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DIGGING INTO THE GENETICS OF HIGH RESPONDERS

Is there a genetic predisposition in women with high risk to develop the ovarian hyperstimulation syndrome?

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Abstract

The main goal for this study is to find either rare or common genes contributing to the development of the ovarian hyperstimulation syndrome (OHSS). To find this gene, there are two populations selected.
The first population exists of four women who developed OHSS after a GnRH antagonist protocol and freeze-all approach with a GnRH agonist trigger. These subjects were not expected to develop this syndrome in these circumstances, which raises questions about the contributing factors to OHSS. Therefore these women are an interesting population to search for a predisposing genetic factor.
The second group are five women with extreme high serum AMH levels. They are selected on this trait because AMH is a predictive factor for the development of OHSS and is strongly associated with polycystic ovary syndrome, which is a clear risk factor to develop OHSS.

With the use of whole exome sequencing, the genes and variants of these nine women were mapped. Next, the genes that are already known to be associated with OHSS or PCOS were selected. Their variants were filtered for a frequency of less than 2% and compared to the variants present in the 1000 genome project. Next, all the other genes that are not yet known to have a link with OHSS or PCOS were selected. The variants more frequent than 2% were again filtered out. The remaining genes were then systematically searched in the PubMed, OMIM and ClinVar database using different pre-specified key words. Based on the information retrieved through this method, only the relevant genes remained.

From the first population, the genes PGR, TP53, SLC9B1P1, PABPC3 and ANKRD30A came out.
The five subjects with high serum AMH levels showed relevant variants in FSHB, LHCGR, THADA, SORBS1, PAPPRG, FBN3, PABPC3 and ADAMTS7.

Finally, a gene seen in all nine subjects was not found. However, PABPC3 did occur in eight women, even with some common variants. This gene is normally only expressed in testis tissue, so this raises many questions about the circumstances in when this gene is expressed and the possible link with PCOS and OHSS.
Eventually, causality of a gene cannot be shown with this study design as it is descriptive by nature, but it does open new aspects of the genetic predisposition of PCOS and OHSS, which may be investigated in the future.

Introduction

1. What is Assisted Reproductive Technology (ART)?

Up to 17% of all couples in their reproductive age are unable to conceive a child following 12 months or more of regular unprotected intercourse[1]. Fifty six percent of these couples seek medical care to remedy this problem[2]. Assisted reproductive technologies (ART) can be one of the medical possibilities. This overarching term covers all procedures and treatments that include the handling of human oocytes, sperm or embryos in vitro with the aim of achieving a pregnancy[1].
In vitro fertilization (IVF) was the first and most common procedure of ART, with the first child being born in 1978[3]. Since then, there has been a great improvement and widespread use of all reproductive techniques, accounting nowadays for 1,3 to 3% of the newborns in European countries[4].

2. Ovarian Stimulation

2.1 Physiology of the menstrual cycle

Ovarian stimulation is a crucial aspect of IVF. This stimulation leads to the development of multiple follicles, which can be aspirated to retrieve the oocytes produced which will be later be fertilized in vitro. The embryos produced by this method are then graded according to their development and division and transferred sequentially, cumulatively increasing the pregnancy rates[5].
To understand the different protocols, it is necessary to comprehend the physiologic mechanisms involved in the normal menstrual cycle. Summarily, one can divide the ovarian cycle in 3 phases: the follicular phase, ovulation and the luteal phase. These phases are regulated by the hypothalamus-pituitary-gonadal axis[6].
The hypothalamus secretes gonadotropin-releasing hormone (GnRH) that stimulates the anterior pituitary gland to secrete follicle stimulating hormone (FSH) and luteinizing hormone (LH) by binding on the GnRH receptor. FSH binds to the FSH receptors located in the granulosa cells, while LH binds to the LH receptors present in both the granulosa and theca cells. This binding leads to the production and secretion of estrogen and progesterone. There are two other important peptides produced by the ovaries: inhibins and activins. These steroids and peptides exert a negative and positive feedback on both the hypothalamus and anterior pituitary gland[6].

2.1.1 The follicular phase

The follicular phase starts when menstruation is initiated and lasts approximately 10 to 14 days. In the luteal phase of the preceding cycle, FSH levels begins to rise. This increase in FSH levels stimulates the recruitment and development of multiple follicles.

In the early follicular phase, LH levels also start to rise. LH is responsible for the uptake of low-density lipoproteins (LDL) by the theca cells. This contains cholesterol, which is converted to androgens, which in turn is transported into the granulosa cells. There they are converted into estrogens. Together with FSH, estrogens are responsible for the proliferation of the granulosa cells and the upregulation of FSH receptors. They also induce the upregulation of LH receptors in the granulosa cells. Estrogen is also responsible for the thickening of the endometrium.
FSH starts to decrease with the rise of estradiol because of the negative feedback on the anterior pituitary gland. The follicle with the most FSH receptors is the most sensitive to FSH and has enough stimulation even with these low levels of FSH to convert the androgens, produced by the theca cells, to estrogens. The other follicles are not stimulated enough and cannot convert the androgens, which leads to atresia of these follicles. This process is responsible for the development of a dominant follicle[6].

2.1.2 Ovulation

In the late follicular phase, estradiol levels rise and have a positive feedback on the secretion of LH resulting in the LH surge 24-36h later. During this peak, the oocyte maturation is finalized and approximately twelve hours after the LH surge, ovulation occurs[6].

2.1.3 The luteal phase

Following ovulation, the remaining theca and granulosa cells form the highly-vascularized corpus luteum. This is the start of the luteal phase. The corpus luteum produces both estrogen and progesterone. The rise of progesterone is more prominent then the rise of estrogen, which leads to a negative feedback and thus a decline of GnRH, LH and FSH. Progesterone is also responsible for the further secretory development of the endometrium in preparation for implantation. Both estrogen and progesterone inhibit folliculogenesis. At the end of the luteal phase, if an implantation does not occur, the levels of estrogen and progesterone decrease because the corpus luteum degenerates and forms a fibrotic corpus albicans. This decrease is called luteolysis and is responsible for menstruation, approximately 14 days after ovulation. If embryo implantation does occur, the corpus luteum is maintained by the production of human chorionic gonadotropin (hCG) by the trophoblast and menstruation does not occur[6].

2.2 Protocols for ovarian stimulation

Ovarian stimulation is performed with exogenous gonadotropins in the early follicular phase before dominance of a follicle is achieved. This supraphysiologic stimulation allows for the development of more than one follicle[5].

Different stimulation protocols have been developed over the years. There is a protocol with no stimulation, the natural cycle, one with minimal oral stimulation, using clomiphene citrate, and a more "aggressive" stimulation with exogenous gonadotropins. The latter has been optimized with the use of GnRH analogues.

2.2.1 Clomiphene citrate

Clomiphene citrate is an anti-estrogen, which lowers the negative feedback on the anterior pituitary gland by binding on the estrogen receptors and so causing a rise in FSH and LH levels. Because the function of estrogen is suppressed, an LH surge is prevented and premature ovulation does not occur[5]. This protocol has a pregnancy rate of 6%, which is significantly lower than those observed with human gonadotropins (20 to 36%). Clomiphene stimulation does not produce enough mature follicles to maximize the pregnancy rate [7].

2.2.2 Exogenous gonadotropins

The two most commonly used exogenous gonadotropins are hMG (human menopausal gonadotropin), which consists of both FSH and hCG-driven LH activity, and recombinant FSH, which consists only of FSH. HMG was discovered and purified from postmenopausal urine in the late 1940s. However, as there were many co-purified proteins, the injections led to hypersensitivity and discomfort. In the 1960s the purification process became more sophisticated and the side effects were reduced. In the 1980s, FSH was purified from hMG by using monoclonal antibodies. This led to a drastic decrease of hypersensitivity and also to the possibility of subcutaneous administration. Following the development of urinary FSH, recombinant FSH was produced by recombinant DNA technology[8].

The gonadotropin stimulation is commenced in the early follicular phase, defined as day 1 of the stimulation cycle, and is continued for a period of approximately 12 days, when the optimal size of follicular development is reached[9].
When this criteria is met, the stimulation is discontinued and hCG is administered[5]. hCG is structurally similar to LH and, therefore, can be used to finalize follicular development and trigger ovulation. After this administration, the oocytes can be aspirated.

2.2.3 The GnRH agonist protocol

GnRH agonists were first thought to be used as a treatment for anovulation because they have the same function as endogenous GnRH: stimulating the GnRH receptors, which leads to a secretion of FSH and LH. Administering this agonist initially led to a rise of FSH and LH, but when it was used over a longer period, FSH and LH started to decrease. Such a decrease is caused by the a desensitization of the GnRH receptors associated with sustained GnRH agonist administration, thus lowering the secretion of FSH and LH and leading to less stimulation of the ovaries instead of more[10].

This phenomenon was very interesting to use in IVF, since, with this desensitization of GnRH receptors, endogenous LH and FSH levels could be decreased and the LH surge could be prevented.

