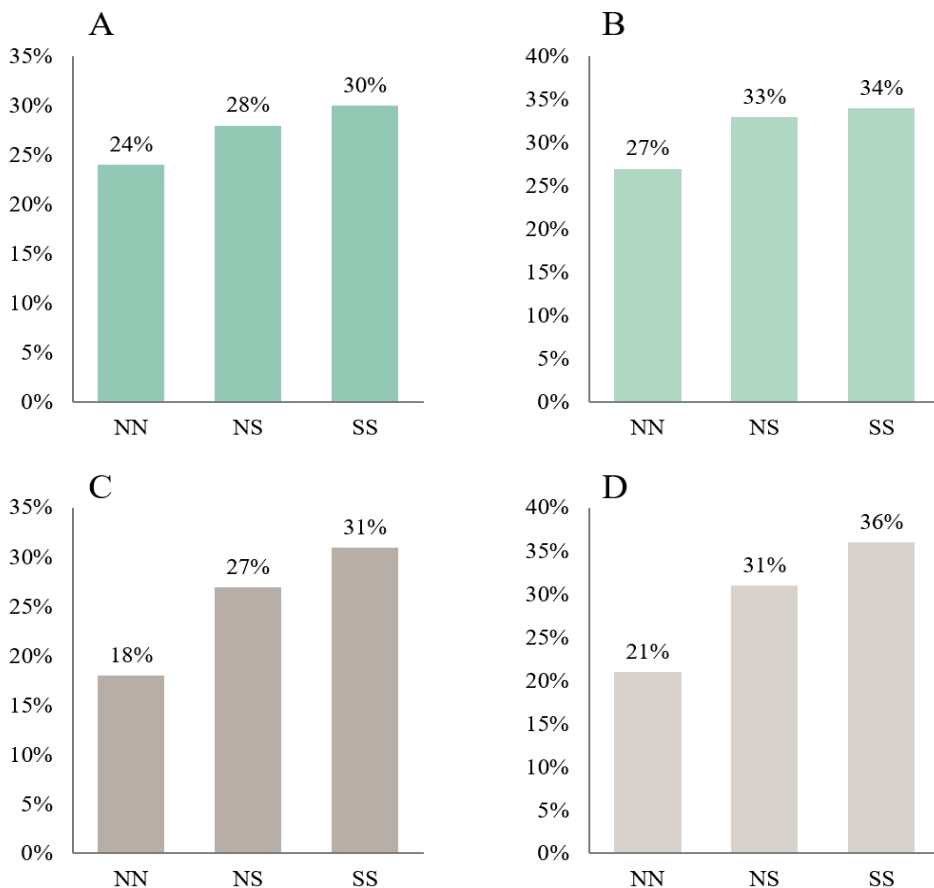
Figure 10. *Pregnancy frequencies for FSHR N680S and LHCGR N312S. A: FSHR, all women. NN: n=181 women, NS: n=322 women, SS: n=103 women; B: FSHR, women receiving embryo transfer. NN: n=158 women, NS: n=273 women, SS: n=91 women; C: LHCGR, all women. NN: n=101 women, NS: n=276 women, SS: n=229 women; D LHCGR, women receiving embryo transfer. NN: n=85 women, NS: n=238 women, SS: n=199 women. N: asparagine. S: serine.*

The results from study II are indicating that the *FSHR* N680S polymorphism alone could not predict pregnancy in women between 20 and 39 years of age (mean 32.1 ± 3.85), but in combination with *LHCGR* N312S, a strong association with pregnancy was found.

Hormonal stimulation of granulosa cells *in vitro* (Study II)

