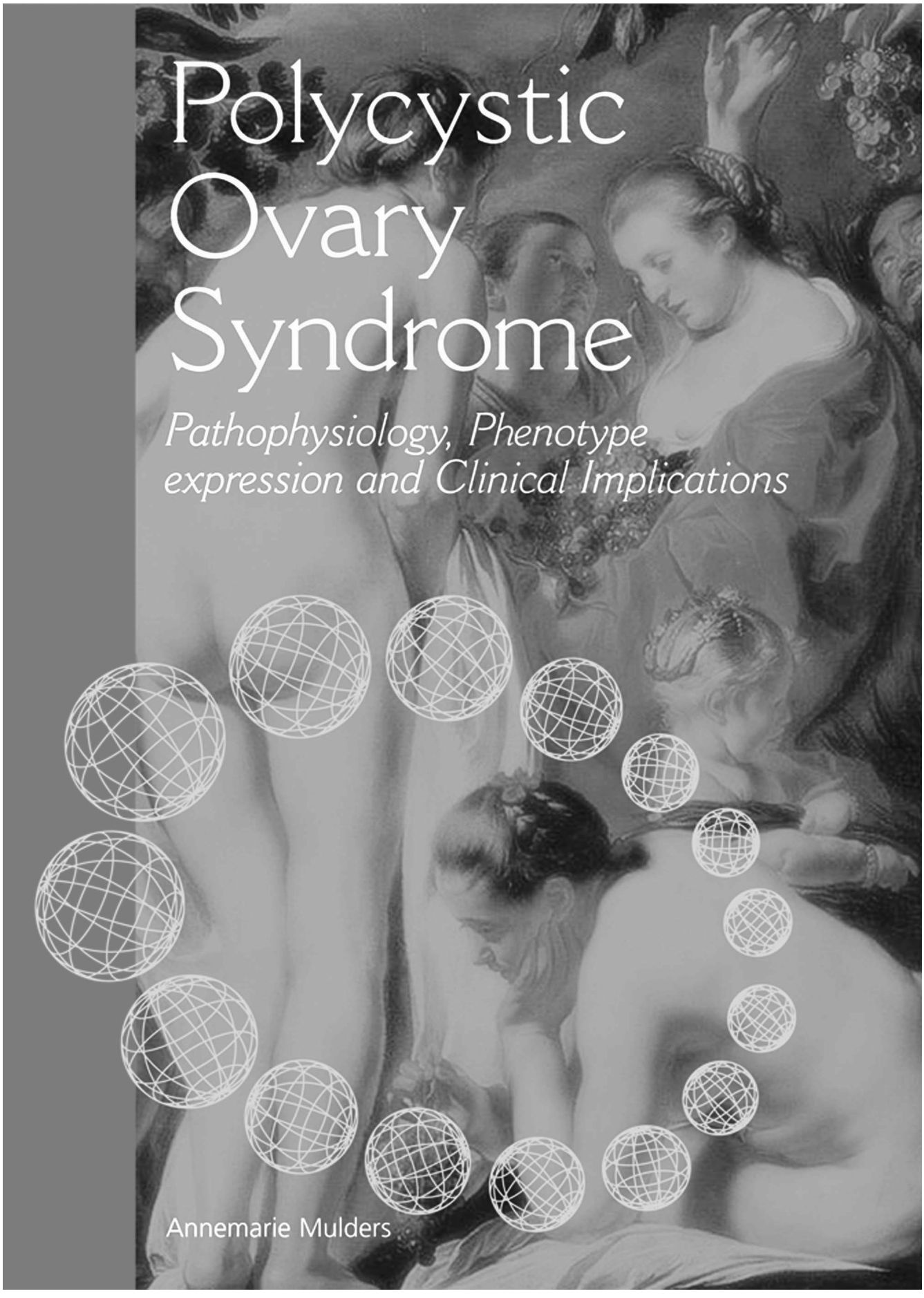
Polycystic
Ovary
Syndrome
Pathophysiology, Phenotype
expression and Clinical Implications
Annemarie Mulders

Polycystic Ovary Syndrome: Pathophysiology, Phenotype Expression and Clinical Implications
Thesis, Erasmus University, Rotterdam, The Netherlands

The work presented in this thesis was performed at the Division of Reproductive Medicine (Head: Prof. Dr. B.C.J.M. Fauser), department of Obstetrics and Gynaecology, Erasmus MC, Rotterdam, The Netherlands.

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Polycystic Ovary Syndrome: Pathophysiology, Phenotype Expression and Clinical Implications

Polycysteus ovarium syndroom: pathofysiologie, fenotype expressie en klinische implicaties

PROEFSCHRIFT

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	Prof.dr. D. Dewailly
Copromotor:	Dr. J.S.E. Laven

Arriving at one goal is the starting point to another

John Dewey (1859 - 1952)

Aan mijn ouders

Voor Guy

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List of abbreviations

AD	androstenedione
Ala	alanine
AMH	anti-Müllerian hormone
Asn	asparagine
AUC	area under the curve
BMI	body mass index (weight/heigth^2)
CI	confidence interval
CIGMA	continuous infusion of glucose with model assessment
CCF	clomiphene citrate failure
CRA	clomiphene citrate resistant anovulation
DHEA	dehydroepiandosterone
DHEAS	dehydroepiandrosterone-sulphate
E_2	17β-estradiol
ESHRE	European Society for Human Reproduction and Embryology
FAI	free androgen index (100 X T/SHBG)
FG score	Ferriman Gallwey score
FSH	follicle-stimulating hormone
FSHR	follicle-stimulating hormone receptor
GnRH	gonadotropin releasing hormone
hCG	human chorionic gonadotropin
HMG	human menopausal gonadotropin
IGF	insulin-like growth factor
IGFBP	IGF binding protein
i.m.	intramuscular
IRMA	immunoradiometric assay
i.v.	intravenous
IVF	in-vitro fertilization
LH	luteinizing hormone
MIS	Müllerian-inhibiting substance
OCP	oral contraceptive pill
OGTT	oral glucose tolerance test
OHSS	ovarian hyperstimulation syndrome
OR	odds ratio
P	progesterone
PCO	polycystic ovaries
PCOS	polycystic ovary syndrome
pFSH	purified follicle-stimulating hormone
POF	premature ovarian failure
PRL	prolactin

RIA	radioimmunoassay
rFSH	recombinant follicle-stimulating hormone
ROC curve	receiver operating characteristic curve
s.c.	subcutaneous
Ser	serine
SD	standard deviation
SHBG	sex hormone-binding globulin
SNP	single-nucleotide polymorphisms
STR	simple tandem repeats
T	testosterone
TGFß	transforming growth factor ß
Thr	threonine
TSH	thyroid-stimulating hormone
TVS	transvaginal sonography
uFSH	urinary follicle-stimulating hormone
WHO	World Health Organization
WHR	waist-to-hip ratio
WMD	weighted mean difference
17-OH-P	17-hydroxyprogesterone

Chapter I

Introduction and objectives

1.1 Introduction

1.1.1 History and classification

The association between oligoamenorrhea, obesity, bilateral polycystic ovaries and hirsutism has been reported for the first time in 1935 by Stein and Leventhal (Stein and Leventhal, 1935). A primary ovarian defect was inferred since bilateral wedge resection of the ovaries restored cycle abnormality and 2 of 7 patients conceived (Stein, 1967). The wide variability of clinical and histological findings associated with polycystic ovaries resulted in the inability to clearly identify any consistent characteristic feature of the syndrome (Goldzieher and Axelrod, 1963). The term Stein Leventhal syndrome was replaced by polycystic ovary syndrome (PCOS). Excessive androgen production has initially been attributed to abnormal adrenal function. Hyperandrogenism due to diminished granulosa cell aromatase activity (responsible for the conversion of androgens to estrogens) of the polycystic ovaries (PCO) has subsequently been demonstrated (Axelrod and Goldzieher, 1962). In 1970, Yen and co-workers (Yen *et al.*, 1970) described abnormalities in the hypothalamic-pituitary-axis evidenced by inappropriate follicle-stimulating hormone (FSH) secretion along with luteinizing hormone (LH) hypersecretion. Further insight in the abnormal physiology of this disorder occurred when hyperandrogenism was demonstrated to be LH dependent (Givens *et al.*, 1974). A subsequent milestone in 1976 was the discovery of the association of ovarian hyperandrogenism and various causes of insulin resistance (Kahn *et al.*, 1976). Thereafter, an association between polycystic ovaries, hyperandrogenism, and hyperinsulinemia was established (Burghen *et al.*, 1980). The ultrasound manifestation of polycystic ovaries was first illustrated by Swanson and co-workers who demonstrated symmetrically enlarged ovaries containing numerous tiny cysts in various diameters which may be arranged in the periphery of the ovary (Swanson *et al.*, 1981).

For clinical purposes, a simple classification for anovulatory infertility was initially proposed (Insler *et al.*, 1968), which was subsequently modified and recommended by the World Health Organization (WHO) (Rowe *et al.*, 2000) and the European Society for Human Reproduction and Embryology (The ESHRE Capri Workshop Group, 1995). According to this classification, which is based on the assessment of gonadotropin and estrogen levels in blood, patients are classified into three main categories. Patients presenting with low levels of gonadotropins and negligible endogenous estrogen activity constitute group I (WHO I), suggesting a central origin of the disease (i.e. at the hypothalamic-pituitary level) . WHO III individuals also present with low estrogen concentrations but gonadotropin levels are elevated, indicating that the primary abnormality is at the ovarian level (i.e. premature ovarian failure (POF)). The great majority of

patients (80%) suffering from chronic anovulation present with serum FSH and estradiol levels within the normal range and are classified as WHO II. Women with PCOS constitute a significant proportion of WHO II women (van Santbrink *et al.*, 1997; Laven *et al.*, 2002). Recently, consensus has been reached regarding the diagnostic criteria of PCOS. According to the revised 2003 Rotterdam criteria women may be diagnosed as PCOS when at least 2 out of the following 3 criteria are present: oligo and/or anovulation, clinical and/or biochemical signs of hyperandrogenism and polycystic appearance of ovaries (The Rotterdam ESHRE/ASRM sponsored PCOS consensus workshop group, 2004a; The Rotterdam ESHRE/ASRM sponsored PCOS consensus workshop group, 2004b).

1.1.2 Phenotypical heterogeneity

PCOS is the most common anovulatory disorder in women. Its cardinal features are hyperandrogenism and polycystic ovary morphology (Laven *et al.*, 2002). Its clinical manifestations include: oligo- or amenorrhea, hirsutism and obesity. It has been recognized that some women with the syndrome will present with polycystic ovaries without clinical evidence of androgen excess. Most cross-sectional studies may find associations between various features such as insulin resistance with hyperandrogenism (Kahn *et al.*, 1976), but a causal relationship usually remains speculative. Moreover, even within affected families individuals may present with a variety of clinical characteristics (Legro *et al.*, 1998). To date, it has become evident that genetic factors are involved in PCOS and molecular studies have identified an array of underlying abnormalities. Despite genetic predisposition, additional environmental factors will lead to differences in phenotypical presentation and infertility. In other words, PCOS clearly is a complex genetic disaese. Hence, the full clinical picture only emerges in women with a genetic predisposition exposed to eliciting supplementary influences (predominantly overweight). Because various genetic as well as environmental factors play a role, each of them with variable influence, these patients constitute a notoriously heterogeneous group.

1.1.3 Short and long-term sequelae

Correct diagnosis of PCOS and division in the subgroups may be relevant because of its possible future short term implications and increased long-term health risks. Patients with PCOS might need medical treatment for hirsutism or complaints related to obesity. However, most patients seek medical help for irregular bleeding and infertility. Infertility treatment strategies are associated with an increased risk of developing ovarian hyperstimulation syndrome (OHSS) and multiple gestations, as will be discussed later.

Additionally, there is clear evidence that PCOS is associated with an increased risk of developing type 2 diabetes (Dahlgren *et al.*, 1992; Ehrmann *et al.*,

1999; Dunaif, 1999; Wild *et al.*, 2000). Moreover, there are several indications that women with PCOS might be at an increased risk of developing cardiovascular disease as well. So far, epidemiological studies have shown no direct evidence of an increased incidence of coronary heart disease events in middle-aged women with a history of PCOS (Wild, 2002). Women with PCOS are also thought to be at an increased risk for endometrial cancer through chronic anovulation with unopposed estrogen exposure of the endometrium. Indeed, in a large epidemiological study the presence of subfertility due to hormonal disorders was associated with an increased risk of endometrial cancer (Klip *et al.*, 2000).

1.2 Phenotype and clinical picture

1.2.1 Folliculogenesis and ovarian dysfunction

In the female gonad a pool of primordial follicles will be formed during embryo development (Baker, 1963). Primordial follicles continuously leave the nongrowing pool through conversion into primary follicles. This process is referred to as initial recruitment (McGee and Hsueh, 2000). The first sign of growth is the transition of the granulosa cells, surrounding the primary oocyte, from a flat to a more cuboidal shape (Hirshfield, 1991). Follicles progress through the secondary stage and early antral stages until they reach the pool of selectable follicles. These follicles have a diameter of 2-5 mm and a clearly formed antrum. Follicles reaching the antral follicle stage inevitably will undergo atresia, unless rescued by the intercycle FSH rise present from pubertal onset, which permits further development (Fauser and van Heusden, 1997). Every month one follicle will be selected and ovulate after the midcycle LH peak, whereas the other follicles will go into atresia. This process is referred to as cyclic recruitment for which FSH is essential (McGee and Hsueh, 2000). It should be noted, however, that this classical concept has been challenged recently by showing stem cells exhibiting meiotic activity in the mouse ovary (Johnson *et al.*, 2004).

In WHO II patients, especially in those presenting with polycystic ovaries, the number of small antral follicles is generally increased due to disturbed dominant follicle selection (Fauser, 1994). Characteristically, in these patients follicle development is initiated from the primordial stage until the antral stage at which follicle development ceases and selection of a dominant follicle and subsequent ovulation is absent (Fauser, 1994). This abnormal condition may be caused by disturbed intra-ovarian regulation of FSH action (Fauser, 1994). There are several factors, produced within or outside the ovary, modifying intra-ovarian FSH activity (Fauser, 1996; Schipper *et al.*, 1997). The combined activity of these regulatory factors together determines the eventual biological response to stimulation. Hence,

the presence or absence of ovarian abnormalities in patients may influence treatment outcome after exogenously adminstered gonadotropins.

Anti-Müllerian hormone (AMH), also known as Müllerian-inhibiting substance (MIS), is a dimeric glycoprotein belonging to the transforming growth factor β (TGFβ) superfamily of growth and differentiation factors. Only recently, insight has been obtained in the potential functions of AMH in the postnatal ovary (Figure 1). AMH is exclusively produced by granulosa cells of primary, secondary and early antral follicles (Durlinger, 2000). Because of the specific expression pattern in preantral (primary and secondary) and antral follicles, it has been suggested that AMH may be involved in follicle development and/or dominant follicle selection. AMH deficient female mice are fertile and show macroscopically normal ovaries (Behringer *et al.*, 1990), but their ovaries are depleted more rapidly of primordial follicles compared to wild-type AMH mice (Durlinger *et al.*, 1999).

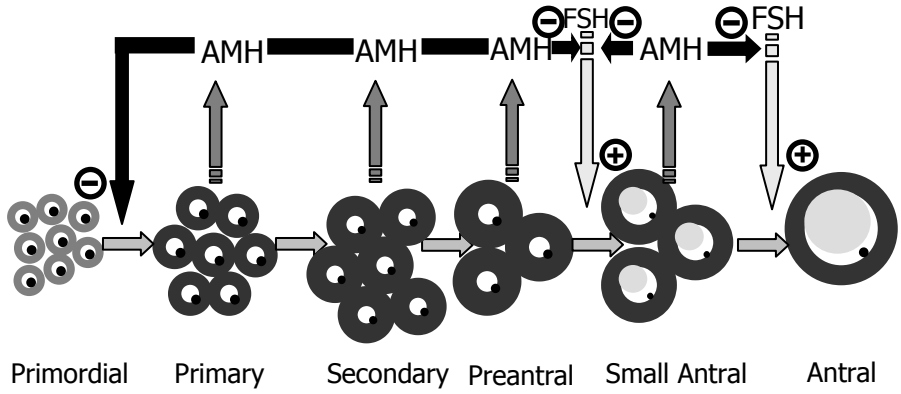

Figure 1 In this figure the dual role of AMH (i.e. the inhibitory effect on the recruitment of primordial follicles and on FSH-induced growth of preantral follicles) during growth and development of ovarian follicles is depicted.

As more preantral and antral follicles could be detected in ovaries of AMH deficient mice it is thought that the more rapid depletion is caused by increased recruitment of primordial follicles. This was confirmed by the observation that addition of AMH to cultures of neonatal mice ovaries, resulted in a 40-50% decrease in the number of growing follicles (Durlinger *et al.*, 2002a). The faster depletion of primordial follicles during early adulthood seemed to induce early cessation of ovulatory cycles in AMH-deficient females (Durlinger *et al.*, 2002b).

Besides influencing initial recruitment, AMH may affect the sensitivity of follicles for FSH, since AMH (-/-) mice of 4 months old had more preantral and antral follicles despite a lower FSH level (Durlinger *et al.*, 1999). Preantral follicle development is not dependent on FSH, but FSH facilitates this early growth

process, indicating that these follicles indeed are sensitive to FSH. From in vitro and in vivo experiments it was concluded that in the absence of AMH these follicles are more responsive to FSH (Durlinger, 2000). This phenomenon might be of importance during cyclic (FSH dependent) recruitment (Durlinger *et al.*, 2001).

Since AMH is predominantly expressed by small follicles in mice (Durlinger *et al.*, 2002b), as well as in the human (Weenen *et al.*, 2004), AMH serum concentrations may be increased in patients with polycystic ovaries. Indeed, in PCOS patients exhibiting the classical features of the syndrome, AMH levels were found to be elevated compared to normal controls (Cook *et al.*, 2002; Pigny *et al.*, 2003). Therefore, it might be hypothesized that AMH, as it reflects the total number of growing follicles, can be used as marker for the extent of disturbed folliculogenesis in WHO II anovulatory women, with or without polycystic ovaries.

1.2.2 Phenotype and clinical presentation

As mentioned previously, PCOS is the most common endocrine disorder in reproductive age women and a major cause of subfertility. Women with PCOS constitute an extremely heterogeneous patient group since various mechanisms might be responsible for the development of the same clinical picture. In order to clearly understand and elucidate the background and pathophysiology of all symptoms displayed in PCOS it might be helpful to focus on these specific characteristics and features. Based upon this approach the various genetic factors involved might be identified. And above all it might be feasible to recognize patients with favourable or unfavourable chances following infertility therapy prior to treatment. In the following section, several characteristic PCOS features used for classification will be briefly described.

It has become clear that oligomenorrheic patients constitute a different subset of WHO II patients compared to amenorrheic subjects in whom anovulation is the rule. Amenorrheic women show significantly higher androgen levels (Laven *et al.*, 2002) and more frequently present with PCO (Hull, 1987; Devoto *et al.*, 1998). Additionally, it was found that amenorrheic patients stand higher chances of remaining anovulatory upon clomiphene citrate ovulation induction treatment, suggesting that ovarian abnormalities indeed are more severe in amenorrheic patients (Imani *et al.*, 1998). Interestingly, if ovulation was achieved, amenorrheic patients had a twofold higher probability of conceiving compared with oligomenorrheic patients (Imani *et al.*, 1999). This disparity suggests that the FSH responsiveness and oocyte quality may be differentially regulated (Imani *et al.*, 1999). Moreover, amenorrheic women had minimal chances of pregnancy before. Therefore, it is less likely in these women that - once normal ovarian function is restored - other subfertility factors will interfere with pregnancy chances.

Obesity frequently coincides with PCOS and obese women classified as WHO II (including PCOS) represent a subgroup with more pronounced endocrine disturbances and likely more profound ovarian dysfunction (Laven *et al.*, 2002). Moreover, applying ovulation induction, obese PCOS women require higher doses of clomiphene citrate (Lobo *et al.*, 1982; Shoham *et al.*, 1990) or gonadotropins (McClure *et al.*, 1992; Imani *et al.*, 2002a) compared to their lean counterparts. Hence, obese WHO II anovulatory patients constitute a subgroup with a less favourable treatment outcome.

Hyperandrogenism is considered to be a key feature in PCOS (Dunaif *et al.*, 1992; The Rotterdam ESHRE/ASRM sponsored PCOS consensus workshop group, 2004a; The Rotterdam ESHRE/ASRM sponsored PCOS consensus workshop group, 2004b). Approximately 95% of hyperandrogenic women will suffer from PCOS (Barnes, 1997) and 75% of hyperandrogenic women with regular cycles will present with polycystic ovaries (Carmina and Lobo, 1999). Elevated androgens seem to be correlated with an increased risk for hirsutism, infertility and cycle disturbances (Balen *et al.*, 1995). In a recently performed analysis, hyperandrogenic WHO II subjects tend to be more obese, have longer bleeding intervals and exhibit polycystic ovaries on ultrasound. Moreover, insulin resistance is more frequently encountered in this group compared with WHO II subjects with normal androgen levels (Laven *et al.*, 2002). Additionally, hyperandrogenism constitutes a powerful predictor for the response to ovulation induction highlighting its significance for ovarian dysfunction in these women.

Severe forms of insulin resistance are associated with polycystic ovaries and hyperandrogenism (Kahn *et al.*, 1976). In addition, PCOS seems to be associated with abnormalities in insulin action (both insulin resistance as well as beta cell dysfunction (Dunaif, 1999)). These abnormalities together with obesity itself, give explanation to the considerably augmented prevalence of glucose intolerance (Dunaif, 1999). Even though hyperinsulinemic patients apparently constitute a different subset of anovulatory infertile subjects, insulin sensitivity is associated with ovarian dysfunction through hyperandrogenism (Laven *et al.*, 2002). Therefore, it is worthwhile to evaluate the role of insulin resistance as a predictor of fertility treatment outcome and as a separate pathway involved in the pathophysiology of the disease.

Upon transvaginal sonography (TVS), the ovarian volume might be increased (Pache *et al.*, 1992a), the ovaries may exhibit an increased stromal echogenicity (Dewailly, 1997), and the follicle number might be augmented in PCOS patients (Obhrai *et al.*, 1990). Recently, it was proposed to modify the ultrasound definition of PCO according to the presence of follicles measuring 2-9 mm in diameter (Jonard *et al.*, 2003). This definition should help to recognize different forms of PCOS and should improve phenotypic analysis amongst others in the frame of family studies (Jonard *et al.*, 2003). In about 63% of all WHO II

anovulatory infertile patients PCO are encountered on TVS (van Santbrink *et al.*, 1997). According to the available literature PCO are now defined by the presence of an increased number of follicles and/or an increased ovarian volume (Jonard *et al.*, 2003; Balen *et al.*, 2003). The ovarian volume seems to be the only characteristic associated with the outcome in clomiphene citrate ovulation induction (Imani *et al.*, 1998). Likewise, the identification of factors predicting ovarian reponsiveness might add to our understanding of the mechanism involved in ovarian dysfunction.

Rather than being of constant value the described parameters are varying (i.e. dynamic) characteristics for each patient. Namely, it has been proven that weight loss results in a significant improvement of menstrual abnormalities, ovulation and fertility rates (Norman *et al.*, 2002). Additionally, the oral contraceptive pill (OCP), commonly used in the treatment of menstrual disorders (Morin-Papunen *et al.*, 2003), has been shown to decrease serum androgens (Falsetti and Galbignani, 1990; Coenen *et al.*, 1996; Dahlgren *et al.*, 1998). Apart from this effect OCP increases insulin sensitivity (Skouby *et al.*, 1987; Godsland *et al.*, 1992). As a consequence, phenotype expression might change for women with PCOS dependent on OCP use. In order to identify all PCOS patients it is amongst others important to quantify the effects of OCP on phenotype expression.

1.3 Phenotype and genetics

1.3.1 Genetic disease and influence

Clinical genetic studies have been hampered by the lack of consensus regarding the definition of polycystic ovary and PCOS. The fact that the disorder is only clinically expressed in women during their reproductive years leads to further difficulties. Hence, assigning a phenotype to premenarchal girls and postmenopausal women is complicated. Moreover, the syndrome is associated with subfertility and therefore large pedigrees with multiple affected women are hard to find (Legro and Strauss, 2002). Besides the controversy about the exact nature of a possible male phenotype (Legro and Strauss, 2002), it has been suggested that premature balding is more prevalent among male relatives of women with PCOS (Franks *et al.*, 1997). Nevertheless, the genetic basis of WHO II/PCOS is demonstrated by many studies showing an increased prevalence in individuals who have a first degree relative with the disease (Ferriman and Purdie, 1979; Lunde *et al.*, 1989; Govind *et al.*, 1999). However, these studies are flawed by aberrant diagnostic criteria and inaccurate diagnostic methods. Despite all shortcomings the existing literature suggests clustering of PCOS in families with a mode of inheritance that is not inconsistent with an autosomal dominant pattern (Legro and Strauss, 2002).

Knowledge of the mode of inheritance (Mendelian or non-Mendelian) is crucial in order to determine the most powerful strategy for a study of the molecular basis of disease (Dekker and van Duijn, 2003). Mendelian disorders are caused by either a dominant or recessive mutation. In non-Mendelian or complex disorders the relationship between genotype and phenoype (the actual disease) is not straightforward, which can be attributed to the interaction of a small number of genes with each other, and with environmental factors (Dekker and van Duijn, 2003). In addition, it seems that the high prevalence of affected individuals and the wide range of related phenotypes involved in PCOS can be explained by the interaction of a small number of key genes with environmental factors (i.e. a complex genetic disorder) (Kashar-Miller and Azziz, 1999; Crosignani and Nicolosi, 2001; Franks *et al*., 2001; Legro and Strauss, 2003).

The extent to which familial clustering of PCOS is due to inheritance or shared environmental factors can be assessed by comparing the extent to which monozygotic twins are concordant for the disease with the concordance of dizygotic twins. Since monozygotic twins are genetically identical while dizygotic twins share on average half of their genes, the difference between the concordance rates signifies the contribution of genetic factors to the etiology of PCOS. In the largest available study both mono- and dizygotic twins were included. Jahanfar *et al*. (1995) found a high degree of discordance among the available twins for polycystic ovaries. However, there was considerable concordance among affected twins with respect to biochemical parameters, including fasting insulin levels and androgens. These results indicate that both genetic and environmental factors might be involved in the etiology of PCOS. Hence, PCOS indeed might have a complex pattern of inheritance.

1.3.2 Genetic approach

In recent years, remarkable progress has been made in unravelling the etiology of several genetic disorders. Progress has been most notable for monogenetic disorders, in which there is a clear-cut relation between the genetic factor and the occurrence of disease. At present, genetic research focuses on chronic disorders with a complex etiology, such as PCOS. In complex disorders the risk associated with a mutation may depend for a large part on interaction with other genetic or environmental risk factors. First, the opportunities for research in the identification of genetic risk factors involved in complex genetic disorders, such as anovulatory infertility, will be discussed.

Family studies

Traditionally, family-based study design has been the backbone of genetic research. Family studies have been of great importance to the identification of new genes. This particularly applies to monogenetic disorders, in which there is a clear-

cut relation between genetic factor and occurrence of disease. Up until now, most of the progress in genetic research has been based on research of monogenetic diseases in large pedigrees, in which the disease is transmitted from one generation to the next.

In family studies the method of linkage analysis is applied. Linkage analysis uses the principle that marker alleles are transmitted in a family together with the disease mutation. Relatives who develop a disease due to the same mutation are expected to share alleles (i.e. variants of a piece of DNA) flanking the disease mutation (Dekker and van Duijn, 2003). Linkage studies have been extremely successful in disclosing the etiology of monogenetic disorders. In complex disorders, several genetic and environmental factors may be implicated. Hence the mutation-associated risk is heavily depending on the presence of other genetic or environmental risk factors. As it is often impossible to clinically distinguish between patients who developed the disease due to a specific mutation and those who have a different etiology, recombination between the disease and marker may be falsely inferred. Therefore, the power to unravel the genetics of complex disorders has proven to be low (Lander and Schork, 1994).

An alternative approach to analysis of complex disorders is the use of affected sibling-pairs (Dekker and van Duijn, 2003). Siblings share a high proportion (50%) of their genetic material including large parts of DNA. The *à priori* probability of a patient sharing for a certain locus no alleles with a sib is 25%; one allele is 50%; two alleles are again 25%. For markers located close to the disease mutation, affected sibs are expected to share more alleles than the average of one allele. The test statistic for the analysis is based on counting alleles shared by a pair of affected siblings. Counts exceeding the expected value under the null hypothesis (one allele shared) are compatible with a disease locus nearby the marker examined. An advantage of the sib-pair design is that two siblings with the same common disease are more likely to have developed the disease due to the same mutation than two distinctly related subjects. Furthermore, siblings do share a large chromosomal region around the disease gene. In principle, the disease gene may thus be detected with a limited number of markers. However, the statistical power of sib-pair studies is limited, particularly if multiple genes are involved (Lander and Schork, 1994). Detecting significant linkage in such disorders requires several hundreds to thousands of sibling pairs. Unfortunately, regarding PCOS no investigative team has yet assembled a sufficient number of families with affected sib-pairs to perform this type of analysis.

Hence, to date, family studies have not proven enormously successful in the identification of genetic factors involved in PCOS. Consequently, the existing

literature is based exclusively on candidate gene studies (Legro and Strauss, 2002).

Population-based studies

There is increasing interest in population-based studies of individuals in an isolated population in order to overcome the aforementioned limitations of family-based research. As in family studies, the basic principle of molecular studies in isolated populations is that besides a disease gene, DNA flanking this gene, is also passed on to the next generation and is thus dispersed throughout the population. Hence, a mutation related to the disease can be ascertained in a genomic screen by identifying chromosomal regions shared by patients. This alignment of marker alleles along a chromosome is called a haplotype.

The first and most successful approach to identify new genes is genome screening, which involves a complete genome search for genes involved in a disorder. Such an approach of a genome wide screening in an isolated population does not need to make an assumption regarding the genes involved, the mode of inheritance, or disease penetrance or prevalence (Legro and Strauss, 2002). This approach starts by genotyping a set of simple tandem repeats (STR) (i.e. a clustered repeat of base pairs) or single-nucleotide polymorphisms (SNP) (i.e. a base pair substitution) polymorphic markers, of which the genomic location is known (Vaessen and van Duijn, 2001). Usually, these markers are more or less equally distributed across the genome, covering all chromosomes. These markers are not necessarily located in a gene, but often are located in non-coding areas not known to be involved in any biological process.

The rationale of genome screening in an isolated population is that marker alleles in the vicinity of a causally related mutation should be found more often in patients with a particular disorder than in controls. However, given the size of the genome of about 2.9 billion base pairs (Lander *et al.*, 2001), the probability that an allele of a random marker is found more often in patients compared to controls by chance is next to zero. Therefore, patients who inherit a disease gene from a common ancestor not only receive the disease mutations, but also adjacent markers on a chromosome. A marker located physically nearby a causal mutation should at least be present more often in cases than in unaffected relatives or unrelated controls, merely flagging the mutation.

Thus, searching the full genome is feasible in an isolated population when using the basic principle that patients who inherit a disease gene from a common ancestor not only receive the disease mutation, but also adjacent marker alleles (Figure 2). This is based on the principle of linkage-disequilibrium (Khoury *et al.*, 1993). Using this principle, disease genes may be localized by screening the genome of patients by using only a comparatively few markers.

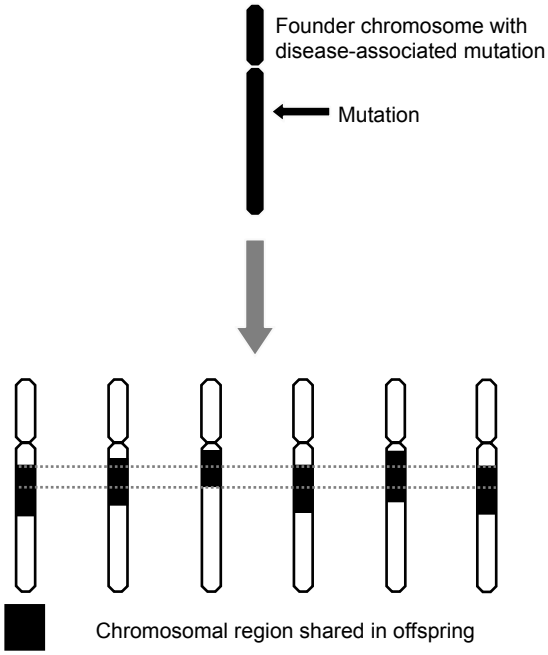

Figure 2 Allele sharing in a geneticially isolated population. Above: founder chromsome with disease-associated mutation. Below: region surrounding the disease locus, shared by patients with the same phenotype. Although they are possibly unaware of this, these affected individuals all descend from a common ancestor.

In the general population, it is not yet statistically feasible to perform this type of a genomic screen. First, there is only a small probability that any two patients from the general population with a complex disorder have inherited a common gene defect from a common ancestor. Secondly, people derived from the general population will only share a small amount of DNA. This requires marker and disease locus to be very close in order to localise the gene in a genome screen.

Successful genome screens have been conducted in genetically isolated populations (Kuokkanen et al., 1997; Leppavuori et al., 1999; Aouizerat et al., 1999; Stefansson et al., 2002). Isolated populations are typically populations that have arisen from a small number of ancestors. As a consequence, these populations show only a limited amount of genetic variation. As a result of the reduced genetic variability in genetically isolated populations, there is a higher probability that patients carry a common mutation inherited from a common ancestor (Vaessen and van Duijn, 2001). The drawback of studies in isolated populations might be the limited value when study results from genetic isolates are extrapolated to other populations. An advantage of studies in populations of more recent isolation is expected to more closely resemble that of the general population. However, it remains to be determined whether disease-related

mutations, even when detected in a recent isolate are also present in the general population (Vaessen and van Duijn, 2001). At present, a genome wide screen in Dutch PCOS patients derived from a population of recent isolation is performed (Mulders *et al.*, *In preparation*).

Still, another strategy can be followed to identify genes implicated in this complex disease (Vaessen and van Duijn, 2001). The most intuitive approach is to target candidate genes, genes that are expected to be involved in the pathophysiology of the disease (Dekker and van Duijn, 2003). A role of these genes in the pathogenesis may be suspected based on the function of the gene product, i.e. the protein (to determine whether mutations in a gene are involved, the gene can be screened for mutations or polymorphisms). These candidate gene studies test for overrepresentation of a specific allele of a candidate gene in a population of unrelated affected individuals, compared with a cohort of unrelated unaffected individuals (Legro and Strauss, 2003). Nevertheless, regarding PCOS there are some disadvantages involved in this specific approach.