There are two protocols with GnRH agonists, a long and a short protocol. In the long protocol, GnRH agonist is usually administered in the luteal phase of the preceding cycle and is continued until hCG administration. Because pituitary quiescence is necessary before starting the ovarian stimulation, GnRH agonists are administered for approximately two weeks prior to starting exogenous stimulation. The short protocol starts during the follicular phase in an attempt to use the initial stimulatory effect of the agonists to recruit additional follicles. Only one day later the stimulation commences[9]. However, a meta-analysis showed that the number of oocytes retrieved and the pregnancy rates were higher in the long protocol compared to the short protocol[11].

2.2.4 The GnRH antagonist protocol

In 2001, GnRH antagonists were registered for use in IVF. These antagonists are responsible for the immediate suppression of the pituitary function. Furthermore, the interruption of the administration of antagonists leads to an immediate recovery of pituitary function. This protocol can be started at any day in the early or midfollicular phase to make sure an LH surge is prevented[9]. It is clear that the GnRH antagonist protocol is shorter than the long GnRH agonist protocol. There is no significant difference in pregnancy rates between the long GnRH agonist protocol and the GnRH antagonist protocol[12].

3. Complications and infertility problems

The two most important complications occurring in IVF are the development of multiple pregnancies and the ovarian hyperstimulation syndrome (OHSS)[8]. The current thesis will be focusing on the latter.

4. Ovarian hyperstimulation syndrome (OHSS)

4.1 Clinical presentation

Ovarian hyperstimulation syndrome (OHSS) is an exaggerated response to ovarian stimulation, characterized by cystic enlargement of the ovaries, abdominal distention and pain, fluid shift from the intravascular space to the third space, which can result in ascites, pericardial and pleural effusions, and even a generalized edema. This may lead to hypovolemia, haemoconcentration, electrolyte imbalances and coagulation disorders and even life-threatening complications such as hemorrhage from ovarian cyst rupture, adult respiratory distress syndrome, thromboembolism, and renal failure[13].
According to Golan et al., OHSS can be sub-classified as either mild, moderate or severe[14]. Each category is subdivided in grades. The classification is listed in table 1.

Category	Description
Mild OHSS	
Grade 1	Abdominal distension and discomfort
Grade 2	Features of grade 1 plus nausea, vomiting, and/or diarrhea. Ovaries are enlarged to 5-12 cm
Moderate OHSS	
Grade 3	Features of mild OHSS plus ultrasonic evidence of ascites
Severe OHSS	
Grade 4	Features of moderate OHSS plus clinical evidence of ascites and/or hydrothorax or breathing difficulties
Grade 5	All of the above plus change in blood volume, increased blood viscosity due to haemoconcentration, coagulation abnormalities, and diminished renal perfusion and function

Table 1. Ovarian hyperstimulation syndrome (OHSS) classification[15].

4.2 Pathophysiology

At the basis of OHSS lies an exaggerated response to the stimulation of the ovaries with exogenous gonadotropins. In some patients, this stimulation leads to a large number of growing follicles and high estrogen levels. These patients are then exposed to a bolus of hCG to finalize oocyte maturation. hCG is structurally alike LH and mimics its effects by binding to the LH receptor. The difference between both hormones is that hCG has a longer half-life (>24h vs. <60min) which leads to sustained luteotropic activity. This is the cause of high vascular permeability, an extravasation of fluid to the third space and, consequently, the clinical presentation of OHSS[16,17].

The key molecules responsible for the high vascular permeability are vascular endothelial growth factor (VEGF) and factors involved in the ovarian renin-angiotensin system[18]. VEGF is produced by the granulosa cells after stimulation with gonadotropins and increases strongly after the administration of hCG[19].
OHSS can occur in two forms. The early-onset pattern of OHSS is associated with gonadotropin stimulation and is seen within nine days after hCG administration and oocyte retrieval. The late-onset pattern occurs after 10 days and is caused by the production of endogenous hCG by the implanted embryo[20].

4.3 Prevalence

The prevalence of early OHSS in both GnRH agonist or GnRH antagonist hCG-triggered protocols varies significantly from 20-23% for the mild form, 2-6% for the moderate form and 0.1-2% for the severe form[21]. Cases of death related to the ovarian hyperstimulation syndrome are under-reported[22].

4.4 Risk factors

The most sufficiently documented factors to determine a patient at risk are polycystic ovaries, estradiol levels above 3000 pg/ml on the day of hCG administration and 13 or more follicles aspirated of 11mm. For the latter two, only extreme high quantities can be assumed as predictors[23].

4.5 Prevention

The risk of OHSS can be reduced by eliminating the use of hCG as trigger. This became possible by the introduction of GnRH antagonists. Because they inhibit the pituitary function directly, the receptors can recover much faster, which is in stark contrast to the effect of desensitization caused by GnRH agonists. This allows clinicians to use GnRH agonists as a trigger instead of hCG in the antagonist protocol leading to a temporary displacement of the GnRH antagonists followed by an endogenous LH surge.
Although the GnRH agonist trigger causes an endogenous flare-up of LH, the levels are not high enough to sustain a normal function of the corpus luteum leading to drastic luteolysis, decreased progesterone levels and, therefore, an underdeveloped endometrium. This leads to luteal phase defect causing menstruation after 5 or 9 days, which is in contrast to the 14 days of the luteal phase that occurs when hCG is used as a trigger[24–26]. Because the endometrium does not develop adequately, implantation rates are significantly lower when the embryo is transferred in the fresh cycle[27].

To rescue the luteal phase and thus to allow for a fresh embryo transfer, Humaidan et al. suggested to administer a small dose of hCG following oocyte retrieval, supporting the corpus luteum, increasing the progesterone levels and leading to a better development of the endometrium[28]. However, in this protocol, the risk of OHSS still exists. Seyhan et al. suggested to instead cryopreserve all the embryos and transfer them in a next natural or artificial cycle[29]. This eliminates the use of hCG completely and further reduces the risk of OHSS[30].

4.6 OHSS and an hCG-free protocol

This last protocol has only been applied for a few years and has seemed thus far very promising in eliminating further the risk of developing OHSS. However, until now there are already seven cases reported of severe OHSS in a GnRH antagonist protocol with GnRH agonist triggering and freeze-all approach[31–34]. These observations lead one to conclude that other crucial components, besides

sustained hCG activity, are also involved in the development of OHSS. The two cases reported by Fatemi et al. showed a curious characteristic. In both cases, menstruation occurred after 14 days instead of 5 or 6 days when using the GnRH antagonist protocol followed by a GnRH agonist trigger[31]. This suggests that there could be a genetic deviation on a higher level in the hormone feedback resulting in a higher quantity of FSH and LH.

Also, there have been several cases of familial spontaneous OHSS[35]. In such cases, OHSS occurs after a spontaneous pregnancy, established without controlled ovarian stimulation. In both spontaneous as iatrogenic OHSS, hCG plays the key factor to cause this syndrome. The fact that it occurs in some women with a normal pregnancy emphasizes even more there is a genetic component involved in the development of OHSS. This is why it is interesting to look for a genetic component in women who developed OHSS after this new protocol where hCG is not a factor anymore.

4.7 Genetic variations related to ovarian stimulation

Genes that are known to be involved in ovarian stimulation are: FSHR, LHR/LHCGR, CYP11A1, CYP19A1, ESR1, ESR2, PGR, VEGFR1, VEGFR2, VEGF, AMH, AMHR, GDF9, BMP15, SOD2, SHBG, FOLR1, MTHFR, p53, PAI and TNFα[36–39].

The polymorphisms described in these articles are both related to poor responders and high responders.

Gene	Name	Function
FSHR	Follicle stimulating hormone receptor	Present on the granulosa cells, induces production of estrogen
LHR/LHCGR	Luteinizing hormone/luteinizing human chorionic gonadotropin receptor	Present on theca and granulosa cells at antral stage, completes oocyte maturation
CYP11A1	Cytochrome P450 C11A1	Cholesterol side-chain cleavage enzyme: first and rate-limiting step in the synthesis of steroid hormones
CYP19A1	Cytochrome P450 C19A1	Aromatase: conversion of androgens in estrogens
ESR1	Estrogen receptor alpha	Expressed in breast cells, caries out the function of estrogen
ESR2	Estrogen receptor beta	Expressed in brain and cardiovascular tissue, granulosa cells, breast cells, caries out the function of estrogen

Table 2. Genetic variations related to ovarian stimulation.