In study II, granulosa cells from n=135 women were isolated and sorted with flow cytometry, and subsequently cultured and stimulated with hormonal preparations *in vitro*. No significant associations were found, except when granulosa cells from women with the *FSHR* N680/*LHCGR* N312 genotype were analyzed separately against all other genotypes. The granulosa cells from the women with the combined homozygous N/N genotype displayed lower cAMP concentration in cell culture supernatants following stimulation with Follitropin alpha (unadjusted: 0.901 pmol cAMP/mg total protein vs. 2.19 pmol cAMP/mg total protein, p=0.034; adjusted: 0.901 pmol cAMP/mg total protein vs. 2.19 pmol cAMP/mg total protein, p=0.035). The stimulation responses for the granulosa cells are presented in Figure 11.
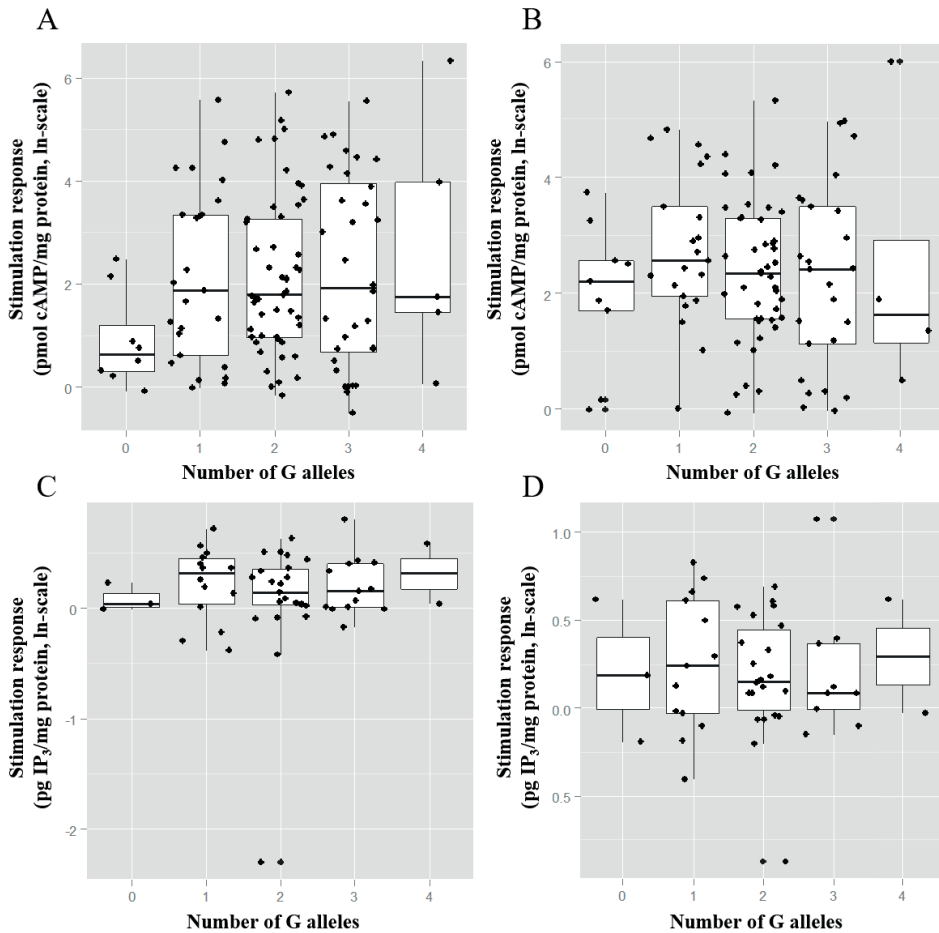
Figure 11. Granulosa cell response measured as cAMP concentration (A, B) and IP$_3$ concentration (C, D) for number of G alleles (0-4 G) for the combined FSHR/LHCGR genotypes. A, C: Cells stimulated with Follitropin alpha; B, D: Cells stimulated with Menotropin. cAMP: 3',5'-cyclic adenosine monophosphate. IP$_3$: Inositol 1,4,5-triphosphate.

The results from study II regarding *FSHR/LHCGR* and pregnancy rate after IVF are to date completely unique, and could, at least to some extent, help in managing the hormonal stimulation of the infertile women. If the women that do not get pregnant that easy (the women with the homozygous *FSHR* N680/*LHCGR* N312 genotype) could be given a different stimulation regimen than the one used today, that will shorten the time to pregnancy, then a lot of suffering for the patient and a lot of money for the society could be saved. In order to further sort out the

mechanisms behind the results obtained in study II, study III was conducted. In study III, an *in vitro* assay was developed where the different *FSHR/LHCGR* isoforms was tested for activity after hormonal stimulation. Also, the pregnancy rates regarding IVF cycles 2-7 from the cohort in study II was further analyzed, in order to see if the results obtained in study II regarding the first IVF cycle also was true for the following cycles for the women that did not get pregnant in the first round of IVF, i.e. if the *FSHR/LHCGR* genotype could predict pregnancy in all IVF cycles.

FSHR/LHCGR polymorphisms and IVF cycles 2-7 (Study III)