A major drawback of the candidate gene approach is that à priori knowledge of the pathogenesis of the disease is required: proteins or genes involved in the disease should be known. For anovulatory infertility, there is limited knowledge of proteins involved in its etiology. Another problem in this type of study is that the candidacy of a gene for a disorder is mostly ill-defined and the number of possible candidate genes is large. As a consequence, the number of incorrect estimates of PCOS candidate genes is likely to be high until the pathophysiology is fully elucidated and genes become obvious candidates. However, wrong guesses are not necessarily without value, since the exclusion of genes does narrow down the field of possible candidates. Additionally, candidate gene studies have been widely criticized because of repeated failure to replicate results. False positive findings may to a great extent be accounted for by multiple testing. Another problem, leading to bias is the phenomenon of population admixture (Dekker and van Duijn, 2003). Whenever a distinct population comprises different subgroups with respect to genetic make-up, bias due to population admixture may occur. Bias will occur if the population consists of subpopulations, which differ in risk of the disease as well as genetic make-up. In a case control study, cases and controls may have been drawn from different subpopulations. Bias due to population stratification can occur in any population study in which cases and controls are not matched for their genealogical history. A way to minimalize the possibility of admixture is the use of genetically homogeneous populations.

The clinical, biochemical and sonographical characteristics provide a basis for investigation of the genetic origins of PCOS by indicating key candidate endocrine/metabolic pathways which are controlled by known genes. This is the

rationale for using the candidate gene approach in this disorder (Table 1). Genes involved in the pathway of androgen production and metabolism include amongst others CYP 17 encoding the major regulator 17-α-hydroxylase/17-20-lyase (Carey *et al.*, 1994; Gharani *et al.*, 1996; Witchel *et al.*, 1998), CYP 19 encoding P450 aromatase (responsible for the conversion of androgens to estrogens) (Urbanek *et al.*, 1999) and CYP11A encoding the cholesterol side-chain cleavage enzyme (Gharani *et al.*, 1997; Urbanek *et al.*, 1999; Diamanti-Kandarakis *et al.*, 2000). Also considered are CYP21 and the androgen receptor (Urbanek *et al.*, 1999; Escobar-Morreale *et al.*, 1999; Mifsud *et al.*, 2000).

TABLE 1 Studies of candidate genes in PCOS (Adapted from Legro, R.S., and Strauss, J.F. (2002) Molecular progress in infertility: polycystic ovary syndrome. *Fertil. Steril.*, **78**, 569-576).

Metabolic pathway	**Gene name**	**Comments**
Steroid hormone synthesis and action	Aromatase	No association (Urbanek *et al.,* 1999)
	17 α-Hydroxy-lyase/17,20-Lyase	Possible association (Carey *et al.,* 1994; Gharani *et al.,* 1996; Witchel *et al.,* 1998)
	Cholesterol side-chain cleavage enzyme	Weak significant association (Gharani *et al.,* 1997; Urbanek *et al.,* 1999; Diamanti-Kandarakis *et al.,* 2000)
	21-Hydroxylase	Significant association (Escobar-Morreale *et al.,* 1999)
	Androgen Receptor	Possible association (Urbanek *et al.,* 1999; Mifsud *et al.,* 2000)
Carbohydrate metabolism/fuel homeostasis	Leptin	No association (Oksanen *et al.,* 2000)
	Leptin receptor	No association (Oksanen *et al.,* 2000)
	Insulin receptor gene	Significant association for nearby genes (Urbanek et al., 1999; Tucci et al., 2001)
	Insulin gene	Possible association (Waterworth *et al.,* 1997; Urbanek *et al.,* 1999)
Gonadotropin action and regulation	LH β-subunit	No association with PCOS (Elter *et al.,* 1999)
	FSH β-subunit	No association (Conway *et al.,* 1999)
	Activin binding protein	Possible linkage (Urbanek *et al.,* 1999)

In recent years there has been a great deal of interest in the metabolic associations of PCOS as characterised by a distinct form of insulin resistance (Dunaif, 1999). In addition to mutations in the insulin gene variable number tandem repeat (Ins-

VNTR) and the insulin receptor gene, the secretory products of adipose tissue including leptin and resistin (Urbanek *et al.*, 2003) also promote insulin resistance and have been considered as candidates for PCOS genes (Moller, 2000). An additional pathway which needs to be considered in identifying potential candidate genes is the regulation and action of gonadotropins. A number of genes have been shown to be involved in follicle development (i.e. follistatin gene, LH and its receptor, FSH and its receptor). Moreover, recent observations have shown that the FSH receptor (FSHR) genotype is associated with different requirements for exogenous FSH in in-vitro fertilization (IVF) patients (Perez *et al.*, 2000). In women with normogonadotropic normo-estrogenic anovulatory infertility the response of the ovary upon exogenous FSH administration varies considerably (van Santbrink and Fauser, 1997; Coelingh Bennink *et al.*, 1998). A less sensitive FSH receptor might be responsible for the increased FSH threshold (i.e the dose at which the first follicle $\geq$ 10 mm emerges) in these women which is only surpassed during clomiphene citrate or gonadotropin induction of ovulation. Therefore, it might be hypothesized that differences in the FSHR genotype might be found in women with anovulatory infertility.

1.4 Phenotype and treatment outcome

1.4.1 Treatment modalities

Medical induction of ovulation using clomiphene citrate (CC) as first line and exogenous gonadropins as second line form the classical ovulation induction treatment algorithm in WHO II anovulatory infertility. CC is generally applied as first line treatment in these women, due to low costs and minor chances of side effects or complications. Traditionally, exogenous gonadotropins are considered second line therapy in case of failure to ovulate or conceive following CC. The final step of treatment strategy is IVF. Major complications during gonadotropin ovulation induction and IVF include OHSS (Golan *et al.*, 1989), multiple pregnancies (Schenker *et al.*, 1981), and a high rate of early pregnancy wastage (Shoham *et al.*, 1991).

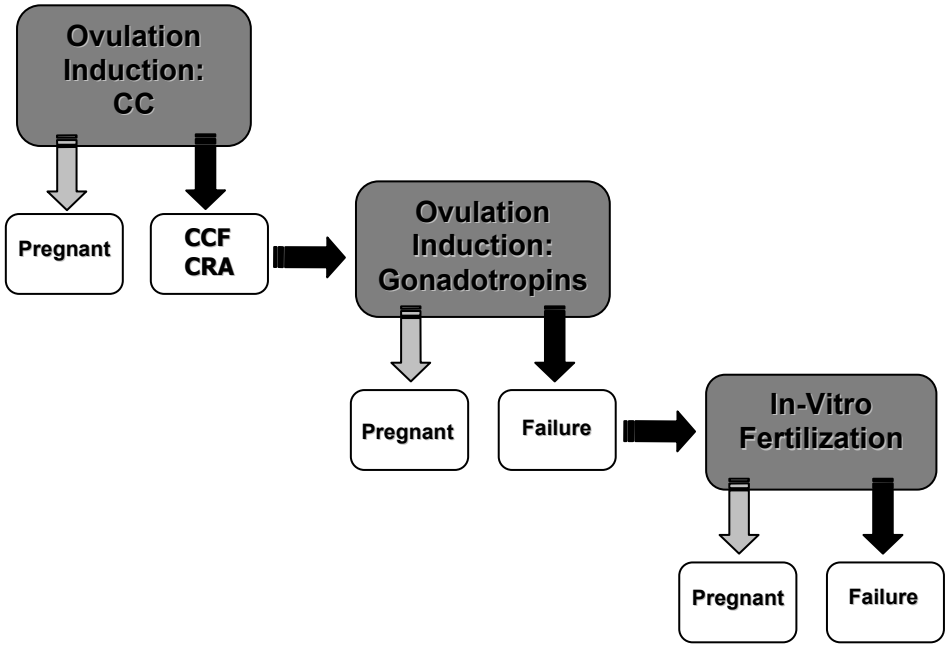

Figure 3 Schematic presentation of the WHO II anovulatory infertility treatment algorithm.

A schematic presentation of the present treatment strategy and possible treatment outcome has been depicted in Figure 3. In this section, the ovulation induction and IVF protocols used in daily clinical practice will be described in brief. Additionally, alternative treatment modalities will be mentioned.

Generally, CC treatment is initiated between day 3 and day 5 after a spontaneous or progestagen-induced withdrawal bleeding. CC interference with estrogen negative feedback may be held responsible for stimulating follicle growth due to rising serum FSH levels (Jacobson *et al.*, 1968; Kerin *et al.*, 1985). The starting dose is 50 mg/day, orally, for 5 subsequent days after initiation of menstruation. Daily doses are increased by 50 mg in the subsequent cycle to increase the endogenous FSH rise and induce follicle maturation. The highest recommended CC dose is 150 mg/day which generally is used in the subsequent cycle after a given WHO II patient persists to remain anovulatory following 100 mg of CC. If ovulation occurs, the CC dose used should remain unaltered for subsequent cycles.

Since their introduction into clinical practice in 1958, exogenous gonadotropin preparations play a central role as the second line modality for ovulation induction in WHO II patients who fail to ovulate or conceive during the previous anti-estrogen

medication (Schwartz and Jewelewicz, 1981; Lunenfeld *et al.*, 1985; Fauser and van Heusden, 1997). The aim of this treatment is to approach normal conditions as closely as possible; i.e. maturation and ovulation of a single dominant follicle and subsequent singleton pregnancy.

Treatment regimens which involve a stepwise increase in dosages of gonadotropin during follicular development ('step-up regimens') are frequently used clinically. The conventional step-up regimen employed relatively high initial doses of gonadotropins leading to high rates of multiple pregnancies (36%) and the potentially life-threatening OHSS (14%) (Dor *et al.*, 1980). The subsequently introduced 'low-dose step-up' regimen is associated with considerably lower complication rates (White *et al.*, 1996), and this regimen is now employed in most European centres.

Studies of the endocrine physiology of normal follicular development have highlighted the essential unphysiological nature of step-up regimens (van Santbrink *et al.*, 1995a). In an attempt to mimic physiology more closely in anovulatory women, a stimulation regimen has been developed which involves reducing (instead of increasing) the dose of gonadotropins administered during the period of follicular development (van Santbrink and Fauser, 1997). In a small prospective randomized trial, the low-dose step-down and the low-dose step-up regimen gave comparable clinical outcomes. However, in the step-down group, a substantially reduced stimulation period was required with a more physiological late-follicular FSH serum profile resulting in an increased number of mono-follicular stimulation cycles (van Santbrink and Fauser, 1997). On the other hand, it has been shown that monitoring of a step-down cycle may need more experience and skills compared with a low-dose step-up regimen (van Santbrink and Fauser, 2003).

In-vitro fertilization (IVF) is the final treatment for WHO II anovulatory infertility. This is the least explored area in terms of understanding ovarian response during treatment and prediction of treatment outcome (complications and live birth) partly due to lack of prospective follow-up studies (Dor *et al.*, 1992; Urman *et al.*, 1992). Previous studies comparing IVF treatment outcome in PCOS versus controls have shown that more oocytes could be retreived, but with a reduced proportion of oocytes fertilized. Despite reduced overall fertilization, IVF pregnancy rates in PCOS patients appear to be comparable to normo-ovulatory women (Dor *et al.*, 1990; Urman *et al.*, 1992; Homburg *et al.*, 1993).

At present, the most commonly used ovarian stimulation regimen in IVF for patients with anovulatory infertility includes pituitary down-regulation with a GnRH agonist in a long protocol which makes use of co-administration of high dose exogenous gonadotropins. This approach to ovarian stimulation involves a complex expensive treatment protocol and intensive monitoring, while the risk of developing complications (i.e OHSS and multiple pregnancies) remains.

Over the past 10 years, increased attention has focused towards alternative treatment options such as the insulin-sensitizing agents (Nestler *et al.*, 2002; Glueck *et al.*, 2002; Lord *et al.*, 2003), aromatase inhibitors (Mitwally and Casper, 2001) or laparoscopic surgery of the ovaries (Farquhar *et al.*, 2001). Diet and exercise may also be a different approach in restoring the ovarian abnormality and should be combined with most of the treatment strategies to increase the chance of spontaneous or stimulated ovulation and conception (Hollmann *et al.*, 1996; Clark *et al.*, 1998). Treatment with the insulin-sensitizing agents has already been shown to be successful in terms of ovulation and pregnancy rates (Lord *et al.*, 2003). For the other treatment modalities it can be concluded that although initial studies are promising, their role in every day clinical practice remains uncertain until data from prospective follow-up studies become available regarding large series of well-defined patient groups.

1.4.2 Prediction of treatment outcome

Clinical characteristics have proven to be predictive of ovulation induction (CC and gonadotropin) treatment outcome in women with WHO II anovulatory infertility (Imani *et al.*, 1998; Imani *et al.*, 1999; Imani *et al.*, 2002a; Imani *et al.*, 2002b; Eijkemans *et al.*, 2003). Since these characteristics appear to be related to ovarian dysfunction they might discriminate between patients with favourable or unfavourable chances following gonadotropin ovulation induction and IVF. Finally, the identification of factors predicting the ovarian response to fertility treatment may help us unravel the complex etiology involved in anovulatory infertility.

1.5 Study objectives

The present thesis is focussing on the heterogeneity of phenotype expression in women suffering from WHO II anovulatory infertility and PCOS.

First, we have evaluated whether AMH can be used as a marker for the extent of ovarian dysfunction in women with normogonadotropic anovulation. Furthermore, we will assess whether AMH might be helpful in elucidating the pathofyiology of disturbed follicle dynamics as found in women with PCOS.

Secondly, the clinical entity WHO II anovulation (including PCOS), characterised by its heterogeneous presentation, has an important focus in the present thesis. The use of OCP has been shown to alter the clinical, endocrine and sonographic characteristics associated with anovulatory infertility. Additionally, another objective is to study the influence of the use of OCP on PCOS phenotype expression.

Thirdly, PCOS seems to cluster in families with a complex pattern of inheritance. In the present thesis the candidate gene approach is followed to identify a possible gene involved. The candidate gene study was performed in a well-defined population of WHO II patients. We chose the gene for follicle-stimulating hormone receptor (FSHR), since the FSHR genotype is associated with different requirements for exogenous FSH.

Finally, shifting the paradigm from diagnosis to prognosis, the optimal treatment for a given WHO II patient may be defined, rendering treatment strategies more patient tailored. Ultimately, the identification of factors predicting the ovarian response to ovulation induction and IVF might add to our understanding of mechanism involved in ovarian dysfunction in these patients. Considering this approach, the prediction of outcome in gonadotropin ovulation induction strategies and IVF in WHO II patients will be amongst the scope of the present thesis.

Chapter II

New markers for ovarian dysfunction and their clinical implications

2.1 Anti-Müllerian Hormone (AMH) serum concentrations in normo-ovulatory and anovulatory women of reproductive age.

Abstract

Anti-Müllerian hormone (AMH) inhibits the recruitment of primordial follicles into the pool of growing follicles. Since serum AMH concentrations correlate with the number of antral follicles as well as age, it may be used clinically as an endocrine marker for ovarian ageing. In normogonadotropic anovulatory infertile women (World Health Organization class II) the number of early antral follicles is usually increased. To investigate whether AMH concentrations are increased, serum levels in 128 WHO II women were compared to those in 41 normo-ovulatory premenopausal women of similar age.

Serum AMH concentrations are significantly (P < 0.001) elevated in WHO II patients (median: 7.6 µg/L [range: 0.1-40.0]) compared to controls (median: 2.1 µg/L [0.1-7.4]). In 106 patients presenting with polycystic ovaries (PCO) (≥ 12 follicles/ovary measuring 2-9 mm and/or an ovarian volume > 10 mL) AMH levels are elevated (9.3 µg/L [1.8-40.0]) compared to 22 patients without PCO (6.4 µg/L [0.1-22.1]) (P < 0.0001). In WHO II patients AMH concentrations correlate with features characteristic for polycystic ovary syndrome (PCOS) such as LH concentrations (r = 0.331; P = 0.0001), testosterone levels (r = 0.477; P = 0.0001), mean ovarian volume (r = 0.421; P = 0.0001) and the number of ovarian follicles (r = 0.308; P = 0.0001). AMH levels correlated well with age in WHO II patients (r = -0.248; P = 0.002) as well as in controls (r = -0.465; P = 0.005). However, the relative decline in AMH with age is less pronounced in WHO II patients. In a subset of patients no significant correlation was found between AMH serum concentrations and the FSH response dose, the duration of stimulation and the total number of ampoules of FSH used.

In conclusion, serum AMH concentrations are elevated in WHO II women, especially in those patients exhibiting PCO. Since AMH concentrations correlated well with other clinical, endocrine and ultrasound markers associated with PCOS, AMH may be used as a marker for the extent of the disease. A less pronounced AMH decrease over time in these women may suggest retarded ovarian ageing. The latter hypothesis however, should be confirmed by longitudinal studies.

2.1.1 Introduction

The dimeric glycoprotein anti-Müllerian hormone (AMH), also referred to as Müllerian- inhibiting substance (MIS), is a member of the transforming growth factor ß (TGFß) superfamily (Cate *et al.*, 1986). During fetal sex differentiation,

AMH is produced by Sertoli cells in the male, in which it induces degeneration of the Müllerian ducts (Munsterberg and Lovell-Badge, 1991). In females, AMH is expressed only postnatally by the ovary, and until recently its function in the female reproductive tract was unknown (Hirobe *et al.*, 1994; Durlinger *et al.*, 2002b).

Female AMH null mice were reported to be fertile and produced normal-sized litters (Lyet *et al.*, 1995). However, ovaries of AMH null mice as well as female mice heterozygous for the AMH null mutation, contained less primordial follicles and more growing follicles, compared with their wild-type littermates (Durlinger *et al.*, 1999). In addition, AMH was able to inhibit the initiation of primordial follicle growth in cultured neonatal mouse ovaries (Dürlinger *et al.*, 1999) and stimulate growth of rat preantral follicles (McGee *et al.*, 2001). Hence, AMH appears to regulate early follicle development directly. Furthermore, the absence of AMH has been shown to enhance follicle-stimulating hormone (FSH) induced follicle growth in female mice (Durlinger *et al.*, 2001).

Recently, serum AMH levels have been shown to decrease over time in young normo-ovulatory women, whereas other markers associated with ovarian ageing did not change during this time interval (de Vet *et al.*, 2002). Although AMH concentrations did correlate with age and FSH, AMH serum levels were most strongly associated with the number of antral follicles. Therefore, AMH might represent a sensitive marker for ovarian ageing (Fanchin *et al.*, 2003a). Indeed, it has been shown that poor response during in-vitro fertilization (IVF), indicative of a diminished ovarian reserve (Beckers *et al.*, 2002), is associated with reduced baseline serum AMH concentrations (Seifer *et al.*, 2002; van Rooij *et al.*, 2002).

Chronic anovulation constitutes a major (20-25%) proportion of infertile couples (The ESHRE Capri Workshop Group, 1995; van Santbrink *et al.*, 1997). According to the World Health Organization (WHO), approximately 80% of patients suffering from chronic anovulation present with serum FSH levels within the normal range, along with normal endogenous estrogen activity. These women are classified as having normogonadotropic normo-estrogenic anovulatory infertility, more commonly referred to as WHO class II (Rowe *et al.*, 2000). Since etiologic factors underlying this condition may vary from one patient to another, WHO II anovulatory women including those with polycystic ovary syndrome (PCOS) constitute a notoriously heterogeneous population (Laven *et al.*, 2002). In WHO II patients the number of small antral follicles is generally increased due to disturbed dominant follicle selection (Fauser, 1994). Because AMH is predominantly expressed by small follicles in mice (Durlinger *et al.*, 2002b) as well as in the human (Fanchin *et al.*, 2003b), AMH serum concentrations may be increased in patients with polycystic ovaries (PCO). Indeed, in PCOS patients exhibiting the classical features of the syndrome, AMH levels were found to be elevated, compared with normal controls (Cook *et al.*, 2002). The current study was

designed to evaluate AMH as a clinically relevant marker for the extent of ovarian dysfunction in WHO II anovulatory women, with or without PCO.

2.1.2 Materials and methods

Subjects

The local Medical Ethics Review Committee approved this study, and informed consent was obtained from all participants. Included in the study were 128 patients attending our fertility clinic between 1994 and 1999 with; (a) infertility, (b) oligomenorrhea (interval between periods > 35 days) or amenorrhea (absence of vaginal bleeding for at least 6 months), (c) serum FSH concentrations within normal limits (1-10 IU/L) (Schipper *et al.*, 1998), (d) positive withdrawal bleeding after progestagen administration in case of amenorrhea, and (e) age between 19 and 41 years. Standardized initial screening (clinical investigation, transvaginal ultrasound (TVS), and fasting blood withdrawal) was performed on a random day between 9-11 AM, irrespective of the interval between blood sampling and the preceding bleeding, as previously described (Imani *et al.*, 1999). A subgroup of these patients was diagnosed as PCOS due to hyperandrogenemia and an increased follicle number and/or ovarian volume. Hyperandrogenemia was defined as an elevated (> 4.5) free androgen index (FAI) (testosterone (T) x 100/sex hormone-binding globulin (SHBG)). Similarly, an increased follicle number was defined as ≥ 12 follicles/ovary measuring 2-9 mm and the ovarian volume was considered to be increased above 10 mL (van Santbrink *et al.*, 1997).

For sonographic imaging we used a 6.5 MHz vaginal transducer (model EUB-415, Hitachi Medical Corp., Tokyo, Japan). The ovaries were localized and scanned as described previously (Pache *et al.*, 1991). Ovarian volume, stroma echogenicity (arbitrarily scored from 1 to 3 per ovary), and the mean follicle number were assessed as described earlier (van Santbrink *et al.*, 1997). Women exhibiting PCO had either an increased ovarian volume (> 10 mL) or an increased number of follicles (≥ 12 follicles measuring 2-9 mm in at least one ovary).

The control group consisted of 41 healthy volunteers selected by advertisement and paid for participation as previously published (Schipper *et al.*, 1998). Inclusion criteria were a regular menstrual cycle (26-30 days), 20-36 years of age, normal body mass index (BMI) (18-25 kg/m^2), and no previous use of medication or oral contraceptives during at least 3 months before the study. Transvaginal ultrasound (TVS) and blood sampling was performed during the early follicular phase (cycle day 3, 4, or 5).

Ovulation induction treatment

In a subgroup of WHO II women (those who failed to ovulate or conceive following clomiphene citrate treatment) gonadotropin treatment was commenced within 3 to 5 days after initiation of a spontaneous or progestagen-induced withdrawal bleeding. Patients received daily s.c. injections of recombinant FSH (Gonal-F®, Ares-Serono, Geneva, Switzerland). During all first cycles a low-dose step-up protocol was used with a starting dose of FSH of one ampoule (75 IU) per day. The daily dose was increased by ½ ampoule if ovarian response (at least one follicle of at least 10 mm) was absent after 14 days. Thereafter the dose was increased by ½ ampoule every 7 days if required. The FSH response dose was defined as the dose at which an ovarian response was observed (Imani *et al.*, 2002a). In case a sufficient ovarian response was observed, the dose was kept constant until administration of 5,000 IU human chorionic gonadotropin (hCG) (Profasi®, Ares-Serono, Geneva, Switzerland).

Hormone assays

Blood samples were obtained by venepuncture and processed within 2 hours after withdrawal. Serum was stored at -20°C and assayed for AMH, LH, FSH, androstenedione (AD), T, SHBG, inhibin B, and estradiol (E_2). Serum AMH levels were assessed using an ultra-sensitive immuno-enzymometric assay (Immunotech-Coulter, Marseilles, France), as described elsewhere (de Vet *et al.*, 2002). The limit of detection (defined as blank +3 SD of the blank) amounted to 0.05 µg/L. For quality control, samples of pooled serum with high and low levels of AMH were assayed in all separate assays. Intra- and interassay coefficients of variation were less then 5% and 8%, respectively.

Serum levels of LH, FSH, and SHBG were measured using luminescence based immuno assays (Immulite, Diagnostic Products Corp., Los Angeles, CA, USA) whereas serum E_2, T, and AD levels were measured using coated tube radioimmunoassays (RIAs) provided by the same supplier. Intra- and interassay coefficients of variation were less than 5% and 15% for LH, less than 3% and 8% for FSH, less than 8% and 11% for AD, less than 3% and 5% for T, less than 5% and 7% for E_2, and less than 4% and 5% for SHBG, respectively.

Dimeric inhibin B levels were assessed using an immuno-enzymometric assay obtained from Serotec (Oxford, UK), as described previously (Schipper *et al.*, 1998). The detection limit of the assay, defined as the amount of inhibin equivalent with the signal of the blank +3 SDs of this signal, was 3.4 ng/L. Intra- and interassay coefficients of variation for inhibin B were less than 9% and 15%, respectively.

Statistical analysis was performed using a commercially available software package (SPSS, SPSS Inc., Chicago, IL, USA). Data were analyzed for normal distribution. Data are presented as the mean ± SD if distributed normally or otherwise as the median and range. To determine differences between groups, Mann-Whitney or Kruskal-Wallis tests were used if data were not normally distributed. In case data were normally distributed, Student's *t* test or ANOVA was used. Correlations were expressed as Spearman's correlation coefficients. Regression statistics were applied to assess the differences in decline of parameters in time. A P level ≤ 0.05 was considered to be statistically significant.

2.1.3 Results

General clinical characteristics, endocrine data as well as ultrasound findings in controls and patients are summarized in Table 1. Briefly, patients were comparable with control subjects as far as age was concerned. Endocrine parameters in control subjects were all well within the normal range for regularly cycling women. Similarly ultrasound scans revealed normal follicle counts in both ovaries in these volunteers.

WHO II patients were either oligo- or amenorrheic, with a median cycle duration of 75 days, being significantly different (P < 0.001) from controls. The BMI was significantly different in WHO II women, compared with controls (P < 0.01). WHO II women presented with elevated LH and T and more PCO on ultrasound scanning.

AMH levels were significantly (P < 0.001) different between controls (median 2.1 μg/L; range 0.1-7.1 μg/L) and WHO II patients (median 7.6 μg/L; range 0.1-40.0 μg/L). When WHO II women were categorized into those with and without PCO (≥ 12 follicles measuring between 2 and 9 mm and/or and ovarian volume > 10 mL), AMH levels were significantly higher (9.3 μg/L; range 1.8-40.0 μg/L) in PCO patients compared with non-PCO (6.4 μg/L; range 0.1-22.1 μg/L; P < 0.001) and controls (2.1 μg/L; range 0.1-7.1 μg/L; P < 0.001) (Figure 1).

There was a significant (r = -0.465; P < 0.002) negative correlation between age and AMH levels in control subjects. Similarly, a significant negative correlation was found in WHO II patients (r = -0.248; P < 0.001). The decrease in AMH levels with increasing age was significantly (P < 0.001) different in WHO II patients, compared with controls (Figure 2).

In WHO II patients, AMH levels were significantly correlated with cycle duration (r = 0.203; P < 0.05), LH (r = 0.331; P < 0.001), T (r = 0.477; P < 0.001), AD (r = 0.321; P < 0.001), FAI (r = 0.224; P < 0.01), mean ovarian volume (r = 0.421; P < 0.001), and mean follicle number (r = 0.308; P < 0.001) but not with inhibin B levels (Figure 3).

TABLE 1 Clinical, endocrine and ultrasound parameters (median and range) in normo-ovulatory control subjects compared to normogonadotropic normo-estrogenic anovulatory infertile women (WHO II).

	Controls (n = 42)	**WHO II (n= 128)**
Clinical parameters		
Age (years)	29.9 (20.6 - 35.6)	28.9 (19.3 - 40.8)
BMI (kg/m^2)	21.5 (18.8 - 24.3)	25.9 (22.1 - 39.8)[a]
Cycle duration (days)	28 (25 - 31)	75 (35 - 183[c])[b]
Endocrine parameters		
LH (IU/L)	3.1 (1.0 - 6.7)	7.0 (1.1 - 23.5)[a]
FSH (IU/L)	6.1 (3.3 - 10.0)	5.5 (2.3 - 10.0)
E_2 (pmol/L)	153 (64 - 404)	241 (73 - 864)
Inhibin B (ng/L)	113 (12 - 213)	129 (21 - 430)
T (nmol/L)	1.5 (0.5 - 2.9)	2.5 (0.7 - 6.5)[a]
AD (nmol/L)	NA	14.1 (4.0 - 49.3)
FAI (100 X T/SHBG)	NA	5.9 (1.4 - 29.3)
Ultrasound parameters		
Mean number of follicles (both ovaries)	14 (6 - 28)	24 (2 - 45)[a]
Ovarian volume (mL)(per ovary)	NA	10.5 (2.5 - 22.9)

[a] $P < 0.01$
[b] $P < 0.001$
[c] Indicating amenorrhea.
NA = not assayed.

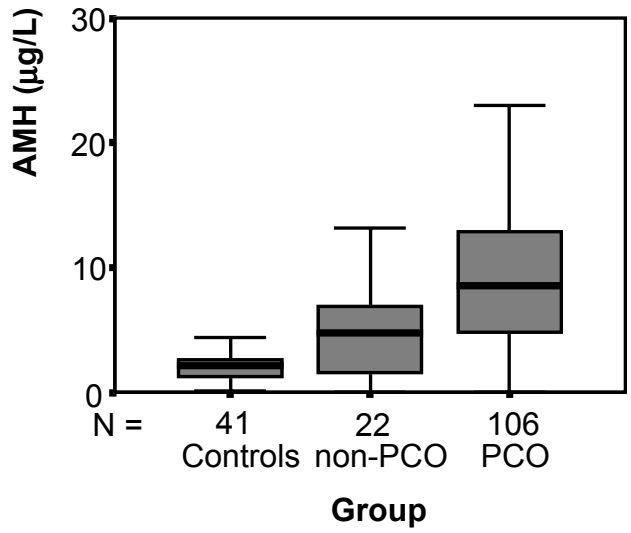

Figure 1 Box and whisker plots depicting the AMH serum levels in normogonadotropic anovulatory infertile women with polycystic ovaries (PCO) and those without (non-PCO), compared with normo-ovulatory controls. Solid lines inside boxes depict the median AMH level, whereas the upper and lower limits of the boxes and whiskers indicate 75th, 25th and 95th, and 5th percentiles.

In 79 WHO II patients who previously failed clomiphene citrate ovulation induction (75%), data with regard to ovulation induction outcome using gonadotropins were available. There was no significant correlation between AMH serum levels and the FSH response dose, being defined as the dose of exogenous FSH at which the first follicle of 10 mm or larger emerged ($r = -0.147$; $P < 0.200$; data not shown). Similarly, there was no correlation between AMH serum concentrations and the duration of stimulation (in days) or the total number of ampoules FSH used (data not shown).

2.1.4 Discussion

The present study clearly shows that AMH levels are increased in normogonadotropic anovulatory infertile patients. The subgroup of WHO II women exhibiting polycystic ovaries presents with the highest AMH serum levels. Furthermore, it seems that AMH levels correlate with the extent of ovarian dysfunction in these women, as represented by elevated LH or T levels and an increased follicle number and/or ovarian volume as established on ultrasound. Finally, it might be hypothesized that the age of menopause is delayed in these anovulatory women, which might be a direct consequence of elevated intra-ovarian AMH production due to an increased number of AMH producing units.

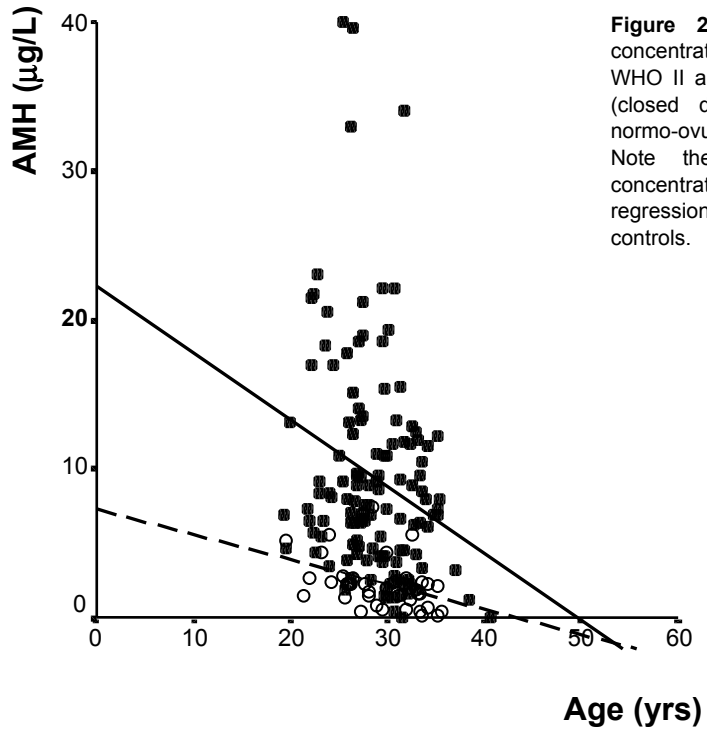

Figure 2 Individual serum AMH concentrations versus age in 128 WHO II anovulatory infertile patients (closed dots), compared with 41 normo-ovulatory controls (open dots). Note the differences (in AMH concentration and slope) between the regression lines of WHO II and controls.

On histological examination it has been shown that PCO exhibit the same number of primordial follicles, whereas the number of developing and subsequent atretic follicles was doubled, compared with normo-ovulatory controls (Hughesdon, 1982). Despite the increased number of developing follicles, inhibin B serum levels (a marker for small developing follicles) were normal in WHO II patients, suggesting an increased number of atretic follicles (Laven *et al.*, 2001a). It appears that follicle development is arrested in PCOS at the stage were dominant follicle selection occurs under normal conditions (Pache *et al.*, 1992b; Schoot *et al.*, 1993; Chandrasekher *et al.*, 1995; van Dessel *et al.*, 1999). Follicle maturation arrest during later stages of development may lead to a build up of many immature follicles, which in it self could explain increased AMH levels. Hence, it might be anticipated that the increased number of follicles, which are generally present in PCO, are the source of increased AMH production (Cook *et al.*, 2002).

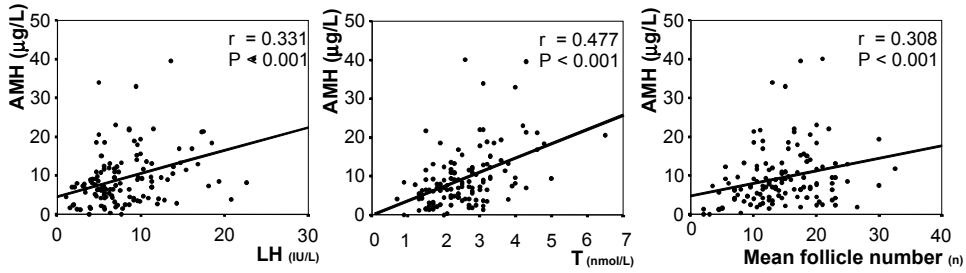

Figure 3 Scatter plot depicting the relationship between the individual AMH serum concentrations and LH, T, and the mean follicle number respectively, in WHO II anovulatory women. Correlation coefficients, Spearman ranks (r), and their respective significance levels (P) are shown in the upper right corner.