PGR	Progesterone receptor	Carries out physiological effects of progesterone, which has a central function in the establishment and maintenance of pregnancy
VEGFR1	Vascular endothelial growth factor receptor 1	Expressed by the endothelial cells, also present in the inner theca of human follicles
VEGFR2	Vascular endothelial growth factor receptor 2	Expressed by the endothelial cells, also present in the inner theca of human follicles. Regulation of vascular permeability, vasculogenesis and angiogenesis
VEGF	Vascular endothelial growth factor	Angiogenic factor responsible for increased vascular permeability
AMH	Anti-müllerian hormone	Produced by granulosa cells. Inhibits recruitment of primordial follicles and reduces sensitivity to FSH
AMHR2	Anti-müllerian hormone receptor type 2	Carries out physiological effects of AMH
GDF9	Growth/differentiation factor 9	A member of transforming growth factor-beta superfamily, which is required for folliculogenesis
BMP15	Bone morphogenic protein 15	A member of transforming growth factor-beta superfamily, which is required for folliculogenesis
SOD2	Superoxide dismutase 2	A mitochondrial enzyme, has a crucial role in protection against damage
SHBG	Sex hormone binding globulin	A protein, which is necessary for the transport of steroids and their access to target tissues
FOLR1	Folate receptor 1	Involved in the processes that facilitate the synthesis and methylation of nucleic acids and proteins
MTHFR	Methelenetetrahydrofolate reductase	
p53	Tumor protein p53	Maintains genomic stability in somatic cells
PAI = SERPINE	Plasminogen activating inhibitor	Primary inhibitor of fibrinolytic system, inactivates tPA and uPA

Table 2. Genetic variations related to ovarian stimulation. (Continued)

5. Polycystic ovary syndrome (PCOS)

5.1 Diagnosis

Polycystic ovary syndrome (PCOS) is an endocrine-metabolic disorder that occurs in women of childbearing age characterized by hyperandrogenism, oligo- or anovulation, insulin resistance and hyperinsulinemia[40]. Polycystic ovaries are also a clinical feature that can occur but according to some diagnostic criteria it is not always necessary to diagnose the disorder. It has been shown that there are women who have polycystic ovaries but have a normal ovulation. In these women the metabolic effects such as hyperandrogenism and insulin resistance are much less prominent[41].
Comorbidities associated to PCOS are hypertension, dyslipidemia, diabetes mellitus type 2, obesity, endothelial dysfunction and chronic low-grade inflammatory state, which lead to cardiovascular risk and increased mortality[42].
Symptoms caused by hyperandrogenism are hirsutism, acne, alopecia, and menstrual irregularity[40].

5.2 Pathophysiology

The cause of the disorder is still unknown but it is a combination of genetic and environmental factors.
These factors could cause anovulation, which leads to a chronic deficiency of progesterone leading to high levels of gonadotropins. These high levels of LH lead to an increased rate of ovarian androgen synthesis.
Not only a deficiency of progesterone, but also insulin resistance leads to a higher pulse frequency of LH due to hyperinsulinaemia. These high levels of insulin directly increase the level of androgens by influencing the StAR (Steroigenic acute regulatory protein) transcription and other key enzymes in the androgen pathway. These raised androgen levels inhibit the growth of a dominant follicle due to conversion of the androgens to 5-alfa-reduced androgens, which cannot be converted to estrogen and, in addition, inhibit aromatase activity.
Two other hormones play a role in the development of PCOS: estrogen and AMH. High levels of estrogen (*e.g.* due to obesity, thyroid disease, etc.) disturb the normal feedback mechanism in the hypothalamus-pituitary-ovary axis, inhibiting especially FSH production resulting in an insufficient FSH-stimulated follicular development and ovulation-inducing LH surge. The high levels of estrogens can also be caused by the high amount of androgens by binding sex hormone binding globulin (SHBG) leading to an increase of free estrogens.
So, a characteristic of PCOS is an increased LH/FSH ratio. This could explain the high number of immature follicles, with hyperplastic theca cells and a small number of granulosa cells, as FSH is not able to induce maturation of the follicles through the FSH-induced LH receptors. Furthermore, AMH, which is also elevated in women with PCOS, can also disrupt the ovarian physiology when in high concentrations by hindering primordial follicle development and sensitivity of the follicle to FSH[42,43].

5.3 Prevalence

According to the European Society for Human Reproduction and Embryology, PCOS occurs in 15-20% of the women[44]. Other sources say that it is much lower (4.6 – 8% or 5 – 13.8%) and dependent on the criteria used[45,46]. With the Rotterdam criteria, which are the most widely used, the prevalence is 2 – 3 times higher than with the other criteria[45].

Diagnostic criteria	
NIH (National Institute of Health)	To include all: 1. Hyperandrogenism and/or hyperandrogenemia 2. Oligo-ovulation 3. Exclusion of related disorders
Rotterdam	To include two: 1. Oligo-ovulation or anovulation 2. Clinical and/or biochemical signs of hyperandrogenism 3. Polycystic ovaries as having 12 or more follicles, measuring between 2 and 9 mm, and/or an ovarian volume > 10 cc
AES (Androgen Excess-PCOS Society)	To include all: 1. Hyperandrogenism: hirsutism and/or hyperandrogenemia 2. Ovarian dysfunction: oligo-ovulation 3. Exclusion of other androgen excess or related disorder

Table 3. Diagnostic criteria[42].

5.4 Anti-Müllerian hormone (AMH)

5.4.1 Physiology

Granulosa cells secrete AMH when primordial follicles are recruited for development. The highest expression of this hormone is in the pre antral and small antral follicle stage. Levels decrease when the dominant follicle is selected and in the FSH dependent stages or atretic follicles it is no longer present[47].

Studies using knockout mice without AMH revealed more details on the function of AMH. These mice recruit primordial follicles much faster until exhaustion of the pool of primary follicles. This means that AMH has an inhibitory effect on follicular recruitment[48]. This is accomplished by reducing the sensitivity of the follicles to FSH, which results in a reduction of LH receptors on the granulosa cells[47,49].

5.4.2 AMH as a marker for ovarian reserve

AMH can be used as an indicator of ovarian reserve, but there are some difficulties involved in the measurement of this hormone. First, there are different forms circulating in the body and some of these forms are biological inactive[50]. Second, the stability of AMH samples is not well known during storage[51].

Third, the sensitivity of the immunoassays due to the interference of complement C1q and C3 is variable[52]. Finally, the use of different types of immunoassays results in an absence of consensual reference values and decision thresholds between different studies[53].
Another indicator of ovarian reserve that is used more often, is the number of small antral follicles, also known as the antral follicle count (AFC). These are seen on ultrasound. But this means it is dependent on the quality of the ultrasound. Also, no international standard has been established[54].

5.4.3 AMH and PCOS

As known, the polycystic ovary syndrome can be defined by the presence of polycystic ovaries according to the Rotterdam criteria. These polycystic ovaries have at least 12 follicles from 2 to 9 mm per ovary or the ovarian volume is more than 10 ml[55]. This increase of growing follicles, although seen during every stage of follicular development, is predominant during the pre-antral and small antral follicle stages[56]. As a reflection of this high number of growing follicles, AMH serum levels are two to four times higher in women with PCOS than in healthy women. This is one of the causes why AMH is so high in women with PCOS.
But there is another reason: the levels of AMH are 75 fold in anovulatory women and 20 fold in normo-ovulatory women with PCOS than in those with normal ovaries. This would mean there is a dysregulation of the granulosa cells, which is caused by AMH itself[57]. One explanation could be the overexpression of the AMH receptor type II (AMHRII) on these granulosa cells.
There has been a positive correlation shown between high serum AMH and androgen levels. This means androgens are involved in the occurrence of high AMH levels seen in PCOS. The cause of this overproduction of androgens could be an intrinsic defect of the theca cells[58,59].
Not all studies have shown a follicular overproduction of AMH in women with PCOS. In fact, Pellatt et al. showed a reduced production of AMH in women with PCOS which could be explained by estrogen[60]. FSH directly stimulates AMH production in small antral follicles until they express aromatase. This leads to the formation of large antral follicles and the production of estrogen by the follicles. Estrogen has a negative feedback on AMH production leading to an indirect inhibitory effect of FSH on the production of AMH[61] (Fig1.).

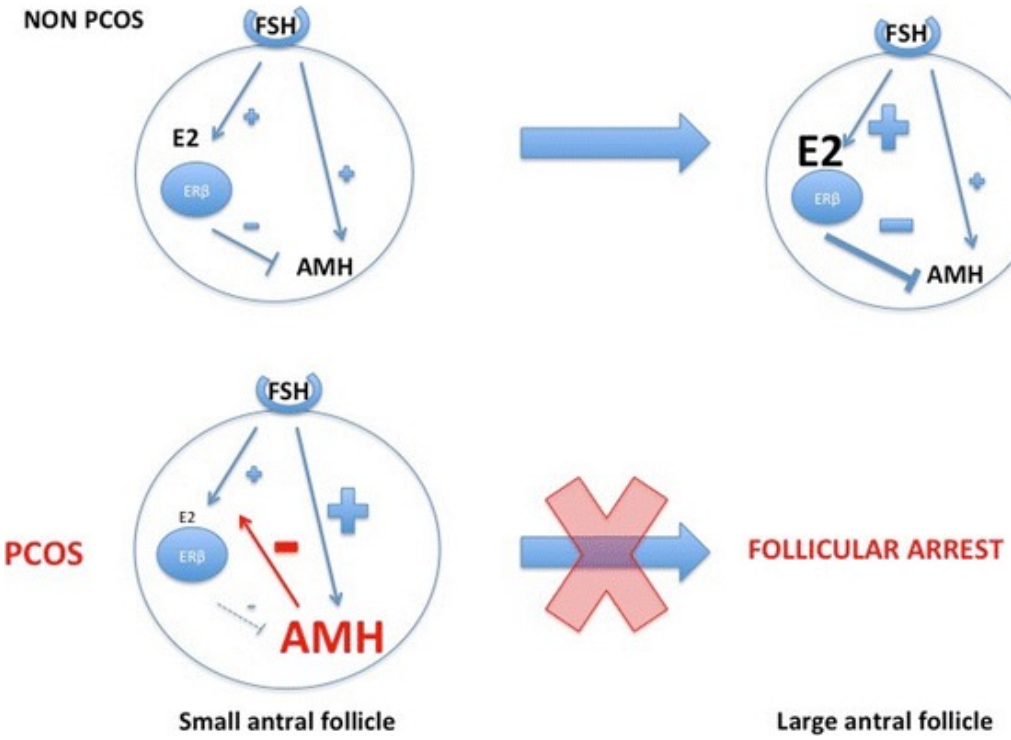

Fig 1. Adapted from Grynberg et al., 2012[62].