In study III, an association with pregnancy rate and the *FSHR* S680/*LHCGR* S312 genotype in women who had failed to receive a pregnancy in the first IVF cycle, and therefore continued with following IVF cycles, was investigated. In the first IVF trial, 370 of the women that failed to get pregnant continued to a second IVF cycle and 25% of those received a pregnancy. A difference in pregnancy rate among those women who got an embryo transferred was evident in the second IVF cycle (69% for *FSHR* S680/*LHCGR* S312 vs. 40% for *FSHR* N680/*LHCGR* N312, p=0.044). Among the women who failed to get pregnant in the second IVF cycle, 217 women continued to a third cycle and of those women 26% received a pregnancy. In the third IVF cycle, no relationship regarding the *FSHR/LHCGR* genotypes was evident (29% for *FSHR* S680/*LHCGR* S312 vs. 27% for *FSHR* N680/*LHCGR* N312, p=0.614); however, when combining the pregnancy outcomes in the second and the third IVF cycles, an association with pregnancy was established among all women (47% for *FSHR* S680/*LHCGR* S312 vs. 35% for *FSHR* N680/*LHCGR* N312, p=0.040). Of the remaining women that did not get pregnant in IVF cycles 1-3, 78 women continued to 4-7 IVF cycles, and among those 29% got pregnant. When all 7 IVF cycles were completed, 340 pregnancies were established (55%), whereas 9% of the women (55) never got pregnant. A flow chart of the women is presented in Figure 12.
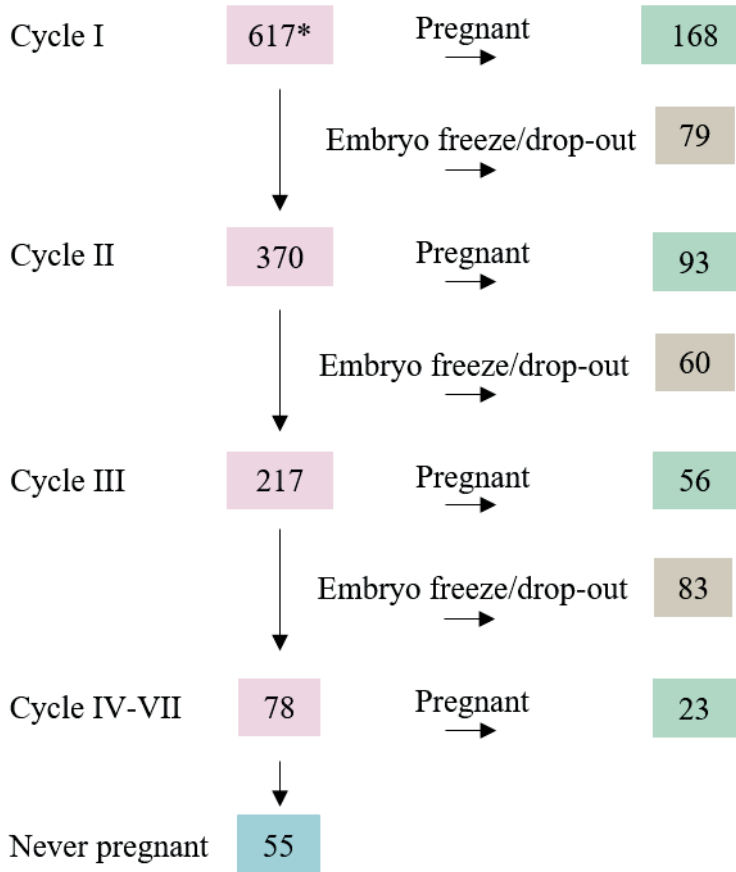
Figure 12. Flow chart of the subjects in study II and III.* In study II 606 women were included in analyzes with clinical parameters due to missing BMI values.

When combining the chance of receiving a pregnancy in all seven IVF cycles, a tendency towards a higher pregnancy rate was evident for women homozygous for *FSHR* S680/*LHCGR* S312, i.e. women with 4 G alleles (unadjusted: HR=1.83, 95% CI: [0.920, 3.63], p=0.085; adjusted for age: HR=1.95, 95% CI: [0.978, 3.87], p=0.058). The tendency towards a higher pregnancy rate for *FSHR* S680/*LHCGR* S312 is presented in Figure 13.
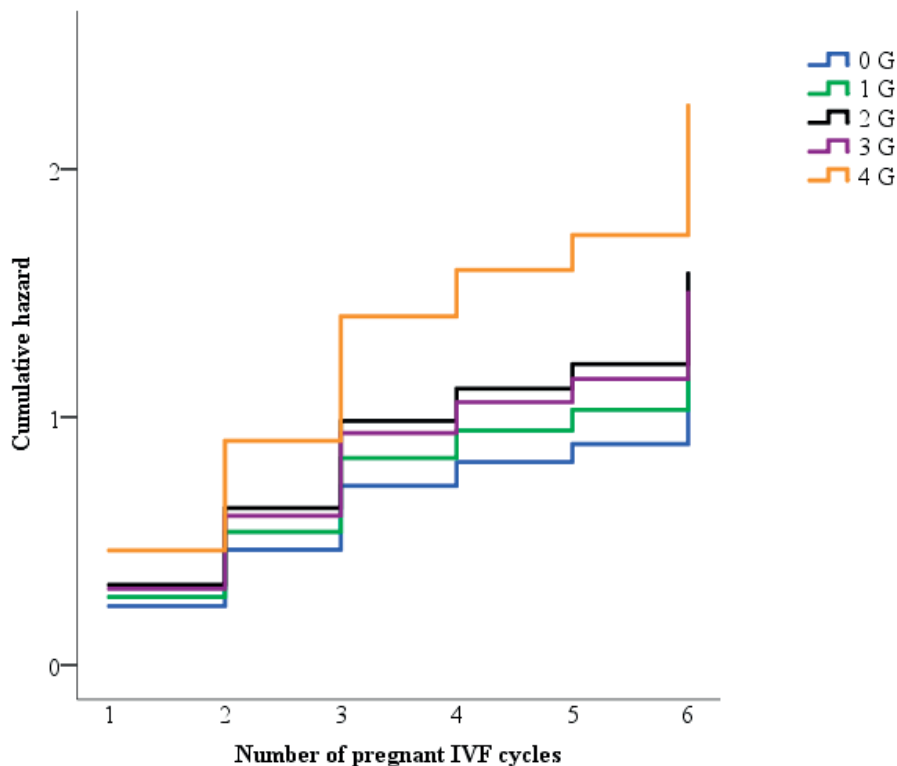
Figure 13. *Age-adjusted cumulative hazard for pregnancy in 7 IVF cycles in relation to number of G alleles for the combined FSHR N680S and LHCGR N312S genotypes (0-4 G alleles). IVF: in vitro fertilization.*