Because AMH constitutes a marker for the number of small follicles, its correlation with ovarian volume and the number of follicles present in the ovary, is not surprising. PCO as observed during ultrasound constitute a sensitive marker for the extent of ovarian dysfunction in anovulatory women as well as for ovulation induction outcome (Imani *et al.*, 2002b; Jonard *et al.*, 2003). Although AMH serum levels were the highest in anovulatory patients with prominent PCO, women without PCO also exhibited elevated levels. In the female, AMH is solely synthesized by granulosa cells of preantral and small antral follicles (Durlinger *et al.*, 2002b). Apparently, smaller follicles, which are not readily detected on ultrasound, do significantly contribute to serum AMH levels. Therefore, AMH might even constitute a more sensitive marker of ovarian dysfunction in WHO II patients than PCO on ultrasound. Indeed, AMH serum levels correlated well with other parameters indicative for the extent of ovarian dysfunction such as elevated LH and T concentrations.

Unfortunately, AMH serum concentrations were not significantly correlated with outcome parameters of ovulation induction using gonadotropins in those women who previously failed clomiphene citrate ovulation induction. Similarly, pregnancy rates and miscarriage rates were similar in patients with moderately and severely elevated AMH serum levels. Hence, elevated AMH serum levels are not associated with adverse treatment outcome indicating a limited predictive power of AMH levels in these patients. It seems therefore that the clinical relevance of AMH serum concentrations is limited in women in whom clomiphene citrate ovulation induction previously failed.

In PCOS patients, aromatase activity may be decreased, because follicles from PCO do not produce large amounts of E_2. It has been shown that follicular fluid has a potent inhibitory effect on E_2 production in PCOS (Agarwal *et al.*, 1996; Schipper *et al.*, 1997). This follicular fluid derived inhibitor decreases aromatase activity by suppressing the P450 aromatase mRNA expression in follicles of PCOS patients (Jakimiuk *et al.*, 1998). Because AMH serum concentrations do correlate with serum levels of T, AD, and SHBG and only weakly with E_2 concentrations, it might be speculated that AMH might constitute this follicular fluid derived inhibitory factor. Indeed, exogenous AMH did inhibit the biosynthesis of aromatase in cultured rat granulosa cells (di Clemente *et al.*, 1994). Moreover, in PCOS women an inverse relationship between E_2 and AMH serum levels has been previously established (Cook *et al.*, 2002).

A surprising finding constitutes the difference in relative decline in AMH serum levels with increasing age between normal controls and WHO II patients, suggesting that the latter group might reach menopause later in life. Because AMH levels correlate with the number of early antral follicles, which might in turn represent the size of the resting pool of follicles, AMH may constitute a marker for ovarian ageing (de Vet *et al.*, 2002; van Rooij *et al.*, 2002). Hence, increased intra-ovarian AMH production in PCO may slow down the process of primordial follicle recruitment and thus retard depletion of the primordial follicle pool. Although, it has been reported that cycle irregularities and hormonal profiles improve with increasing age (Dahlgren *et al.*, 1992; Elting *et al.*, 2000; Bili *et al.*, 2001) data regarding the age of menopause in these women are lacking. However, menopausal age in these women is difficult to establish because most of them will regulate their cycles up to advanced age using oral contraceptive pills. Whether AMH can be used as a reliable marker in PCOS should be further substantiated. Moreover, the challenging concept of retarded ovarian ageing in PCOS needs further confirmation by properly designed longitudinal follow-up studies.

In conclusion, serum AMH concentrations are elevated in WHO II women, which appears to be related to the increased number of small preantral and early antral follicles, especially in those patients exhibiting PCO. Because AMH concentrations correlated well with other clinical, endocrine and ultrasound parameters indicative of ovarian dysfunction in these patients, AMH may constitute a novel marker for the extent of the disease. Elevated AMH serum levels in WHO II and especially PCOS patients might indicate an increased ovarian reserve.

2.2 Changes in anti-Müllerian hormone serum concentrations over time suggest delayed ovarian ageing in normogonadotropic anovulatory infertility

Abstract

Anti-Müllerian hormone (AMH), produced by growing preantral and early antral ovarian follicles, has been shown to be a useful marker for ovarian ageing. Serum AMH concentrations are elevated during reproductive life in anovulatory women, especially in those patients exhibiting polycystic ovaries (PCO). The current study was designed to investigate whether the decrease in AMH serum concentrations over time is different comparing women with normogonadotropic anovulation (World Health Organization (WHO) group II)(including polycystic ovary syndrome (PCOS)) and normo-ovulatory controls.

AMH serum levels were assessed on 2 occasions in 98 patients suffering from WHO II anovulatory infertility as well as in 41 normo-ovulatory premenopausal women. Median time interval between both visits was 2.6 years [range: 0.3-9.0] for WHO II patients compared to 1.6 years [range: 1.0-7.3] in controls. Serum AMH concentrations were significantly ($P < 0.0001$) elevated at both occasions in WHO II patients (AMH_1: median = 7.5 µg/L [range: 0.1-35.8] and AMH_2: median = 6.7 µg/L [range: 0.0-30.6]) compared to controls (AMH_1: median = 2.1 µg/L [range: 0.1-7.4] and AMH_2: median = 1.3 µg/L [range: 0.0-5.0]). Regression analysis, corrected for age, indicated a significant relative decrease in serum AMH concentrations over time for both groups ($P < 0.001$). However, the decline in serum AMH in WHO II patients was significantly less compared to controls ($P = 0.03$).

The present longitudinal study shows that serum AMH concentrations decrease over time both in women presenting with WHO II anovulatory infertility as well as in normo-ovulatory controls. The decrease in WHO II patients is less pronounced despite distinctly elevated concentrations. This observation may suggest retarded ovarian ageing and hence a sustained reproductive life-span in these patients.

2.2.1 Introduction

The dimeric glycoprotein anti-Müllerian hormone (AMH), a member of the transforming growth factor β (TGF-β) superfamily, is produced exclusively in the gonads (Lee and Donahoe, 1993) and is involved in the regulation of growth and development (Cate *et al.*, 1986). During male fetal sexual differentiation, AMH (also known as Müllerian-inhibiting substance (MIS)) is synthesized by testicular Sertoli cells and induces degeneration of the Müllerian ducts (Jost, 1947; Josso *et al.*,

1993; Lee and Donahoe, 1993). AMH expression in the ovary starts at the end of the third trimester of pregnancy (Rajpert-De Meyts *et al.*, 1999), where it is produced in the granulosa cells of early developing follicles (Baarends *et al.*, 1995).

Ovaries of AMH knock-out mice as well as female mice heterozygous for the AMH deletion showed an accelerated exhaustion of the primordial follicle stock (Durlinger *et al.*, 1999), suggesting important roles for AMH in depletion of the primordial follicle pool. Moreover, AMH was able to inhibit the initiation of primordial follicle growth in cultured neonatal mouse ovaries (Durlinger *et al.*, 2002a), and AMH has been shown to inhibit follicle-stimulating hormone (FSH) induced follicle growth in female mice (Durlinger *et al.*, 2001). Recent data suggest that AMH expression in the human ovary is similar to that observed in mouse and rat (Weenen *et al.*, 2004), suggesting important roles for AMH in the regulation of human early follicle growth as well.

AMH serum levels decline with increasing age in normo-ovulatory women (de Vet *et al.*, 2002) and are strongly correlated with the number of antral follicles. Hence, AMH may be used as a marker for ovarian ageing (de Vet *et al.*, 2002; van Rooij *et al.*, 2002; Fanchin *et al.*, 2003a). In fact, poor response during ovarian hyperstimulation for in-vitro fertilization (IVF) (indicative of ovarian ageing (Beckers *et al.*, 2002)) has been shown to be associated with reduced early follicular phase AMH serum concentrations (van Rooij *et al.*, 2002; Seifer *et al.*, 2002; Fanchin *et al.*, 2003b).

Chronic anovulation is a common cause of infertility and it is diagnosed in around 20-25% of couples with fertility problems (The ESHRE Capri Workshop Group, 1995; Laven *et al.*, 2002). Most of these women present with irregular menstrual cycles and normal serum FSH concentrations (World Health Organization [WHO] Group II) (Rowe *et al.*, 2000). Recent data have shown that serum levels of AMH are elevated in WHO II and polycystic ovary syndrome (PCOS) patients (Cook *et al.*, 2002; Pigny *et al.*, 2003; Laven *et al.*, 2004). Moreover, it seems that AMH levels correlate well with the extent of ovarian dysfunction in anovulatory women (Laven *et al.*, 2004). Finally, the decline in AMH serum levels with increasing age in this cross-sectional data set differs when comparing anovulatory women and normo-ovulatory controls (Laven *et al.*, 2004).

Since AMH constitutes an important regulator of primordial follicle pool depletion (Durlinger *et al.*, 1999), an increased intra-ovarian AMH production may slow down the process of depletion of the primordial follicle pool. Due to retarded exhaustion of the primordial stock of follicles, the age of menopause might be delayed in these anovulatory women. The current longitudinal cohort study was designed to investigate whether the decrease in AMH serum concentrations over time is different comparing women with WHO II anovulation (including PCOS) and ovulatory controls.

2.2.2 Materials and methods

Subjects

The local Medical Ethics Review Committee approved this study and informed consent was obtained from all participants. Ninety eight patients attending our fertility clinic between 1993 and 2003 with: (a) infertility, (b) oligomenorrhea (interval between periods > 35 days) or amenorrhea (absence of vaginal bleeding for at least 6 months), (c) serum FSH concentrations within normal limits (1-10 IU/L) (van Santbrink *et al.*, 1997), and (d) between 16 and 41 years of age were included in the present study. A subgroup of these patients was diagnosed as having PCOS due to hyperandrogenism and/or polycystic ovaries (PCO) on ultrasound (The Rotterdam ESHRE/ASRM sponsored PCOS consensus workshop group, 2004b). PCO were diagnosed in the case of an increased follicle count (> 11 follicles in one or both ovaries) and/or an increased ovarian volume (> 10.0 mL) of at least one ovary (Balen *et al.*, 2003). All WHO II women participated in previously published studies (Imani *et al.*, 1998; Imani *et al.*, 1999; Mulders *et al.*, 2003a; Mulders *et al.*, 2003b).

The control group consisted of 41 normo-ovulatory women, as described before (de Vet *et al.*, 2002). All control women participated in previous studies between 1993 and 1999 (van Santbrink *et al.*, 1995a; Schipper *et al.*, 1998; Hohmann *et al.*, 2001). Inclusion criteria were regular menstrual cycle (26-30 days), 20-36 years of age, body mass index (BMI) (19-26 kg/m^2), absence of endocrine disorders or any other relevant disease, and no use of medications or oral contraceptives during the 3 months prior to the start of the study.

For the anovulatory patients, repetitive standardized screening (clinical investigation, fasting blood withdrawal and transvaginal sonography (TVS)), was performed on a random day between 9 and 11 AM, as previously described (Imani *et al.*, 1998). For each individual anovulatory patient, the length of the interval between visits is dependent on the time between each step of the treatment regimen (Imani *et al.*, 1998). For the normo-ovulatory controls, repetitive TVS and blood sampling were performed during the early follicular phase (cycle day 3, 4, or 5) (de Vet *et al.*, 2002). For each control, the interval length between visits is dependent on the time between participation in both studies (de Vet *et al.*, 2002).

Hormone assays

Blood samples were obtained by venepuncture and processed within 2 hours after withdrawal, as described previously. Serum was stored at -20°C until assayed. The hormone assays used have all been described elsewhere (Imani *et al.*, 1998; de Vet *et al.*, 2002). Serum AMH levels were measured by using an ultra-sensitive enzyme-linked immunosorbent assay (Immunotech-Coulter, Marseilles, France, as

described elsewhere (Long *et al.*, 2000)). This assay uses the same components as the normal assay, but some procedural adaptations result in increased sensitivity, making it possible to determine lower serum concentrations of AMH as they exist in women. Intra- and interassay coefficients of variation were less than 5% and 15% for LH, less than 3% and 8% for FSH, less than 8% and 11% for androstenedione (AD), less than 3% and 5% for testosterone (T), less than 5% and 7% for estradiol (E_2), less than 4% and 5% for sex hormone-binding globulin (SHBG), less than 9% and 15% for inhibin B, and less than 5% and 8% for AMH, respectively (Imani *et al.*, 1998; de Vet *et al.*, 2002).

Data analysis

Results are presented as the mean ± SD if distributed normally, or otherwise as the median and range. To assess differences between groups, Mann-Whitney or Kruskal-Wallis tests were used. Associations between different parameters were assessed by Spearman's rank correlation coefficient. To establish whether variables changed over time, the Wilcoxon matched pairs signed rank sum test was used. To determine the rate of change over time, regression analysis was used. After log-transformation, the ratio (value visit 2:visit 1) of variables was plotted against the time interval between visit 1 and 2. The relative decline per year was introduced in the present analysis since ovarian follicle depletion occurs at a constant rate of proportional decline for women under 38 years of age (Faddy *et al.*, 1992). A possible difference in rate of decline between WHO II and controls was tested for in the analysis. All regression analyses were corrected for age. Data were analysed using the commercially available software package SPSS (Chicago, IL, USA). A P value of 0.05 was chosen as the threshold level for statistical significance.

2.2.3 Results

Clinical, endocrine and ultrasound characteristics during the first assessment in the WHO II and normo-ovulatory control group are summarized in Table 1. During the second assessment, BMI ($P < 0.001$), cycle duration ($P < 0.001$), serum E_2 concentrations ($P < 0.001$) and mean number of follicles ($P < 0.001$) were significantly elevated in WHO II patients when compared with the controls. Age ($P = 0.03$) and serum FSH concentrations ($P < 0.001$) were significantly elevated during the second assessment for the normo-ovulatory controls. Serum T concentrations decreased significantly ($P < 0.001$) over time for the WHO II patients (T_1: median = 2.4 nmol/L (range: 0.3-6.8); T_2: median = 2.1 nmol/L (range: 0.5-4.6)).

Serum AMH concentrations were significantly (P < 0.001) elevated on both occasions in WHO II patients (AMH_1: median = 7.5 µg/L (range: 0.1-35.8) and AMH_2: median = 6.8 µg/L (range: 0.0-30.6)) compared with controls (AMH_1: median = 2.1 µg/L (range: 0.1-7.4) and AMH_2: median = 1.3 µg/L (range: 0.0-5.0)) (Figure 1). Levels of AMH were negatively correlated with age at visit 1 (WHO II: r = -0.30, P = 0.003; controls: r = -0.47, P = 0.002) and visit 2 (WHO II: r = -0.25, P = 0.01; controls: r = -0.57, P < 0.001) (Figure 2). Additionally, associations between AMH and mean follicle number are shown for both groups at visit 1 and visit 2 (Figure 2).

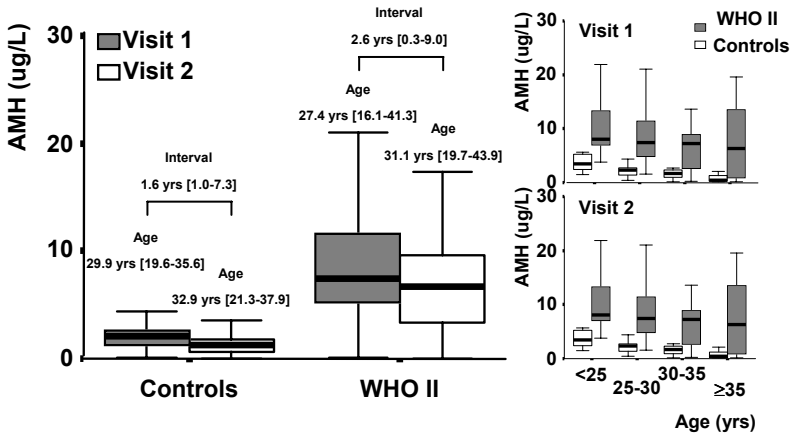

Figure 1 The left panel shows box-and whiskers-plots depicting the AMH levels in 98 women with WHO II anovulation and 41 normo-ovulatory women at two different (visit 1 and visit 2) assessments. The right panel shows box-and whiskers-plots depicting the AMH levels for both groups by age category Solid lines inside boxes depict the median AMH level, whereas the upper and lower limits of the boxes and whiskers indicate 75th, 25th, 95th, and 5th percentiles.

A statistical analysis on the serum levels of AMH from participants in whom levels at both visits were assessed within 2 years after withdrawal was performed to exclude bias due to longterm storage (Lee *et al.*, 1996). A one-sample *t*-test showed a significant decrease in AMH levels. Storage time did not significantly influence the slopes of the regression lines of follicle number compared with AMH level. The slope of the regression line for follicle number versus AMH serum levels was 0.36, 0.30 and 0.29 in groups with a storage time of 2 years, 2-3 years and > 4 years, respectively (P = 0.8).

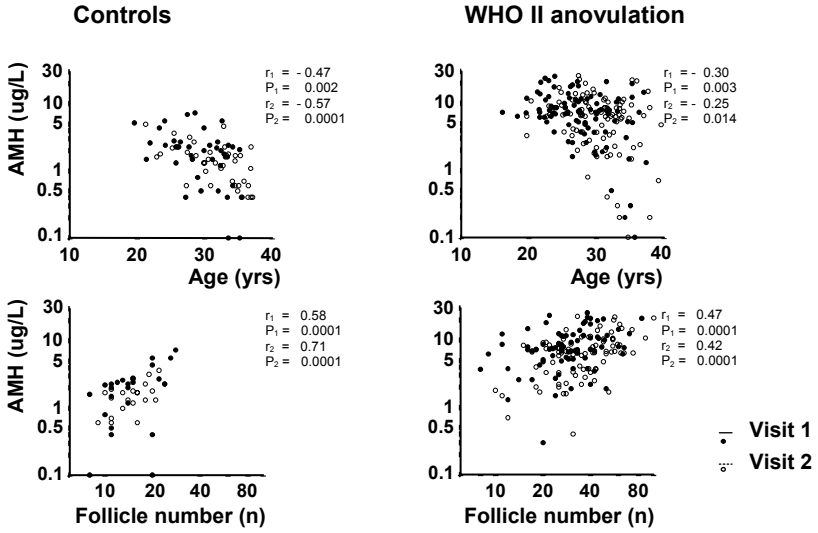

Figure 2 Scatter plots depicting the correlations between the individual AMH serum concentrations versus age and number of follicles, respectively, in 98 women with WHO II anovulation and 41 normo-ovulatory women at visit 1 (closed circles) and visit 2 (open circles). Spearman's correlation coefficients and corresponding P values are depicted.

AMH serum levels declined in both groups (Figure 3). The rate of decline, as shown by the regression lines, in WHO II patients differed significantly from controls (P = 0.03) (Figure 3). The regression coefficient (β) (representing the logarithm of 1 minus the relative decrease per year) for the normo-ovulatory controls was -0.16, compared to only -0.08 for women with WHO II anovulation. This implies a yearly decrease in AMH concentrations of 15% of the level of the year before in controls, compared to only 8% in WHO II women. Since there was a significant difference in age between the WHO II women and the controls, age was corrected for in the regression model. Correction for possible differences in age did not change either the data or the outcome. Figure 4 shows the fitted curves for the decline of AMH serum levels (mean (95% confidence interval (CI))) over time for both groups.

TABLE 1 Clinical, endocrine and ultrasound characteristics (medians with (range)) during the first assessment in 98 patients with WHO II anovulatory infertility compared with 41 normo-ovulatory controls.

	WHO II anovulation	Normo-ovulatory controls	P value
Clinical parameters			
Age (years)	27.4 (16.1 - 41.3)	29.9 (19.6 - 35.6)	0.04
BMI (kg/m^2)	27.9 (17.7 - 50.6)	22.3 (18.8 - 27.3)	< 0.001
Cycle duration (days)	93 (13 - 199[a])	28 (25 - 31)	< 0.001
Endocrine parameters			
FSH (IU/L)	4.4 (0.1 - 15.7)	6.2 (3.3 - 13.5)	< 0.001
LH (IU/L)	6.8 (0.1 - 24.4)	NA	
E_2 (pmol/L)	207 (44 - 1137)	153 (64 - 404)	< 0.001
Inhibin B (ng/L)	102 (1 - 621)	113 (12 - 213)	0.9
Testosterone (nmol/L)	2.4 (0.3 - 6.8)	NA	
FAI (100 x T/SHBG)	5.5 (0.5 - 27.8)	NA	
AMH (µg/L)	7.5 (0.1 - 35.8)	2.1 (0.1 - 7.4)	< 0.001
Ultrasound parameters			
Mean number of follicles (both ovaries)	29 (5 - 84)	15 (6 - 28)	< 0.001

[a] Indicating amenorrhea.
NA = not available.

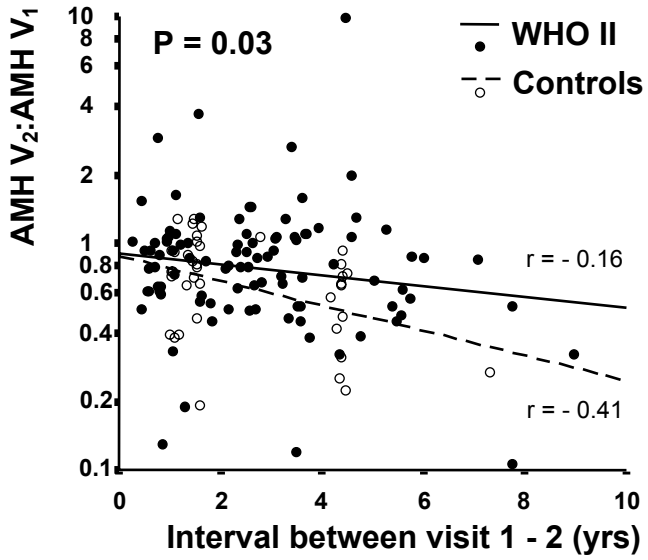

Figure 3 Scatter plot depicting the change of the ratio of AMH (value visit 2:visit 1), in relation to the time interval between visit 1 and visit 2 in 98 women with WHO II anovulation (closed circles, solid line) and 41 normo-ovulatory women (open circles, dotted line). The decline in serum AMH over time was significantly less in WHO II patients (P = 0.03).

There were no significant differences in the rate of change of AMH between the WHO II women with and without PCOS (data not shown).

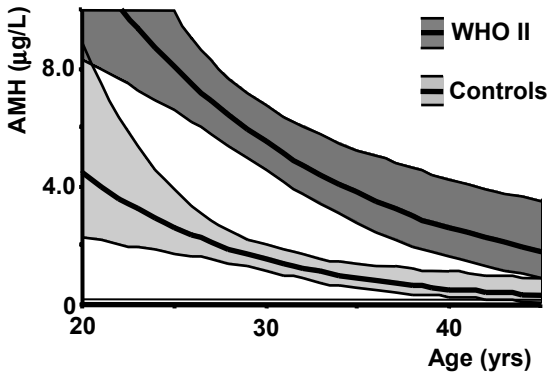

Figure 4 Serum levels of AMH in relation to age in 98 women with WHO II anovulation and 41 normo-ovulatory women. Solid lines indicate the fitted regression line. Shaded areas indicate the 95% confidence intervals around the fitted lines. Note the difference in the decline of the regression lines between WHO II women and normo-ovulatory controls. The intersection with the cut-off value of AMH (arbitrarily defined as 0.2 µg/L) of WHO II women and normo-ovulatory controls was 74 and 42 years respectively.

2.2.4 Discussion

The present data once more show that AMH serum concentrations are increased in women with normogonadotropic normo-estrogenic anovulatory infertility compared with normal controls with regular menstrual cycles. Similar results in a previous publication from our group were based on a cross-sectional set of data (Laven *et al.*, 2004). However, the current longitudinal analysis indicates for the first time that serum AMH concentrations decline less rapidly over time in women with WHO II anovulatory infertility compared with normo-ovulatory controls. This may indicate a sustained reproductive life-span in these anovulatory patients.

Polycystic ovaries (PCO) differ from normal ovaries in that follicle development is arrested at the stage where dominant follicle selection would have taken place under normal conditions (Pache *et al.*, 1992b; van Santbrink *et al.*, 1995a; Fauser and van Heusden, 1997). Upon histological examination, it has been shown that the number of developing and subsequently atretic follicles was doubled compared with normo-ovulatory controls (Hughesdon, 1982; Webber *et al.*, 2003). Moreover, the number of primordial follicles per section did not differ between women with and without PCO (Hughesdon, 1982; Webber *et al.*, 2003). However, since the total ovarian volume is increased in PCO, it might be speculated that the primordial follicle pool is enlarged in these women. Indeed, recent histological studies using more sophisticated morphometric techniques suggest that the increased density of small preantral follicles in PCO possibly could result from a higher initial population of primordial follicles (Webber *et al.*, 2003). Alternatively, the rate of follicle depletion in women with PCO may also vary. At present, evidence regarding dissimilarities involved in regulation of ovarian ageing in women with and without PCO is lacking. However, data currently available suggest that the intrinsic ovarian abnormality associated with abberant follicular dynamics in the PCO might cause a reduced rate of atresia (Webber *et al.*, 2003).

Menopause represents the clinical hallmark of follicle pool exhaustion and the definitive end of reproductive life. In addition, the commencement of menopause at an earlier age is associated with an earlier initiation of subfertility, sterility and transition to cycle irregularity, and vice versa (te Velde and Pearson, 2002). For normo-ovulatory women, it has been demonstrated that menstrual cycle irregularities associated with increasing age are dependent on the number of remaining follicles (Richardson *et al.*, 1987). The basis of ovarian ageing in women is depletion of the primordial follicle pool (Richardson *et al.*, 1987; Nikolaou and Templeton, 2003). Critical aspects involved in the process of ovarian ageing are the number of primordial follicles present in the initial stock and the factors that regulate the rate of loss of this stockpile (Wise *et al.*, 1996). It seems likely that the

ovary is the predominant pacemaker in reproductive ageing (te Velde *et al.*, 1998; te Velde and Pearson, 2002). Studies in mice suggested an important role of AMH in depletion of the primordial follicle pool (Durlinger *et al.*, 1999; Durlinger *et al.*, 2002a). AMH, produced in growing ovarian follicles, has been shown subsequently to be an excellent marker for ovarian ageing (de Vet *et al.*, 2002; Seifer *et al.*, 2002; Fanchin *et al.*, 2003a). Recently, AMH levels were found to be elevated in anovulatory and PCOS patients compared with normal controls (Cook *et al.*, 2002; Pigny *et al.*, 2003; Laven *et al.*, 2004).

In anovulatory women for whom the number of all classes of follicles including the total number of primordial follicles seems to be increased (Pache *et al.*, 1992a; Webber *et al.*, 2003), the age-related menstrual cycle irregularities (Kok *et al.*, 2003) and follicle pool exhaustion might occur later. Moreover, it may be speculated that the process of ovarian ageing is indeed delayed in women with PCO, since levels of AMH, an important inhibitor of primordial follicle pool depletion (Durlinger *et al.*, 1999), are increased. Indeed, cross-sectional data have suggested that women with PCOS may reach menopause at a later age (Dahlgren *et al.*, 1992). Furthermore, it has been reported previously that cycle irregularities improve with increasing age (Dahlgren *et al.*, 1992; Elting *et al.*, 2000; Bili *et al.*, 2001), possibly associated with a decrease in the follicle cohort size (Elting *et al.*, 2003). Although no information is available regarding the age of menopause in women with WHO II anovulation, it has been shown in a cross-sectional study that advanced age is associated with lower LH and androgen levels in this group (Bili *et al.*, 2001), as could be confirmed for the androgens in the current longitudinal study. Both oocyte quantity and quality dictate the subsequent reproductive events including decrease of fertility, increased abortion rate, the end of fertility, the beginning of cycle irregularity and, when almost no follicles are left the occurrence of menopause (te Velde *et al.*, 1998). As a consequence, it might be hypothesized that women with WHO II anovulatory infertility when compared with normo-ovulatory controls still might be able to conceive at an advanced age. However, from this point of view oocyte quality is not taken into account. Finally, the possibility that a deviant AMH synthesis or receptor is causally related to PCOS, cannot be ruled out at this stage.

In summary, the current longitudinal study confirms that AMH serum levels are elevated in anovulatory women presenting with PCO, and demonstrates for the first time that the decline in AMH with age is less pronounced compared with controls. Considering important and well documented roles of intra-ovarian AMH in the pace of follicle pool depletion and resulting female reproductive ageing, it may be proposed that the reproductive life span is extended in PCOS. Nevertheless, most women included in the present analysis have not yet reached the age of menopause. In order to substantiate further a delayed exhaustion of the primordial

stock in women with WHO II anovulation, collection of additional follow-up data is required.

Chapter III

Phenotype expression and environment

3.1 Influence of oral contraceptive pills on phenotype expression in women with polycystic ovary syndrome

Abstract

Polycystic ovary syndrome (PCOS) is characterised by a heterogeneous phenotype including chronic anovulation, hyperandrogenism and polycystic ovaries. It has been shown that the use of oral contraceptive pills (OCP) alters clinical, endocrine and sonographic features characteristic for the syndrome. The genetic and environmental homogeneity of women descendant from a founder population provides the opportunity to assess the sole effect of OCP on phenotype expression of PCOS.

All patients with normogonadotrophic anovulatory infertility (World Health Organization, group II), descendant from a restricted area as identified by ZIP codes, underwent a standardised clinical, sonographic and endocrine screening. The previous diagnosis WHO II anovulation could be reconfirmed for a total number of 101 women. At present, a total of 81 (80%) women could be diagnosed as having PCOS (according to the revised 2003 criteria). From these women a total of 54 (67%) did not use OCP, whereas 27 (33%) did. Corrected for age, the latter group presented with significantly decreased serum concentrations of estradiol (E_2) ($P < 0.001$), progesterone (P) ($P = 0.02$), 17-hydroxyprogesterone (17-OH-P) ($P < 0.001$), testosterone (T) ($P = 0.04$) and androstenedione (AD) ($P = 0.01$). Serum concentrations of cortisol ($P < 0.001$) and sex hormone-binding globulin (SHBG) ($P < 0.001$) were significantly decreased in these women. These differences resulted in a significantly decreased free androgen index (FAI) (100 x T/ SHBG) for women presently on OCP ($P < 0.001$).

Use of OCP influences phenotype expression (the observable trait) of individual women known to suffer from PCOS by reducing hyperandrogenism. Despite taking OCP, women diagnosed in the past as PCOS, still fulfilled the revised 2003 criteria for the syndrome, as PCO morphology was still present. Hence, it may be suggested that PCOS women presenting with PCO may suffer from a more severe form of the syndrome. Moreover, the present study suggests that it is permitted to rely on historical data regarding diagnosis of PCOS for women presently on the OCP since its cardinal features appear to be relatively constant. Hence, OCP use does not appreciably affect the PCOS phenotype.

3.1 Introduction

Polycystic ovary syndrome (PCOS) is the most common anovulatory disorder in women (Rowe *et al.*, 2000). Besides oligo- or amenorrhea its cardinal features are hyperandrogenism and polycystic ovary morphology (Laven *et al.*, 2002; The Rotterdam ESHRE/ASRM sponsored PCOS consensus workshop group, 2004). Since many underlying causes might lead to a similar clinical phenotype, this condition constitutes a notoriously heterogeneous patient group. A clear familial clustering can be observed in PCOS, suggesting that genetic factors are involved in its etiology. Although previous studies suggested an autosomal dominant pattern of inheritance (Lunde *et al.*, 1989; Carey *et al.*, 1993; Legro *et al.*, 1998; Govind *et al.*, 1999) more recent research indicates that a complex mode of inheritance is more likely (Hague *et al.*, 1988; Legro and Strauss, 2003). The high prevalence of affected individuals and the wide range of related phenotypes involved in PCOS might be explained by the interaction of a small number of key genes with environmental factors (Franks *et al.*, 2001).

Some genes for complex conditions, such as PCOS, are expected to be more easily identified in isolated populations compared to a general outbred population (Wright *et al.*, 1999). As a consequence of drift and founder effects, a large number of patients in isolated populations have likely inherited the disease susceptibility from a common genetic ancestor (Te Meerman *et al.*, 1995). Hence, the number of genes possibly involved in PCOS might be reduced in such a population. Although there is an ongoing debate whether genetically isolated populations are more suitable than outbred populations for genetic association studies, several studies in isolates have been successful (Kuokkanen *et al.*, 1997; Leppavuori *et al.*, 1999; Aouizerat *et al.*, 1999; Stefansson *et al.*, 2002).

The oral contraceptive pill (OCP) is commonly used in the treatment of menstrual irregularities and hyperandrogenism in women with PCOS (Morin-Papunen *et al.*, 2003). OCP has been shown to inhibit significantly the ovarian androgen production and to increase serum androgens binding capacity by increasing sex hormone-binding globulin (SHBG) (Falsetti and Galbignani, 1990; Coenen *et al.*, 1996; Dahlgren *et al.*, 1998). Apart from these positive effects, OCP slightly decreases insulin sensitivity (Skouby *et al.*, 1987; Godsland *et al.*, 1992) and for this reason their use may augment the already impaired glucose metabolism of women with PCOS (Dunaif, 1999). Additionally, OCP appears to alter the sonographic profile of women with PCOS (Falsetti *et al.*, 2001). Hence, the use of oral contraceptives affects clinical, endocrine and sonographic parameters characteristic for the disease, and complicates diagnosis of PCOS.

In order to perform a genetic association study, PCOS patients derived from an isolated population were recruited. At present, a number of these women uses OCP for contraceptive purposes and control of cycle irregularities. The genetic and environmental homogeneity offers an opportunity to assess the influence of OCP on phenotype expression in a genetically and environmentally homogeneous population.

3.1.2 Materials and methods

Patient recruitment and screening

The local Medical Ethics Review Committees of all participating hospitals approved this study and informed consent was obtained from all participants.

All previously diagnosed PCOS patients descendant from a restricted area in the south of the Netherlands were permitted to participate in the study. The individuals were selected by means of their ZIP code and a previous assessment of endocrine features (1994-2002). Medical charts of the identified individuals were handsearched. In case the diagnosis WHO II anovulation or PCOS was confirmed, based upon a clinical, endocrine and sonographic evaluation performed in the past (The Rotterdam ESHRE/ASRM sponsored PCOS consensus workshop group, 2004), women were recruited to participate. Subsequently the clinician involved in the study contacted the patient. Only after the subject had signed the informed consent the study started.