Another contribution of AMH to PCOS is in the fact that when there are normal levels of AMH this inhibits the FSH receptors and aromatase leading to a protective effect on the small follicles from premature aromatase expression[60]. When AMH is present in excess or when it lasts longer in large follicles, it exceeds its protective function and could lead to a defect in the selection of the dominant follicle. This phenomenon is called 'follicular arrest'.
Another factor that could contribute to follicular arrest is LH. This hormone stimulates the production of AMH in granulosa cells of women with PCOS. Also, it does not lead to the down regulation of the AMHRII receptors in anovulatory PCOS, in contrast with normo-ovulatory PCOS or normal women[63]. These two factors could contribute to anovulation and, therefore, to follicular arrest[64].

Three characteristics lie at the basis of the development of PCOS:

1. Inhibition of the terminal follicular growth, resulting in follicular arrest (no dominant follicle)[65].
2. Increased number of small growing follicles[66].
3. Follicular apoptosis defect aggravating the excess of growing follicles[67].

Serum AMH can be used in the diagnosis for PCOS, as it is theoretically a better parameter than AFC because it reflects also the number of follicles that are not seen on ultrasound[68]. However, as stated previously, there are technical difficulties that lead to an absent stratification of the results. Dumont et al. recommends a cut off at 35 pmol/L with the enzyme immunoassay AMH-EIA because it had a good specificity (97%) and a better sensitivity (92%) than AFC[54].

They suggest using serum AMH as a marker for PCO-like abnormality (PCOM and/or high AMH) as the third item of the Rotterdam classification rather than polycystic ovary morphology. Serum AMH is also correlated with the severity of PCOS symptoms and the level is higher when hyperandrogenism or oligo-anovulation is present[58,69].

5.4.4 AMH and ovarian response to controlled ovarian stimulation

Given the correlation between AMH and the number of follicles responsive to FSH, serum AMH is a good predictive marker for the ovarian response to exogenous FSH stimulation, allowing physicians to determine which women are at high risk of developing OHSS[70]. But, here too, there is a problem with the quantification and standardization of the AMH assays leading to no consensus on the threshold.
Nonetheless, Yates et al. have shown that an individualized AMH-tailored controlled hyperstimulation leads to lower fertilization failure, less occurrence of OHSS, improved embryo transfer rates and higher pregnancy and life birth rates in comparison with classic stimulating protocols[71].
Given the link between AMH and PCOS and OHSS, it is reasonable to posit that AMH genetic variation could also be involved in the genesis of PCOS or OHSS.

5.5 Genomics of PCOS

Genetic variants related to PCOS that have been described in the literature are listed in table 4.

Gene	Name	Function
AKR1C1	Aldo-keto reductase family 1, member 1	Involved in androgen biosynthesis[72]
AR	Androgen receptor	Binds androgens[40]
ADRB3	Beta-3-adrenergic receptor 3	Involved in obesity and insulin resistance[73]
CAPN10	Calpain 10	Associated with Type II Diabetes Mellitus[74]
CYP11A	Cytochrome p450, family 11, subfamily A	Involved in androgen biosynthesis. Encodes the cholesterol side chain cleavage enzyme[72]
CYP11B2	Cytochrome p450, family 11, subfamily B, protein 2	Encodes for aldosterone synthetase. Involved in the renin-angiotensin system[40]

Table 4. Genetic variants related to PCOS.

CYP17A1	Cytochrome p450, family 17, subfamily A, protein 1	Involved in androgen biosynthesis[72]
CYP19	Cytochrome P450, family 19	Encodes for aromatase, which turns androgens into estrogens[40]
CYP21	Cytochrome P450, family 21	Encodes 21-hydroxylase enzyme, which converts 17-hydroxyprogesterone into 11-deoxycortisol[40]
DENND1A	Denn/Madd domain-containing protein 1A	Member of the connecdenn family involved in the regulation of endocytosis, expressed higher in theca cells in PCOS[72]
DRD1-5	Dopamine receptor D1-D5	Binds dopamine, involved in the hypothalamic control of gonadotropin secretion[40]
FSHB	Follicle stimulating hormone, ß polypeptide	ß subunit of follicle stimulating hormone[40]
FSHR	Follicle stimulating receptor	Responsible for the ovarian maturation[75]
GNRHR	Gonadotropin-releasing hormone receptor	Binds GnRH on the pituitery[40]
GYS2	Glycogen synthetase 2	Glycogen synthesis in the liver[76]
HMGA2	High mobility group at-hook 2	Transcription factor involved in the expression of DENND1A [72,77]
HSD2B	3ß-hydroxysteroid reductase type II	Involved in androgen biosynthesis[72]
HSD3B2	3-beta-hydroxysteroid dehydrogenase 2	Catalyzes the oxidation and isomerization of steroid precursors[40,78]
IGF 1 and 2	Insulin-like growth factor	Important in the normal development of ovaries[79]
IL6	Interleukin 6	Associated with insulin sensitivity[40]
IL6R	Interleukin receptor	Involved in inflammation[40]
INS	Insulin	Involved in glucose homeostasis[80]
INSR	Insulin receptor	Regulates growth and metabolic responses to insulin[81]
IRS1 and IRS2	Insulin receptor substrate 1 and 2	Docking protein involved in binding and activating other molecules for signal transduction[82,83]

Table 4. Genetic variants related to PCOS. (Continued)

LEP	Leptin	Regulates the coordination of food intake
LEPR	Leptin receptor	Binds leptin and carries out its effect[84]
LHB	Luteinizing hormone, beta polypeptide	Responsible for LH specificity[40]
LHCGR	Luteinizing human chorionic gonadotropin receptor	Necessary for the response of the follicle on the LH surge[72]
PON1	Paraoxonase 1	An anti-oxidant high-density lipoprotein associated enzyme. Expression decreases by pro-inflammatory mediators and androgens[40]
PPARG	Peroxisome proliferator-activated receptor-gamma	Involved in insulin resistance[85]
RAB5B	Ras-associated protein	Involved in the signaling pathway of DENND1A[72]
RETN	Resistin	Involved in insulin resistance at the level of adipose tissue[86]
SF1, DAX-1 = STAR	Splicing factor 1, steroidogenic acute regulatory protein	Involved in androgen biosynthesis[72]
SHBG	Sex hormone-binding globulin	Regulates access to target tissues from estradiol and testosterone[40]
SORBS1	Sorbin and SH3-domains containing protein 1	Involved in insulin resistance[87]
SUMO1P1 (=ZNF217)	Small ubiquitin-like modifier 1	Posttranslationally modifies different cellular proteins[88]
THADA	Thyroid adenoma-associated gene	SNP's associated with type II diabetes mellitus[89]
TNF	Tumor necrosis factor-alfa	Inflammatory marker[40]
TOX3	Tox high mobility group box family member 3	Transcription factor involved in pathway of DENND1A[72]
UGT2B15	UDP-glucuronyltransferase 2B15	Involved in androgen inactivation[40]
YAP1	Yes-associated protein 1	Transcription factor involved in pathway of DENND1A[72]
ZNF217	Zinc finger protein 217	Transcription factor involved in pathway of DENND1A[72]

Table 4. Genetic variants related to PCOS. (Continued)

Aims of the study

Following from the literature study above there were two interesting items to investigate: the potential genetic predisposition to ovarian hyperstimulation syndrome and a genetic predisposition to elevated AMH levels/polycystic ovary syndrome. As said, the first is an important and severe complication in some women undergoing IVF. The second is a cause of infertility and, therefore, a reason to appeal to IVF.

Two specific aims arose:

1. Given the current understanding of OHSS, such a complication should not develop following a GnRH antagonist protocol with a GnRH agonist trigger and freeze-all approach. For this reason, it is peculiar that a few women did still develop this complication in such a situation. Hence, describing the genetic predisposition for OHSS in these rare cases of women who developed OHSS after this procedure could give more insight in the causal pathway of OHSS.

2. Because there is a link of high AMH levels with the existence of PCOS in women, it is interesting to investigate whether the genetic predisposition for PCOS starting from a population of women with high levels of AMH. This could give more insight in the pathophysiology of PCOS, the most important risk factor of OHSS.