Hormonal stimulation of eukaryotic cells *in vitro* (Study III)

In study III, receptor activation studies in COS-1 cells were performed with each receptor *per se*, but also with the combination of *FSHR/LHCGR*. When *FSHR* and *LHCGR* were treated one at a time with 150 mIU/mL Follitropin alpha, Menotropin or Lutropin alpha, no differences in cAMP production were noted between the receptor variants (*FSHR* N680S: $p=0.211$, $p=0.531$ and $p=0.580$, respectively; *LHCGR* N312S: $p=0.823$, $p=0.101$ and $p=0.354$, respectively). The results from the receptor activation studies with each receptor *per se* are presented in Figure 14.
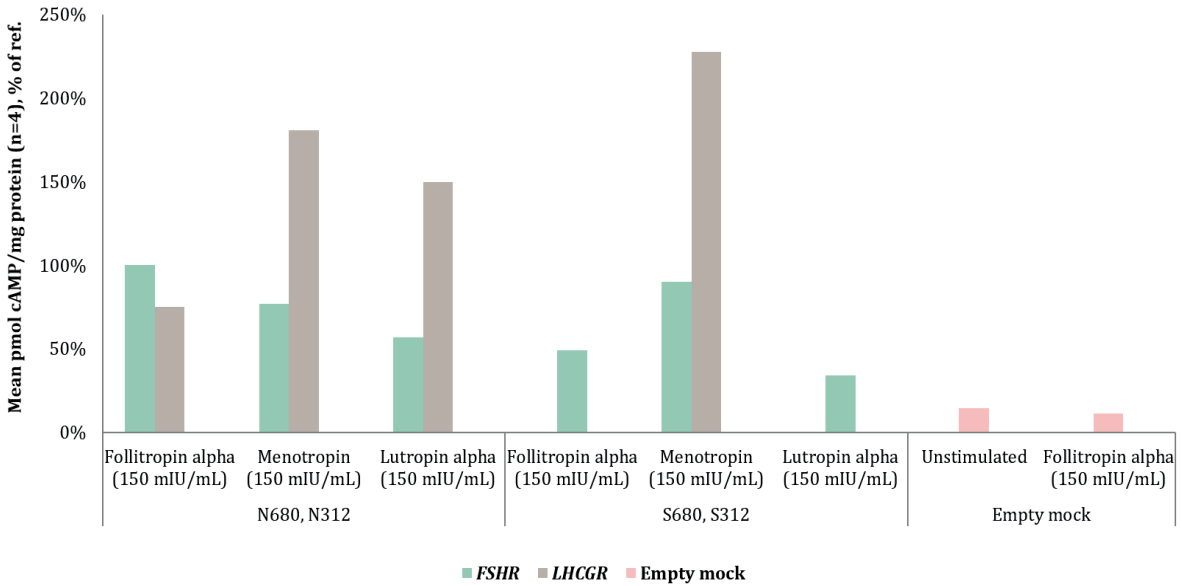
Figure 14. *Stimulation response for FSHR, LHCGR and a mock vector, measured as pmol cAMP/mg protein. Cells stimulated with 150 mIU/mL Follitropin alpha were set as reference and the values for non-stimulated cells were subtracted from each experiment. The experiment was repeated 4 times. FSHR: follicle-stimulating hormone receptor. LHCGR: luteinizing hormone/human chorionic gonadotrophin receptor. N: asparagine. S: serine.*

For the receptor activation studies performed with the combination of *FSHR/LHCGR* receptor variants, a higher cAMP response was noted in cell culture supernatants from cells with the *FSHR* S680/*LHCGR* S312 compared to cells with the *FSHR* N680/*LHCGR* N312 variant, when cells were treated with all individual concentrations of Follitropin alpha, i.e. 150 mIU/mL, 300 mIU/mL and 450 mIU/mL ($p=0.001$, $p=0.004$ and $p=0.014$, respectively). No differences in cAMP concentration in cell culture supernatants were noted when cells were treated with different concentrations of Menotropin or Lutropin alpha (Menotropin: $p=0.120$, $p=0.088$ and $p=0.117$, respectively; Lutropin alpha: $p=0.615$, $p=0.435$ and $p=0.746$, respectively). When merging all three concentrations as one group for each hormonal compound, a difference in cAMP concentration was revealed for Follitropin alpha (mean rank for *FSHR* N680/*LHCGR* N312: 6.00, mean rank for *FSHR* S680/*LHCGR* S312: 17.50, $p<0.0001$), but also for Menotropin (mean rank for *FSHR* N680/*LHCGR* N312: 6.89, mean rank for *FSHR* S680/*LHCGR* S312: 14.08, $p=0.007$). No difference was noted for Lutropin alpha though, when hormonal stimulation concentrations were merged (mean rank for *FSHR* N680/*LHCGR* N312: 7.67, mean rank for