All participants underwent a randomly performed clinical, sonographic and endocrine screening in order to reconfirm the diagnosis of WHO II anovulation and PCOS (The Rotterdam ESHRE/ASRM sponsored PCOS consensus workshop group, 2004) in a standardised fashion (Imani *et al.*, 1998). In case of use of OCP the screening was performed on the last day of the pill free interval (Van Heusden and Fauser, 2002). Clinical screening parameters included cycle history (age at menarche, duration and (ir)regularity of the cycle nowadays and in the past, use of orally administered contraceptives, use of other cycle - intervening medication, treatment of infertility). A familial questionnaire was completed concerning oligoamenorrhea, hirsutism, type II diabetes, cardiovascular disease, premature baldness in males, and subfertility. The following parameters were mapped by physical examination: weight, length, waist and hip circumference, extent of hirsutism (Ferriman Gallwey score (FG score) (Ferriman and Gallwey, 1961)), markers of insulin resistance (acanthosis nigricans (Burke *et al.*, 1999)), BMI (kg/m²) and waist-to-hip ratio (WHR). Through transvaginal sonography (TVS) the ovarian volume (mL) and the total number of follicles (both ovaries) (Balen *et al.*, 2003) were assessed.

Hormone assays

Blood samples, obtained by venepuncture, were processed within 2 hours after withdrawal and stored at -20˚C until assay. Endocrine screening (fasting levels) included serum assays for cortisol, prolactin (PRL), thyroid-stimulating hormone (TSH), FSH, LH, estradiol (E_2), testosterone (T), androstenedione (AD), dehydroepiandosterone (DHEA), dehydroepiandosterone-sulphate (DHEAS), SHBG, progesterone (P), 17-hydroxyprogesterone (17-OH-P), inhibin B, anti-Müllerian hormone (AMH), glucose and insulin. The free androgen index (FAI = testosterone (T) x 100/sex hormone-binding globulin (SHBG)) could be calculated subsequently. Serum FSH, LH, PRL, cortisol, TSH, AD, DHEAS, SHBG, and insulin were assessed by using a chemiluminescent immunoassay (Immulite; Diagnostic Products Corp., Los Angeles, CA). E_2 and T were measured by using coated tube radioimmunoassay kits (Diagnostic Products Corp., Los Angeles, CA). Serum DHEA was measured by using a radioimmunoassay (Diagnostic Laboratories, Webster, Texas). All other assays have been described previously (Lamberts *et al.*, 1987; Imani *et al.*, 1998; Imani *et al.*, 1999; de Vet *et al.*, 2002).

Data analysis

Data were tested for normality of distribution. Results are presented as the mean ± SD in case distributed normally or otherwise as the median and range. To assess differences between groups Chi square or Mann-Whitney tests were used. Data were analysed using the commercially available software package SPSS (Chicago, IL, USA). A P value of 0.05 was chosen as threshold level for statistical significance.

3.1.3 Results

From the restricted area defined by zip code the computer selected a total of 1602 women who all had undergone a previous endocrine assessment in any of the participating hospitals. All medical records were handsearched for criteria fitting the diagnosis WHO II anovulation (N = 252). Subsequently, a total of 118 women agreed to participate in the current analysis. A total of 17 women did not fulfil the criteria to confirm WHO II anovulation at present (i.e. no discernable WHO category (N=10), WHO I anovulation (N=1), WHO III anovulation (N=3) and increased serum prolactin (N=3)).

The diagnosis WHO II anovulation could be reconfirmed for 101 women. A total of 81 (80%) women suffered from PCOS (according to the revised 2003 criteria) (The Rotterdam ESHRE/ASRM sponsored PCOS consensus workshop group, 2004). From these women a total of 54 (67%) did not use OCP, whereas 27

(33%) women did. Clinical, endocrine and sonographic characteristics of these PCOS patients are summarized in Table 1.

TABLE 1 Clinical, endocrine and sonographic characteristics of 81 PCOS women, separately for those presently without or with the use of OCP. * P value corrected for difference in age.

	No OCP (n = 54)	**OCP (n = 27)**	**P value**
Clinical parameters			
Age (years)	34.7 ± 5.8	30.8 ± 5.6	0.005
Menarche (years)	12.9 ± 1.8	13.3 ± 1.9	0.4*
Cycle duration (history)(days)	70 ± 59	74 ± 51	0.9*
Time to first liveborn pregnancy (months)	29.0 ± 25.3	23.1 ± 14.3	0.1*
BMI (kg/m^2)	29.5 ± 8.4	27.1 ± 7.3	0.3*
Waist-to-hip ratio	0.88 ± 0.11	0.84 ± 0.07	0.4*
Ferriman Gallwey Score	8 ± 5	7 ± 4	0.5*
Endocrine parameters			
Cortisol (nmol/L)	360 ± 127	531 ± 202	< 0.001*
LH (IU/L)	7.9 ± 9.0	3.5 ± 2.6	0.1*
FSH (IU/L)	6.5 ± 11.1	5.3 ± 2.7	0.5*
E_2 (pmol/L)	327 ± 263	136 ± 64	0.001*
P (nmol/L)	7.6 ± 12.7	1.3 ± 2.3	0.02*
17-OH- P (nmol/L)	3.5 ± 3.2	1.2 ± 0.9	< 0.001*
T (nmol/L)	1.3 ± 0.7	1.0 ± 0.7	0.04*
AD (nmol/L)	9.1 ± 4.2	7.3 ± 3.4	0.01*
DHEA (nmol/L)	43.5 ± 25.7	43.7 ± 18.1	0.4*
DHEAS (µmol/L)	3.98 ± 1.81	3.53 ± 1.59	0.08*
SHBG (nmol/L)	37.6 ± 20.5	92.2 ± 64.5	< 0.001*
FAI (100 x T/SHBG)	4.4 ± 2.9	1.4 ± 1.0	< 0.001*
Glucose:Insulin ratio	0.77 ± 0.55	0.61 ± 0.43	0.2*
Ultrasound parameters			
Mean ovarian volume/ovary (mL)	7.3 ± 3.3	10.0 ± 13.9	0.6*
Mean follicle number/ovary (n)	15 ± 6	14 ± 6	0.3*

TABLE 2 Clinical, endocrine and sonographic characteristics of 37 women for whom the past diagnosis of PCOS could be reconfirmed in the present study, separately for those presently without or with OCP.

	No OCP (n = 27)	OCP (n = 10)	P value
Clinical parameters			
Age (years)	33.4 ± 4.5	31.0 ± 4.5	0.2
Menarche (years)	12.9 ± 1.8	13.5 ± 2.6	0.5
Cycle duration (history)(days)	84 ± 70	88 ± 61	0.9
Time to first liveborn pregnancy (months)	30.9 ± 29.5	25.3 ± 8.5	0.4
BMI ($kg/m^{2)}$	31.3 ± 9.8	29.9 ± 9.6	0.7
Waist-to-hip ratio	0.87 ± 0.11	0.85 ± 0.07	0.5
Ferriman Gallwey Score	7 ± 5	8 ± 6	0.7
Endocrine parameters			
Cortisol (nmol/L)	344 ± 126	634 ± 226	0.003
LH (IU/L)	6.0 ± 4.1	2.9 ± 2.4	0.03
FSH (IU/L)	4.8 ± 2.3	5.6 ± 1.9	0.3
E_2 (pmol/L)	287 ± 160	128 ± 55	< 0.001
P (nmol/L)	6.5 ± 12.0	2.0 ± 3.9	0.3
17-OH- P (nmol/L)	3.6 ± 4.0	1.4 ± 1.1	0.01
T (nmol/L)	1.2 ± 0.6	1.1 ± 0.6	0.4
AD (nmol/L)	9.3 ± 4.5	7.6 ± 2.9	0.3
DHEA (nmol/L)	41.9 ± 21.3	52.5 ± 17.9	0.2
DHEAS (µmol/L)	3.8 ± 1.9	3.7 ± 1.6	0.9
SHBG (nmol/L)	34.3 ± 19.9	112.8 ± 75.0	0.009
FAI (100 x T/SHBG)	4.8 ± 3.2	1.4 ± 0.9	< 0.001
Glucose:Insulin ratio	0.70 ± 0.55	0.44 ± 0.18	0.04
Ultrasound parameters			
Mean ovarian volume/ovary (mL)	7.6 ± 3.6	5.8 ± 2.8	0.2
Mean follicle number/ovary (n)	17 ± 6	17 ± 3	0.6

Age was significantly different (P = 0.005) comparing women without (mean±SD: 34.7±5.8 years) and with the use of OCP (mean±SD: 30.8±5.6 years). Corrected for age women taking OCP had significantly increased serum concentrations of cortisol (P < 0.001) and SHBG (P < 0.001). Serum concentrations of E_2 (P < 0.001), P (P = 0.02), 17-OH-P (P < 0.001), T (P = 0.04) and AD (P = 0.01) were significantly decreased. These differences resulted in a significantly decreased FAI for women currently taking OCP (mean±SD: 1.4±1.0) compared to women without (mean±SD: 4.4±2.9) (P < 0.001). Figure 1 shows the distribution of hirsutism, hyperandrogenism and polycystic ovaries in women with PCOS presently without and with OCP, respectively.

In the present study it was possible to compare the diagnosis PCOS (according to the revised 2003 criteria) as set in the past and at present for a total of 41 women. The diagnosis could be reconfirmed for a total of 37 women. One participant did not fulfil the PCOS criteria in the past, however at present she did. For a total of 3 women the diagnosis PCOS could not be reconfirmed (2 of them were presently taking OCP). At the time of first diagnosis in the local hospital none of these women were on OCP. Clinical, endocrine and sonographic characteristics of the women for whom the diagnosis PCOS could be reconfirmed are summarized in Table 2. A total of 27 (73%) women did not use OCP at present whereas 10 (27%) of them did. Women taking OCP had significantly increased serum concentrations of cortisol (P = 0.003) and SHBG (P < 0.001). Serum concentrations of LH (P = 0.030), E_2 (P < 0.001), 17-OH-P (P = 0.012), FAI (P < 0.001) and the G:I ratio (P = 0.035) were significantly decreased for those using OCP. Figure 2 shows the distribution of hirsutism, hyperandrogenism and polycystic ovaries in women with PCOS presently without and with OCP, respectively.

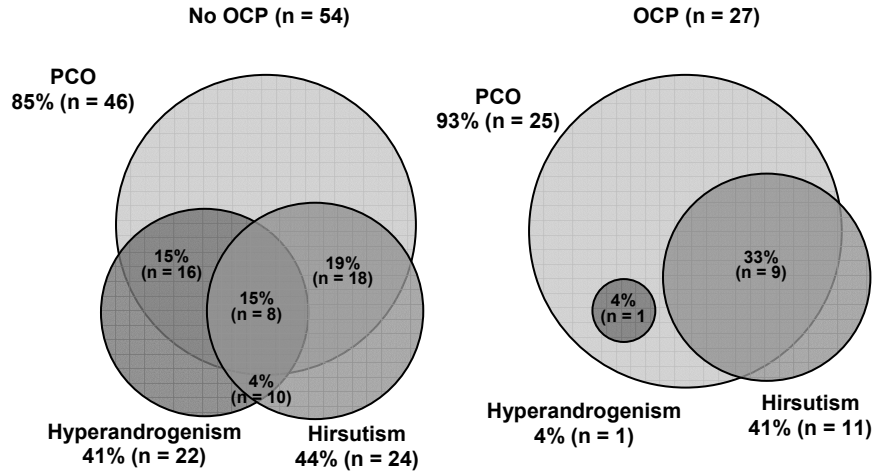

Figure 1 Distribution of hirsutism (FG score ≥ 8), hyperandrogenism (FAI ≥ 4.5) and polycystic ovaries (PCO) in women with PCOS, presently without (n = 54) and with (n = 27) OCP. Figures given outside and inside circles are percentages of women within the specified subgroup. Figures between parentheses are numbers of patients within the specified subgroup.

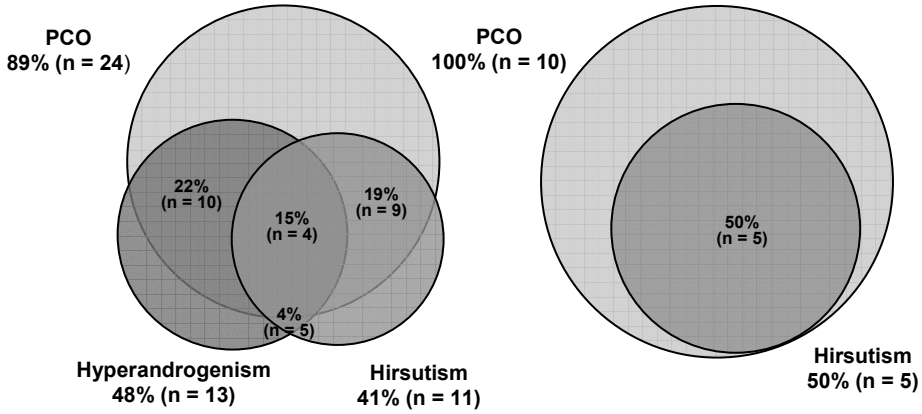

Figure 2 Distribution of hirsutism (FG score ≥ 8), hyperandrogenism (FAI ≥ 4.5) and polycystic ovaries (PCO) in women for whom the past diagnosis PCOS could be reconfirmed in the present study, separately for those currently without (n = 27) and with (n = 10) OCP. Figures given outside and inside circles are percentages of women within the specified subgroup. Figures between parentheses are numbers of patients within the specified subgroup.

Finally, clinical, endocrine and sonographic characteristics of the 54 PCOS patients without OCP participating in the present study were compared to PCOS controls. All controls (n=361) visited the infertility outpatient clinic of the Erasmus Medical Center and participated in previous studies (Laven *et al.*, 2002; Eijkemans *et al.*, 2003). Age (P < 0.001), serum concentrations of T (P < 0.001), AD (P < 0.001), and mean ovarian volume (P < 0.001) were significantly different between both groups. Corrected for age the control group still had significantly increased serum concentrations of T (P < 0.001), AD (P < 0.001) and an increased ovarian volume (P < 0.001). The presence of polycystic ovaries (P = 0.6) and obesity (P = 0.6) was equally distributed between both groups. PCOS controls suffered significantly more often from amenorrhea (P = 0.02) and hyperandrogenism (P = 0.004). Figure 3 shows the distribution of obesity, hyperandrogenism and polycystic ovaries in PCOS women descendant from the founder population and the PCOS controls, respectively.

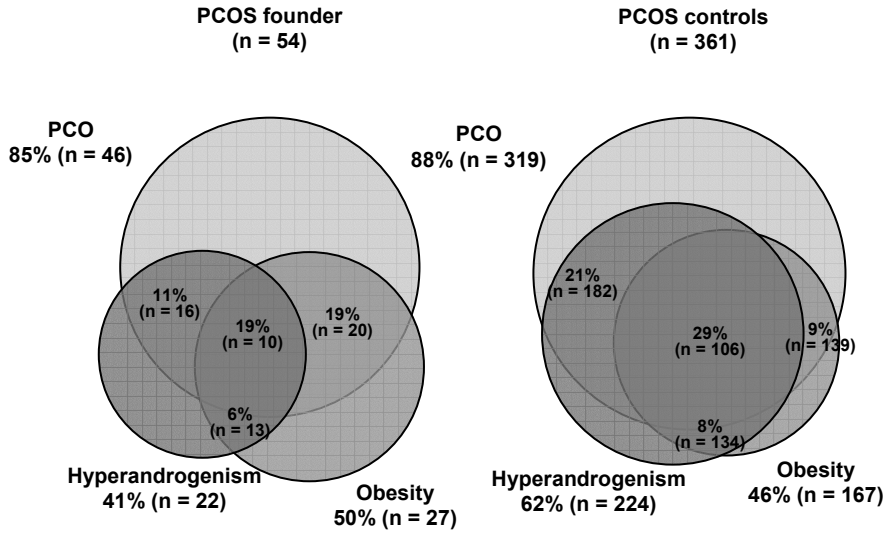

Figure 3 Distribution of obesity (BMI > 27), hyperandrogenism (FAI ≥ 4.5) and polycystic ovaries (PCO) separately for PCOS women descendant from the founder population (n = 54) and PCOS controls (n = 361). Figures given outside and inside circles are percentages of women within the specified subgroup. Figures between parentheses are numbers of women within the specified subgroup.

3.1.4 Discussion

In the present analysis it could be established that the use of the OCP influences phenotype expression in women known to suffer from PCOS. Parameters characteristic for the extent of the disease such as hyperandrogenism are less severe for those women using OCP. This is shown for women who could be diagnosed as having PCOS (according to the revised criteria) at present, as well as for those for whom the past diagnosis could be reconfirmed currently (Table 1 and 2). Moreover, women still fulfilled the PCOS criteria despite taking OCP. These data suggest that OCP use does not appreciably affect the PCOS phenotype, despite the observed decrease in hyperandrogenism.

OCP are frequently used for the long-term management of women with PCOS (Cagnacci *et al.*, 2003). The reasons for OCP use in PCOS are contraception, protection against the development of endometrial hyperplasia, control of irregular cycles and suppression of excessive androgen secretion to control hirsutism and acne (The ESHRE Capri Workshop Group, 2001; Morin-Papunen *et al.*, 2003). Indeed, the OCP reduces the amount of androgens produced by the ovary, causes a decrease of the free androgen fraction and leads to reduction of peripheral androgen effects (Cagnacci *et al.*, 2003). After cessation of OCP use the original PCOS features usually return within a few months (The ESHRE Capri Workshop Group, 2001).

Administration of the OCP in women with PCOS has been shown to significantly suppress ovarian androgen synthesis and to increase serum androgens binding capacity (Falsetti and Pasinetti, 1995; Dahlgren *et al.*, 1998). Hence, treatment with the OCP significantly reduces serum concentrations of both total and free androgens (Dahlgren *et al.*, 1998; Gjonnaess, 1999). In terms of anti-androgenicity, OCP containing cyproterone acetate (a specific anti-androgen) or a 3rd generation progestin are mostly used (Cibula *et al.*, 2001; Mastorakos *et al.*, 2002). A combination of ethinyl estradiol (EE) and cyproterone acetate however, is effective in reducing hirsutism, but may decrease insulin sensitivity (Venturoli *et al.*, 1999; Morin-Papunen *et al.*, 2000). In addition, an OCP containing a 3rd generation progestin significantly decreases androgen production and concentrations without reducing insulin sensitivity (Cibula *et al.*, 2002). In the present study the free androgen index was significantly decreased for women using OCP. However, ultrasonographic evaluation showed the presence of polycystic ovaries (PCO) and hence these women still fulfilled PCOS criteria.

Apart from the aforementioned positive effects on androgen production and free androgen levels, the OCP is believed to slightly deteriorate insulin sensitivity (Skouby *et al.*, 1987; Kasdorf and Kalkhoff, 1988), and for this reason their use may aggravate the already impaired glucose metabolism in women with PCOS.

OCP does induce impairment of glucose tolerance and does elevate insulin levels in healthy women, but these effects depend largely on the dose of estrogen and the dose and type of progestin (Godsland *et al.*, 1992). However, studies evaluating the role of the OCP on glucose tolerance or insulin sensitivity in women with PCOS have generated conflicting results (Korytkowski *et al.*, 1995; Dahlgren *et al.*, 1998; Morin-Papunen *et al.*, 2000; Cibula *et al.*, 2002; Morin-Papunen *et al.*, 2003). Differences in outcome are primarily based on the various methods used to assess glucose intolerance (insulin resistance or hyperinsulinaemia) as well as the difference in the estrogen/progestin components. Moreover, it has been shown that the magnitude and variability of the metabolic effects observed with OCP use are primarily dependent on the progestin component (Spellacy, 1982; Godsland *et al.*, 1992). In the current study, no differences are observed in the fasting glucose/insulin ratio of PCOS women with or without the use of OCP. However, when comparing women for whom the diagnosis PCOS could be reconfirmed the glucose/insulin ratio is significantly reduced for those using OCP. Indeed, the current observation may suggest an increased sensitivity to develop glucose intolerance for women with PCOS when taking oral contraceptives.

It has been suggested that the use of oral contraceptives as a consequence of suppressed ovarian function results in reduction of ovarian dimension and follicle number, as assessed by abdominal ultrasound examination, in adolescents with hyperandrogenism (Venturoli *et al.*, 1988). Additionally, the presence of polycystic ovaries significantly decreased after a long term treatment (60 cycles) with OCP in adults with PCOS (Falsetti *et al.*, 2001). On the other hand, it is shown that polycystic ovary morphology is restored after cessation of therapy (Falsetti *et al.*, 2001). However, in the current study women taking OCP still present with PCO. These dissimilarities may exist due to differences in ultrasound examination and duration of treatment with OCP. Moreover, polycystic ovaries appear to be a relatively constant feature comparing women presently without or with OCP use (Figure 1 and 2). Indeed, these data may suggest that women with polycystic ovary appearance may suffer from a more severe disease condition (Imani *et al.*, 1998; Laven *et al.*, 2002; Mulders *et al.*, 2003a).

Finally, even after correction for age, the PCOS phenotype in women descendant from the founder population seems to be associated with less increased serum levels of androgens. As a consequence, the frequency of hyperandrogenism is decreased in women participating in the present study when compared to a PCOS control group with a heterogeneous genetic background. Hence, for the former group it could be hypothesized that genes involved in androgen metabolism are less probably involved in the development of the disease. However, these suggestions need to be interpreted with caution.

Use of OCP influences phenotype expression (the observable trait) of individual women known to suffer from PCOS by reducing hyperandrogenism.

Despite taking OCP, women diagnosed in the past as PCOS, still fulfilled the revised 2003 criteria for the syndrome, as PCO morphology was still present. Hence, it may be suggested that PCOS women presenting with PCO may suffer from the most stubborn form of the syndrome. Moreover, the present study suggests that it is permitted to rely on historical data regarding diagnosis of PCOS for women presently on the OCP since its cardinal features appear to be relatively constant. In addition, OCP use does not appreciably affect the PCOS phenotype.

Chapter IV

Phenotype expression and genetic background

4.1 Follicle-stimulating hormone receptor polymorphism in women with normogonadotropic anovulatory infertility.

Abstract

In the present cross-sectional study the incidence of different FSH receptor (FSHR) genotypes in normogonadotropic anovulatory infertile women (WHO II) and normo-ovulatory controls was assessed. Furthermore, the FSHR genotypes were correlated with baseline characteristics and ovarian responsiveness during ovulation induction in women with WHO II anovulatory infertility.

Thirty normo-ovulatory controls were compared to 148 WHO II women. In WHO II patients and controls a standardized evaluation including: cycle history, body mass index and transvaginal ultrasound scanning of ovaries was performed. Fasting blood samples were obtained for endocrine evaluation. Ovarian responsiveness to FSH in WHO II women was assessed during ovulation induction and DNA was analyzed to determine the FSHR genotype.

The Thr/Thr 307 genotype was significantly less (52% vs 23%; $P < 0.05$) and the Ser/Ser 680 polymorphism was significantly more prevalent (40% vs 16%; $P < 0.05$) in WHO II patients compared to controls. WHO II patients with the Ser/Ser 680 polymorphism presented with higher median FSH serum levels (5.2 IU/L; range 2.4-9.7) compared to the Asn/Asn 680 (4.6 IU/L; range 1.4-5.8) and Asn/Ser 680 (4.5 IU/L; range 1.8-9.7) variants ($P < 0.05$). However, ovarian responsiveness to FSH was similar comparing different polymorphisms in anovulatory women.

WHO II patients exhibit a different FSHR genotype compared to normo-ovulatory controls and although this is associated with increased baseline FSH serum levels altered ovarian sensitivity to exogenous FSH during ovulation induction could not be established.

4.1.1 Introduction

Follicle-stimulating hormone (FSH) plays a crucial role during folliculogenesis by stimulating granulose-cell estrogen production through induction of aromatase activity (Fauser and van Heusden, 1997). The action of FSH is mediated by the FSH receptor, which belongs to the large family of G-protein-coupled receptors. These receptors are characterized by a transmembrane domain consisting of seven membrane transversing α-helices connected by three extracellular and three intracellular loops (Simoni *et al.*, 1997; Themmen and Huhtaniemi, 2000). The FSH receptor gene is located at chromosome 2p21 to 16 (Simoni *et al.*, 1997; Themmen and Huhtaniemi, 2000; Simoni *et al.*, 2002).

Several naturally occurring mutations in the FSH receptor gene have been found. In a sample of Finnish women with hypergonadotropic ovarian dysgenesis, a loss-of-function mutation was found that resulted from a (Ala189Val) missense mutation that segregated perfectly with the phenotype (Aittomaki *et al.*, 1995). Patients with the lowest remaining FSH receptor activity have hypergonadotropic primary amenorrhea with atrophic ovaries (Aittomaki *et al.*, 1995), whereas carriers of mutations that less severely affect receptor function present with secondary normal-sized ovaries, and follicular development up to the antral stage (Beau *et al.*, 1998; Tapanainen *et al.*, 1998; Touraine *et al.*, 1999; Themmen and Huhtaniemi, 2000). However, inactivating mutations of the FSH receptor are rarely found in premature ovarian failure (POF) (Layman *et al.*, 1998; Takakura *et al.*, 2001; Doherty *et al.*, 2002).

The only activating FSH receptor mutation was identified in a hypophysectomized man who remained fertile despite undetectable gonadotropin levels (Gromoll *et al.*, 1996). Symptoms of activating FSH receptor mutations might resemble the phenotype of patients with McCune-Albright disease (Laven *et al.*, 2001b). In these patients, the constitutive activation of $G_s\alpha$ leads to symptoms of combined luteinizing hormone (LH) and FSH hyperactivity. In addition, enlarged ovaries with multiple cysts have been described in women with FSH-producing pituitary tumours (Christin-Maitre *et al.*, 1998).

Recently, two polymorphisms of the FSH receptor gene have been identified. One is located in the extracellular domain at position 307, occupied either by alanine (Ala) or threonine (Thr). The second one is located in the intracellular domain at position 680, occupied either by asparagine (Asn) or serine (Ser). Both polymorphic sites are within exon 10 and give rise most frequently to two discrete allelic variants of the FSH receptor: Thr307/Asn680 and Ala307/Ser680 (Perez *et al.*, 2000; Simoni *et al.*, 2002). No distinct differences could be found in the distribution of these two allelic variants in infertile men or women compared with normal persons (Conway *et al.*, 1999; Simoni *et al.*, 1999).

From the combination of the two polymorphisms in both positions, two more allelic variants are possible: Thr307/Ser680 and Ala307/Asn680. Their frequency distribution in persons of different ethnic background has not been systematically analyzed. Whether FSH receptor polymorphisms have pathophysiologic significance with regard to ovarian dysfunction or ovarian response to stimulation is uncertain. Some investigators did not find differences in the distribution of these polymorphisms in polycystic ovary syndrome (PCOS) and POF (Conway *et al.*, 1999; Tong *et al.*, 2001), whereas others did (Sudo *et al.*, 2002).

In normogonadotropic normo-estrogenic anovulatory infertility (World Health Organization (WHO) class II), the response of the ovary to exogenous FSH administration varies considerably among patients (van Santbrink and Fauser, 1997; Coelingh Bennink *et al.*, 1998). In a recent study, the individual FSH

response dose for gonadotropin induction of ovulation in anovulatory infertile women could be predicted on the basis of initial screening characteristics, such as the initial FSH serum level (Imani *et al.*, 2002a). Recent observations in patients undergoing in-vitro fertilization (IVF) suggest that the FSH receptor genotype is associated with different requirements for exogenous FSH (Perez *et al.*, 2000).

We analyzed the frequency distribution of the two FSH receptor polymorphisms and their combination into four discrete allelic variants and compared their occurrence in normogonadotropic anovulatory infertile women with that in normo-ovulatory controls of different ethnic origin. In addition, we studied the correlation between the observed FSH receptor genotype and the response to exogenous FSH for ovulation induction in normogonadotropic anovulatory infertile women.

4.1.2 Materials and methods

Patients

This study was conducted as a part of a research line that was approved by the Institutional Review Board of the Erasmus Medical Center. Informed consent was obtained from all participants.

We included 148 white Dutch patients who attended our infertility outpatient clinic between 1994 and 1999. No immigrants (persons of Mediterranean, Latin American, or southeast Asian origin) were included.

Inclusion criteria for patients were infertility, oligomenorrhea (interval between periods > 35 days) or amenorrhea (absence of vaginal bleeding for at least 6 months), serum FSH concentrations within normal limits (1 to 10 IU/L) (van Santbrink *et al.*, 1997; Schipper *et al.*, 1998), positive withdrawal bleeding after progestagen administration in patients with amenorrhea, and age 20 to 40 years. Standardized initial screening (clinical examination, transvaginal ultrasonography, and fasting blood sampling) was performed on a random day between 9 AM to 11 AM, as described elsewhere (van Santbrink *et al.*, 1997).

The control group consisted of 30 healthy volunteers selected by advertisement and paid for participation, as described elsewhere (Macklon and Fauser, 2000). Like the patients, controls were Dutch and not immigrants. Inclusion criteria were a regular menstrual cycle (26 to 30 days), age 20 to 35 years, normal body mass index (BMI) (18 to 25 kg/m^2), no history of endocrine disease, and no use of medication or oral contraceptives for at least 3 months before study entry. Transvaginal ultrasonography and blood sampling were performed during the early follicular phase (cycle day 3, 4, or 5). The controls are described in detail elsewhere (Macklon and Fauser, 2000).

Ovulation induction

In a subgroup of 89 women who failed to ovulate or conceive after clomiphene citrate administration, gonadotropin treatment was commenced within 3 to 5 days after initiation of spontaneous or progestagen-induced withdrawal bleeding. Patients received daily s.c. injections of recombinant (r)FSH (Gonal-F®; Ares-Serono, Geneva, Switzerland). During all first cycles, a low-dose step-up protocol was used with a starting dose of FSH of one ampoule (75 IU) per day. The daily dose was increased by 0.5 ampoule if ovarian response (≥ 1 follicle ≥ 10 mm) was lacking after 14 days. Thereafter, the dose was increased by 0.5 ampoule every 7 days if required. The FSH response dose was defined as the dose at which an ovarian response was observed. If sufficient ovarian response was observed, the dose was kept constant until administration of hCG (Profasi®; Ares-Serono).

Hormone assays

Blood samples were obtained by venepuncture and processed within 2 hours after withdrawal. Serum was stored at -20°C and assayed for LH, FSH, androstenedione (AD), testosterone (T), sex hormone-binding globulin (SHBG), inhibin B, estradiol (E_2) and progesterone (P). Serum LH and FSH were measured by immunofluorometric assay (Amerlite; Ortho-Clinical Diagnostics, Amersham, United Kingdom), whereas serum E_2, P, T, AD, and SHBG levels were measured by radioimmunoassay (RIA) provided by Diagnostic Products Corp. (Los Angeles, CA), as described elsewhere (Imani *et al.*, 2000). Intra- and interassay coefficients of variation were less than 5% and 15% for LH, less than 3% and 8% for FSH, less than 8% and 11% for AD, less than 3% and 5% for T, less than 5% and 7% for E_2, less than 16% and 17% for P, and less than 4% and 5% for SHBG.

Dimeric inhibin B levels were assessed by using an immuno-enzymometric assay obtained from Serotec (Oxford, United Kingdom), as described elsewhere (Schipper *et al.*, 1998). The detection limit of the assay, defined as the amount of inhibin equivalent with the signal of the blank plus 3 SDs of this signal, was 3.4 ng/L. Intra- and interassay coefficients of variation for inhibin B were less than 9% and 15%, respectively.

DNA isolation and analysis

Genomic DNA was obtained from peripheral blood leukocytes, as described elsewhere (Gromoll *et al.*, 1996). Polymerase chain reaction amplification of fragments of exon 10 encompassing amino acid positions 307 and 680 were analyzed by single-stranded conformation polymorphism gel electrophoresis, as described elsewhere (Simoni *et al.*, 1999; Gromoll *et al.*, 2000; Simoni *et al.*, 2002). The results of single-stranded conformation polymorphism analysis were confirmed by direct sequencing of about 10% of randomly chosen DNA samples.

Statistical analysis

Statistical analysis was performed by using a commercially available software package (SPSS; SPSS Inc, Chicago, IL). Data were analyzed for normal distribution. Data are presented as the mean (±SD) if distributed normally or as the median and range if distributed non-normally. To detect differences between groups, Mann-Whitney or Kruskal-Wallis tests were used if data were not normally distributed. Normally distributed data were subjected to one-way analysis of variance. $P \leq 0.05$ was considered statistically significant.

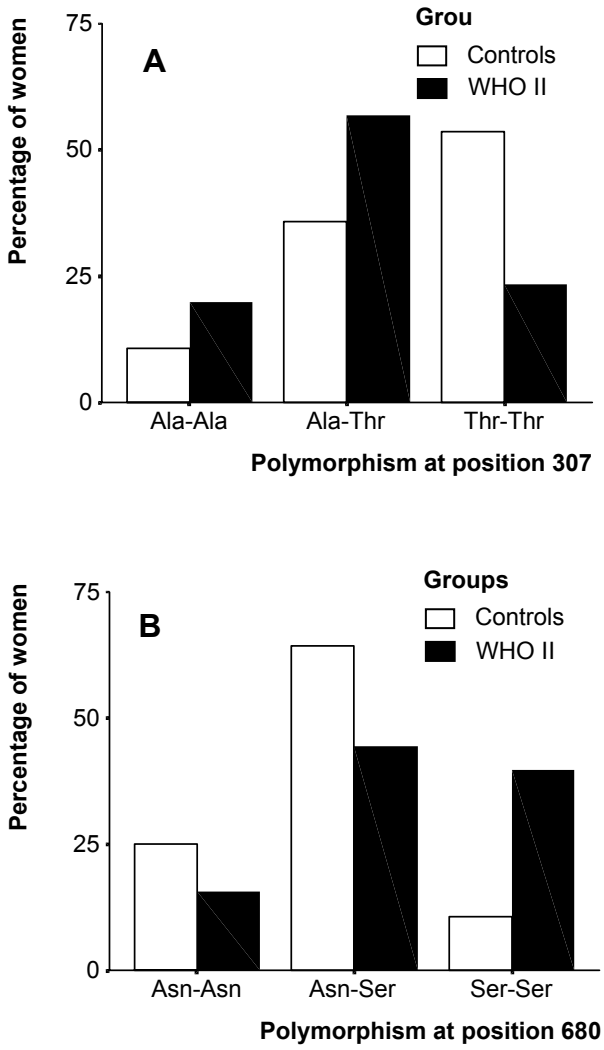

Figure 1 Distribution of the three possible FSH receptor genotypes at positions 307 (A) and 680 (B) in exon 10 of the FSH receptor gene among normogonadotropic anovulatory women and normo-ovulatory controls. The difference in distribution between anovulatory patients and normo-ovulatory controls was significant for both polymorphisms ($P < 0.05$ for position 307 and $P < 0.05$ for position 680).

4.1.3 Results

The Ala/Ala 307 variant was found in approximately 16% of controls, whereas the Ala/Thr and Thr/Thr variants were found in 32% and 52% of controls. As for the polymorphism at position 680, 23% of controls had Asn/Asn, 61% has Asn/Ser, and 16% had Ser/Ser.