Material and methods

Concerning the two aims, the study design is separated in two parts. The first part focuses on OHSS. The second part is focused on the high serum AMH levels and their link with PCOS.

1. Study design and subjects

1.1 Ovarian hyperstimulation syndrome

To study the genetic predisposition of OHSS, four specific subjects were examined. The exome of each subject was sequenced and then analyzed for mutations in specific genes related to ovarian stimulation, listed in table 2 and other genes where potentially relevant variations occurred. All subjects have signed a written informed consent. Approval for the study was received from the Ethics Committee of the UZ Brussel.

The four subjects are women who developed OHSS after a GnRH antagonist protocol with a GnRH agonist trigger and freeze-all approach. One of the subjects came from our Center. The other three from the Novafertil IVF Center in Konya, Turkey by asking their blood samples via the investigators after they signed a specific informed consent.

1.2 High serum AMH levels

The exome of five patients was sequenced. Afterwards, the exome was completely analyzed for mutations, first in known genes related to the polycystic ovary syndrome listed in table 4, then in all the other genes with potentially relevant variations. All subjects signed a written informed consent. The Ethics Committee of the UZ Brussel had approved this study.
The five subjects were selected on basis of their AMH levels, measured with the ROCHE kit. The women with the highest levels of AMH measured since September 2014 consenting to participate were chosen. This way we elected the most extreme cases of AMH levels, in which a genetic predisposition would be more likely.

2. Exome sequencing

Whole exome sequencing (WES) was performed at the Brussels Interuniversity Genomics High Throughput core (BRIGHT core) according to the standard procedures. First, DNA was fragmented to fragments of on average 250 bp by sonification (using the Covaris M220 device). The KAPA Hyper Prep Kit was used to create DNA libraries. Afterwards, the Roche SeqCap EZ v3.0 kit was employed to perform the target enrichment (of the exome), followed by clonal amplification on the Illumina cBot using the TruSeq PE Cluster Kit v4-cBot-HS kit. On the Illumina HiSeq 1500, paired-end sequencing was performed using the TruSeq SBS kit v4-HS (250 cycles) to obtain a 75x minimum average coverage.

3. Data analysis

Raw WES data is mapped to the human reference genome (by BWA). The mapped reads were processed using the Genome Analysis Toolkit (GATK) pipeline and Alamut Batch to annotate the detected variants. This part was performed by a bio-informatician of the BRIGHT core.

All detected variants in the exomes were analyzed. First, we looked specifically at variants in the genes listed in table 2 and table 4. These two tables arose from literature reviews regarding the genetics of each disorder[36,40,72]. Afterwards, the link of every gene with the disorder was looked up, separately.

The frequency of these variants was assessed within the 1000 Genome project to exclude frequent variants. Because OHSS following agonist triggering and a freeze-all strategy is a rare disorder, only variants in genes up to just 2% of the population were included. Next, variants were eliminated that are (most probably) not affecting the protein sequence.

Afterwards, every gene with a variant that occurred in more than 50% of the subjects was analyzed. First, previous information on the gene was looked up in the database Online Mendelian Inheritance in Man (OMIM). Next, PubMed was used to search for the relevance of these variations using MeSH terms. The following strategy was applied: 'gene', 'gene' AND "fertility", 'gene' AND "infertility", 'gene' AND "gynecology", 'gene' AND "ovulation induction", 'gene' and "ovarian hyperstimulation syndrome", 'gene' AND "polycystic ovary syndrome", 'gene' AND "endocrinology", 'gene' AND "genetics". The last step was to re-check every variant in the ClinVar database. This way, a systematic review was done of every gene and its variants present in less than 2% of the general population.

Results

The results are divided into two sections for each aim. First, the variants of the known genes to be related to OHSS and PCOS, present in less than 2% of the general population, are listed. Second, the variations of the other genes, possibly related but not yet known, are listed.
This last section is subdivided into two parts: the variants present in all subjects and the variants that might have a connection with OHSS or PCOS present in at least 50% of the subjects but not seen in all. The genes seen in all subjects are listed separately, as a common, contributing variant is searched.

1. Ovarian hyperstimulation syndrome

1.1 Selection of known genes related to ovarian stimulation

First, all the genes known to be related to OHSS were mapped and their variants were listed. There are genes of this preselection that did not show any variants and are not included. These concern specifically CYP11A1, CYP19A1, ESR1, AMHR2, GDF9, BMP15, SOD2 and FOLR1.
In table 5, the variants present in the genes related to OHSS are listed, but these variants are not part of the final selection because they are more frequent than 2% in the general population.

Gene	Codon substitution	Amino acid change	Subjects	Hetero-/homozygous
AMH	146G>T	Ser49Ile	A, B, C, D	Homozygous
	1544T>C	Val515Ala	D	Homozygous
ESR2	1421A>C	Lys474Thr	D	Heterozygous
FSHR	2039G>A	Ser680Asn	A, B, C, D	Homozygous (A and C)
	919G>A	Ala307Thr	A, B, C, D	Heterozygous (B and D)
LHR/ LHCGR	935A>G	Asn312Ser	A, B, C, D	Heterozygous (A and B) Homozygous (C and D)
	50_55dupTGCAGC	Leu17_Gln18dup	A	Heterozygous
MTHFR	665C>T	Ala222Val	A, C, D	Homozygous (A)
	1286A>C	Glu429Ala	C, D	Heterozygous (C and D)
SERPINE1	43G>A	Ala15Thr	D	Heterozygous
	49G>A	Val17Ile	D	Heterozygous
SHBG	1066G>A	Asp356Asn	A	Heterozygous
TP53	215C>G	Pro72Arg	A, C, D	Heterozygous
KDR	1416A>T	Gln472His	A	Homozygous
	889G>A	Val297Ile	A, C	Heterozygous

Table 5. Variations found in the known genes related to PCOS, filtered out because of a frequency >2%.

After filtering the genes for minor allele frequency (MAF) less then 2% in the general population, PGR and TP53 remained with one variant in subject C and D respectively. PGR facilitates the effects of progesterone like release of oocyte maturation and also implantation and maintenance of the pregnancy. But, progesterone also inhibits the FSH mediated estrogen production and increases the effect of FSH on the granulosa cells[36]. The other gene that remained was TP53. This gene encodes for protein 53, which regulates the cell cycle in somatic cells, including cells in the ovaries[37].

Gene	Codon substitution	Amino acid change	Subject	Hetero-/homozygous
PGR	97G>T	Ala33Ser	C	Heterozygous
TP53	868C>T	Arg290Cys	D	Heterozygous

Table 6. Variations in the known genes related to OHSS.

1.2 Selection of the possible related genes

1.2.1 Present in all four subjects

Table 7 lists all the genes with variants seen in all four subjects, but in less then 2% of the general population. These genes could be related to OHSS or not. The relevant ones are listed in table 14. They were selected based on a possible link with a risk factor of OHSS.

Gene	Codon substitution	Amino acid change	Subject	Hetero-/homozygous
ANKRD36	1504G>A	Gly502Ser	A, B, C, D	Heterozygous
	1517T>C	Leu506Pro	A, B, C, D	Heterozygous
	4495G>A	Val1499Met	A, B, C, D	Heterozygous
	4496T>C	Val1499Ala	A, B, C, D	Heterozygous
	4505C>A	Pro1502Gln	A, B, C, D	Heterozygous
	4522A>G	Lys1508Glu	A, B, C, D	Heterozygous
	4529C>T	Ala1510Val	A, B, C, D	Heterozygous
	4468G>A	Glu1490Lys	A, B, D	Heterozygous
	4478A>G	Tyr1493Cys	A, B, D	Heterozygous
	4479T>G	Tyr1493*	A, B, D	Heterozygous
	4481G>A	Arg1494Lys	A, B, D	Heterozygous
	1163T>G	Val388Gly	D	Heterozygous
	1672G>A	Asp558Asn	D	Heterozygous
	1677C>G	Asp559Glu	D	Heterozygous
	1681G>T	Asp561Tyr	D	Heterozygous
	1964C>G	Ser655*	D	Heterozygous
FRG1B	253A>G	Asn85Asp	A	Heterozygous
	409T>C	Cys137Arg	A	Heterozygous
	410G>A	Cys137Tyr	A, C	Heterozygous
	409T>C	Cys137Arg	C	Heterozygous
	238G>C	Ala80Pro	D	Heterozygous
	477G>C	Glu159Asp	D	Heterozygous
NBPF1	403A>G	Lys135Glu	A, B, C, D	Heterozygous
	60C>G	p.Ile20Met	A	Heterozygous
	3177G>C	p.Arg1059Ser	B	Heterozygous
	19C>T	p.Pro7Ser	B	Heterozygous

Table 7. Genetic variations found in the genes with a possible but unknown link in all four subjects.