FSHR S680/*LHCGR* S312: 7.20, p=0.898). The results from the receptor activation studies with the combined *FSHR/LHCGR* variants are presented in Figure 15.
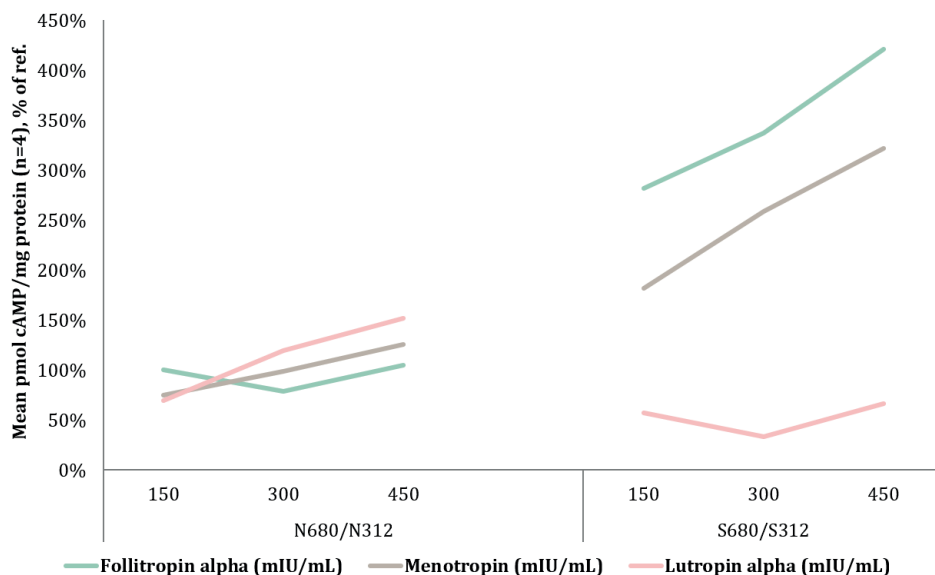
Figure 15. *cAMP (pmol/mg protein) in response to co-expressed variants of FSHR and LHCGR. Cells stimulated with 150 mIU/mL Follitropin alpha were set as reference and the values for non-stimulated cells were subtracted from each experiment. The experiment was repeated 4 times. N: asparagine. S: serine.*

Impact of *FSHR/LHCGR* polymorphisms on female reproductive function (Studies II & III)

In conclusion, the main finding of studies II and III was that women homozygous for S680 in the *FSHR* gene in combination with a homozygous genotype of S312 in the *LHCGR* gene had a four-fold increased chance of pregnancy in the first IVF cycle, and a two times higher chance of pregnancy in the second and third IVF cycle. For all IVF cycles in total, a tendency towards a higher pregnancy for those women was noted. The findings in studies II and III regarding higher pregnancy rate in all IVF cycles in women with the *FSHR* S680/*LHCGR* S312 variant was also corroborated with *in vitro* findings, since COS-1 cells expressing this receptor combination produced a higher amount of cAMP in response to stimulation with Follitropin alpha and Menotropin. The granulosa cells from the women with the *FSHR* N680/*LHCGR* N312 genotype in study II displayed a lower cAMP response

68

in response to Follitropin alpha stimulation when compared to granulosa cells from women with other genotypes, which also fits into this model. In study I, a lower serum FSH concentration, and higher E2, SHBG and total testosterone concentrations were found for men with the *FSHR* T307/N680 variant, indicating a higher FSHR activity in individuals with this genotype. This was also in line with the previous finding that women with the *FSHR* S680 genotype required a higher amount of hormonal stimulation for adequate follicle development (Perez Mayorga, *et al.*, 2000). Lower serum FSH concentrations could however not be found for women in studies II and III with this genotype, which perhaps could be explained by the fact that they were of a wide age span, but also the fact that these women were infertile, which was not the case for the men in study I. Women with the *LHCGR* S312 variant demanded a higher amount of hormonal stimulation compared to women with other genotypes, indicating that also the *LHCGR* N312S polymorphism could be a marker of the ovarian response in hormonal stimulation prior to IVF. The fact that no significant results regarding the *FSHR* N680S polymorphism and the ovarian response was found could be explained by the differences in cohort sizes, since in the study conducted by Perez Mayorga *et al.* 2000, only 161 women were evaluated. In study II, where no association with hormone sensitivity and the *FSHR* N680S polymorphism alone was found, 373 women were included, and in study III, 370 women were analyzed for differences in hormonal stimulation according to *FSHR* genotype. Also in the Perez Mayorga *et al.* study from 2000, only women were included where the infertility of the couple was derived from the male side, which gives a cohort of women that would be representative for the general population, which was not the case for the women included in studies II and III.