In normogonadotropic anovulatory infertile women, the overall frequency distribution for polymorphism at position 307 was 20% for Ala/Ala, 57% for Ala/Thr, and 23% for Thr/Thr; for polymorphism at position 680, 16% for Asn/Asn, 44% for Asn/Ser, and 40% for Ser/Ser. The distribution of both polymorphisms differed significantly between anovulatory patients and normo-ovulatory controls ($P < 0.01$ for position 307 and $P < 0.01$ for position 680) (Figure 1). However, the prevalence of the combined genotype (polymorphisms at position 307 and 680) did not significantly differ ($P < 0.09$) between controls and anovulatory infertile women (Figure 2).

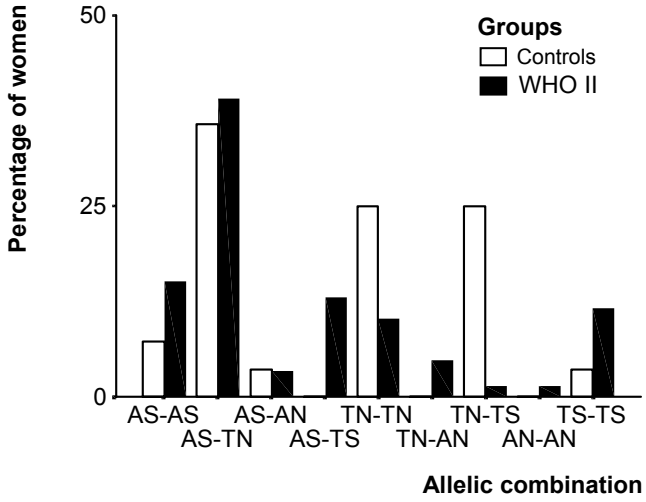

Figure 2 The similarity in distribution of several FSH receptor genotypes among normogonadotropic anovulatory women and normo-ovulatory controls. Since the Ala307/Ser680-Thr307/Asn680 and Ala307/Asn680-Thr307/Ser680 genotype cannot be distinguished by the methods used, they are considered together and designated as Ala307/Ser680-Thr307/Asn680.

Table 1 shows the prevalence of the different alleles of the FSH receptor in controls and anovulatory patients. The distribution of alleles did not significantly differ between (P = 0.8) or within groups (P = 0.7 in controls and P = 0.9 in anovulatory patients).

Table 2 shows clinical, endocrine, and ultrasonographic variables of anovulatory patients with the various FSH receptor polymorphisms. Except for the initial serum FSH concentration, no statistically significant differences were found among the three polymorphisms for position 307 or 680. The FSH serum concentrations were 5.2 IU/L in patients with the Ser/Ser 680 variant; this value was significantly higher than FSH serum levels in patients with the Asn/Ser 680 variant (4.5 IU/L) and those with the Asn/Asn 680 variant (4.6 IU/L).

Data on ovulation induction were available for 89 (60%) anovulatory women. The frequency of polymorphisms at position 307 in clomiphene citrate-resistant patients was 16% for those with the Ala/Ala variant, 54% for those with the Ala/Thr variant, and 30% for those with the Thr/Thr variant. The frequency of the polymorphisms at position 680 in these patients was 9% (Asn/Asn), 40% (Asn/Ser), and 51% (Ser/Ser). The frequencies of polymorphisms at position 307 or 680 did not differ between clomiphene citrate-resistant patients and those who failed to conceive during previous successful ovulation induction with clomiphene citrate. In addition, the distribution of different alleles and allelic combinations were similar between clomiphene citrate-resistant patients and those who ovulated after clomiphene citrate administration (data not shown).

TABLE 1 Distribution of allelic FSH receptor variants in normogonadotropic anovulatory patients and normo-ovulatory controls.

	Allele (%)			
Group	**AS**	**TN**	**AN**	**TS**
Anovulatory patients (n = 148)	126 (43%)	97 (33%)	17 (5%)	56 (19%)[a]
Controls (n = 30)	17 (28%)	32 (53%)	2 (3%)	9 (15%)

Note: Data are expressed as numbers of participants (percentage).
[a] Differences in distribution of the four distinct alleles between controls and anovulatory patients were not statistically significant (P < 0 .8).

TABLE 2 Clinical, endocrine, ultrasonographic, and stimulation characteristics of anovulatory infertile patients among polymorphic variants of the FSH receptor.

	Polymorphism 307			Polymorphism 680		
	Ala/Ala (n = 30)	**Ala/Thr** (n = 84)	**Thr/Thr** (n = 34)	**Asn/Asn** (n = 24)	**Asn/Ser** (n = 66)	**Ser/Ser** (n = 58)
Clinical parameters						
Age (years)	28.8 (22.3 - 35.8)	28.1 (19.6 - 35.8)	27.7 (21.8 - 35.3)	27.6 (21.8 - 35.3)	28.3 (19.4 - 35.3)	28.8 (22.3-35.8)
BMI (kg/m^2)	26.5 (17.9 - 42.6)	24.7 (17.7 - 52.3)	25.5 (17.3 - 39.8)	26.5 (17.9 - 39.8)	26.5 (17.7 - 52.9)	24.6 (17.3-42.6)
Cycle duration (days)	90 (35 - 199)	60 (35 - 199)	51 (35 - 199)	75 (39 - 199)	60 (35 - 199)	57 (35-199)
Endocrine parameters						
FSH (IU/L)	4.8 (3.2 - 9.0)	4.7 (1.8 - 9.7)	4.9 (1.4 - 7.1)	4.6 (1.4 - 5.8)	4.5 (1.8 - 9.7)	5.2 (2.4 - 9.7)[a]
LH (IU/L)	8.3 (1.1 - 23.5)	8.1 (1.4 - 23.5)	6.9 (2.4 - 20.6)	7.6 (2.9 - 23.6)	7.3 (2.1 - 22.5)	7.6 (2.9 - 20.6)
T (nmol/L)	2.3 (0.6 - 5.0)	2.5 (0.7 - 6.8)	2.3 (0.6 - 4.3)	2.5 (0.6 - 4.0)	2.4 (0.7 - 6.8)	2.3 (0.6 - 5.0)
FAI (100 x T/SHBG)	5.8 (0.6 - 26.9)	4.7 (1.4 - 34.0)	4.4 (0.8 - 18.1)	5.0 (0.8 - 34.0)	4.8 (1.4 - 26.9)	4.3 (0.6 - 29.3)
E_2 (pmol/L)	188 (81 - 1868)	223 (39 - 1062)	222 (47 - 864)	218 (47 - 864)	227 (81 - 1062)	204 (39 - 1868)
FSH/E_2	0.03 (0.01 - 0.07)	0.02 (0.01 - 0.18)	0.02 (0.01 - 0.11)	0.03 (0.01 - 0.11)	0.02 (0.01 - 0.07)	0.03 (0.01 - 0.18)[b]
Inhibin B (ng/L)	122 (25 - 326)	127 (9 - 541)	138 (37-506)	138 (59 - 316)	126 (33 - 541)	134 (9 - 506)
Ultrasound parameters						
Ovarian volume	8.1 (3.5 - 17.9)	9.5 (2.6 - 23.0)	8.3 (5.4 - 21.5)	8.0 (5.4 - 19.9)	9.5 (2.6 - 23.0)	9.2 (2.6 - 22.9)
Mean follicle number	13.5 (4.5 - 25.0)	13.5 (2.0 - 34.5)	12.5 (5.5 - 23.5)	11.0 (5.5 - 23.5)	13.0 (4.5 - 34.5)	15.0 (2.0 - 30.0)
Women with PCO (%)	30 (20%)	81 (55%)	38 (25%)	23 (15%)	68 (46%)	58 (39%)
FSH Treatment parameters						
Duration of stimulation (days)	11 (8 - 22)	12 (6 - 28)	12 (8 - 29)	11 (8 - 24)	12 (6 - 27)	14 (6 - 29)
Total number of ampoules	12.3 (7.0 - 33.5)	14.5 (5.0 - 59.0)	15.0 (6.0 - 49.0)	15.0 (6.0 - 38.0)	13.5 (5.0 - 44.0)	13.5 (5.0 - 59.0)

Note: Data are medians (ranges). Comparisons are made between three different genotypes within either polymorphism 307 or 680. [a] $P < 0.01$ [b] $P < 0.05$

The FSH response dose could be determined in 77 (87%) of the 89 clomiphene citrate-resistant patients. There were no statistically significant differences among patients with different subtypes of FSH receptor variants for the polymorphism at position 307 or 680 in terms of the FSH dose at the beginning of the stimulation cycle, the number of ampoules of FSH used, the duration of stimulation, the median daily dose of FSH, or the response dose (Table 2). Moreover, the number of cancelled cycles (due to poor response or hyperstimulation) was the same in all three groups for both receptor polymorphisms.

Patients with polycystic ovaries (defined as an ovarian volume ≥ 10.8 mL, mean number of follicles > 10, or mean ovarian stroma score > 3) (van Santbrink *et al.*, 1997) and hyperandrogenemia, defined as a free androgen index (T level X 100/SHBG level) exceeding 4.5, were classified as having PCOS. In 61 women with PCOS, a similar distribution as in anovulatory patients was found; this distribution differed significantly from that in controls. In patients with PCOS and polymorphism at position 307, the overall frequency distribution was 21% for position Ala/Ala, 58% for Ala/Thr, and 21% for Thr/Thr; corresponding values for anovulatory women without PCOS were 22%, 51%, and 27%, respectively. The frequencies among patients with polymorphisms at position 680 and PCOS were 15% for Asn/Asn, 50% for Asn/Ser, and 35% for Ser/Ser; values in patients with this polymorphism but no PCOS were 19%, 38%, and 44%, respectively. For both polymorphisms, the distribution differed significantly among anovulatory women with PCOS, anovulatory women without PCOS on one hand and normo-ovulatory controls on the other hand ($P < 0.01$ for position 307 and $P < 0.01$ for position 680).

Figure 3 summarizes the data for patients with the polymorphism at position 680. No statistically significant differences in clinical, endocrine, or ultrasonographic variables were found among anovulatory women with PCOS, anovulatory women without PCOS, and controls. Stimulation characteristics in patients with PCOS and those without PCOS were similar (data not shown).

4.1.4 Discussion

We found differences in distribution of the different FSH receptor genotypes between normogonadotropic anovulatory patients (those with WHO class II disease and PCOS) and normo-ovulatory controls. The FSH receptor variants Ala/Thr 307 and Ser/Ser 680 were significantly more prevalent among anovulatory women. The Thr/Thr 307 polymorphism was significantly more prevalent in controls. Earlier reports failed to establish differences in prevalence of FSH receptor genotypes in fertile or infertile men (Simoni *et al.*, 1999) and in infertile women (Conway *et al.*, 1999).

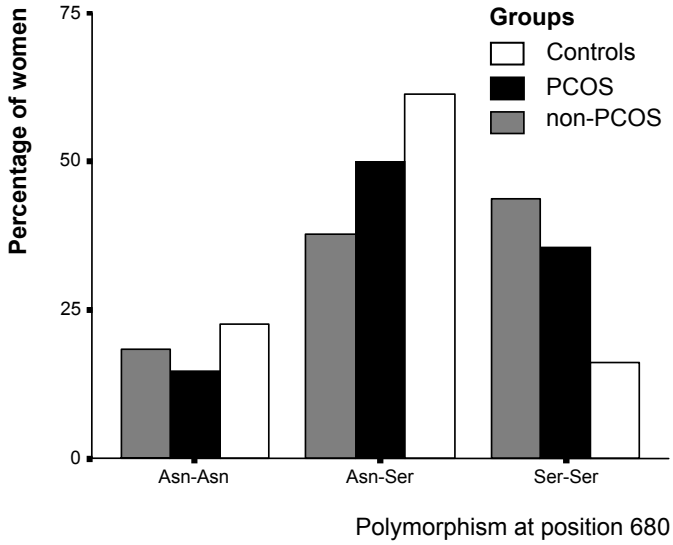

Figure 3 Distribution of the three possible FSH receptor genotypes at both positions 307 and 680 in exon 10 of the FSH receptor gene among women without the polycystic ovary syndrome (*non-PCOS*), women with PCOS (*PCOS*), and normo-ovulatory controls. The distribution of both polymorphisms significantly differed between women with PCOS and those without PCOS on one hand and controls on the other hand.

Few studies have addressed the distribution of the allelic variants in patients with PCOS, and results are conflicting. Two studies revealed no differences in prevalence between a limited number of patients with PCOS and normo-ovulatory women (Conway *et al.*, 1999; Tong *et al.*, 2001). Recently, Sudo *et al.* (2002) reported a significant increase in the Ala/Thr 307 and Asn/Ser 680 genotype in a large cohort of women with PCOS. Because the distribution of the polymorphism at position 680 among our normo-ovulatory controls is similar to the previously reported incidence in normal women (Conway *et al.*, 1999; Asatiani *et al.*, 2002; Sudo *et al.*, 2002), the observed difference in this large cohort of anovulatory women and patients with PCOS seems to be real.

The FSH receptor polymorphism combination Ser/Ser 680 was associated with higher basal FSH levels compared with the Asn/Asn 680 and Asn/Ser 680 variants. This might indicate that the Ser/Ser 680 FSH receptor polymorphism is associated with decreased FSH sensitivity. In a recently published prediction model, the individual FSH response dose during ovulation induction therapy was determined in part by the initial basal FSH serum concentration (Imani *et al.*, 2002a). Moreover, in normo-ovulatory IVF patients, ovarian response to exogenous FSH stimulation was determined by the FSH receptor genotype. Normo-ovulatory patients exhibiting the Ser/Ser 680 allelic variant had higher levels of basal FSH and required a significantly higher daily FSH dose for successful hyperstimulation in a routine IVF/intracytoplasmic sperm injection (ICSI) program (Perez *et al.*, 2000).

Similarly, in our study, the highest basal FSH serum levels in anovulatory infertile patients were associated with the Ser/Ser 680 allelic variant. However, neither the response dose nor the total number of ampoules FSH used or duration of stimulation differed among patients with the different FSH receptor genotypes. This lack of association between FSH receptor genotype and ovarian sensitivity might be due to differences in stimulation protocols. Because ovulation induction protocols in anovulatory patients aim for mono-follicular development, the amount of exogenous FSH used may be within the physiologic range. In contrast, exogenous FSH administered during IVF stimulation protocols extends the physiologic range since multi-follicular development is the goal.

Because previous in vitro experiments failed to establish significant differences in binding characteristics and receptor activation between several FSH receptor polymorphisms (Simoni *et al.*, 1999; Sudo *et al.*, 2002), differences in FSH receptor activity might become apparent only after supraphysiologic stimulation (i.e. IVF stimulation protocols). However, the association between the FSH receptor phenotype and the basal FSH serum levels might also be coincidental. Moreover, the FSH receptor polymorphism might merely constitute a genetic marker for a nearby gene (not the FSH receptor) that increases the risk for anovulation.

More clinical and experimental data are necessary to establish the exact relationship between the dose of exogenous FSH and FSH receptor genotypes. Although about 50% of anovulatory infertile patients in our study were clomiphene citrate-resistant and not normo-ovulatory, which might make them not readily comparable to normo-ovulatory women, a dose dependent relationship between the FSH receptor polymorphism and the magnitude of the post-receptor signal cannot be ruled out.

In conclusion, normogonadotropic anovulatory patients and women with PCOS have a different FSH receptor genotype compared with normo-ovulatory controls. Despite these differences, FSH receptor genotypes are not associated with altered ovarian sensitivity to exogenous FSH during ovulation induction in anovulatory patients.

Acknowledgement

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Chapter V

Phenotype expression and clinical implications

5.1 Prediction of chances for success or complications in gonadotropin ovulation induction in normogonadotropic anovulatory infertility

Abstract

A prospective follow-up study was designed to investigate whether initial clinical, endocrine, and sonographic screening characteristics of women with normogonadotropic anovulatory infertility are capable of predicting individual outcome of gonadotropin induction of ovulation applying a step-down dose regimen. Previous studies from our group focussed on the prediction of outcome from first line CC treatment in these women. It would be of distinct clinical benefit in case patient characteristics could also predict individual chances for complications (i.e. multi-follicular growth) or success (i.e. ongoing pregnancy) prior to initiation of gonadotropin induction of ovulation.

One hundred and fifty four women attending our infertility unit between March 1993 and December 1999 were included in the present study. All these women presented with (a) a history of infertility, (b) oligomenorrhea or amenorrhea, (c) normal follicle-stimulating hormone (FSH) and estradiol (E_2) serum concentrations and (d) previously unsuccessful treatment with clomiphene citrate (i.e. CC resistant anovulation (CRA) or failure to conceive in 6 ovulatory CC-cycles (CCF)). Daily FSH injections were initiated on day 3-5 after a spontaneous or progestagen-induced withdrawal bleeding. A dose finding low-dose step-up regimen was applied during the first treatment cycle in order to identify the individual FSH response dose. In all subsequent cycles a step-down protocol was applied with an individually adjusted starting dose.

The total number of step-down cycles was 441, with 47% of cycles presenting with mono-follicular development (1 follicle > 15 mm on the day of hCG). Initial serum levels of luteinizing hormone (LH), testosterone (T) and androstenedione (AD) were significant predictors in univariate logistic regression analysis for the probability of multi-follicular development ($P < 0.05$). The area under the receiver operating characteristic curve (AUC) of the multivariate model (including AD and mean follicle number) to predict the chances of multiple dominant follicle development was 0.62. FSH treatment resulted in a total of 67 (44%) ongoing pregnancies with a cumulative ongoing pregnancy rate of 58.3%. Comparing those women who did versus those who did not reach an ongoing pregnancy in a multivariate Cox regression analysis, initial serum insulin-like growth factor-I (IGF-I), T and women's age entered into the final model (AUC = 0.67).

The individual treatment outcome – both multi-follicular growth and ongoing pregnancy - following gonadotropin induction of ovulation may be predicted by initial screening characteristics.

5.1.1 Introduction

Chronic anovulation is a common cause of infertility in women. Most of these women present with irregular menstrual cycles and normal serum follicle-stimulating hormone (FSH) concentrations (World Health Organization [WHO] group II) (The ESHRE Capri Workshop Group, 1995; Rowe *et al.*, 2000). Depending on the criteria used, polycystic ovary syndrome (PCOS) has been diagnosed in approximately 60% of these women (van Santbrink *et al.*, 1997; Laven *et al.*, 2002).

Exogenous gonadotropin preparations have been used extensively for stimulation of ovarian function since their introduction into clinical practice in 1958 (Lunenfeld, 2003). During the last two decades, its use in ovulation induction has been restricted to anovulatory infertile patients who failed to ovulate or conceive during preceding first line clomiphene citrate treatment (Schwartz and Jewelewicz, 1981; Insler, 1988; Fauser and van Heusden, 1997). Exogenous gonadotropins elicit increased ovulation rates (up to 90 %) compared with clomiphene citrate (CC), with comparable cumulative conception (van Santbrink *et al.*, 1995b; White *et al.*, 1996; Imani *et al.*, 1999). However, FSH treatment is associated with higher chances for complications such as ovarian hyperstimulation syndrome (OHSS) and multiple pregnancies, especially in PCOS (Wang and Gemzell, 1980; Hamilton-Fairley and Franks, 1990). As these complications are related to the number of developing follicles (Blankstein *et al.*, 1987), adjusted dose regimens along with intense monitoring of ovarian response aiming at mono-follicular cycles have been implemented (Hamilton-Fairley *et al.*, 1991; van Santbrink *et al.*, 1995b; Balasch *et al.*, 1996). Additionally, previous retrospective studies have suggested that individual patient characteristics such as luteinizing hormone (LH) concentrations (Homburg *et al.*, 1988; Hamilton-Fairley *et al.*, 1991), overweight (Hamilton-Fairley *et al.*, 1992; White *et al.*, 1996; Yarali *et al.*, 1999), age (Hamilton-Fairley *et al.*, 1992) and insulin resistance (Homburg *et al.*, 1996; Fulghesu *et al.*, 1997; Dale *et al.*, 1998) may also be involved in chances for complications or success following gonadotropin induction of ovulation.

Distinct individual differences in the amount of FSH required to elicit an ovarian response (referred to as the ‘FSH threshold’) may underlay chances for hyperresponse and severe complications (Brown, 1978; Fauser and van Heusden, 1997). Recently, a model based on initial screening characteristics has been introduced, to predict the individual ovarian FSH response (threshold) dose for

gonadotropin induction of ovulation (Imani *et al.*, 2002a; van Santbrink *et al.*, 2002).

The present prospective follow-up study was designed to investigate whether these initial clinical, endocrine, and sonographic screening characteristics of women with normogonadotropic anovulatory infertility can also predict individual outcome of gonadotropin induction of ovulation applying a step-down dose regimen. Previous studies have focussed on the prediction of outcome from first line CC treatment (Imani *et al.*, 1998; Imani *et al.*, 1999; Imani *et al.*, 2000) in these women. It would be of distinct clinical benefit if patient characteristics could also predict individual chances for complications (i.e. multi-follicular growth) or success (i.e. ongoing pregnancy) prior to initiation of second line gonadotropin induction of ovulation.

5.1.2 Materials and methods

Subjects

One hundred and fifty four women attending the infertility unit between March 1993 and December 1999 were included in the present study. Study approval was obtained from the human subjects committee of the Erasmus Medical Center and informed consent was obtained from all subjects. Inclusion criteria were [1] oligomenorrhea (bleeding interval between 35 days and 6 months) or amenorrhea (bleeding interval > 6 months), [2] normal serum FSH (1-10 IU/L), estradiol (E_2) (> 100 pmol/L) (van Santbrink *et al.*, 1995a; Schipper *et al.*, 1998), prolactin (< 44 µg/L) and thyroid-stimulating hormone (TSH) (0.2-4.2 mU/L) concentrations, [3] spontaneous menses or positive bleeding response to progestagen withdrawal, [4] body mass index (BMI) (weight divided by square height) > 18 kg/m^2, [5] between 19 and 40 years of age, [6] previous unsuccessful CC treatment (i.e. anovulation during at least three consecutive cycles with an increasing dose up to 150 mg/day (CC resistant anovulation (CRA)) or failure to conceive in six ovulatory CC cycles (CCF) (Imani *et al.*, 1998; Imani *et al.*, 1999; Imani *et al.*, 2000)), [7] a total motile sperm count (TMC = ejaculate volume (mL) $\times$ sperm concentration (10^6/mL) $\times$ percentage of progressive motile spermatozoa) above 1 million, and [8] a negative history for any tubal pathology along with negative antibody test for *Chlamydia* (*C. trachomatis* IgG and IgA < 1.0) or proven normal tubal patency on hysterosalpingography or laparoscopic inspection.

Standardized initial clinical, sonographic and endocrine screening took place prior to administration of exogenous gonadotropins using a low-dose step-up regimen, as described before (Imani *et al.*, 1998; Imani *et al.*, 1999; Imani *et al.*, 2002a; Imani *et al.*, 2002b). Clinical screening included age, duration and type (primary versus secondary) of infertility, cycle history, ovarian response to previous

CC medication, BMI, waist-to-hip ratio (WHR), any other previous medication and/or surgery. Transvaginal sonographic (TVS) screening included assessment of the ovarian stroma echogenicity (arbitrarily classified from 1 (normal) to 3 (markedly increased) per ovary), ovarian volume (mL) and total number of antral (2-8 mm) follicles (both ovaries), as described previously (van Santbrink *et al.*, 1997). Sonographic monitoring was performed by a single observer (BI), using an ultrasound machine (model EUB-415, Hitachi Medical Corp., Tokyo, Japan) with a 6.5 MHz transvaginal transducer.

Endocrine screening included serum assays for FSH, LH, E_2, AD, T, and sex hormone-binding globulin (SHBG), as described previously (Imani *et al.*, 1998). In addition, serum was also assayed for fasting insulin and glucose, free and total insulin-like growth factor-I (IGF-I), IGF binding protein-I (IGFBP-I), IGFBP-III and leptin concentrations as described previously (Imani *et al.*, 2000). The method of blood withdrawal, the assays used and the intra- and interassay coefficients of variation valid for this study have all been previously described (Imani *et al.*, 1999; Imani *et al.*, 2000).

Study protocol

Exogenous FSH, using either daily intramuscular (i.m.) injections of urinary FSH (Metrodin HP®, Serono Benelux BV, The Hague, The Netherlands) or subcutaneous (s.c.) injections of recombinant FSH (Gonal-F®, Serono Benelux BV), was initiated on day 3-5 after a spontaneous or progestagen-induced withdrawal bleeding. The treatment protocol and assessment of ovarian response have been described before (Imani *et al.*, 2002a).

Low-dose step-up protocol: During the first cycle a low-dose step-up regimen was applied to assess the individual FSH response (threshold) dose (Imani *et al.*, 2002a) (i.e sonographic visualization of a follicle ≥ 10 mm (Pache *et al.*, 1990; van Santbrink *et al.*, 1995b)). The starting dose of FSH was one ampoule (75 IU) per day. The first increase in FSH dose by half an ampoule (37.5 IU) per day was based on the absence of sonographic visualization of a follicle ≥ 10 mm after 7 days. If ovarian response remained absent during the following 7 days, the FSH dose was increased again (i.e. incremental steps of 37.5 IU/day every 7 days). If there was an ovarian response, the exogenous FSH dose was not changed until administration of human chorionic gonadotropin (hCG).

Step-down protocol: Arbitrarily, a dose of half an ampoule (37.5 IU) of FSH above the response dose (Imani *et al.*, 2002a) was used, to start the subsequent step-down cycle in most patients. Initially, in some patients the starting dose was based on BMI alone. The first decrease in dose by half an ampoule (37.5 IU) per day was based on visualization of at least 1 follicle ≥ 10 mm in diameter, as described previously (van Santbrink and Fauser, 1997). A further dose decrease (each time by 37.5 IU/day) was performed every 3 days in case follicular growth

continued, to a minimum dose of one ampoule (75 IU) per day until the day hCG could be administered. The initial dose was increased by half an ampoule (37.5 IU) per day if ovarian response remained absent after 5 days. If follicular growth remained absent over the following 10 days (two incremental steps of 37.5 IU/day), further medication was withheld and the cycle was cancelled.

hCG (Pregnyl®, NV Organon, Oss, The Netherlands) was administered intramuscularly as a single dose of 5,000 IU on the day upon which one or two follicles ≥ 18 mm could be visualized. If three or more follicles ≥ 16 mm were present, stimulation was cancelled. Ovulation after hCG administration was assessed by the sonographic signs of collapse of the dominant follicle and mid-luteal progesterone (P) concentrations above 25 nmol/L. No luteal support was provided.

Data analysis

Data of clinical characteristics in patient groups are presented as means ± SD if distributed normally. Otherwise data are presented as median and range.

Univariate and multivariate logistic regression analyses were used for comparison of patients who developed multi-follicular growth (> 1 follicle > 15 mm). Because multiple cycles from the same patient were included, the data analysed were interrelated. Therefore, P values were corrected by Huber's method (using S-plus software) to take account of clustering of data (Huber, 1967). Variables that had P values lower than 0.10 were candidates for stepwise multi-variable analysis. Multivariate analyses for multi-follicular growth were performed using the method of backward stepwise selection. The multivariate odds ratio is provided for the factors included in the final model. For instance, multivariate odds ratio (95% CI) of serum AD = 1.04 (1.02-1.07). The best estimate is that a 1 unit increase in a given patient's AD level increases her odds for multi-follicular growth following exogenous gonadotropin induction of ovulation by 4% relatively. There is a 95% probability that this increase will be between 2 and 7%.

Univariate and multivariate Cox regression was used for analysis of ongoing pregnancy rates during exogenous gonadotropin induction of ovulation. The number of gonadotropin induced cycles was the time variable. Censoring was defined as definitive discontinuation of gonadotropin therapy without an ongoing pregnancy or end of follow-up. The log rank test has been used to test statistical significance in life-table analyses. The prognostic impact of variables was expressed as a fecundability ratio, which is equivalent to the hazard ratio in survival analysis. Multivariate analyses for cumulative ongoing pregnancy rates were performed using the method of forward stepwise selection to gain a better insight in the interdependence between initial screening parameters. To build a multivariable model, multiple imputation of missing values was used (Little, 1992). Variables that had P values lower than 0.10 were candidates for stepwise multivariable analysis.

Data were analysed using the commercially available software package SPSS (Chicago, IL, USA). A P value of 0.05 was chosen as the threshold level for statistical significance.

5.1.3 Results

A total number of 154 patients fulfilled the inclusion criteria and underwent 544 cycles with exogenous FSH for induction of ovulation. All participating women underwent a mean number of 3.5 FSH-stimulation cycles before discontinuing treatment. Twenty percent of the women underwent > five cycles. For a total of 103 women, a low-dose step-up regimen was applied during the first cycle. In 32% of the step-up cycles, multi-follicular growth (> 1 follicle > 15 mm) occurred. In 11% of the cycles, no hCG was administered due to potential risks for multiple pregnancy and OHSS. The step-down protocol was applied in a total number of 441 cycles. In 199 (45%) cycles, multi-follicular growth (> 1 follicle > 15 mm) occurred. If there were > two follicles > 15 mm (65 cycles: 15%), no hCG was administered due to potential risks for multiple pregnancy and OHSS.

TABLE 1 Initial clinical, endocrine, and ultrasound screening characteristics (mean ± SD) of women with normogonadotropic anovulatory infertility, separately for cycles with mono- (n = 208) or multi-follicular (n = 199) growth after FSH induction of ovulation using a step-down dose regimen.

	Overall (n = 407)	Mono-follicular growth (n = 208)	Multi-follicular growth (n= 199)	P[a]	AUC[b]
Clinical parameters					
Age (years)	29.0 ± 4.3	29.3 ± 4.3	28.7 ± 4.3	NS	-
Amenorrhea (%)	115 (28)	53 (26)	62 (31)	NS	-
BMI (kg/m^2)	26.4 ± 5.7	26.7 ± 5.7	26.0 ± 5.7	NS	-
Waist-to-hip ratio	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	NS	-
CC resistant anovulation (%)	192 (50)	89 (45)	103 (55)	0.08	0.55
Predicted FSH response dose (IU)	120 ± 46	117 ± 38	123 ± 53	NS	-
Endocrine parameters					
LH (IU/L)	8.3 ± 4.3	7.8 ± 4.4	8.8 ± 4.0	0.05	0.59
FSH (IU/L)	5.0 ± 1.5	5.1 ± 1.6	4.8 ± 1.4	0.07	0.55
E_2 (pmol/L)	254 ± 142	249 ± 152	259 ± 130	NS	-
AD (nmol/L)	17.2 ± 8.0	16.0 ± 6.7	18.5 ± 9.0	0.02	0.57
T (nmol/L)	2.8 ± 1.1	2.6 ± 1.0	3.0 ± 1.2	0.001	0.60
IGF-I (ng/mL)	210 ± 77	206 ± 79	214 ± 74	NS	-
Leptin (ng/mL)	20.1 ± 15.3	20.7 ± 15.9	19.4 ± 14.7	NS	-
Ultrasound parameters					
Total stroma score[c]	3.6 ± 1.2	3.5 ± 1.2	3.7 ± 1.2	0.06	0.55
Mean ovarian volume (mL)	11.2 ± 4.9	11.0 ± 4.9	11.4 ± 4.8	NS	-
Mean follicle number	13.6 ± 5.9	12.9 ± 6.2	14.4 ± 5.5	0.08	0.57

[a] P value of univariable logistic regression predicting multi-follicular growth, correcting for clustering of cycles within women by Huber's method. [b] Area under the ROC curve (univariate analysis). [c] Arbitrarily defined as one to three per ovary (both ovaries added), as published previously (van Santbrink *et al.*, 1997). Footnote: A total of 32 cycles were excluded from analyses because of poor response (i.e. no follicles > 15 mm and no hCG administered). Another two cycles were excluded due to the existence of an ovarian cyst.

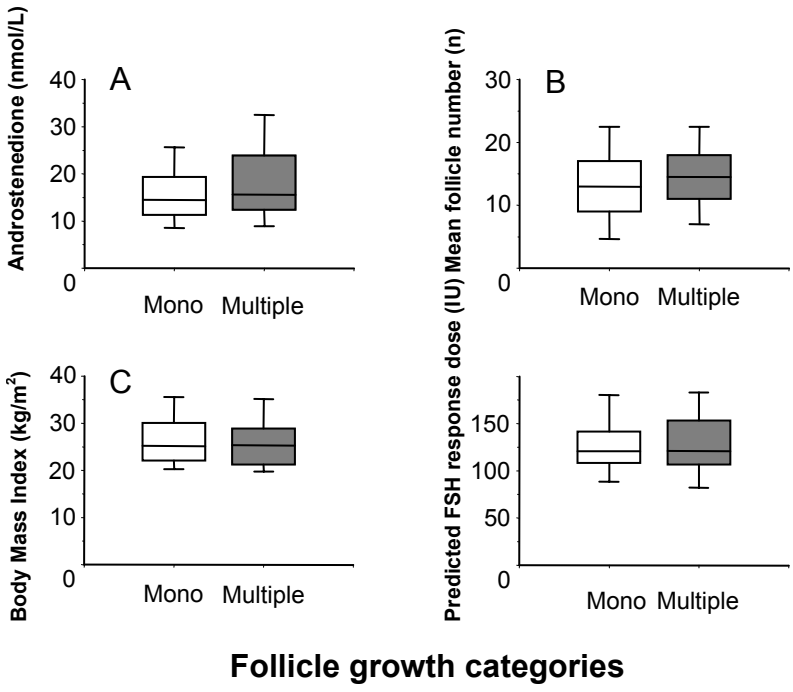

Figure 1 AD concentrations (A), mean follicle number (B), BMI (C) and predicted FSH response dose (D) in mono-follicular (n = 208) or multi-follicular (n = 199) cycles during gonadotropin ovulation induction applying a step-down regimen in 154 normogonadotropic oligoamenorrheic infertile patients for whom previous treatment with clomiphene citrate was unsuccessful. Data are presented as box and whisker plots: boxes encompass values between the 25th and the 75th percentile, horizontal lines represent median values, and whiskers give the 95% range of values. Only initial serum AD and mean number of follicles were included in the final model.

Initial serum LH, T and AD were significantly different for patients who developed one single dominant versus multiple follicles during ovarian stimulation. The area under the ROC curves of univariable logistic regression for the prediction of multi-follicular growth are depicted in Table 1. Backward stepwise multivariate analyses of initial screening characteristics was performed for the prediction of developing multi-follicular growth after gonadotropin induction of ovulation using a step down regimen (Table 2).