NBPF10	295G>T	Val99Phe	A, B, C	Heterozygous
	10451G>C	Arg3484Pro	A, B	Heterozygous
	1874A>C	Asp625Ala	D	Homozygous
	3979A>T	Met1327Leu	D	Homozygous
NBPF12	124T>A	Cys42Ser	A	Heterozygous
	106A>G	Arg36Gly	B, C, D	Heterozygous
	107G>T	Arg36Ile	C	Heterozygous
	373G>C	Glu125Gln	D	Heterozygous
	411C>A	Asp137Glu	D	Heterozygous
PPIAL4G	71A>T	Gln24Leu	A, B, C, D	Heterozygous
	16A>G	Ile6Val	C, D	Heterozygous
PRSS1	652G>T	Asp218Tyr	A, B, C, D	Heterozygous
	674A>G	Lys225Arg	A, B, D	Heterozygous
	8C>T	Pro3Leu	B	Heterozygous
	40C>G	Leu14Val	B	Heterozygous
	443C>T	Ala148Val	D	Heterozygous
	637G>A	Val213Ile	D	Heterozygous
PRSS3	587G>C	Cys196Ser	A, B, C, D	Heterozygous
	496A>C	Met166Leu	A B, C	Heterozygous
	646A>C	Lys216Gln	C	Heterozygous
	418G>A	Gly140Arg	D	Heterozygous
RBMXL1	273A>T	Arg91Ser	A	Heterozygous
	127C>T	Arg43Cys	A, B, C	Heterozygous
	614T>A	Val205Asp	D	Heterozygous
	596T>C	Leu199Pro	D	Heterozygous
	586A>G	Arg196Gly	D	Heterozygous
	481T>C	Ser161Pro	D	Heterozygous
	473G>T	Gly158Val	D	Heterozygous
SLC25A5	352G>A	Ala118Thr	A, B, C, D	Heterozygous
	730C>T	Arg244Cys	B, C	Heterozygous
SLC9B1P1	79G>A	Glu27Lys	A, B, C, D	Heterozygous
TRBV7-6	310C>G	Arg104Gly	A, B, C, D	Heterozygous
	225C>A	Asp75Glu	A, B	Heterozygous
	221A>T	Gln74Leu	A, B	Heterozygous
	208T>A	Tyr70Asn	A	Heterozygous
	207T>G	Asn69Lys	A	Heterozygous
	205A>C	Asn69His	A	Heterozygous
	242A>G	Asn81Ser	D	Heterozygous
TTN	98075C>G	Thr32692Arg	A	Heterozygous
	36509A>T	Glu12170Val	A	Heterozygous
	58992T>A	Asp19664Glu	B	Heterozygous
	107576T>C	Met35859Thr	C	Heterozygous
	37330G>A	Val12444Met	D	Heterozygous

Table 7. Genetic variations found in the genes with a possible but unknown link in all four subjects. (Continued)

1.2.2 Related to ovarian stimulation according to literature

The following genes were not seen in all patients but they are selected because they have a possible link to OHSS. ANKRD30A is a distant homologue of the POTE (prostate, ovaries, testis and placenta-expressed genes) family[90]. PABPC3 is only expressed in the testis and could be related to azoospermia[91]. The FBN3 gene encodes for connective molecules but they also influence members of the TGFß family, which affect the glucose metabolism[92].

Gene	Codon substitution	Amino acid change	Subject	Hetero-/homozygous
ANKRD30A	2299G>T	Ala767Ser	A	Heterozygous
	1278T>G	Cys426Trp	C, D	Heterozygous
	1285C>G	Arg429Gly	C, D	Heterozygous
	1286G>T	Arg429Leu	C, D	Heterozygous
	1232G>T	Arg411Met	D	Heterozygous
FBN3	719T>C	Ile240Thr	A	Heterozygous
	922C>G	Leu308Val	C	Heterozygous
	4738G>A	Val1580Ile	D	Heterozygous
PABPC3	583A>G	Ile195Val	A, B, D	Heterozygous
	617G>A	Arg206His	A, B, D	Heterozygous
	619C>T	Leu207Phe	A, B, D	Heterozygous
	652T>G	Leu218Val	A, B, D	Heterozygous
	1033G>T	Glu345*	A, B, D	Heterozygous
	1093G>T	Val365Leu	A	Heterozygous
	1115A>G	Glu372Gly	A	Heterozygous
	1120C>T	Arg374Cys	A	Heterozygous
	1129T>C	Tyr377His	A	Heterozygous
	431A>G	His144Arg	D	Heterozygous
	571C>A	Pro191Thr	D	Heterozygous

Table 8. Genetic variants of unknown genes, which are relevant according to literature.

2. Extremely high serum AMH levels

2.1 Selection of genes known to be related to polycystic ovary syndrome

Table 10 lists all variants found in the known genes to be related to PCOS. However, these genes are not in the final selection because they are more frequent than 2% in the general population. The genes that remain after this filter are listed in table 8.

The genes part of the preselection in table 3 and that did not show any variants are CYP17A1, CYP19, DENND1A, DRD1, DRD2, DRD3, DRD4, DRD5, GNRHR, HMGA2, HSD3B2, IGF1, IGF2, INS, IRS2, LHB, RAB5B, SF1, STAR, SUMO1, TOX3, UGT2B15 and YAP1.

Gene	Codon substitution	Amino acid change	Subject	Homo-/heterozygous
ADRB3	190T>C	Trp64Arg	A, B	Heterozygous
AKR1C1	441A>G 783A>G	Thr147Thr Leu261Leu	A, B, C, D, E C, D, E	Homozygous Heterozygous
AR	1418_1420dupGCG	Gly473dup	A, B, D, E	Heterozygous
CAPN10	1510A>G	Thr504Ala	C, E	Heterozygous
CYP11A1				
CYP11B2	518A>G 1157T>C 1303G>A	Lys173Arg Val386Ala Gly435Ser	A, B, C, D, E B C	Heterozygous Heterozygous Heterozygous
FSHR	2039G>A 919G>A	Ser680Asn Ala307Thr	A, B, D, E A, B, D, E	Heterozygous Heterozygous
INSR	5C>G	Ala2Gly	B, C, D, E	Homozygous
IRS1	2911G>A	Gly971Arg	E	Heterozygous
LHCGR	935A>G 50_55dupTGCAGC	Asn312Ser Leu17_Gln18dup	A, C, E C, D	Homozygous Heterozygous
PON1	575A>G 163T>A	Gln192Arg Leu55Met	A, C A, B, D, E	Heterozygous Heterozygous (A and B) Homozygous (D and E)
SHBG	1066G>A	Asp356Asn	B	Heterozygous
SORBS1	182T>C 709A>G 1454A>G 524G>T	Leu61Pro Thr237Ala Tyr485Cys Gly175Val	A, B, C, D, E C, E D E	Homozygous Heterozygous Heterozygous Heterozygous
THADA	4153A>T 2095G>A 4018_4020delTCT	Thr1385Ser Val699Ile Ser1340del	A B, C, D D	Heterozygous Heterozygous (B and D) Homozygous (C) Heterozygous
ZNF217	2708G>A 2666A>G 2215G>A	Arg903Gln Asp889Gly Val739Ile	C C C	Heterozygous Heterozygous Heterozygous

Table 10. Variations found in the known genes related to PCOS, filtered out because of a frequency >2%.

FSHB encodes for the beta subunit of FSH[93]. LHCGR encodes for the receptor HCG and LH binds to and carries out their effects[94]. PPARG, SORBS1 and THADA are all genes that play a possible role in insulin resistance[85,87,89,95].

Gene	Codon substitution	Amino acid change	Subject	Hetero-/homozygous
FSHB	59G>T	Ser20Ile	Y	Heterozygous
LHCGR	854T>C	Leu285Ser	W	Heterozygous
PPARG	598G>A	Arg200Thr	X	Heterozygous
SORBS1	1225A>G	Thr409Ala	W	Heterozygous
THADA	4018_4020delTCT	Ser1304del	Y	Heterozygous

Table 11. Variations found in the known genes related to PCOS and in less than 2% of the population.

2.2 Selection of genes possibly related to PCOS

2.2.1 Present in all five subjects

All five subjects show variants seen in the genes listed in table 9. These genes are seen in less then 2% of the general population. Not all of these genes seem to have a link with PCOS. The ones with a possible link are listed in table 14. Their possible relevance is further discussed in the discussion.

Gene	Codon substitution	Amino acid change	Subject	Hetero-/homozygous
CAPN12	1237C>G	Arg413Gly	V, W, X, Y, Z	Heterozygous
	1234dupG	Ala412fs	V	Heterozygous
	1229C>G	Ala410Gly	V, Y, Z	Heterozygous
	1226C>G	Ala409Gly	V	Heterozygous
	1219T>G	Trp407Gly	V, Y, Z	Heterozygous
	1776C>G	Ile592Met	W	Heterozygous
	1773_1774delGA	Glu591fs	X	Heterozygous
IGHV7-81	149C>G	Thr50Ser	V, W, X, Y, Z	Heterozygous
	140G>C	Ser47Thr	V, W, X, Y, Z	Heterozygous
NBPF10	10451G>C	Arg3484Pro	V, W, X, Y, Z	Heterozygous
	274C>T	Leu92Phe	V	Heterozygous
	659C>T	Ser220Phe	V	Heterozygous
	775C>T	Pro259Ser	V, W	Heterozygous

Table 12. Variants found in the unknown genes present in all five subjects.