A recent study suggested that intracellular cAMP production was faster in granulosa cells from women with *FSHR* N680 that from women with *FSHR* S680 (Casarini, *et al.*, 2014). However, since this study only comprised four women, no firm conclusions can be drawn. The observed higher cAMP concentration in cell culture supernatants from COS-1 cells transfected with the *FSHR* S680/*LHCGR* S312 variant in study III and the granulosa cells from women with the *FSHR* N680/*LHCGR* N312 genotype displaying a lower cAMP concentration in response to Follitropin alpha stimulation in study II are supporting the theory that the activity of the FSHR and LHCGR is different depending on receptor variant. Previous studies have failed to show any differences in cAMP concentrations or show a higher activity for the *FSHR* N680 variant (Casarini, *et al.*, 2014; Nordhoff, *et al.*, 2011; Ryan, *et al.*, 2007; Simoni, *et al.*, 1999; Sudo, *et al.*, 2002), which could be explained by e.g. the fact that different cell types were used, or by the fact that only the *FSHR* was studied. Since both FSH and LH are required for oocyte maturation (Segaloff & Ascoli, 1993), it is not surprising that not only genetic variants of either *FSHR* or *LHCGR* are involved in outcomes after IVF, but instead when analyzing a combination of the receptors and when a more

defined population of women is designed, a clear effect is seen both *in vivo* and *in vitro* in studies II and III. Since the distribution of LHCGR on granulosa cells is not as dense and stable as the FSHR distribution during the menstrual cycle (Camp*, et al.*, 1991), many LH effects have been considered as indirect in the follicular phase. However, it has been demonstrated that *LHCGR* gene expression is highest in granulosa cells from preovulatory follicles close to ovulation; in fact, it was also confirmed that *LHCGR* gene expression was observed in granulosa cells from small antral follicles with a diameter of approximately 5 mm in diameter, but to 10% of the level from the follicles close to ovulation (Jeppesen*, et al.*, 2012). In this study, *LHCGR* gene expression was also visible in mature follicles, but to a level that was 5 times lower compared with the level seen in the follicles close to ovulation. A positive association between *FSHR* and *LHCGR* gene expression was also shown in small antral follicles, but when the diameter of the follicle started to increase, the association was not maintained. In contrast, it appeared as the *FSHR* gene expression was decreased when the *LHCGR* gene expression was increased, which is in consistency with the hormonal regulation of the menstrual cycle, where FSH is important in follicle recruitment in the early follicular phase, and LH is governing the late follicular phase where selection of the follicle has taken place and when the follicle develops to a large antral, preovulatory follicle. Since it is clear that there is an expression of *FSHR* and *LHCGR* simultaneously in granulosa cells, the theory of dimerization between G protein-coupled receptors, where e.g. the FSHR and the LHCGR can act as dimers or at least cross-talk, is more than reasonable. This theory is clearly supported by the results obtained in studies II and III.

The mechanism behinds the observed higher cAMP concentration could also be attributed to, as has been mentioned before, an altered glycosylation status of the receptors, since a shift from N to S potentially could lower the grade of glycosylation of the FSHR and LHCGR (Weerapana & Imperiali, 2006), and in that way possibly make the receptors more attractive for either the G protein, or some of the other proteins in the early signaling cascade, initiated by FSH/LH stimulation. It could also be the case that this effect only is seen when the FSHR and LHCGR are in dimer complexes with each other. The *in vitro* effect was studies for both cAMP and IP_3 in the granulosa cells in study II, but an effect was visible only for cAMP, indicating that only this specific signaling pathway is affected when cells are stimulated with high doses of exogenous FSH preparations. Therefore, only cAMP was evaluated *in vitro* in study III, in which a clear connection with cAMP concentration in cell culture supernatants and *FSHR/LHCGR* variants was established.

In summary, the results from studies II and III indicate that the combination of genetic variants of the *FSHR/LHCGR* clearly have an impact on the female reproductive function, in terms of an altered pregnancy rate after IVF. The results

have been corroborated by *in vitro* studies both on human granulosa cells and COS-1 cells, but it remains to be elucidated whether this effect also is seen in the normal state, when IVF not is needed and no super-physiological concentrations of exogenous FSH/LH formulations are used. Since most women in studies II and III were given Follitropin alpha, it also remains to be elucidated whether women with certain *FSHR/LHCGR* genotypes benefit from hormonal compounds other than Follitropin alpha. The results from studies II and III may in the future be a help in the management of the infertile women, in order to prevent treatments with non-satisfying effect.

CONCLUSIONS

The studies presented in this thesis contribute to clarify the connection between *FSHR/LHCGR* polymorphisms and human reproductive function. Hopefully, the presented results may contribute to the development of new targets for the management of human infertility.

The conclusions from studies I-III are:

- In Swedish young men from the general population, the *FSHR* T307/N680 genotype was associated with lower serum FSH concentration and higher serum E2, SHBG and total testosterone concentrations. The men in this genotype group also presented with higher sperm concentration and total sperm counts, as well as larger testicles. The mechanism behind the observed results is currently not known, but could be explained by a higher activity of this isoform of the FSHR in men.

- In Swedish women undergoing IVF, women with the *FSHR* S680/*LHCGR* S312 genotype had a four-fold increased chance of pregnancy in the first IVF cycle, and a twice as high chance of pregnancy in the second and third IVF cycle compared to women with other genotypes. Women homozygous for *LHCGR* S312 also had higher pregnancy rates after the first IVF cycle, regardless of *FSHR* variant, and demanded higher doses of FSH for follicle recruitment. In contrast, women with the *FSHR* N680/*LHCGR* N312 variant had a pregnancy rate of only 10% in the first IVF cycle, compared to 39% for the *FSHR* S680/*LHCGR* S312 genotype. The clinical finding were validated experimentally.