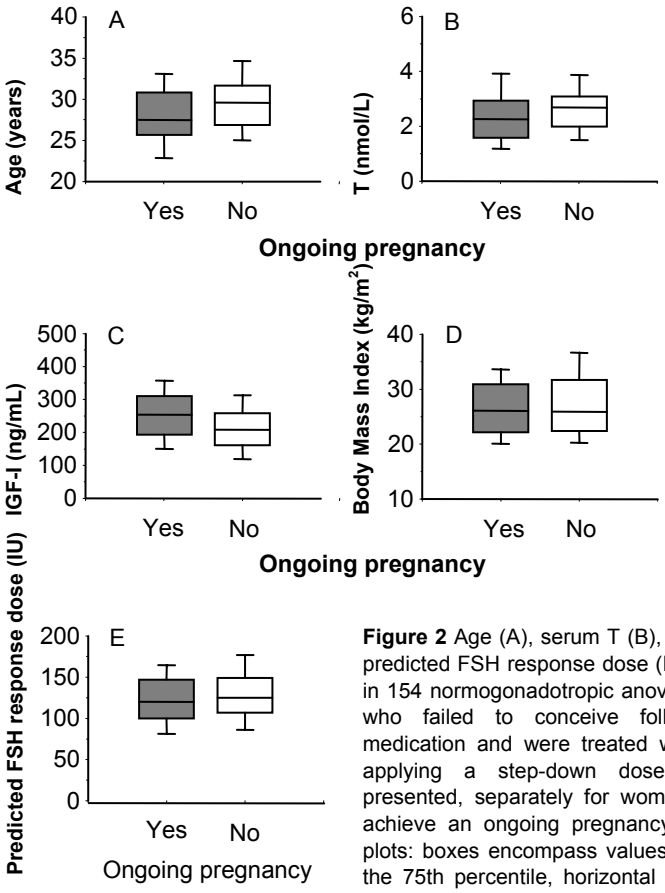

Figure 2 Age (A), serum T (B), IGF-I (C), BMI (D), and predicted FSH response dose (E) as prognostic factors in 154 normogonadotropic anovulatory infertile patients who failed to conceive following preceding CC medication and were treated with ovulation induction applying a step-down dose regimen. Data are presented, separately for women who did or did not achieve an ongoing pregnancy, as box and whisker plots: boxes encompass values between the 25th and the 75th percentile, horizontal lines represent median values, and whiskers give the 95% range of values. Only age, serum T, and IGF-I were included in the final model.

Initial serum AD and mean number of follicles were included in the final model (Area under the ROC curve = 0.62). The impact of initial screening characteristics as a prognostic factor in 154 normogonadotropic anovulatory infertile patients on prediction of single dominant follicle development has been depicted in Figure 1.

Seventy-nine women (51%) conceived. Twelve (15%) miscarried and the remaining ongoing pregnancies ended in 60 singleton, five twin, one triplet and one quadruplet pregnancy. The differences found in initial parameters between those who did or did not have an ongoing pregnancy are depicted in Table 3.

TABLE 2 Multivariate analyses of screening parameters for the prediction of developing multi-follicular growth or ongoing pregnancy following exogenous gonadotropin induction of ovulation in women with normogonadotropic anovulatory infertility.

Prediction of multi-follicular growth (Logistic regression)			
		Odds Ratio	95% Confidence Interval
	AD (nmol/L) (range 4.5 -49.3)	1.04	1.01 - 1.07
	Number of follicles (n) (range 2.0 - 34.5)	1.04	1.00 - 1.09
Final model (Area under the ROC curve)		0.62	0.56 - 0.67
Prediction of ongoing pregnancy (Cox regression)			
		Fecundability Ratio	95% Confidence Interval
	Age (years) (range 19.0 - 40.0)	0.96	0.92 - 1.0
	T (nmol/L) (range 0.6 - 6.8)	0.71	0.55 - 0.92
	IGF-I (ng/mL) (range 64 - 440)	1.004	1.001 - 1.007
Final model (Area under the ROC curve)		0.67	0.57 - 0.77

In univariate Cox regression analysis, initial serum T and IGF-I were significantly different between patients who did or did not have an ongoing pregnancy following gonadotropin induction of ovulation. Multivariate Cox regression analyses of screening parameters for prediction of ongoing pregnancy following gonadotropin induction of ovulation using a step-down regimen in 154 anovulatory infertile patients was performed (Table 2). In the final model, the multivariate fecundability ratio (95% CI) of age was 0.96 (0.92-1.0). This means that a 1 year increase in a given patient's age reduces her monthly probability of ongoing pregnancy following exogenous gonadotropin induction of ovulation by 4%. The impact of individual screening characteristics on treatment outcome (i.e. ongoing pregnancy) is shown in Figure 2.

Age (cut-off of 30 years), ovarian response during preceding CC medication (CRA versus CCF), and initial serum LH concentration (cut-off level of 7.0 IU/L) in univariate analyses for cumulative ongoing pregnancy rates are depicted in Figure 3. The life-table analysis of cumulative ongoing pregnancy rates of the overall group of patients is depicted in Figure 3. A cumulative ongoing pregnancy rate of 58.3% was reached within eight induced cycles.

5.1.4 Discussion

The present study was designed to evaluate whether initial screening characteristics may predict treatment outcome of gonadotropin induction of ovulation, applying a step-down dose regimen in women with anovulatory infertility who failed to ovulate or conceive during previous CC treatment. Although gonadotropin ovulation induction has been the focus of a vast number of investigators during the last four decades, prospective follow-up studies designed to identify screening characteristics capable of predicting treatment outcome are extremely scarce. The current study shows for the first time that AD concentrations and follicle number as assessed by ultrasound upon initial screening can predict the occurrence of multi-follicular cycles (and presumably chances for complications such as multiple gestation and OHSS). Secondly, chances for ongoing pregnancy can be predicted on the basis of age of the woman, T and IGF-I concentrations.

TABLE 3 Initial clinical, endocrine, and ultrasound screening characteristics (mean ± SD) and sperm parameters of the spouse (median and range) of 154 normogonadotropic anovulatory infertile women separated for patients who did or did not reach an ongoing pregnancy after exogenous gonadotropin induction of ovulation using a step down regimen. [a] Cox regression analyses. [b] Area under the ROC curve (univariate analysis). [c] Arbitrarily defined as one to three per ovary (both ovaries added).

	Overall group (n = 154)	No ongoing pregnancy (n = 87)	Ongoing pregnancy (n = 67)	P[a]	AUC[b]
Clinical parameters					
Age (years)	28.5 ± 4.6	29.1 ± 5.0	27.8 ± 3.7	0.08	0.61
Duration of Infertility (years)	2.5 ± 2.2	2.5 ± 2.4	2.4 ± 1.9	NS	-
Primary infertility (%)	108 (70)	62 (71)	46 (69)	NS	-
Amenorrhea (%)	51 (34)	27 (32)	24 (36)	NS	-
Body mass index (kg/m^2)	27.1 ± 6.1	27.4 ± 6.1	26.7 ± 6.1	NS	-
Waist-to-hip ratio	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	NS	-
CC resistant anovulation (%)	73 (50)	41 (51)	32 (48)	NS	-
Endocrine parameters					
LH (IU/L)	8.0 ± 4.6	7.9 ± 4.2	8.1 ± 5.2	NS	-
FSH (IU/L)	4.9 ± 1.6	5.0 ± 1.7	4.9 ± 1.5	NS	-
E_2 (pmol/L)	241 ± 125	254 ± 125	224 ± 124	0.07	0.57
AD (nmol/L)	15.8 ± 7.7	15.8 ± 6.7	15.9 ± 9.0	NS	-
T (nmol/L)	2.5 ± 1.0	2.7 ± 1.1	2.4 ± 1.0	0.01	0.60
SHBG (nmol/L)	49 ± 28	52 ± 30	45 ± 24	0.06	0.58
FAI (100 x T/SHBG)	7.8 ± 9.2	8.5 ± 11.6	6.8 ± 4.4	NS	-
IGF-I (ng/mL)	231 ± 81	213 ± 76	251 ± 82	0.01	0.58
Free IGF-I (ng/mL)	2.7 ± 1.7	2.6 ± 1.7	2.9 ± 1.6	0.1	0.58
Leptin (ng/mL)	20.4 ± 15.5	21.9 ± 17.8	18.8 ± 12.3	NS	-
Ultrasound parameters					
Total stroma score[c]	3.7 ± 1.3	3.7 ± 1.4	3.8 ± 1.1	NS	-
Mean ovarian volume (mL)	10.8 ± 4.8	11.1 ± 5.2	10.5 ± 4.3	NS	-
Mean follicle number	13.7 ± 6.2	13.9 ± 6.7	13.4 ± 5.4	NS	-
Total motile sperm count (TMC)	60 (0 - 662)	53 (0 - 428)	72 (1 - 662)	NS	-
% Normal morphology	25 (0 - 90)	25 (0 - 52)	24 (8 - 90)	NS	-

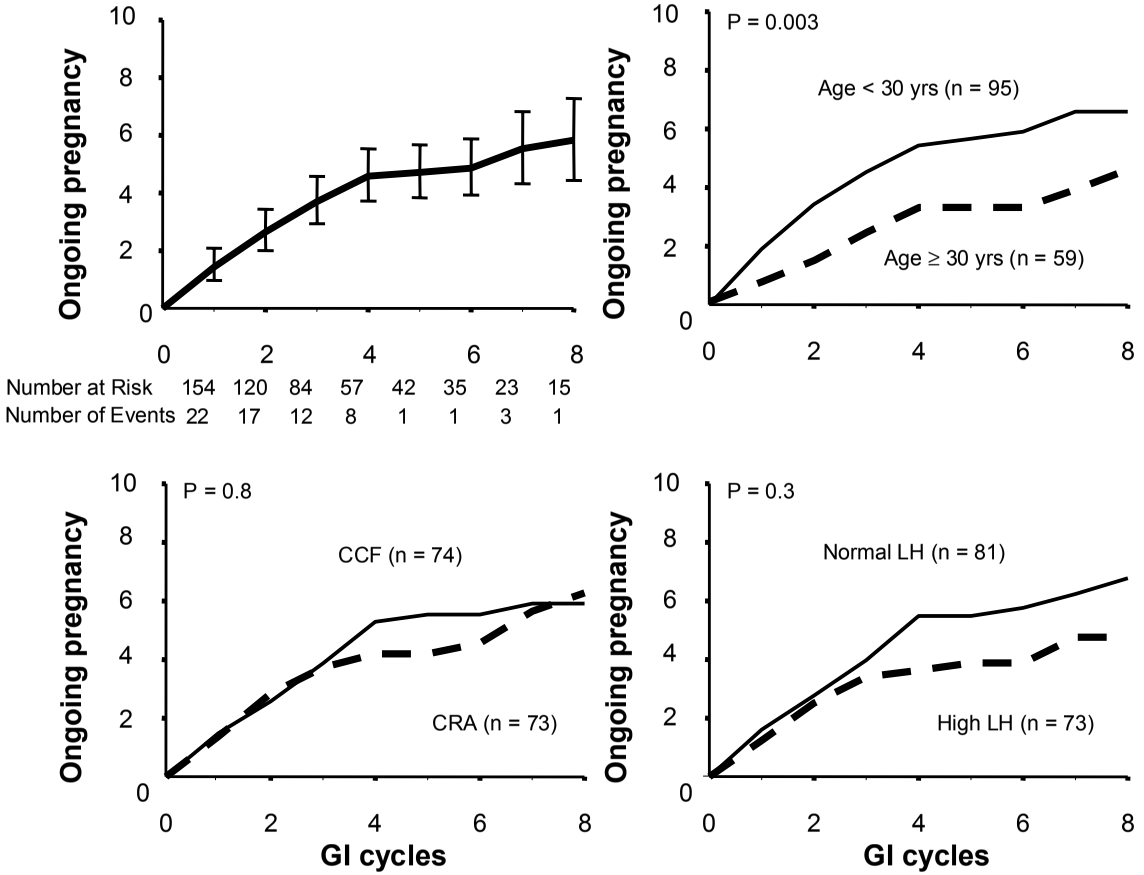

Figure 3 Life-table analysis of cumulative ongoing pregnancy rates in 154 normogonadotropic oligoamenorrheic infertile patients who failed to ovulate or conceive following preceding CC medication and were treated with gonadotropin induction of ovulation. Ongoing pregnancy rates (including absolute number of patients at risk and number of events (i.e. ongoing pregnancies)) are presented for the total study group (upper left panel) (vertical lines represents 95% confidence intervals). Univariate analysis of ongoing pregnancies are shown for the following initial screening characteristics: patient's age (cut-off level at 30 years) (upper right panel), ovarian response to preceding CC medication (CRA versus CCF) (lower left panel) and initial serum LH concentrations (cut-off level at 7.0 IU/L) (lower right panel). *n* represents the initial number of patients at risk.
P = log rank test P value.

These models might be of clinical importance, since they discriminate between women with low or high chances for success (i.e. ongoing pregnancy) or complications (i.e. multi-follicular development). The probability to reach a conception resulting in an ongoing pregnancy lower than 30% was predicted in 10% out of the 154 patients. The apparent c–statistic of the model for ongoing pregnancy was 0.67, indicating a moderate discriminative ability. For instance, a woman with AD concentrations and follicle numbers of respectively 10.0 and 7.0 has a chance for multi-follicular development of 35%. This is in contrast with a chance of 66% for a woman with an AD level of 25.0 and a mean follicle number of 22.5. In practical terms, this may mean that differences are not large enough for practitioners to counsel their patients in terms of multi-follicular growth. It should be emphasized that the predictive power is relatively limited with an AUC of the ROC curve of 0.62 for prediction of multi-follicular cycles. Moreover, these observations need to be confirmed in an independent patient population (external validation) before this approach could be introduced in clinical practice.

Women with PCOS seem to be at risk for multi-follicular development in response to gonadotropin stimulation (MacDougall *et al.*, 1992a). The observed association between mean follicle number and multiple follicle development confirms previous findings applying a low-dose step-up protocol (van der Meer *et al.*, 1998). Others recently described a correlation between initial ovarian volume and subsequent response (Lass *et al.*, 2002). Indeed, sonographic parameters also predict patients remaining anovulatory following CC (Imani *et al.*, 1998).

As previously reported, ovarian response to CC and initial serum FSH were major factors predicting an augmented FSH response dose (Imani *et al.*, 2002a; van Santbrink *et al.*, 2002), which may represent the severity of ovarian abnormalities in these patients (Imani *et al.*, 2002a). Similar factors also predict ovarian response in terms of mono- or multi-follicular development in the current study. Women with low levels of initial serum FSH and resistant to CC (CRA) have a higher chance of developing multi-follicular growth compared with women with normal FSH serum concentrations who failed to conceive during ovulatory CC cycles (CCF). On the other hand, the FSH response dose itself is not predictive of chances for multiple follicle growth. Further studies regarding the association of these factors are required to clarify this issue. Pathophysiological mechanisms underlying mono- and multi-follicular development are still unknown. Additional studies should also investigate the possible cycle-to-cycle variability in the occurrence of multi-follicular development.

In the current study, CRA patients appear to be more likely to develop multiple follicle growth and therefore an increased chance of cancellation of the stimulated cycle. However, the present study demonstrates for the first time that patients who remain anovulatory following CC have similar chance for ongoing pregnancy during gonadotropin induction of ovulation compared with women who

failed to conceive despite the preceding CC-induced ovulatory cycles. These observations may have important clinical implications, since there seems no reason to withhold FSH from these women as second line treatment, as often stated. CRA patients have no chance of conception due to persistent anovulation, whereas CCF patients ovulate but do not conceive following consecutive ovulatory CC-induced cycles, presumably due to impaired cervical mucus quality or endometrial receptivity associated with direct anti-estrogenic effects of CC in the reproductive tract (Imani *et al.*, 1999). These effects are counterbalanced once exogenous FSH preparations are used.

In the present analysis, a marked impact of initial serum T and IGF-I on ongoing pregnancy rates was observed in women with anovulatory infertility. Differences in patient selection could possibly explain why these associations were not observed by others (White *et al.*, 1996). However, many authors have reported that both T (Nelson *et al.*, 1999) and the IGF system (van Dessel *et al.*, 1999) are involved in ovarian dysfunction in PCOS. It could be postulated that alterations in ovarian androgen synthesis will lead to aberrant folliculogenesis during gonadotropin stimulation. The predictive power of initial serum IGF-I was the highest. Age entered in the last step of the multivariate analysis. The association of advanced age with reduced treatment outcome (i.e. ongoing pregnancy) following CC-induced or FSH-induced cycles has previously been reported (McClure *et al.*, 1993; Imani *et al.*, 2002b), as for many other interventions, including IVF. This may be due to an increased incidence of chromosomally abnormal oocytes resulting in reduced fertilization and an increased incidence of aneuploid embryos (Macklon *et al.*, 2002).

Surprisingly, the current study suggests that obesity, the main factor involved in prediction of exogenous FSH requirement for ovarian response, is not a predictor for multi-follicular growth. Moreover, factors associated with obesity such as BMI, WHR or serum leptin level also do not predict treatment outcome of exogenous FSH induction of ovulation, as previously reported by others (Dale *et al.*, 1993; Dale *et al.*, 1998). Weight reduction in women with PCOS may normalize insulin resistance, androgen metabolism and improve outcome of ovulation induction (Norman *et al.*, 2002). It has also been suggested that the incidence of spontaneous miscarriage increases with increasing BMI in women with PCOS (Hamilton-Fairley *et al.*, 1992; White *et al.*, 1996). It should be noted, however, that some of these conclusions were drawn after inclusion of a selected group of non-obese women (BMI < 27). Moreover, accurate information regarding preceding CC treatment is often lacking. The present analysis does not confirm a possible association of increased BMI with pregnancy outcome, as already described by others (McClure *et al.*, 1993).

Initial serum LH concentrations predict chances for multiple follicle development but not ongoing pregnancy in the present study. In addition, no

significant increase in the chance of spontaneous miscarriages was observed in patients with an elevated initial serum LH level. The findings are in contrast with retrospective previous reports indicating a higher percentage of miscarriages in these patients (Homburg *et al.*, 1988; Balen *et al.*, 1993a). However, the small number of miscarriages occurring in most studies preclude definitive conclusions in this regard. In addition, in previous studies, initial LH concentrations were not predictive for chances of ovulation and conception after CC medication (Imani *et al.*, 1998; Imani *et al.*, 1999).

In summary, the present longitudinal follow-up study demonstrates that normogonadotropic anovulatory women who exhibit elevated AD concentrations along with an increased number of ovarian follicles prior to ovarian stimulation are at an increased risk of multiple follicle development during gonadotropin ovulation induction. They exhibit higher chances for cancellation of the FSH-induced cycle. Furthermore, this study suggests that gonadotropin ovulation induction should be considered as a treatment strategy with high chances of ongoing pregnancy, particularly in young patients (both CRA and CCF) who exhibit elevated initial IGF-I concentrations and T levels within the normal range. Those women, who are more likely to fail to conceive following exogenous FSH stimulation, may benefit from alternative treatment modalities such as insulin-sensitizing agents, laparoscopic ovarian surgery or assisted reproduction. However, these options should be evaluated in future prospective studies.

Acknowledgement

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5.2 Patient predictors for outcome of gonadotropin ovulation induction in women with normogonadotropic anovulatory infertility: a meta-analysis

Abstract

A systematic review was conducted to determine if initial screening characteristics of women with normogonadotropic anovulatory infertility, predict clinically significant outcomes of ovulation induction with gonadotropins, and to obtain pooled estimates of their predictive value through meta-analysis. Relevant studies were identified by a search strategy which consisted of MESH headings and a check of bibliographies. Only those studies in which pre-treatment screening characteristics (such as body mass index (BMI), serum luteinizing hormone (LH) and androgens, insulin sensitivity and ultrasound appearance of ovaries) were related to outcome parameters (such as total amount of FSH administered, cancellation, ovulation, pregnancy and miscarriage), were included in this analysis. Studies reporting relationships between initial screening characteristics and outcome parameters of ovulation induction as measures of association (e.g. Odds ratios) were pooled if at least 2 studies report an association. The measures of association were pooled using inverse variance weighting.

Thirteen studies fulfilled the inclusion criteria. A positive association was seen in all studies between the level of obesity (definition applied as assessed by individual studies) and total amount of FSH administered (Weighted Mean Difference (WMD) of 771 IU (95%CI: 700 - 842)). Pooled Odds ratios of 1.86 (95% CI: 1.13 - 3.06) and 0.44 (95% CI: 0.31 - 0.61) were found between obesity with cancellation and ovulation, respectively. Pooled analysis did not show a significant association between obesity and pregnancy rate. The pooled Odds ratio for obese versus non-obese women and miscarriage rate was significant (3.05 (95% CI: 1.45 - 6.44)). Association measures between insulin resistance (definition applied as assessed by individual studies) and total amount of FSH administered produced a weighted mean difference (WMD) of 351 (95% CI: 73 - 630) IU. A pooled Odds ratio of 0.29 (95% CI: 0.10 - 0.80) was found for insulin resistance with pregnancy rate. The pooled Odds ratio for insulin resistance (hyperinsulinemia versus normoinsulinemia) and miscarriage rate was not significant. A pooled Odds ratio of 1.04 (95% CI: 1.01 - 1.07) was found for LH (IU/L) with pregnancy rate. The pooled Odds ratio for LH and miscarriage rate was not significant. Finally, pooled analysis did not find a significant association between testosterone (T) and pregnancy rate. The included studies did not address possible links between initial sonographic parameters and treatment outcome.

This analysis suggests that the most clinically useful predictors of gonadotropin ovulation induction outcome in normogonadotropic women are

obesity and insulin resistance. These findings might be of clinical relevance since they discriminate between women with low or high chances for success. However, the best available evidence testing measures of association between screening characteristics and treatment outcome is limited.

5.2.1 Introduction

Chronic anovulation presents with amenorrhea or oligomenorhea and can be classified on the basis of serum follicle-stimulating hormone (FSH) and estradiol (E_2) levels. Hypogonadotropic anovulation, low levels of gonadotropins and negligible estrogen activity, is also referred to as World Health Organization (WHO) group I. Hypergonadotropic anovulation is characterized by elevated gonadotropin levels and low E_2 (WHO group III) (Lunenfeld and Insler, 1974; The ESHRE Capri Workshop Group, 1995; Rowe *et al.*, 2000). Normogonadotropic anovulation (FSH and E_2 levels within the normal range) (WHO group II) represents the most common form of ovarian dysfunction and is a frequent cause of infertility (Rowe *et al.*, 2000; Laven *et al.*, 2002).

Clomiphene citrate (CC) has been used worldwide as the medication of first choice for the treatment of these women, because it is safe, convenient, cheap, and reasonably effective. The risk of developing ovarian hyperstimulation syndrome (OHSS) and multiple gestation is limited (2-3%) (Imani *et al.*, 1999). However, a significant proportion (23%) of women remains anovulatory following CC (Imani *et al.*, 1998). A cumulative pregnancy rate of 73% is reported in ovulatory CC women (Imani *et al.*, 1999).

Induction of ovulation using exogenous gonadotropins is generally indicated in patients with normogonadotropic anovulatory infertility who have failed to ovulate or conceive during previous CC treatment (Schwartz and Jewelewicz, 1981; Lunenfeld *et al.*, 1985; Insler, 1988; Kelly and Jewelewicz, 1990; Franks and Gilling-Smith, 1994; Fauser and van Heusden, 1997). Since the early 1960s many anovulatory patients have been treated with human menopausal gonadotropin (hMG) and human chorionic gonadotropin (hCG) to induce ovulation. This treatment modality has been proven to be effective, but the risks of OHSS (Stephenson, 1991; Navot *et al.*, 1992) and multiple pregnancies are considerably increased (Schenker *et al.*, 1981; Fauser and van Heusden, 1997).

Recent studies have focused on the prediction of ovulation induction outcome based upon initial screening characteristics of WHO II anovulatory infertile women (Imani *et al.*, 1998; Imani *et al.*, 1999; Imani *et al.*, 2002b; Mulders *et al.*, 2003a). It could be demonstrated that some clinical, sonographic, and endocrine characteristics are predictive of ovulation and conception during CC treatment (Imani *et al.*, 1998; Imani *et al.*, 1999; Imani *et al.*, 2000; Imani *et al.*, 2002b).

Outcome parameters of gonadotropin treatment in these women correlated with woman's age, ovarian response to preceding CC medication, body mass index (BMI), the mean follicle number assessed by ultrasound, serum levels of FSH, testosterone (T), androstenedione (AD), and initial insulin-like growth factor-I (IGF-I) (Imani *et al.*, 2002a; Mulders *et al.*, 2003a).

The aim of gonadotropin ovulation induction in anovulatory infertility is healthy live birth, preferably from a singleton pregnancy. This is often hard to achieve despite the recent introduction of low-dose incremental or decremental regimens (Fauser and van Heusden, 1997). An individualized treatment regimen, based on valid outcome predictors, might optimize ovulation induction strategies by improving the balance between success and complications. The existing literature concerning the prediction of outcome is limited and ambiguous. Some studies observe significant associations, whereas others fail to do so. Therefore, a systematic review was undertaken to establish more firmly which screening parameters are predictive of outcome of gonadotropin induction of ovulation.

5.2.2 Methodology

The objectives of this review were to determine whether screening criteria applied to women with normogonadotropic anovulatory infertility, predict clinically significant outcomes of ovulation induction with gonadotropins, and to obtain pooled estimates of their predictive value through meta-analysis.

Criteria for considering studies for this review

Studies reporting gonadotropin ovulation induction in women with normogonadotropic anovulatory infertility (WHO II, including polycystic ovary syndrome (PCOS)) were considered for inclusion if they provided specific information on: the regimens and type of gonadotropin administered, e.g. standard protocol, step-up protocol, step-down protocol, hMG, urinary derived (u)FSH and recombinant (r)FSH. The following primary outcome measures were sought: mono-follicular growth (arbitrarily defined as one follicle > 15 mm on the day of hCG (van Santbrink and Fauser, 1997)), total amount of gonadotropins administered on the day of hCG in international units (IU), cancellation rate (cycle where there is no hCG administered), ovulation rate (as confirmed by an increased serum progesterone (P) level (> 20 nmol/L) in the luteal phase), pregnancy rate (per cycle or per patient)(defined as a positive urinary pregnancy test) and miscarriage rate (sonographic assessment of absence of an intrauterine gestational sac with heart beat at 12 weeks amenorrhea). The following screening characteristics were also sought: age (years), cycle history (oligo- or amenorrhea), BMI (kg/m^2), response during previous CC treatment (clomiphene citrate-resistant anovulation

(CRA)/clomiphene citrate failure (CCF)), ovarian volume (mL), total number of follicles (both ovaries) (Pache *et al.*, 1992a), ovarian stroma echogenicity (Dewailly *et al.*, 1994), serum levels of T, AD, luteinizing hormone (LH), LH/FSH, fasting insulin and glucose. Inclusion was limited to studies in which outcome parameters were related to pre-treatment screening characteristics.

Search strategy for the identification of studies

Studies reporting the prediction of outcomes following gonadotropin induction were initially identified through a handsearch (no specific criteria: papers at hand were considered). The wide variety of keywords used in these reports provided the foundation for the final search strategy. It consisted of: (1) a Medline search by means of MESH headings (in the following order): (follicle stimulating hormone [majr] OR menotropins [majr]) AND "female genital diseases and pregnancy complications" [majr] and (2) a check of the bibliographies of identified studies.

Identification

Through the MESH headings search strategy ((follicle stimulating hormone [majr] OR menotropins [majr]) AND "female genital diseases and pregnancy complications" [majr]) 631 titles were identified (1986 to October 2002). For 474 titles it was clear that population or intervention did not fulfil the selection criteria. To verify whether it was appropriate to exclude such articles based solely on titles, one of us (AM) read 10 of the 474 articles. None fulfilled the inclusion justifying this identification strategy. The remaining 157 articles were then read by one author (AM). Twenty-three studies fulfilled the selection criteria. All of their bibliographies were checked. This identified one additional study for inclusion.

Twenty-four potentially relevant studies were read by all authors and 13 were included. There were no disagreements between authors regarding the inclusion of studies.

Methods of the review

The following information was extracted from the potentially relevant studies: study characteristics, specified as observational, cohort, cross-over, consecutive or randomized, multicentre or not, method of randomization, number of patients/cycles (randomized, excluded and analysed), duration, timing and location of the study. Patient characteristics were recorded: definition of normogonadotropic anovulatory infertility (WHO II including PCOS) (clinical, biochemical, ultrasonographic markers or combination of the former), definition and duration of infertility, age, investigative work-up, other causes of infertility and previously administered treatment(s), in particular whether previous treatment with CC had been tried and how CRA or CCF was defined. Finally, the outcome measures and their specific definitions were also recorded: total amount of exogenous FSH

administered (IU), duration of administration of exogenous FSH (days), the number of cancelled cycles, the number of cycles with multi- or mono-follicular growth, the number of ovulatory cycles, the number of patients pregnant and not pregnant, miscarriage rate, multiple pregnancy rate and OHSS rate. A study had to give either a direct measure of association between predictor and outcome variables or present data that allowed for the calculation of such a measure. Studies reporting relationships between initial screening characteristics and outcome parameters of ovulation induction as measures of association (Odds ratio: OR) and studies from which measures of association could be derived from the data given were included. For example, if a study reported the mean and standard deviation (SD) of an outcome variable (e.g. cancellation rate) for obese and for lean women separately, the OR of cancellation rate for obesity could be calculated assuming a normal distribution of the outcome variable in both groups, by the formula: ln(OR) = ($mean_{obese}$ - $mean_{lean}$)/(pooled variance in $_{obese}$ and $_{lean}$).

5.2.3 Results

Studies excluded

Eleven potentially relevant studies were excluded (Table 1). These included application of modified stimulation schemes (Norfolk (1, 3, 5) regimen or administration of 150 IU every other day) (Ginsburg and Hardiman, 1991; Remorgida *et al.*, 1991), application of modified controlled ovarian hyperstimulation followed by intrauterine and/or intraperitoneal insemination for PCOS and normo-ovulatory patients (Zullo *et al.*, 1996), and comparison of two different stimulation regimens for a different subset of patients (repetitive cycles, not equally distributed) (Shoham *et al.*, 1991).

Six studies stated insufficient data to allow analysis: only P levels were noted for significant and non-significant prediction of duration of treatment by screening parameters (Coelingh Bennink *et al.*, 1998), insufficient data were provided to calculate OR (respectively age versus conception) (Ginsburg and Hardiman, 1991), no clear statement of the background of LH levels supplied (Hamilton-Fairley *et al.*, 1991), only data of LH pre-treatment versus ovulation for a subset of patients (Polson *et al.*, 1987), no original data of LH levels (separately for pregnant and non-pregnant women) (Strowitzki *et al.*, 1994). Finally, data for different subsets of patients (WHO I and II) were not provided separately (Fluker *et al.*, 1994).

In one study (Abdel *et al.*, 1990), Pearson's correlation statistics showed a significant positive correlation between the BMI and the dose of gonadotropins ($r = 0.4666$; $P < 0.001$). This dose correlated negatively with ovarian volume ($r = -0.1958$; $P = 0.01$). Since these correlation coefficients could not be incorporated in the pooled analysis, these data were not included.

One study (Imani *et al.*, 2002a) provided significant correlations between the amount of exogenous FSH required for ovarian response (sonographic visualization of a follicle ≥ 10 mm (Pache *et al.*, 1990)) and initial clinical, sonographic and endocrine screening characteristics. Four of these parameters (i.e. ovarian response to CC medication (CRA), BMI, initial serum levels of FSH and free IGF-I) were included in the multivariate model to predict the FSH response dose (i.e. the amount of exogenous FSH required for ovarian response). Since this study only reported one specific outcome parameter that was not considered in the review, this study was excluded from the pooled analysis.

Methodological quality of included studies

A total of 13 studies were included in the current review (Table 2) (Sagle *et al.*, 1991; Hamilton-Fairley *et al.*, 1992; McClure *et al.*, 1992; Dale *et al.*, 1993; McClure *et al.*, 1993; Farhi *et al.*, 1993; Balasch *et al.*, 1996; White *et al.*, 1996; Fulghesu *et al.*, 1997; Dale *et al.*, 1998; Strowitzki *et al.*, 1998; Yarali *et al.*, 1999; Vicino *et al.*, 2000). Some researchers performed several studies concerning outcome of ovulation induction in the same patient group of interest (Sagle *et al.*, 1991; McClure *et al.*, 1992; McClure *et al.*, 1993; White *et al.*, 1996). However, these studies appeared to include a different subset of patients (Dale *et al.*, 1993); Dale *et al.*, 1998) or the focus (screening characteristic versus outcome parameter) of the studies was different (Sagle *et al.*, 1991; Hamilton-Fairley *et al.*, 1992; McClure *et al.*, 1992; McClure *et al.*, 1993; White *et al.*, 1996).

Description of participants

The definitions of normogonadotropic anovulatory infertility (WHO II) and PCOS varied between centres, as detailed in Table 2. Patients suffering from WHO II anovulatory infertility were included (Farhi *et al.*, 1993; Balasch *et al.*, 1996; Yarali *et al.*, 1999). The most comprehensive definition of PCOS specified as a combination of clinical features (oligoamenorrhea), biochemical parameters (increased androgen concentrations) and polycystic appearance of ovaries on ultrasound scan (enlarged ovaries with multiple small follicles), was used in a number of studies (McClure *et al.*, 1992; Fulghesu *et al.*, 1997; Vicino *et al.*, 2000). Various combinations of clinical, biochemical and ultrasonographic findings were also used: ultrasound and clinical, or biochemical (Sagle *et al.*, 1991; Hamilton-Fairley *et al.*, 1992; Dale *et al.*, 1993; Dale *et al.*, 1998; Strowitzki *et al.*, 1998), clinical and ultrasound and/or biochemical (McClure *et al.*, 1993), or clinical and ultrasound (White *et al.*, 1996). According to these definitions oligoamenorrhea is not present in all patients per se (McClure *et al.*, 1992; Dale *et al.*, 1993; Farhi *et al.*, 1993; Balasch *et al.*, 1996; Dale *et al.*, 1998; Strowitzki *et al.*, 1998).

TABLE 1 Characteristics of studies regarding gonadotropin ovulation induction in women with normogonadotropic anovulatory infertility who were excluded (reasons for exclusion are depicted in bold in "Comments" section) for the current meta-analysis.