PAPBC3	583A>G	Ile195Val	V, W, X, Y, Z	Heterozygous
	619C>T	Leu207Phe	V, W, X, Y, Z	Heterozygous
	652T>G	Leu218Val	V, W, X, Y, Z	Heterozygous
	1033G>T	Glu345*	V, W, X, Y, Z	Heterozygous
	1093G>T	Val365Leu	X, Y	Heterozygous
	1115A>G	Glu372Gly	X, Y	Heterozygous
	1120C>T	Arg374Cys	X, Y	Heterozygous
	1129T>C	Tyr377His	X, Y	Heterozygous
	1141G>C	Glu381Gln	Y	Heterozygous
PPIAL4G	71A>T	Gln24Leu	V, W, X, Y, Z	Heterozygous
	16A>G	Ile6Val	W, X, Y	Heterozygous
TAS2R30	142C>G	Leu48Val	V, W, X, Y, Z	Heterozygous
	131T>C	Val44Ala	V, W, X, Y, Z	Heterozygous
	555T>A	Phe185Leu	W, X, Z	Heterozygous
	535C>G	Leu179Val	W, X, Z	Heterozygous
	530T>C	Met177Thr	W, X, Z	Heterozygous
	526A>G	Asn176Asp	W, X, Z	Heterozygous
	521A>T	His174Leu	W, X, Z	Heterozygous
	508A>C	Ser170Arg	W, X, Z	Heterozygous
	484G>A	Val162Met	Z	Heterozygous
TAS2R31	133G>C	Asp45His	V	Heterozygous
	869T>A	Phe290Tyr	W, X, Y, Z	Heterozygous

Table 12. Variants found in the unknown genes present in all five subjects. (Continued)

2.2.2 Related to ovarian stimulation according to literature

Table 13 summarizes the variants seen in ADAMTS7, which is a desintegrin and metalloproteinase with thrombospondin motifs. This gene is not seen in all five patients but is possibly involved in the physiology of ovulation[96].

Gene	Codon substitution	Amino acid change	Subject	Hetero-/homozygous
ADAMTS7	3436G>C	Glu1146Gln	V, Z	Heterozygous
	3433T>C	Ser1145Pro	V, Y, Z	Heterozygous
	653G>A	Arg218His	X	Heterozygous
	22delC	Arg8fs	X	Heterozygous
	3459G>T	Leu1153Phe	Y	Heterozygous
	3453C>A	Asn1151Lys	Y	Heterozygous
	3438G>C	Glu1146Asp	Z	Heterozygous

Table 13. Variants found in ADAMTS7.

Discussion

After selecting a very specific population and studying their genes, different, both common and rare gene variations were found, but none of them were conclusive. Neither were the variants found in the genes with a known link with the disorders.
That does not mean they could not have a possible meaning or influence on both OHSS and PCOS. Aside these common genes in each population, there were also genes where the variants, less frequent than 2%, did not occur in every subject but at least in 50% of the subjects, that could have a link with the two disorders.
Table 14 summarizes all the relevant genes seen in every subject, in at least three subjects or part of the genes known to be associated with OHSS or PCOS.

Gene	Codon substitution	Amino acid change	ExAC may 2017 (%)	Highest ExAC or GoNl (%)	Patient	Relevance
PGR	97G>T	Ala33Ser	/	/	C	Facilitates effect of progesterone[97]
TP53	868C>T	Arg290Cys	0.00	0.01 (EAS)	D	There are known variants to contribute to the number of oocytes retrieved[37]
FBN3	719T>C	Ile240Thr	0.31	0.42 (NFE)	A	Encodes for connective molecules. Link described with PCOS[98,99]
	922C>G	Leu308Val	0.80	1.24 (FIN)	C	
	4738G>A	Val1580Ile	0.58	1.40 (dutch)	D	
SLC9B1P1	79G>A	Glu27Lys	NA	NA	A, B, C, D	Expressed in testis[100]
ANKRD30A	2299G>T	Ala767Ser	0,51	1.70 (dutch)	A	Expressed in the ovaries, distant homologue of POTE family[90]
	1278T>G	Cys426Trp	0.00	0.00	C, D	
	1285C>G	Arg429Gly	0.00	0.01 (AFR)	C, D	
	1286G>T	Arg429Leu	0.02	0.20 (dutch)	C, D	
	1232G>T	Arg411Met	0.07	0.23 (AFR)	D	
PABPC3	583A>G	Ile195Val	0.08	0.36 (AFR)	A, B, D	Expressed specifically in the testis[91]
	617G>A	Arg206His	0.30	1.81 (AFR)	A, B, D	
	619C>T	Leu207Phe	0.19	0.60 (AFR)	A, B, D	
	652T>G	Leu218Val	0.06	0,14 (AFR)	A, B, D	
	1033G>T	Glu345*	0.02	0.05 (FIN)	A, B, D	
	1093G>T	Val365Leu	/	/	A	
	1115A>G	Glu372Gly	/	/	A	
	1120C>T	Arg374Cys	/	/	A	
	1129T>C	Tyr377His	/	/	A	
	431A>G	His144Arg	/	/	D	
	571C>A	Pro191Thr	0.04	0.16 (AFR)	D	

Table 14. Relevant genes after selection, with the current frequency and frequency per population group noted.

FSHB	**59G>T**	**Ser20Ile**	**0.23**	**0.35 (NFE)**	**Y**	**Possibly involved in the isolated FSH deficiency**[101]
LHCGR	854T>C	Leu285Ser	/	/	W	Facilitates effects of LH and hCG[102]
PPARG	598G>A	Arg200Thr	/	/	X	Involved in insulin resistance[85]
SORBS1	1225A>G	Thr409Ala	0.40	1.00 (dutch)	W	Involved in insulin resistance[87]
THADA	4018_4020delTCT	Ser1304del	1.28	1.67 (NFE)	Y	Possibly involved in insulin resistance[89]
PABPC3	583A>G	Ile195Val			V, W, X, Y, Z	Expressed in the testis[91]
	619C>T	Leu207Phe			V, W, X, Y, Z	
	652T>G	Leu218Val			V, W, X, Y, Z	
	1033G>T	Glu345*			V, W, X, Y, Z	
	1093G>T	Val365Leu			X, Y	
	1115A>G	Glu372Gly			X, Y	
	1120C>T	Arg374Cys			X, Y	
	1129T>C	Tyr377His			X, Y	
	1141G>C	Glu381Gln			Y	
ADAMTS7	3436G>C	Glu1146Gln	/	/	V, Z	Influence on peri-ovulatory changes in theca cells and surrounding tissue[96]
	3433T>C	Ser1145Pro	0.01	0.38 (OTH)	V, Y, Z	
	653G>A	Arg218His	0.04	0.06 (NFE)	X	
	22delC	Arg8fs	/	/	X	
	3459G>T	Leu1153Phe	/	/	Y	
	3453C>A	Asn1151Lys	/	/	Y	
	3438G>C	Glu1146Asp	/	/	Z	

Table 14. Relevant genes after selection, with the current frequency and frequency per population group noted. (Continued)

1. Remaining genes known to be associated with OHSS or PCOS

The first gene in the subjects with OHSS that showed a variant less frequent than 2% is PGR. The variant found in subject C has not been described before, so the effect is not known. This receptor is more related to pregnancy outcome than ovarian stimulation outcome. Nonetheless, there could be a link with PCOS as progesterone influences the effect of FSH on the granulosa cells and this effect is impaired in this syndrome, maybe through this receptor[97]. However, it has been suggested that PGR is related to progesterone resistance in an autosomal recessive way. Consequently, the effect of a single (heterozygous) alteration remains unsure.

Lledo et al. described a variant in the TP53 gene that could be related to a higher number of oocytes in women, although the effect of p53 in ovaries is not fully understood[37]. That variant was not seen in our subjects A, C and D but p.Arg290Cys could have a similar effect leading to a high number of growing follicles and high estrogen levels which increases the risk for the development of OHSS.

The genes with variants seen in the subjects with high AMH are FSHB, LHCGR, THADA, SORBS1 and PPARG.
The FSHB variant is heterozygously found in subject Y and was reported by the ClinVar database of uncertain significance for isolated FSH deficiency. It concerns the c.59G>T, p.Ser20Ile variant. Isolated FSH deficiency is a disorder where the FSH cannot stimulate the gonads leading to infertility, in both male and female[101]. Unfortunately, the variant is not traceable in any article so the effect and the way it is inherited remain unknown. Mutations in FSHB have been associated with hypogonadotropic hypogonadism with an autosomal recessive inheritance pattern. As it is heterozygous in our subject and the effect is of uncertain significance, not much can be concluded from this information. It is probably a recessive disorder, so homozygous presence of the variant is necessary to cause a phenotype. Although, this variant could have a partial effect on the hormone leading to a slightly different binding to the receptor, resulting in a higher or lower effect of the hormone. In this case, a lower effect would be expected as it leads to a lack of maturation of the follicles, present in PCOS. The variant is only present in one subject, which excludes a probable common causal link with PCOS.