- A lower response to FSH treatment in terms of a lower cAMP concentration in cell culture supernatants was revealed for granulosa cells from women with the *FSHR* N680/*LHCGR* N312 genotype with less chance of pregnancy. In COS-1 cells the same pattern was observed; COS-1 cells transfected with the *FSHR* S680/*LHCGR* S312 variant displayed higher amount of cAMP in culture medium in response to stimulation with FSH and a mixture of FSH/LH. The increased pregnancy rate after IVF in

women with this specific genotype could therefore be dependent on the higher activity seen *in vitro* for this receptor combination.

- A new method for sorting of granulosa cells was developed, resulting in almost 100% purity.

FUTURE PERSPECTIVES

The studies within this thesis conclude that further studies regarding polymorphic genes that have an impact on male and female reproduction and their association with IVF outcomes are needed. When work on this thesis begun, conflicting results had been produced, and no firm conclusions could be drawn regarding the impact of especially the *FSHR* N680S polymorphism on human reproductive function. The work conducted in the studies within this thesis have contributed substantially to the clarification of the connection between the polymorphic *FSHR* and *LHCGR* genes and, in particular, their involvement in outcomes after IVF. However, further studies are needed in order to confirm and develop the results presented in this thesis, both regarding the impact on the male reproductive function, but especially regarding the observed relationship with *FSHR* N680S/*LHCGR* N312S and female reproduction in terms of pregnancy outcomes after IVF. It is possible that the population with this genotype combination need to be further defined by the use of additional polymorphisms in the *FSHR* and/or *LHCGR* genes, or in other genes as e.g. the *FSHB* gene.

The intrafollicular milieu of women with a more defined combination of genotypes would also be interesting to take a closer look at, both regarding follicles from women that not have been stimulated with FSH prior to an IVF treatment, and from women that have, since a potential difference between these two groups reflects the impact that hormonal stimulation has on the machinery of human follicles.

In men, e.g. a connection with *FSHR* genotypes and FSH stimulation in order to increase sperm output in infertile men potentially could be drawn, since there seem to be a difference in the reproductive hormonal profile and in sperm parameters between men with different *FSHR* genotypes. Future studies would need to be designed in a way where a homogenous population, both regarding age and ethnicity, and that is large enough, is used in order to find a possible connection between *FSHR* genotypes and FSH treatment. Possibly, a connection with *FSHR* genotypes and the FSH dose could be found, but in order to develop genotype-adjusted FSH doses a large homogenous group of infertile men would need to be evaluated in a randomized study.

Regarding the finding that women with the homozygous *FSHR* S680/*LHCGR* S312 genotype have a higher pregnancy rate compared to women with other genotypes, a study confirming these results would be needed. As for future studies conducted on males, also in females the study populations used need to be defined regarding ethnicity and age, since the genes studied not are evenly distributed among all populations, and since age affects the reproductive hormonal profile and outcomes after an IVF trial.

Since most women in the studies were given recombinant FSH for follicle stimulation and development, it is not possible to say whether the result would be different if more of the women were given a different hormonal compound. It is hence not possible to say whether women with different genotypes would benefit from different hormonal compounds. Further research in this area is therefore necessary.

An attempt to explain the association with *FSHR* S680/*LHCGR* S312 and the observed higher pregnancy rate have been sought by the use of *in vitro* stimulation experiments of human granulosa cells as well as COS-1 cells with different receptor variants, but these studies could further be developed. Using human granulosa cell lines would be preferable in these future studies, since these cells constitute the natural environment for the FSHR and the LHCGR. Elucidating whether a dose-response relationship with different hormonal compounds and genotypes exists would be necessary in order to define the population of women with the *FSHR* N680/*LHCGR* N312 genotype further, with the final goal to find a genotype-adjusted hormonal treatment in IVF that optimizes the chance of receiving a positive pregnancy test. In order to make this possible, the *in vitro* studies need to be followed by randomized studies where women with different genotypes are treated with different hormonal compounds and/or doses. If the hormonal regimens used in IVF today could be adjusted according to genotype so that women that not get pregnant that easy would be given a fair starting point in their treatments, a lot of suffering for the infertile couple, and a lot of money for the society, could be saved.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Mekanismerna bakom den hormonella regleringen av den kvinnliga och manliga reproduktionsfunktionen är endast delvis kända. Man har dock länge vetat att follikelstimulerande hormon (FSH) spelar en viktig roll, både för äggutvecklingen hos kvinnan och för spermieutvecklingen hos mannen. Stimulering med FSH används också för att behandla kvinnor som ska genomgå assisterad befruktning i syfte att öka antalet tillgängliga ägg. Olika individer skiljer sig dock markant från varandra beträffande behandlingssvar. Kvinnans ålder och vikt spelar en roll, men då skillnaderna kvinnor emellan trots detta är betydande har man sökt svar även i kvinnans genetiska uppsättning. Man har i tidigare studier på kvinnor som genomgått *in vitro*-fertilisering (IVF) visat att individer som har en dubbel uppsättning av aminosyran serin i aminosyraposition 680 i FSH-receptorn producerar färre antal ägg än kvinnor med andra genetiska uppsättningar. Dessa kvinnor krävde dessutom högre FSH-doser för att äggutvecklingen skulle bli optimal. Dock kunde inte resultaten från dessa inledande studier valideras, eftersom efterföljande studier inte kunde hitta samma samband. Inga direkta slutsatser kunde därför dras. Skillnaden mellan studiernas resultat kan exempelvis härledas till att för få kvinnor ingått i studierna, vilket gör att de resulat som hittats inte kan appliceras på befolkningen i stort. Beträffande män hade inte lika omfattande studier av olika varianter i FSH-receptorn och deras betydelse för den manliga reproduktionsfunktionen gjorts när arbetet med denna vetenskapliga avhandling inleddes. En hypotes om att varianter av FSH-receptorgenen kunde ha betydelse för den kvinnliga och manliga könscellsutvecklingen fanns dock, och om denna kunskap kunde utvecklas så att exempelvis FSH-dosen och vilket preparat som används inför en IVF-behandling kunde individanpassas och på det sättet optimeras skulle mycket lidande och tid för patienten sparas, men även betydande kostnader för samhället.