Study	Methodology	Participants	Interventions	Screening parameters[a]	Outcome parameters[a]	Numbers	Comments
Abdel *et al.* (1990)	Randomized controlled trial	PCOS; All CRA.	Electrocautery or gonadotropins Step-up regimen: hMG or uFSH; starting dose: 75 IU; first dose ↑ after 1 wk: 75 IU; subsequent cycle starting dose: individually adjusted; hCG (5000 IU): 1 follicle ≥ 18 mm + increased E_2; cancellation criteria: 3 follicles ≥ 15 mm; OHSS: not reported.	BMI (kg/m^2); T (nmol/L); Ovarian volume (ml).	Total amount of FSH administered (ampoule: 75 IU); Conception.	59 patients; 233 cycles.	Monitoring through transabdominal ultrasound? **Significant Pearson's correlation between BMI and ovarian volume with the dose of gonadotropins.** Correlation for testosterone with pregnancy is provided for both treatments (n = 88 patients). Correlations for age, BMI and mean ovarian volume with viable/non-viable pregnancies are provided for both treatments (n = 88 patients).
Coelingh Bennink *et al.* (1998)	Prospective multicenter randomized rrial	WHO II; CRA + CCF.	Step-up regimen: rFSH s.c. versus uFSH i.m.; starting dose: 75 IU; ovarian response: 1 follicle ≥ 12 mm; first dose ↑ after 14 days: 37.5 IU; subsequent dose ↑ after 1 week: 37.5 IU; subsequent cycle: first dose ↑ after 1 week; maximum dose: 225 IU/day; hCG (10000 IU): 1 follicle ≥ 18 mm or 2-3 ≥ 15 mm; cancellation criteria: > 3 follicles ≥ 15 mm or no response after 42 days; OHSS: reported.	Cycle history; Age (years); Duration infertility (years); Type of infertility (p/s); BMI (kg/m^2); LH/FSH.	Duration of stimulation (days)	172 patients; 172 cycles.	Inclusion criteria: Age: 18- 39 (years); BMI: 19-32 (kg/m^2). Definition of CRA: dose ? Statistical testing for secondary outcome parameters based solely on first cycle data. **Significant (only P-level noted) and non-significant prediction of duration of treatment by screening parameters (no data provided).**
Fluker *et al.* (1994)	Retrospective observational study	WHO II: Oligomenorrheic/ Hyperandrogenic/ Luteal phase defect (endo/histol); CRA + CCF. (WHO I)	Step-up regimen: hMG i.m./i.v.; starting dose: 75-450 IU/day; hCG (10000 IU): 1-4 follicles ≥ 16 mm + E_2 > 3600-4500 pmol/L; luteal support; OHSS: not reported.	Age (years)	Cumulative Conception Rates (conception: gestational sac or histological criteria)	118 patients; 396 cycles.	18 year span of the study. Hyperandrogenic sub-group WHO2: 39 of 49 cycles concomitant corticosteroids. h**MG (i.m.: 385 cycles and i.v.: 83 cycles): mode of administration not clearly for WHO I and II separately.** Luteal support. **Prediction of cumulative conception rates based upon data of both patient groups (WHO I+II).**

Study	Methodology	Participants	Interventions	Screening parameters[a]	Outcome parameters[a]	Numbers	Comments
Ginsberg *et al.* (1991)	Cohort study	WHO II; All failed to respond to CC.	Norfolk (1, 3, 5) regimen: modified step-up; hMG; hCG (5000 IU): 1 follicle ≥ 18 mm; OHSS: not reported.	Age (years)	Conceptionrate	93 patients	12 year span of the study. Definition of failure to respond to CC not clearly stated. **Norfolk regimen: modified step-up. Insufficient data provided to calculate OR (age with conceptionrate).**
Hamilton-Fairley *et al.* (1991)	Observational Study	WHO II: oligoamenorrhea + TVS-PCO[b,c] + (↑ LH, ↑T and/or both); All CRA.	Step-up regimen: hMG i.m. or uFSH i.m.; starting dose: 75 IU; ovarian response: 1 follicle ≥ 12 mm; first cycle: first dose ↑ after 14 days: 37.5 IU; further dose ↑ after 7 days: 37.5 IU; subsequent cycle: starting dose: 52.5-75 IU/day; first dose ↑ after 1 week; maximum dose: 225 IU/day, hCG (5000 IU): 1 follicle ≥ 18 mm; cancellation criteria: > 3 follicles ≥ 15 mm; OHSS: reported.	LH (IU/L)	Ovulation (progesterone ≥ 30 nmol/L); Pregnancy (serum hCG > 25 IU/L and presence of an intrauterine gestational sac); Early pregnancy loss (failure of fetus to develop > 8 weeks gestation).	100 patients; 401 cycles.	Exclusioncriteria: BMI > 28. **Data LH (CD?). Data LH per pt / cy?**
Imani *et al.* (2002)	Prospective observational study	WHO II; CRA + CCF.	First cycle: Step-up regimen: uFSH i.m. versus rFSH s.c.; starting dose: 75 IU; ovarian response: 1 follicle ≥ 10 mm; first dose ↑ after 7 days: no ovarian response: 37.5 IU; subsequent dose ↑ after 1week: 37.5 IU; hCG (5000 IU): 1-2 follicles ≥ 18 mm; cancellation criteria: ≥ 3 follicles ≥ 16 mm.	Cycle duration; CRA/CCF; BMI (kg/m^2) FSH (IU/L); FAI[d] (100 X T/SHBG); Insulin (mU/L); Free IGF-1 (ng/ml); IGFBP-1 (ng/ml); Leptin (ng/ml); Ovarian volume (ml).	FSH response dose (ovarian response equal to sonographic vizualization of a follicle ≥ 10 mm)	90 patients; 90 cycles.	Significant correlation between response during CC treatment (CRA), BMI, FSH and free IGF-1 with FSH response dose (multi - variate model provided). **Correlations for 1 specific outcome parameter (i.e. FSH response dose) are provided; no further data on treatment outcome available.**
Polson *et al.* (1987)	Prospective observational study	WHO II; All CRA.	Step-up regimen: uFSH s.c.; infusionpump: pulse every 90 minutes: 5 IU; starting dose: 75 IU daily; first dose ↑ after 2 weeks: 37.5 IU; subsequent dose ↑ after 1 week: 37.5 IU; maximum dose: 225 IU/day, hCG (3000 IU): 1 follicle ≥ 16 mm; subsequent cycle: starting dose: 75 IU daily; first dose ↑ after 1 week: 37.5 IU; luteal support; OHSS: not reported.	LH (IU/L); FSH (IU/L).	Ovulation (TVE and > progesterone)	10 patients; 33 cycles.	All patients previously underwent a variety of treatments (i.e. romocriptine (9 patients), tamoxifen (1), hMG (5), ovarian wedge resection (2), GnRH (2), CC (10). Dose CRA not stated. **No pre-treatment LH data on cycle level for the total group. Data of LH pre-treatment with ovulation are only provided for ovulatory cycles (12 out of 33).**

Study	Methodology	Participants	Interventions	Screening parameters[a]	Outcome parameters[a]	Numbers	Comments
Remorgida *et al.* (1991)	Prospective crossover study	WHO II; CRA + CCF.	GnRH agonist plus gonadotropins (A) versus gonadotropins alone (B). A: GnRh + uFSH: GnRh: starts CD 2; FSH: CD 5+7+9: dose 75 IU; FSH stop in case of follicular selection: 1 follicle > 12 mm and E_2 > 150 pg/ml; hCG (5000 IU): ≥ 1 follicle ≥ 18 mm and E_2 > 250 pg/ml. B: uFSH: FSH: CD 3+5+7: dose 150 IU; CD 8: individualized therapy: dose adjustment: 0-4 ampoules per day; hCG (5000 IU): ≥ 1 follicle ≥ 18 mm and E_2 > 250 pg/ml, cancellation criteria: > 3 preovulatory follicles and/or E_2 > 2000 pg/ml; luteal support; OHSS: not reported.	Cycle history; Age (years); BMI (kg/m^2); Testosterone (ngl/mL); LH/FSH; SHBG (nmol/L).	Duration of stimulation (days); Total amount of FSH administered (ampoule:75 IU).	4 patients; 4 cycles.	Previous treatment: GOI[e]: all patients hyperresponded; GnRH: all patients underwents at least 3 cycles and no ovulation was observed. **Modified ovarian stimulation regimen.**
Shoham *et al.* (1991)	Observational Study	PCOS and WHO II; All CRA.	Step-up regimen: hpFSH i.m. Low dose: starting dose: 75 IU; first dose ↑ after 1 week: 37.5 IU; subsequent cycle: starting dose: 37.5 IU in case of hyperresponse first cycle. Conventional: starting dose: 75 IU; first dose ↑ after 1 week: 75 IU. cancellation criteria: > 3 follicles ≥ 16 mm; OHSS: not reported.	Cycle history; Duration of infertility (years); Age (years); BMI (kg/m^2); Testosterone (nmol/L); LH (IU/L); LH/FSH.	Duration of stimulation (days); Total amount of FSH administered (ampoule: 75 IU).	8 patients; 24 cycles	Inclusion: 7 PCOS patients and 1 WHO II patient. Dose CRA not stated. Variable previous treatment history for all patients. **Comparison of 2 different stimulation regimens:2 patients: low-dose protocol only; 6 patients: both low-dose and conventional protocol**.
Strowitzky *et al.* (1994)	Prospective observational study	PCOS: TVS-PCO[c] + ≥ 2 criteria; CRA + CCF.	Step-up regimen: uFSH i.m.; starting dose: 75 IU; first dose ↑ after 10-12 days: 37.5 IU; no further dose ↑; hCG (10000 IU): 1 follicle ≥ 16 mm; cancellation criteria: > 3 major follicles or no response; OHSS: reported.	LH (IU/L)	Pregnancy (hCG measurement)	20 patients; 27 cycles.	Included: 1 patient with a regular cycle; 6 couples with andrological factors requiring homologous insemination in 4. Previous treatment: definition of CRA: dose ?; maximum of 30 CC cycles; severe OHSS for 7 patients during conventional GOI[e]. **No significant differences between pregnant and non-pregnant women concerning LH levels (no data provided).**

Study	Methodology	Participants	Interventions	Screening parameters[a]	Outcome parameters[a]	Numbers	Comments
Zullo *et al.* (1996)	Retrospective observational study	PCOS: TVS-PCO[c] + ≥ 1 criterion and normo - ovulatory	Modified controlled ovarian hyperstimulation (COH) followed by IUI and/or IPI. COH: uFSH; starting dose: 75 IU; first dose ↑ after 7 days: 75 IU; subsequent dose ↑ after 3 days: 37.5 IU; hCG (10000 IU); 1-4 follicles ≥ 18 mm + > E_2[f]; OHSS: reported.	Waist:hip ratio (WHR)	Duration of stimulation (days); Total amount of FSH administered (ampoule:75 IU); Number of follicles > 18 mm (day hCG); Ovulation (TVE and > progesterone); Pregnancy.	60 patients (PCOS and normo-ovulatory); 53 cycles (PCOS only).	Previous treatment: not reported. **Modified COH followed by IUI/IPI.** Numbers: 111 cycles: 53 PCOS cycles?

Footnotes:
[a] Screening parameters and outcome parameters outlined: only those parameters which are discussed as (possibly) related.
[b] Presence of polycystic ovaries based on published criteria (Adams et al., 1985; Adams et al., 1986).
[c] TVS-PCO: transvaginal sonography -polycystic ovaries.
[d] FAI: free androgen index defined as 100 x T/SHBG.
[e] GOI: gonadotropin ovulation induction.
[f] > E_2: serum estradiol levels of between 200 and 1500 pg/ml per follicle > 15 mm.

TABLE 2 Characteristics of studies regarding gonadotropin ovulation induction in women with normogonadotropic anovulatory infertility who were included in the current meta-analysis.

Study	Methodology	Participants	Interventions	Screening parameters[a]	Outcome parameters[a]	Numbers	Comments
Balasch *et al.* (1996)	Prospective multicenter study	WHO II; CRA + CCF.	Step-up protocol: uFSH i.m. or hpFSH s.c.; starting dose: 75 IU; ovarian response: 1 follicle ≥ 11 mm; first dose ↑ after 14 days: 37.5 IU; subsequent dose ↑ after 1week: 37.5 IU; subsequent cycle starting dose: 37.5-112.5 IU; maximum dose: 225 IU/day; hCG (10000 IU): 1 follicle ≥ 17 mm; cancellation criteria: ≥ 4 follicles > 14 mm or no response (maximum daily dose: 3 ampoules); luteal support; OHSS: Golan 1989.	LH (IU/L); LH/FSH.	Pregnancy (intrauterine gestational sac)	234 patients; 534 cycles.	Luteal support. No significant correlations for LH and LH/FSH with viable pregnancy/ spontaneous abortion: no data provided.
Dale *et al.* (1993)	Retrospective observational study	PCOS: TVS-PCO[b,c] + ≥ 2 criteria; CRA + CCF.	Step-up regimen: pFSH i.m.; starting dose: 75 IU; ovarian response: 1 follicle ≥ 10 mm; first dose ↑ after 2 weeks: 37.5 IU; hCG (9000 IU): 1 follicle ≥ 18 mm; cancellation criteria: ≥ 4 follicles > 16 mm; OHSS: 2 stages moderate/severe.	BMI (kg/m^2); Insulin resistance (CIGMA-test[d]).	Total amount of FSH administered (IU); Cancellation; Pregnancy (gestational sac or histological criteria).	50 patients; 66 cycles.	Included: 8 patients with a regular cycle; 15 patients previously underwent ovarian wedge resection or electrocautery.
Dale *et al.* (1998)	Prospective observational study	PCOS: TVS-PCO[b,c] + ≥ 3 criteria; CRA + CCF.	Step-up regimen: uFSH i.m.; starting dose: 75 IU; first dose ↑ after 14 days: 37.5 IU; subsequent dose ↑ after 1 week: 37.5 IU; hCG (5000 IU): leading follicle ≥ 18 mm + ≤ 3 follicles ≥ 15 mm; cancellation criteria: ≥ 4 follicles ≥ 15 mm; OHSS: 2 stages moderate/severe.	BMI (kg/m^2)	Total amount of FSH administered (IU); Cancellation.	42 patients; 70 cycles.	19 patients previously underwent ovarian wedge resection or electrocautery. Significant and non-significant correlations for BMI versus outcome (i.e. obese versus non-obese patients).
Farhi *et al.* (1993)	Retrospective observational study	Anovulatory infertility + PCO-TVS[b,c]; CRA + CCF.	Step-up regimen: uFSH i.m. versus hMG i.m. versus hMG i.mm + GnRH; starting dose: 75 IU; first dose ↑ after 5 days: 75 IU; hCG (10000 IU): 1follicle ≥ 16 mm + ↑ E_2, cancellation criteria: > 3 follicles > 17 mm or E_2 > 2000 pg/ml; OHSS: reported.	Cycle history; Age (years); BMI (kg/m^2); Testosterone (ng/ml); LH (IU/L).	Pregnancy	89 patients; 195 cycles.	5 year span of the study. Included: 13 patients with a regular cycle.

Study	Methodology	Participants	Interventions	Screening parameters[a]	Outcome parameters[a]	Numbers	Comments
Fulghesu *et al.* (1997)	Prospective observational study; consecutive series.	PCOS; CCF (+CRA?).	Step-up regimen: uFSH i.m.; starting dose: 150; first dose ↑ after 7 days: 75 IU if E_2 = insufficient; subsequent dose ↑ after 5 days; maximum dose: 225 IU/day; hCG (5000 IU); 1 follicle ≥ 18 mm; OHSS: Golan 1989.	BMI (kg/m^2); Hyperinsulinism (OGTT).	Total amount of FSH administered (IU)	34 patients; 52 cycles.	Definition CRA not clearly stated. Inclusion 8 patients: 1 open tube. Significant correlation between obesity with increased ovarian volume? (no data provided).
Hamilton-Fairley *et al.* (1992)	Retrospective observational study	WHO II; olam + TVS-PCO$_{ul}$ + (↑ LH, ↑T and/or both); All CRA.	Step-up regimen: hMG i.m. or uFSH i.m.; starting dose: 75 IU; ovarian response: 1 follicle ≥ 12 mm; first cycle: first dose ↑ after 14 days: 37.5 IU; further dose ↑ after 7 days: 37.5 IU; subsequent cycle: starting dose: first dose ↑ after 1week; maximum dose: 225 IU/day; hCG (5000 IU): 1 follicle ≥ 18 mm; cancellation criteria: > 3 follicles ≥ 16 mm; OHSS: not reported.	BMI (kg/m^2)	Total amount of FSH administered (IU); (Uni) Ovulatory cycle (Progesterone ≥ 30 nmol/L); Pregnancy (serum hCG > 25 IU/L and presence of an intrauterine gestational sac); Early pregnancy loss (failure of fetus to develop > 8 weeks gestation).	100 patients; 405 cycles.	Exclusion: BMI > 28
McClure *et al.* (1992)	Observational Study	PCOS; CRA + CCF.	Step-up regimen: hMG i.m.; starting dose: 75 IU (maximum 150 IU); first dose ↑ after 5-7 days: 37.5 IU; subsequent cycle: starting dose: 37.5 -225 IU/day; hCG (3000 IU): 1 follicle ≥ 16 mm; cancellation criteria: > 3 follicles > 14 mm + > E_2; luteal support (failing CL 9 days); OHSS: not reported.	BMI (kg/m^2)	Total amount of FSH administered (IU)	71 patients; 224 cycles.	Luteal support (failing corpus luteum 9 days) N.?; Significant correlation between BMI with total amount of FSH administered (IU) (no data provided: only P-value). No significant correlation between BMI and pregnancy outcome (no data provided: only P-value).
McClure *et al.* (1993)	Retrospective observational study	PCOS; All CRA.	Step-up regimen: hpFSH i.m.; starting dose: 75 IU (37.5-225); first dose ↑ after 5 - 7 days; maximum dose: 225 IU/day; hCG (3000 IU): 1 follicle ≥ 16 mm; cancellation criteria: > 3 follicles > 14 mm + > E_2; luteal support; OHSS: not reported.	Age (years); BMI (kg/m^2);LH (IU/L).	Miscarriage (pregnancies ending < 20 weeks)	44 patients; 75 cycles.	Definition CRA not clearly stated (dose ?). Luteal support.

Study	Methodology	Participants	Interventions	Screening parameters[a]	Outcome parameters[a]	Numbers	Comments
Sagle *et al.* (1991)	Prospective randomized controlled trial	WHO II: oligoamenorrhea + TVS-PCO[c] + (↑ LH, ↑T and/or both); All CRA.	Step-up regimen: hMG i.m. or uFSH i.m.; starting dose: 75 IU; ovarian response: 1 follicle > 12 mm; first dose ↑ after 2 weeks: 37.5 IU; subsequent dose ↑ after 1 week: 37.5 IU; subsequent cycle: starting dose: 75 IU; hCG (5000 IU): 1follicle ≥ 18 mm; cancellation criteria: > 3 follicles ≥ 15 mm; OHSS: not reported.	LH (IU/L)	Ovulation (midluteal P > 30 nmol/L)	30 patients; 74 cycles.	Exclusion criteria: BMI > 28.
Strowitzki *et al.* (1998)	Retrospective Study	PCOS: TVS-PCO[c] + ≥ 2; CC treatment unsuccesful.	Step-up regimen: rFSH s.c. or uFSH i.m. or hpFSH i.m.; starting dose: 75 IU; first dose ↑ after 10 -12 days: 37.5 IU; no further dose ↑; hCG (10000 IU): ≥ 1 follicle ≥ 16 mm; cancellation criteria: > 3 follicles > 14 mm or no response; OHSS: reported.	Obesity: BMI (kg/m^2)	Total amount of FSH administered (IU); Cancellation; Ovulation (TVS); Pregnancy (hCG); Miscarriage.	68 patients; 116 cycles.	Definition CC respons not clearly stated. Inclusion: 1 regular cycle. Definition obesity? No correlations obesity with outcome (insufficient data provided: no BMI levels of obese versus lean groups).
Vicino *et al.* (2000)	Randomized controlled trial	PCOS; All CRA.	Electrocautery or gonadotropins. Step-up regimen: hpFSH i.m.; starting dose: 75 IU; first dose ↑ after 7 days: 37.5 IU; subsequent dose ↑ after 2 days: 37.5 IU; subsequent cycle: starting dose individually adjusted; maximum dose: 225 IU/day; hCG (5000 IU): maximum of 2 follicles ≥ 16 mm; cancellation criteria: > 2 follicles ≥ 16 mm or > 6 follicles ≥ 12 -16 mm or no response.	BMI (kg/m^2); Testosterone (ng/ml); Androstenedione (ng/ml); LH (IU/L).	Total amount of FSH administered (IU); Cancellation; Ovulation (TVS and progesterone > 10 ng/ml); Pregnancy (intrauterine gestational sac and fetal heartbeat).	21 patients; 107 cycles.	Correlations BMI with duration of stimulation and amount of follicles significant as well.

Study	Methodology	Participants	Interventions	Screening parameters[a]	Outcome parameters[a]	Numbers	Comments
White *et al.* (1996)	Observational Study	WHO II: oligomenorrhea + TVS-PCO[b,c] + androgen excess	Step-up regimen: hMG or uFSH i.m.; starting dose: 75 IU; ovarian response: 1 follicle > 10 mm; first dose ↑ after 14 days: 37.5 IU; subsequent dose ↑ after 1 week: 37.5 IU; subsequent cycle: starting dose subtreshold level: 37.5-75 IU; dose ↑: 25-37.5 IU; maximum dose: 225 IU/day, hCG (5000 IU): 1 follicle ≥ 18 mm + endometrial thickness ≥ 8 mm; cancellation criteria: > 3 follicles ≥ 15 mm; OHSS: mild/moderate.	White *et al.* (1996)	Observational Study	WHO II: oligomenorrhea + TVS-PCO[b,c] + androgen excess	Step-up regimen: hMG or uFSH i.m.; starting dose: 75 IU; ovarian response: 1 follicle > 10 mm; first dose ↑ after 14 days: 37.5 IU; subsequent dose ↑ after 1 week: 37.5 IU; subsequent cycle: starting dose subtreshold level: 37.5-75 IU; dose ↑: 25-37.5 IU; maximum dose: 225 IU/day, hCG (5000 IU): 1 follicle ≥ 18 mm + endometrial thickness ≥ 8 mm; cancellation criteria: > 3 follicles ≥ 15 mm; OHSS: mild/moderate.
Yarali *et al.* (1999)	Prospective randomized trial	WHO II; CRA + CCF.	Step-up regimen: uFSH i.m. or rFSH s.c.; starting dose: 75 IU; first dose ↑ after 14 days: 37.5 IU; subsequent dose ↑ after 1 week: 37.5 IU; subsequent cycle: starting dose subtreshold level: 37.5-75 IU; dose ↑: 18.7-37.5 IU; maximum dose: 225 IU/day; hCG (10000 IU): 1 follicle ≥ 17 mm; cancellation criteria: > 4 follicles ≥ 15 mm or no response after 35 days; OHSS: not reported.	BMI (kg/m^2)	Ovulation (↑ progesterone)	51 patients; 96 cycles.	Logistic regression for prediction of ovulation in first cycle and duration of treatment: insufficient data: only some p-values provided.

Footnotes:
[a] Screening parameters and outcome parameters outlined: only those parameters which are discussed as related.
[b] Presence of polycystic ovaries based on published criteria (Adams et al., 1985; Adams et al., 1986).
[c] TVS-PCO: transvaginal sonography-polycystic ovaries
[d] CIGMA: continuous infusion of glucose with model assessment

The extent of the infertility work-up was stated in all studies (Sagle *et al.*, 1991; Hamilton-Fairley *et al.*, 1992; McClure *et al.*, 1992; Dale *et al.*, 1993; McClure *et al.*, 1993; Farhi *et al.*, 1993; Balasch *et al.*, 1996; White *et al.*, 1996; Fulghesu *et al.*, 1997; Dale *et al.*, 1998; Strowitzki *et al.*, 1998; Yarali *et al.*, 1999; Vicino *et al.*, 2000). This consisted most commonly of a semen analysis and a hysterosalpingography and/or laparoscopic inspection. Twelve studies included only couples with a normal semen analysis (Sagle *et al.*, 1991; Hamilton-Fairley *et al.*, 1992; McClure *et al.*, 1992; Dale *et al.*, 1993; McClure *et al.*, 1993; Farhi *et al.*, 1993; Balasch *et al.*, 1996; Fulghesu *et al.*, 1997; Dale *et al.*, 1998; Strowitzki *et al.*, 1998; Yarali *et al.*, 1999; Vicino *et al.*, 2000). In all studies tubal patency (at least one open tube) was confirmed (Sagle *et al.*, 1991; Hamilton-Fairley *et al.*, 1992; McClure *et al.*, 1992; McClure *et al.*, 1993; Farhi *et al.*, 1993; Balasch *et al.*, 1996; White *et al.*, 1996; Fulghesu *et al.*, 1997; Strowitzki *et al.*, 1998; Vicino *et al.*, 2000). In one study, donor sperm (one patient) was used because of co-existing male factor infertility (Hamilton-Fairley *et al.*, 1992).

Except for one study (White *et al.*, 1996), the patients of interest either had remained anovulatory after CC treatment, or had failed to conceive despite ovulating during CC treatment. A wide variation in the definition of CC resistant anovulation and failure was used. In two studies, ovarian wedge resection or ovarian electrocauterization had been performed before gonadotropin induction of ovulation (Dale *et al.*, 1993; Dale *et al.*, 1998).

In three studies, patients with increased BMI levels were excluded from treatment: BMI > 28 kg/m^2 (Hamilton-Fairley *et al.*, 1992; White *et al.*, 1996) and BMI > 30 kg/m^2 (Sagle *et al.*, 1991).

Description of interventions

The step-up regimen was applied according to the following protocol: a starting dose of 75 IU per day and a first dose increase of 75 IU per day after 5-7 days (Farhi *et al.*, 1993). Various alternatives of this protocol were reported (McClure *et al.*, 1992; McClure *et al.*, 1993; Fulghesu *et al.*, 1997; Vicino *et al.*, 2000). Others used the step-up regimen according to the following protocol: starting dose of 50-75 IU per day, a first dose increase of 37.5 IU per day after 14 days and a subsequent dose increase of 37.5 IU per day (Sagle *et al.*, 1991; Hamilton-Fairley *et al.*, 1992; Dale *et al.*, 1993; Balasch *et al.*, 1996; White *et al.*, 1996; Dale *et al.*, 1998; Yarali *et al.*, 1999). Strowitzky *et al.* (1998) utilized a variation of this regimen. In some studies the starting dose was adjusted in the subsequent (> 1) cycle performed (McClure *et al.*, 1992; Balasch *et al.*, 1996; White *et al.*, 1996; Yarali *et al.*, 1999; Vicino *et al.*, 2000).

The following preparations of gonadotropins were used: hMG (Sagle *et al.*, 1991; Hamilton-Fairley *et al.*, 1992; McClure *et al.*, 1992; Farhi *et al.*, 1993; White *et al.*, 1996), uFSH (Sagle *et al.*, 1991; Hamilton-Fairley *et al.*, 1992; McClure *et al.*,

1992; Dale *et al.*, 1993; Farhi *et al.*, 1993; Balasch *et al.*, 1996; White *et al.*, 1996; Fulghesu *et al.*, 1997; Dale *et al.*, 1998; Strowitzki *et al.*, 1998; Yarali *et al.*, 1999; Vicino *et al.*, 2000) and rFSH (Strowitzki *et al.*, 1998; Yarali *et al.*, 1999). Only one study compared gonadotropin-only treatment (uFSH/hMG) with a combined regimen (hMG and the concomitant administration of a GnRH agonist) (Farhi *et al.*, 1993). Besides gonadotropin induction of ovulation, electrocautery was also performed in one study (Vicino *et al.*, 2000).

Description of outcome measures

In six studies the number of cycles with mono-folllicular development was not reported (McClure *et al.*, 1992; McClure *et al.*, 1993; Farhi *et al.*, 1993; White *et al.*, 1996; Fulghesu *et al.*, 1997; Vicino *et al.*, 2000). The definition of mono-follicular growth varied from one follicle > 15 mm (Dale *et al.*, 1998) and one follicle > 17 mm (Balasch *et al.*, 1996) in diameter on the day of hCG. In five studies the definition used was not clearly stated (Sagle *et al.*, 1991; Hamilton-Fairley *et al.*, 1992; Dale *et al.*, 1993; Strowitzki *et al.*, 1998; Yarali *et al.*, 1999). Only seven studies reported the number of cycles with mono-follicular growth (Sagle *et al.*, 1991; Hamilton-Fairley *et al.*, 1992; Dale *et al.*, 1993; Balasch *et al.*, 1996; Dale *et al.*, 1998; Strowitzki *et al.*, 1998; Yarali *et al.*, 1999).

The total amount of gonadotropins administered per cycle (Sagle *et al.*, 1991; McClure *et al.*, 1992; Dale *et al.*, 1993; McClure *et al.*, 1993; Farhi *et al.*, 1993; White *et al.*, 1996; Strowitzki *et al.*, 1998; Yarali *et al.*, 1999; Vicino *et al.*, 2000) or the mean total quantity of gonadotropins to induce ovulation or achieve follicular maturation (Hamilton-Fairley *et al.*, 1992; Balasch *et al.*, 1996; Fulghesu *et al.*, 1997; Dale *et al.*, 1998) was provided for all studies in ampoules or IU. For one study (McClure *et al.*, 1992) the total amount of gonodotropins administered was deduced from a figure illustration. A conversion to IU was made for those studies reporting the total dose in ampoules (Sagle *et al.*, 1991; Hamilton-Fairley *et al.*, 1992; Farhi *et al.*, 1993; White *et al.*, 1996; Strowitzki *et al.*, 1998; Vicino *et al.*, 2000).

Criteria for cycle cancellation were based on the number of follicles developed and/or serum E_2 levels. In five studies, cycles were cancelled in case of multi-follicular growth (≥ 4 follicles ≥ 15 mm) (Sagle *et al.*, 1991; Hamilton-Fairley *et al.*, 1992; Dale *et al.*, 1993; White *et al.*, 1996; Dale *et al.*, 1998; Yarali *et al.*, 1999). In four studies, cycles were cancelled because of multi-follicular growth or absence of response (Balasch *et al.*, 1996; Strowitzki *et al.*, 1998; Yarali *et al.*, 1999; Vicino *et al.*, 2000). Finally, three studies cancelled treatment cycles based on multi-follicular growth and/or increased serum E_2 levels (McClure *et al.*, 1992; McClure *et al.*, 1993; Farhi *et al.*, 1993). One study did not provide information on criteria for cycle cancellation (Fulghesu *et al.*, 1997). Only nine studies reported the number of cancelled cycles (Sagle *et al.*, 1991; McClure *et al.*, 1992; Dale *et al.*, 1993;

Balasch *et al.*, 1996; White *et al.*, 1996; Dale *et al.*, 1998; Strowitzki *et al.*, 1998; Yarali *et al.*, 1999; Vicino *et al.*, 2000).

Criteria for ovulation were based on the assessment of serum P levels (Hamilton-Fairley *et al.*, 1992; Dale *et al.*, 1993; White *et al.*, 1996; Dale *et al.*, 1998; Yarali *et al.*, 1999), or ultrasound (Strowitzki *et al.*, 1998) or both (Sagle *et al.*, 1991; Fulghesu *et al.*, 1997; Vicino *et al.*, 2000). Four studies did not provide information concerning confirmation of ovulation (McClure *et al.*, 1992; McClure *et al.*, 1993; Farhi *et al.*, 1993; Balasch *et al.*, 1996). A total of 11 studies reported the number of ovulatory cycles (Sagle *et al.*, 1991; Hamilton-Fairley *et al.*, 1992; McClure *et al.*, 1992; Dale *et al.*, 1993; Farhi *et al.*, 1993; Balasch *et al.*, 1996; White *et al.*, 1996; Fulghesu *et al.*, 1997; Strowitzki *et al.*, 1998; Yarali *et al.*, 1999; Vicino *et al.*, 2000).

Explicit details of the definition of pregnancy were given by using serum hCG (Sagle *et al.*, 1991; Strowitzki *et al.*, 1998; Yarali *et al.*, 1999), ultrasound (Balasch *et al.*, 1996; Vicino *et al.*, 2000), serum hCG and ultrasound (Hamilton-Fairley *et al.*, 1992; McClure *et al.*, 1993) or ultrasound and/or histological verification (Dale *et al.*, 1993; Dale *et al.*, 1998). Two studies specifically stated the presence of a clinical pregnancy (Sagle *et al.*, 1991; Yarali *et al.*, 1999). Finally, one study only provided data on the definition of a clinical pregnancy (i.e. intrauterine gestational sac and fetal heart beat) (Vicino *et al.*, 2000). In four studies the definition of pregnancy was not stated (McClure *et al.*, 1992; Farhi *et al.*, 1993; White *et al.*, 1996; Fulghesu *et al.*, 1997). Pregnancy rate (per cycle and per patient) was provided for all studies except one (McClure *et al.*, 1993).

The definition of miscarriage, spontaneous abortion or ongoing pregnancy rate was not clearly stated in seven studies (Farhi *et al.*, 1993; Balasch *et al.*, 1996; White *et al.*, 1996; Fulghesu *et al.*, 1997; Strowitzki *et al.*, 1998; Yarali *et al.*, 1999; Vicino *et al.*, 2000). In two studies the definition of early pregnancy loss was stated (Sagle *et al.*, 1991; Hamilton-Fairley *et al.*, 1992). In four studies the definition of miscarriage was based on the division in first and second trimester abortions (McClure *et al.*, 1992; Dale *et al.*, 1993; McClure *et al.*, 1993; Dale *et al.*, 1998). Data on miscarriage rates were stated in all studies except one (Vicino *et al.*, 2000).

Description of screening characteristics related to treatment outcome

Except for one (Sagle *et al.*, 1991), all studies stated data of age (means ± SD) for patients. In only four studies exact data of cycle history (i.e. oligomenorrhea or amenorrhea) for all patients were mentioned (Sagle *et al.*, 1991; Hamilton-Fairley *et al.*, 1992; Farhi *et al.*, 1993; Balasch *et al.*, 1996).

Details of BMI levels for patients were given in nine studies (Dale *et al.*, 1993; McClure *et al.*, 1993; Farhi *et al.*, 1993; Balasch *et al.*, 1996; White *et al.*, 1996; Fulghesu *et al.*, 1997; Dale *et al.*, 1998; Yarali *et al.*, 1999; Vicino *et al.*,

2000). Some studies divided patients into non-obese (lean) and obese (BMI > 25 kg/m^2 (Hamilton-Fairley *et al.*, 1992; Dale *et al.*, 1993; White *et al.*, 1996; Fulghesu *et al.*, 1997; Dale *et al.*, 1998), BMI > 27 (Vicino *et al.*, 2000), or probably BMI > 30 (Yarali *et al.*, 1999)). One study (Strowitzki *et al.*, 1998) described patients as lean or obese without any further information. The fraction of obese (BMI > 25) and non-obese patients was calculated where only continuous data of BMI were provided (McClure *et al.*, 1992; Farhi *et al.*, 1993; White *et al.*, 1996), assuming a normal distribution.