LHCGR codes for a G protein-coupled receptor for LH and hCG in the theca cells. It is necessary for the pre-ovulatory follicle to respond to the LH surge resulting in ovulation[102]. Inactivating mutations in this gene in women are associated with higher LH levels, oligomenorrhea and enlarged ovaries[103].
The variant p.Leu285Ser of LHCGR present in subject W could have a similar effect as this subject is characterized with traits of PCOS, e.g. oligomennorhea and enlarged ovaries.

But it could also have an activating effect leading to a high production of androgens in the theca cells caused by LH, which is also a symptom of PCOS.

THADA, SORBS1 and PPARG are genes that are all related to insulin resistance, which is seen in PCOS.
THADA encodes thyroid adenoma-associated protein, which is expressed in different tissues including pancreas, adrenal cortex, adrenal medulla, testis, and thyroid. In benign thyroid adenomas, chromosomal changes of the region containing this gene have been observed[104].
The variant p.Ser1304del is present in 2% of the population. So it finds itself at the edge of the inclusion criteria. It is not certain if this variant causes insulin resistance owing to an associated hyperinsulinemia which may increase androgen production, a phenomenon that contributes to the anovulation seen in PCOS[43].
For the same reason as mentioned with THADA, SORBS1 is included: this gene and its variant could contribute to the pathogenesis of PCOS.
PPARG gene is expressed in several cells and tissues like macrophages, intestines, adipose tissue and ovaries. It influences adipocyte differentiation, insulin sensitivity, atherosclerosis and lipid metabolism[85].
p.Arg200Thr is the variant found in the PPARG gene in subject X. This variant has not been described before. This variant could increase insulin resistance, which, as mentioned above, leads to anovulation and high estrogen levels. These signs, anovulation and high estrogen levels, are both risk factors for developing OHSS. Of course, this is an effect that is remains unproven, but should be considered in future studies.

The genes described above were selected as they were described before with an association with PCOS or OHSS. All genes had only one variant in one subject, so a common, solely-responsible variant was not found. This does not mean they do not contribute to the development of high-risk conditions for ovarian stimulation, but it does imply that none of the variants can serve as a target for prevention of OHSS.

2. Relevant genes that were not yet associated with PCOS or OHSS

After filtering for frequency in the general population, all genes that occurred were researched. There are five genes that could have a relevant link with PCOS or OHSS according to literature. Out of the subjects with OHSS, FBN3, SCL9B1P1, ANKRD30A and PABPC3 were selected. Form the subjects with high serum AMH levels PABPC3 and ADAMTS7 resulted.

FBN3 is controversially associated with PCOS. A variant in allele 8 of this gene was mentioned to influence both reproductive as metabolic signs corresponding with PCOS[99]. But according to other studies, the effect of FBN3 and its variants could not be shown[105].

As there were only four variants present with a MAF less than 2% of which only one was present in the population with high AMH levels and an extreme phenotype of PCOS, the association with PCOS or OHSS remains very unclear. There is definitely no constant variant for this gene, causing a high risk to develop OHSS. The circumstances in which these variants are expressed are also inconclusive. No effect of these variants is expected.

There are two genes very interesting to mention, which are SLC9B1P1 (also known as NHEDC1) and PABPC3. Both are only expressed in testis tissue and not in other tissue, including ovaries[91,100]. Perhaps SLC9B1P1 with the p.Glu27Lys variant is only expressed after stimulation and not in normal conditions as it is seen in subject A to D.
PABPC3 is peculiar because eight variants are seen in almost every subject: A, B, D and V to Z. Subjects V to Z were not stimulated but the common trait of all subjects is the presence of a form of PCOS. Eventually, an effect of these variants is not expected, however this is uncertain for pathophysiological circumstances, which applies to all genes.
ANKRD30A is a distant homologue of the POTEfamily[90]. It has not been linked in any other way with PCOS or OHSS, but the fact this is expressed on these tissues could lead one to posit a possible influence. The gene is not expressed in ovaries or uterus but it is in breast, testis and placenta[106].

The last gene that was seen in four subjects with high AMH levels is ADAMTS7 (a desintegrin and mettaloproteinase with thrombospondin motifs, type 1, motif 7). This is a member of a family of zinc-dependent proteases. It is expressed in rheumatoid arthritic cartilage and synovium, also slightly in osteoarthritic cartilage, in smooth muscle cells in coronary arteries and carotid atherosclerotic plaques[107,108].
This gene was interesting to include in the selection because, according to Willis et al. the messenger RNA (ribonucleic acid) of ADAMTS subtypes (including ADAMTS7), in the theca cells are regulated by progesterone and prostaglandins in the peri-ovulatory period[96]. This family encodes for proteases involved in the changes of extracellular tissue[109]. It is suggested that each subtype has a time-specific role in the changes of the theca cells and surrounding tissue and that the regulation is situated downstream from the LH/FSH surge in the cells and contributes this way to ovulation.
So far, only a positive correlation of progesterone and prostaglandins and increase of mRNA of the ADAMTS family has been described[96]. A clear phenotype of these proteases in the theca cells is not yet determined.

One final gene that is important to mention is SPRY4 (Sprouty, Drosophila, Homolog of, 4), because it's variant was immediately reported by the ClinVar database. This variant, p.Lys177Arg, is only seen in Subject V, who is heterozygous for the variant. In spite of the variant being only present in one

subject, it is necessary to mention it as it is pathogenic for hypogonadotropic hypogonadism with anosmia, also known as Kallman syndrome. This disease is characterized by a congenital deficiency of the GnRH secretion, leading to failure of sexual maturation and olfactory deficiency. At the basis lies a defect of the migratory process of the neurons responsible for this secretion from the nasal placode into the forebrain. For certain variants it has been described to be a dominant disorder, e.g. the c.910G>A was heterozygous present in a female with the Kallman syndrome and 155 controls did not have this variant[110,111].
The link with PCOS and OHSS is not this clear, as these women have a state of low gonadotropins leading to low levels of progesterone and estrogen in comparison with PCOS and OHSS where the women tend to have high levels of estrogen. Both disorders are related with anovulation and infertility, so there are aspects where the two disorders overlap.

The main strength of this study is the use of whole exome sequencing. With this method, the data retrieved is massive. It is easy to search every gene known to be involved in the disorder, but every other gene can also be analyzed. This broadens the outcome of the study.
To filter the data, the 1000 genome project was used, which is a database with exomes of many different people. With this control, the very frequent variants could be filtered out. For this study, this database as a control is adequate as the search was aimed for a rare and common gene in a carefully selected population.
However, the relevance of each variant is not examined with this control. There are men, children and women included, but also different disorders, e.g. people with infertility, diabetes, etc. This means that it is not seen if a variant is seen frequently in a specific population, for example women with diabetes.
A possible solution could be to use Sanger sequencing in specific selected controls, e.g. women who did not develop OHSS after the same protocol. With this method, the genes can be controlled for other traits such as anovulation, polycystic ovaries, etc.
Another problem with this study is the fact that PCOS is a very heterogeneous disorder. To identify a common gene is difficult with only five patients. Therefore, the starting point for this part of the study was subjects with extremely high serum AMH levels. This stratifies the criteria to include the subjects. Nonetheless, their phenotype can still be different, which means the possible found gene is not conclusive as it is not clear to what part of the disorder the variant would contribute.
A larger population selected on their extremely high AMH levels could one day clarify if genes described in the literature are of application to the diagnosis of PCOS.

Conclusion

By using whole exome sequencing, it was possible to investigate many of genes. After applying a filter of MAF < 2% to narrow it down to the rarest variants, by using the 1000 genome project as a control, different genes remained. First, we looked at the genes that were already described in the literature with a link with ovarian stimulation, OHSS or PCOS.
With this method, we tried to find a common gene that could possibly contribute to at least one of the two disorders.

A common, possible causal variant was not found. But there are some genes that could have a possible link.
From the genes that were described before to be associated with OHSS or PCOS, there were some variants found but only seen in one of the subjects. Specifically, genes PGR, TP53, FSHB, LHCGR, SORBS1, THADA and PPARG.

From all the other genes with their rare variants, the most particular gene found was PABPC3. Here, different, common variants were seen in almost all subjects, three subjects with OHSS and all subjects with high AMH levels. This gene is only expressed in the testis, not in ovaries, uterus, breasts or placenta, which makes it very interesting.
This study does not make clear in which conditions this gene is expressed. But the fact that it is seen in subjects, all with a form of PCOS, some stimulated and some not, suggests a possible link with this disorder.

The other genes with a possible link are FBN3, SLC9B1P1, ADAMTS7 and ANKRD30A.

Finally, with this kind of study, a causal link could not be established. The results are a description of the genes seen in a specific selected population, with either PCOS or history of OHSS. In the future, this study can serve as a reference for other genetic studies regarding these disorders. It opens many new questions about each gene and variant which can be answered in future studies.

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