För att analysera FSH-receptorvarianters betydelse för den manliga reproduktionsfunktionen analyserades 313 unga svenska män från normalbefolkningen för FSH-receptorvarianter, och resultatet associerades med hormon- och reproduktionsparametrar. Detta var den första studie som gjorts på normala, unga män; alla tidigare studier hade omfattat infertila män i olika åldrar. Vidare undersöktes 617 kvinnor som genomgått en IVF-behandling på

Reproduktionsmedicinskt centrum i Malmö för FSH-receptorvarianten som analyserats i tidigare studier, kombinerat med en variant av genen för receptorn för luteiniserande hormon (LH), och resultatet associerades med bland annat graviditetsutfall. Från 135 av de 617 kvinnorna isolerades en celltyp kallad granulosaceller från follikelvätskan, eftersom dessa celler uttrycker både FSH-receptorn och LH-receptorn och därför kan användas för att studera receptorernas mekanismer och aktivitet på ett molekylärt sätt. Granulosacellerna odlades i laboratotium under förhållanden som liknar de som finns i kroppen, för att sedan kunna stimuleras med FSH-preparat och på så sätt kunna utvärdera receptorernas aktivitet. Resultatet kunde sedan kopplas till kvinnans FSH- respektive LH-receptorvarianter. Då mängden granulosaceller som fanns att tillgå var begränsat användes även en njurcellinje som härstammar från apor för att studera receptorernas mekanismer. Eftersom njurcellinjen helt saknar FSH- och LH-receptorer kunde dessa introduceras i cellernas genetiska uppsättning i valda kombinationer och stimuleras med olika FSH-preparat, varefter resultatet kunde analyseras med avseende på aktivitet och receptorvariant.

Hos de 313 unga männen var serin i aminosyraposition 680 i FSH-receptorn associerad med bland annat lägre testosteron, lägre spermiekoncentration och mindre testikelstorlek. Kvinnorna som genomgått assisterad befruktning blev däremot oftare gravida (fyra gånger högre chans) om de var bärare av serin i dubbel uppsättning i både FSH- och LH-receptorn i den första IVF-cykeln, medan en näst intill dubbelt så hög chans till graviditet i IVF-cykel två och tre uppmättes. Granulosaceller från bärare av aminosyran asparagin i FSH- och LH-receptorgenen visade lägre svar på FSH-stimulering i laboratoriet, jämfört med granulosaceller från kvinnor med andra genuppsättningar. Även de utökade cellstudierna med njurceller från apa visade på samma samband; en högre aktivitet hos FSH- och LH-receptorvarianterna med serin uppmättes vid stimulering med två olika FSH-preparat, vilket är i linje med den högre graviditetschansen för kvinnor med denna FSH-variant.

Sammanfattningsvis verkar alltså varianter av FSH-receptorn påverka den manliga och kvinnliga reproduktionsfunktionen så att manliga bärare av serin i FSH-receptorgenen har något sämre reproduktiva parametrar än bärare av andra varianter av FSH-receptorgenen, medan en kombination av serin i både FSH- och LH-receptorgenen hos kvinnor som genomgått assisterad befruktning verkar ha en positiv effekt på graviditetsutfallet, vilket stöds av resultat från cellstudierna. Då den hormonella regleringen är komplex och påverkas av många gener bör resultaten presenterade i denna vetenskapliga avhandling utvärderas i fler vetenskapliga studier, där exempelvis ytterligare gener som anses påverka reproduktionsfunktionen hos människan tas i beaktande. Det bör också utredas huruvida olika genvaraínter kan påverka män och kvinnor olika, vilket resultatet från denna avhandling antyder.

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The impact of gonadotropin receptor polymorphisms on human reproductive function

Ida Lindgren holds a MSc in Biomedicine from Lund University, Sweden, since 2010. The focus of her thesis is the effect of polymorphisms in the gonadotropin receptor genes on human reproductive function. The main results are that FSH receptor polymorphisms have an effect on male reproductive hormone levels and sperm parameters, and that in women, a combination of FSH receptor and LHCG receptor polymorphisms predict the pregnancy chance after *in vitro* fertilization.

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