In nine studies, CRA was defined as anovulation during at least 3 consecutive cycles with an increasing dose up to at least 150 mg/day for a period of 5 days (Sagle *et al.*, 1991; Hamilton-Fairley *et al.*, 1992; Dale *et al.*, 1993; Farhi *et al.*, 1993; Balasch *et al.*, 1996; Dale *et al.*, 1998; Strowitzki *et al.*, 1998; Yarali *et al.*, 1999; Vicino *et al.*, 2000). One study decreased the threshold for CRA to 100 mg/day (Fulghesu *et al.*, 1997). In one study the dose for CRA was not clearly stated (McClure *et al.*, 1992). CCF was defined as failure to conceive after at least 6 ovulatory CC cycles (McClure *et al.*, 1992; Farhi *et al.*, 1993; Fulghesu *et al.*, 1997; Strowitzki *et al.*, 1998; Yarali *et al.*, 1999). One study decreased the number of cycles for CCF to 3 (Balasch *et al.*, 1996). In two other studies the number of ovulatory cycles to fulfil the criteria for CCF were not stated (Dale *et al.*, 1993; Dale *et al.*, 1998). In two studies (McClure *et al.*, 1993; Strowitzki *et al.*, 1998), patients were said to be (un)responsive or resistant to CC, however the dose as well as the duration of CC treatment were not stated. In only six studies the exact number of patients suffering from CRA and CCF were mentioned (Sagle *et al.*, 1991; Hamilton-Fairley *et al.*, 1992; McClure *et al.*, 1992; Dale *et al.*, 1993; McClure *et al.*, 1993; Vicino *et al.*, 2000).

The definition of polycystic ovaries was based on the Adams criteria (i.e. increased number of follicles and either an increased ovarian volume or increased stromal area or both) (Sagle *et al.*, 1991; Hamilton-Fairley *et al.*, 1992; Farhi *et al.*, 1993; White *et al.*, 1996; Dale *et al.*, 1998; Strowitzki *et al.*, 1998; Vicino *et al.*, 2000), or based on the presence of an increased number of follicles and/or ovarian stroma (McClure *et al.,* 1992; McClure *et al.*, 1993). Others did not report (clear) information on the definition of polycystic ovaries (Dale *et al.*, 1993; Balasch *et al.*, 1996; Fulghesu *et al.*, 1997; Yarali *et al.*, 1999). In seven studies the number of patients with polycystic ovaries was stated (Sagle *et al.*, 1991; Hamilton-Fairley *et al.*, 1992; Dale *et al.*, 1993; Farhi *et al.*, 1993; White *et al.*, 1996; Dale *et al.*, 1998; Strowitzki *et al.*, 1998).

Details of baseline T levels were provided in nmol/L (Sagle *et al.*, 1991; Hamilton-Fairley *et al.*, 1992; Dale *et al.*, 1993; Fulghesu *et al.*, 1997; Dale *et al.*, 1998) or converted to SI units when expressed as ng/mL (Farhi *et al.*, 1993; Strowitzki *et al.*, 1998; Yarali *et al.*, 1999; Vicino *et al.*, 2000). Data regarding AD

levels were provided in nmol/L (Dale *et al.*, 1993; Fulghesu *et al.*, 1997; Dale *et al.*, 1998; Vicino *et al.*, 2000) or converted to SI units (Vicino *et al.*, 2000).

Details concerning baseline LH levels for patients or subgroups of patients were provided in IU/L in eight studies (Sagle *et al.*, 1991; Hamilton-Fairley *et al.*, 1992; Dale *et al.*, 1993; Farhi *et al.*, 1993; Fulghesu *et al.*, 1997; Dale *et al.*, 1998; Strowitzki *et al.*, 1998; Vicino *et al.*, 2000) or converted when expressed as mIU/mL (Vicino *et al.*, 2000). Baseline LH/FSH ratios were provided by eight studies (Sagle *et al.*, 1991; Farhi *et al.*, 1993; Balasch *et al.*, 1996; Fulghesu *et al.*, 1997; Dale *et al.*, 1998; Strowitzki *et al.*, 1998; Yarali *et al.*, 1999; Vicino *et al.*, 2000).

Dale *et al.* (1998) assessed insulin resistance and glucose tolerance by means of a continuous infusion of glucose with CIGMA (continuous infusion of glucose with model assessment) model assessment test. Fulghesu *et al.* (1997) classified patients as hyperinsulinemic or normoinsulinemic based on the insulinemic response to glucose load (OGTT). Both studies (Fulghesu *et al.*, 1997; Dale *et al.*, 1998) provided data of fasting glucose (nmol/L) and insulin levels (mIU/L). Conversion to IU units was performed where necessary.

Pooling of data

Data from studies reporting relationships between initial screening characteristics and outcome parameters of ovulation induction as measures of association (OR) were pooled if at least two studies reported an association of similar screening parameter and outcome characteristic. The measures of association were pooled using the inverse of the variance as weight. Heterogeneity was tested for using the Q statistic as defined by DerSimonean and Laird, which has a χ^2 distribution with df = [number of pooled studies - 1] (DerSimonian and Laird, 1986). Random effects estimates were calculated using the likelihood method described by Hardy and Thompson (Hardy and Thompson, 1998), when at least three studies were available. Association measures were extracted from studies for the following outcome parameters: total amount of FSH administered (Table 3), cancellation rate (Table 4), ovulation rate (Table 5), pregnancy rate (Table 6 and 7) and miscarriage rate (Table 8).

Results of pooling

A total number of seven studies reported an association (all positive) between obesity and total amount of gonadotropins administered (IU) (Figure 1) (Hamilton-Fairley *et al.*, 1992; McClure *et al.*, 1992; Dale *et al.*, 1993; Fulghesu *et al.*, 1997; Dale *et al.*, 1998; Strowitzki *et al.*, 1998; Vicino *et al.*, 2000). The weighted mean difference (WMD) (obese versus non-obese) for total dose used was 771 (95% CI: 700 - 842) IU. Significant heterogeneity was detected between studies ($P < 0.001$). The random effects estimate of the difference between obese and non-obese patients was 629 (95% CI: 317 - 931) IU. Two studies reporting on insulin

resistance versus total amount of FSH administered (Fulghesu *et al.*, 1997; Dale *et al.*, 1998) produced a WMD (hyperinsulinemic versus normoinsulinemic) of 351 (95% CI: 73 - 630) IU.

Four studies reported an association between obesity and cancellation rate (Dale *et al.*, 1993; Dale *et al.*, 1998; Strowitzki *et al.*, 1998; Vicino *et al.*, 2000) (Figure 2). The pooled OR (obese versus non-obese) was 1.86 (95% CI: 1.13 - 3.06). Despite conflicting directions of association, the test for heterogeneity was not significant (P = 0.2).

Four studies (Hamilton-Fairley *et al.*, 1992; Strowitzki *et al.*, 1998; Yarali *et al.*, 1999; Vicino *et al.*, 2000) reported an association between obesity and ovulation rate, with a pooled OR (obese versus non-obese) of 0.44 (95% CI: 0.31 - 0.61) (Figure 3).The test for heterogeneity was not significant (P = 0.4). Two studies (Sagle *et al.*, 1991; White *et al.*, 1996) reported an association between LH and ovulation rate. Pooling of the results was not possible because one studied reported LH as continuous variable (Sagle *et al.*, 1991) and the other provided data of LH in two categories (White *et al.*, 1996). Association measures for respectively T (White *et al.*, 1996) and insulin resistance (Fulghesu *et al.*, 1997) with ovulation were calculated from the data provided.

Pregnancy was analyzed per cycle and per patient. Four studies reported an association (three positive and one negative) between obesity and pregnancy rate per cycle, pooled OR (obese versus non-obese) 1.13 (95% CI: 0.70 - 1.84) (Hamilton-Fairley *et al.*, 1992; Dale *et al.*, 1993; Strowitzki *et al.*, 1998; Vicino *et al.*, 2000). The test for heterogeneity was not significant (P = 0.4). Five studies reported an association (two positive and three negative) between obesity and pregnancy rate per patient (Hamilton-Fairley *et al.*, 1992; Farhi *et al.*, 1993; White *et al.*, 1996; Strowitzki *et al.*, 1998; Vicino *et al.*, 2000). The pooled OR (obese versus non-obese) was 1.22 (95% CI: 0.77 - 1.93). The test for heterogeneity was not significant (P = 0.16). Three studies (Farhi *et al.*, 1993; White *et al.*, 1996; Vicino *et al.*, 2000) reported an association between T and pregnancy rate per patient. The pooled Odds ratio (per nmol/L) was 0.94 (95% CI: 0.80 - 1.09). Four studies (Farhi *et al.*, 1993; Balasch *et al.*, 1996; White *et al.*, 1996; Vicino *et al.*, 2000) reported an association between LH and pregnancy rate per patient (Figure 4). The pooled OR (per IU/L) was 1.04 (95% CI: 1.01 - 1.07). The test for heterogeneity was not possible in the latter two cases. Association measures between insulin resistance and pregnancy rate per cycle as well as per patient (Fulghesu *et al.*, 1997; Dale *et al.*, 1998) were calculated. Both studies (Fulghesu *et al.*, 1997; Dale *et al.*, 1998) reported a negative association between insulin resistance and pregnancy rate, with pooled OR (hyperinsulinemic versus normoinsulinemic) of 0.29 (95% CI: 0.10 - 0.80) and 0.24 (95% CI: 0.08 - 0.74).

TABLE 3 Possible clinical and endocrine features involved in the total amount of gonadotropins administered (IU) during gonadotropin induction of ovulation in normogonadotropic anovulatory infertility (see also Figure 1).

Study	N (cycles)	Mean IU (SD)	Mean difference in IU (95% CI)			
			Obesity[a]	T	LH	Insulin resistance[b]
Balasch *et al.* (1996)	534	1185 (900)	-	-	-	-
Dale *et al.* (1993)	66	1702 (925)	759 (346 - 1172)	-	-	741 (290 - 1192)
Dale *et al.* (1998)	70	1611 (949)	449 (46 - 852)	-	-	-
Farhi *et al.* (1993)	195	1979 (1027)	-	-	-	-
Fulghesu *et al.* (1997)	52	1462 (638)	263 (-59 - 585)	-	-	113 (-241 - 466)
Hamilton-Fairley *et al.* (1992)	405	1360 (719)	1013 (848 - 1177)	-	-	-
McClure *et al.* (1992)[c, d]	181	1483 (640)	892 (706 - 1079)	-	-	-
McClure *et al.* (1993)	-	-	-	-	-	-
Sagle *et al.* (1991)	75	1269 (475)	-	-	-	-
Strowitzki *et al.* (1998)	116	1110 (567)	33 (-173 - 238)	-	-	-
Vicino *et al.* (2000)	107	1444 (578)	908 (801 - 1014)	-	-	-
White *et al.* (1996)	429	1140 (785)	-	-	-	-
Yarali *et al.* (1999)	96	1145 (762)	-	-	-	-
Pooled estimates	**2326**	**1358**	**-**	**-**	**-**	**-**
Weighted mean difference (WMD) (95% CI)			**771 (700 - 842)**	**-**	**-**	**351 (73 - 630)**
Random effects model			**629 (317 - 931)**	**-**	**-**	**-**
Test for heterogeneity			**P < 0.001**	**-**	**-**	**-**

Footnotes: [a] Obesity versus total amount of gonadotropins administered expressed as: weighted mean difference (WMD) in IU: WMD based on obese versus non-obese patients (applied cut-off varied from study to study: range 25-30 kg/m^2). [b] Insulin resistance versus total amount of gonadotropins administered expressed as: weighted mean difference (WMD) in IU: WMD based on hyperinsulinemic versus normoinsulinemic patients (applied definition varied between studies). [c] Total dose in IU deducted from figure illustration: data not stated in results/table (McClure *et al.*,1992). [d] Only ovulatory cycles included for the present analysis (i.e. total amount of gonadotropins administered) (McClure *et al.*,1992).

Table 4 Possible clinical and endocrine features involved in the observed cancellation rate during gonadotropin induction of ovulation in normogonadotropic anovulatory infertility (see also Figure 2).

Study	N (cycles)	N cancelled (%)	Mean difference in IU (95% CI)			
			Obesity[a]	T	LH	Insulin resistance[b]
Balasch *et al.* (1996)	534	93 (17%)	-	-	-	-
Dale *et al.* (1993)	66	11 (17%)	0.69 (0.18 - 2.61)	-	-	21.10 (2.51 - 176.62)
Dale *et al.* (1998)	70	11 (16%)	0.82 (0.23 - 2.89)	-	-	-
Farhi *et al.* (1993)	195	-	-	-	-	-
Fulghesu *et al.* (1997)	52	-	-	-	-	-
Hamilton-Fairley *et al.* (1992)	405	-	-	-	-	-
McClure *et al.* (1992)	224	14 (6%)	-	-	-	-
McClure *et al.* (1993)	-	-	-	-	-	-
Sagle *et al.* (1991)	75	3 (4%)	-	-	-	-
Strowitzki *et al.* (1998)	116	30 (26%)	1.89 (0.81 - 4.41)	-	-	-
Vicino *et al.* (2000)	107	39 (36%)	3.84 (1.68 - 8.80)	-	-	-
White *et al.* (1996)	429	76 (18%)	-	-	-	-
Yarali *et al.* (1999)	96	11 (16%)	-	-	-	-
Pooled estimates	**2369**	**288 (17%)**	**-**	**-**	**-**	**-**
	Fixed effects OR (95% CI)		**1.86 (1.13-3.06)**	-	**-**	**-**
	Test for heterogeneity		**P = 0.2**	**-**	**-**	**-**

Footnotes: [a] OR based on obese versus non-obese patients (applied cut-off varied from study to study: range 25-30 kg/m^2).[b] OR based on hyperinsulinemic versus normoinsulinemic patients (applied definition varied between studies).

TABLE 5 Possible clinical and endocrine features involved in the observed ovulation rate during gonadotropin induction of ovulation in normogonadotropic anovulatory infertility (see also Figure 3).

Study	N cycles	N ovulatory cycles (%)	Mean difference in IU (95% CI)			
			Obesity[a]	T	LH	Insulin resistance[b]
Balasch *et al.* (1996)	534	419 (79%)	-	-	-	-
Dale *et al.* (1993)	66	49 (74%)	-	-	-	21.10 (2.51 - 176.62)
Dale *et al.* (1998)	70	-	-	-	-	-
Farhi *et al.* (1993)	195	146 (75%)	-	-	-	-
Fulghesu *et al.* (1997)	52	44 (85%)	-	-	-	0.87 (0.18 - 4.09)
Hamilton-Fairley *et al.* (1992)	405	292 (72%)	0.39 (0.24 - 0.63)	-	-	-
McClure *et al.* (1992)	224	181 (81%)	-	-	-	-
McClure *et al.* (1993)	-	-	-	-	-	-
Sagle *et al.* (1991)	75	61 (81%)	-	-	1.12 (1.02 - 1.24)[c]	-
Strowitzki *et al.* (1998)	116	86 (74%)	0.53 (0.23 - 1.23)	-	-	-
Vicino *et al.* (2000)	107	68 (64%)	0.26 (0.11 - 0.60)	-	-	-
White *et al.* (1996)	429	305 (71%)	-	0.68 (0.44 -1.05)[d]	1.02 (0.65 - 1.59)[d]	-
Yarali *et al.* (1999)	51	39 (77%)	0.78 (0.36 - 1.71)[e]	-	-	-
Pooled estimates	**2324**	**1690 (75%)**	**-**	**-**	**-**	**-**
	Fixed effects OR (95% CI)		**0.44 (0.31-0.61)**	-	[f]	**-**
	Test for heterogeneity		**P = 0.2**	**-**	**-**	**-**

Footnotes: [a] OR based on obese versus non-obese patients (applied cut-off varied from study to study: range 25-30 kg/m^2). [b] OR based on hyperinsulinemic versus normoinsulinemic patients (applied definition varied between studies). [c] Indirect calculation of OR: based on continuous data (deducted from figure illustration) (Sagle *et al.*, 1991). [d] OR calculated based on subdivision of T and LH serum levels (respectively T > 2.6 nmol/L or < 2.7 nmol/L and LH > 11.0 IU/L or < 11.1 IU/L) (White *et al.*, 1996). [e] Only first cycle data included in the present analysis (Yarali *et al.*, 1999). [f] Pooled OR not calculated because continuous (Sagle *et al.*, 1991) and categorical (White *et al.*, 1996) data were provided by either studies.

TABLE 6 Possible clinical and endocrine features involved in the observed pregnancy rate per cycle during gonadotropin induction of ovulation in normogonadotropic anovulatory infertility.

Study	N cycles	N pregnancies (%)	Mean difference in IU (95% CI)			
			Obesity[a]	T	LH	Insulin resistance[b]
Balasch *et al.* (1996)	534	93 (17%)	-	-	-	-
Dale *et al.* (1993)	66	12 (18%)	1.35 (0.38 - 4.72)	-	-	0.14 (0.03 - 0.69)
Dale *et al.* (1998)	70	16 (23%)	-	-	-	-
Farhi *et al.* (1993)	195	35 (18%)	-	-	-	-
Fulghesu *et al.* (1997)	52	11 (21%)	-	-	-	0.48 (0.13 - 1.85)
Hamilton-Fairley *et al.* (1992)	405	45 (11%)	1.7 (0.87 - 3.30)	-	-	-
McClure *et al.* (1992)	224	45 (20%)	-	-	-	-
McClure *et al.* (1993)	-	-	-	-	-	-
Sagle *et al.* (1991)	75	10 (13%)	-	-	-	-
Strowitzki *et al.* (1998)	116	21 (18%)	0.60 (0.23 - 1.59)	-	-	-
Vicino *et al.* (2000)	107	8 (8%)	0.45 (0.09 - 2.35)[c]	-	-	-
White *et al.* (1996)	429	49 (11%)	-	0.93 (0.51 - 1.69)[d]	1.61 (0.88 - 2.94)[d]	-
Yarali *et al.* (1999)	96	21 (22%)	-	-	-	-
Pooled estimates	**2369**	**366 (15%)**	**-**	**-**	**-**	**-**
Fixed effects OR (95% CI)			**1.13 (0.70 - 1.84)**	-	**-**	**0.29 (0.10 - 0.80)**
Test for heterogeneity			**P = 0.4**	**-**	**-**	**-**

Footnotes: [a] OR based on obese versus non-obese patients (applied cut-off varied from study to study: range 25-30 kg/m^2). [b] OR based on hyperinsulinemic versus normoinsulinemic patients (applied definition varied between studies). [c] Vicino *et al.* (2000) provides continuous data of BMI for pregnant versus non-pregnant women: assume BMI is normally distributed among pregnant versus non-pregnant women: calculate the fraction obese versus non-obese (2x2 table constructed): indirect calculation of OR (based on number of cycles): 0.45. [d] OR calculated based on subdivision of T and LH serum levels (respectively T > 2.6 nmol/L or < 2.7 nmol/L and LH > 11.0 IU/L or < 11.1 IU/L) (White *et al.*, 1996).

TABLE 7 Possible clinical and endocrine features involved in the observed pregnancy rate per patient during gonadotropin induction of ovulation in resistant normogonadotropic anovulatory infertility (see also Figure 4).

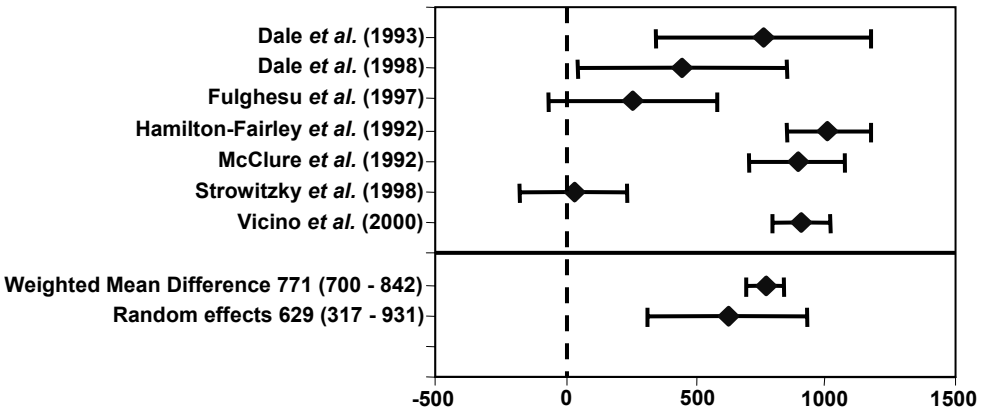
Study	N patients	Mean N cycles per patient	N pregnancies (%)	OR (95% CI)			
				Obesity[a]	T	LH	Insulin resistance[b]
Balasch *et al.* (1996)	234	2.3	93 (40%)	-	-	1.07 (1.06 - 1.16)	-
Dale *et al.* (1993)	50	1.3	12 (24%)	-	-	-	0.10 (0.02 - 0.56)
Dale *et al.* (1998)	42	1.7	16 (38%)	-	-	-	-
Farhi *et al.* (1993)	89	2.2	35 (39%)	2.95 (1.09 - 7.96)[c]	0.90 (0.73 - 1.12)	1.00 (0.96 - 1.04)	-
Fulghesu *et al.* (1997)	34	1.5	11 (32%)	-	-	-	0.44 (0.10 - 1.92)
Hamilton-Fairley *et al.* (1992)	100	4.1	45 (45%)	2.25 (0.89 - 5.67)	-	-	-
McClure *et al.* (1992)	71	3.2	45 (63%)	-	-	-	-
McClure *et al.* (1993)	-	-	-	-	-	-	-
Sagle *et al.* (1991)	30	2.5	10 (33%)	-	-	-	-
Strowitzki *et al.* (1998)	68	1.7	21 (31%).	0.59 (0.21 - 1.69)	-	-	-
Vicino *et al.* (2000)	21	5.1	8 (38%)	0.39 (0.06 - 2.70)	0.95 (0.70 - 1.29)	0.73 (0.35 - 1.53)	-
White *et al.* (1996)	91	4.7	49 (54%)	0.79 (0.35 - 1.80)[c]	1.0 (0.73 - 1.37)	1.01 (0.96 - 1.08)	-
Yarali *et al.* (1999)	51	1.9	21 (41%)	-	-	-	-
Pooled estimates	**881**	**2.7**	**366**	**-**	**-**	**-**	**-**
			Fixed effectsOR (95% CI)	**1.22 (0.77-1.93)[d]**	**0.94 (0.80 -1.09)[e]**	**1.04 (1.01 -1.07)[f]**	**0.24 (0.08 - 0.71)**
			Test for heterogeneity	**P = 0.16**	**-**	**-**	**-**

Footnotes: [a] OR based on obese versus non-obese patients (applied cut-off varied from study to study: range 25- 30 kg/m^2). [b] OR based on hyperinsulinemic versus normoinsulinemic patients (applied definition varied between studies). [c] Farhi *et al.* (1993) and White *et al.* (1996) both provide continuous data of BMI for pregnant versus non-pregnant women: assume BMI is normally distributed among pregnant versus non-pregnant patients: calculate the fraction obese and non-obese patients (2x2 table constructed): indirect calculation of OR (based on number of patients): 2.95 and 0.79 respectively. [d] Second best analysis performed (per patient), because not all studies provided data per cycle: analysis based on obese versus non-obese patients (applied cut-off varied from study to study: range 25-30 kg/m^2); obesity versus pregnancy rate expressed as: OR. [e] For T levels expressed as SI units (nmol/L) pooled analysis was performed (Farhi *et al.*, 1993; White *et al.*, 1996; Vicino *et al.*, 2000). [f] For LH levels expressed as SI units (IU/L) pooled analysis was performed (Farhi *et al.*, 1993; Balasch *et al.*, 1996; White *et al.*, 1996; Vicino *et al.*, 2000).

TABLE 8 Possible clinical and endocrine features involved in the observed miscarriage rate during gonadotropin induction of ovulation in normogonadotropic anovulatory infertility (see also Figure 5).

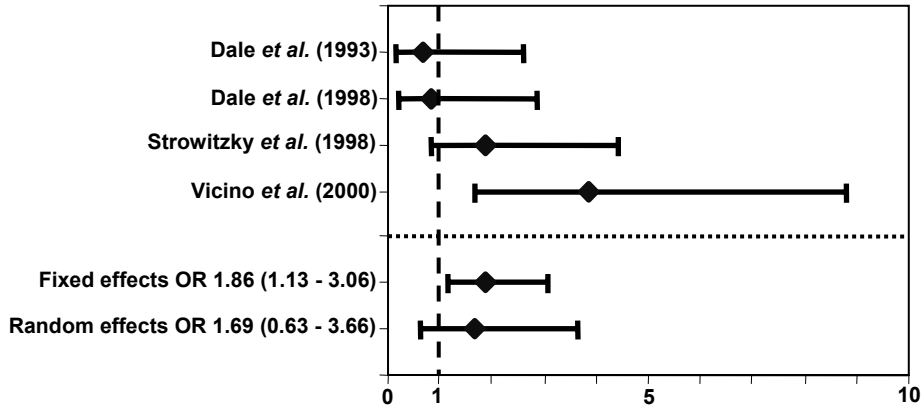
Study	N (pregnancies)	Mean N miscarriages (%)	Mean difference in IU (95% CI)			
			Obesity[a]	T	LH	Insulin resistance[b]
Balasch *et al.* (1996)	93	10 (11%)	-	-	-	-
Dale *et al.* (1993)	12	4 (33%)	-	-	-	∞
Dale *et al.* (1998)	16	7 (44%)	-	-	-	-
Farhi *et al.* (1993)	35	13 (37%)	-	-	-	-
Fulghesu *et al.* (1997)	11	2 (18%)	-	-	-	1.25 (0.06 - 26.9)
Hamilton-Fairley *et al.* (1992)	45	17 (38%)	4.13 (1.11 - 15.32)	-	-	-
McClure *et al.* (1992)	45	12 (27%)	-	-	-	-
McClure *et al.* (1993)	50	14 (28%)	1.44 (0.35 - 5.84)[c]	-	1.004 (0.93 - 1.09)	-
Sagle *et al.* (1991)	10	4 (40%)	-	-	-	-
Strowitzki *et al.* (1998)	21	3 (14%)	4.0 (0.30 - 53.47)	-	-	-
Vicino *et al.* (2000)	-	-	-	-	-	-
White *et al.* (1996)	49	13 (27%)[d]	4.12 (1.08 - 15.71)[c]	0.86 (0.54 - 1.37)	1.03 (0.93 - 1.14)	-
Yarali *et al.* (1999)	21	4 (19%)	-	-	-	-
Pooled estimates	**408**	**288 (17%)**	**-**	**-**	**-**	**-**
	Fixed effects OR (95% CI)		**3.05 (1.45 - 6.44)**	**-**	**3.05 (1.45 - 6.44)**	**1.8 (0.3 - 10.3)**
	Test for heterogeneity		**P = 0.17**	**-**	-	**-**

Footnotes: [a] OR based on obese versus non-obese patients (applied cut-off varied from study to study: range 25-30 kg/m^2). [b] OR based on hyperinsulinemic versus normoinsulinemic patients (applied definition varied between studies). [c] McClure *et al.* (1993) and White *et al.* (1996) both provide continuous data of BMI for pregnant versus non-pregnant women: assume BMI is normally distributed among pregnant versus non-pregnant patients: calculate the fraction obese and non-obese patients (2x2 table constructed): indirect calculation of OR (based on number of patients): 1.44 and 4.12 respectively. [d] Miscarriage: Ectopic pregnancy included (White *et al.*, 1996). [e] For LH levels expressed as SI units (IU/L) pooled analysis was performed (McClure *et al.*, 1993; White *et al.*, 1996).



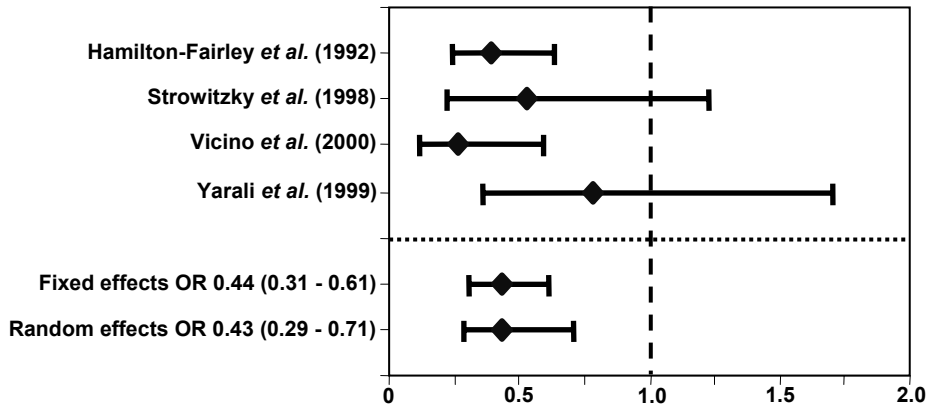
Difference in total amount administered (IU) for obese versus non-obese women

Figure 1 Association measures between obesity and total amount of gonadotropins administered (IU) for ovulation induction in normogonadotropic anovulatory infertility (median and 95% CI). The weighted mean difference (WMD) was generated using inverse variance weighting. Heterogeneity was tested for and random effects estimates were calculated using the likelihood method as described by Hardy and Thompson (1998).



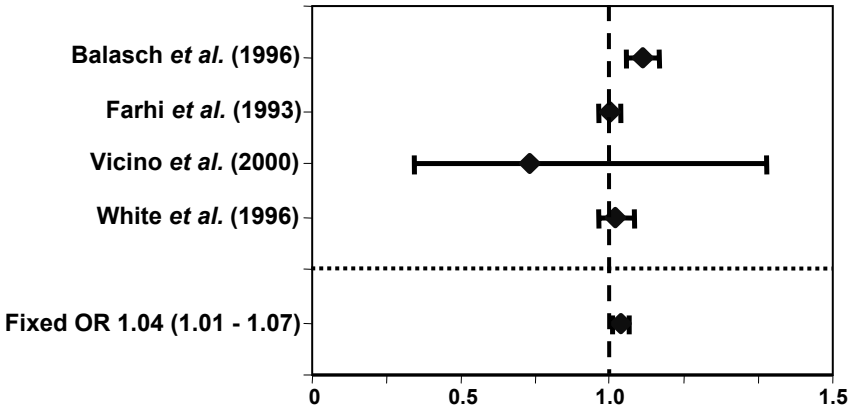
Odds ratio of cancellation rate for obese versus non-obese women

Figure 2 Four studies reported an association between obesity and cancellation rate. The pooled OR and 95% CI (obese versus non-obese) was calculated by inverse variance weighting.



Odds ratio of ovulation rate for obese versus non-obese women

Figure 3 Four studies reported an association between obesity and ovulation rate, with a pooled OR and 95% CI (obese versus non-obese) generated calculated by inverse variance weighting. Note: the range of the *x*-axis is different from Figure 2.



Odds ratio of pregnancy rate (per patient) for LH (per IU/L)

Figure 4 Association measures between LH (per IU/L) and pregnancy rate per patient was provided by a total number of 4 studies. The pooled OR and 95% CI (obese versus non-obese) was calculated by inverse variance weighting.

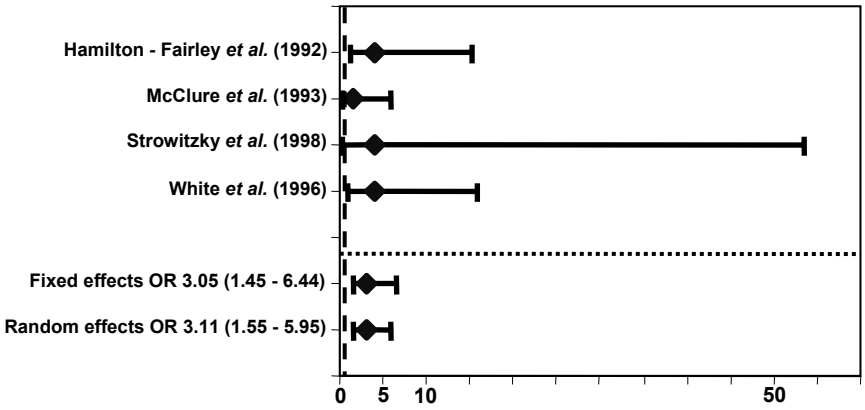


Figure 5 Four studies reported an association between obesity and miscarriage rate. The pooled OR and 95% CI (obese versus non-obese) was calculated by inverse variance weighting. Note: the range of the *x*-axis is different from Figure 2.

Four studies reported an association between obesity and miscarriage rate (Figure 5) (Hamilton-Fairley *et al.*, 1992; McClure *et al.*, 1993; White *et al.*, 1996; Strowitzki *et al.*, 1998). The pooled OR (obese versus non-obese) was 3.05 (95% CI: 1.45 - 6.44). The test for heterogeneity was not significant (P = 0.17). Two studies (McClure *et al.*, 1993; White *et al.*, 1996) reported an association between LH and miscarriage rate. The pooled OR (per IUI/L) was 1.013 (95% CI: 0.95 - 1.08). Two studies (Fulghesu *et al.*, 1997; Dale *et al.*, 1998) reported an association between insulin resistance and miscarriage rate. The pooled OR (hyperinsulinemic versus normoinsulinemic) was 1.75 (95% CI: 0.30 - 10.3). An association of age (McClure *et al.*, 1993) versus T (White *et al.*, 1996) and miscarriage rate was calculated from the data provided.

None of the studies provided a measure of association between CRA/CCF or the presence of polycystic ovaries and treatment outcome.

In summary, significant associations were found for the total amount of FSH administered, cancellation rate, ovulation rate and miscarriage rates with BMI. Furthermore, significant associations were found for the total amount of FSH and pregnancy rate with insulin resistance.

5.2.4 Discussion

This systematic review and meta-analysis demonstrates how few studies have provided measures of association between screening characteristics in women with normogonadotropic anovulatory infertility and gonadotropin ovulation induction treatment outcome. The studies included used various criteria for patient inclusion and intervention and are prone to bias. The best available evidence suggests that obesity and insulin resistance are both associated with adverse treatment outcomes, including increased FSH requirements, increased cancellation and miscarriage rates and most importantly decreased ovulation and pregnancy rates.

Obesity frequently coincides with normogonadotropic anovulation and represents an important clinical feature associated with PCOS (Laven *et al.*, 2002). Differences in pharmacokinetic characteristics of gonadotropin preparations (Mannaerts *et al.*, 1993) as well as the amount of exogenous gonadotropins required to achieve follicular maturation (Hamilton-Fairley *et al.*, 1992; McClure *et al.*, 1992; Dale *et al.*, 1993; Vicino *et al.*, 2000; Imani *et al.*, 2002a) related to body weight, have been reported. Obesity is associated with reduced circulating levels of sex hormone-binding globulin (SHBG), mildly elevated androgen levels (Poretsky *et al.*, 1999) and hyperinsulinemia (Norman *et al.*, 2002). Insulin resistance (associated with PCOS as well (Dunaif, 1999)) is also related to the total amount of gonadotropins administered, as previously reported (Homburg *et al.*, 1996).

So far, the impact of obesity on the cycle cancellation rates in women with anovulatory infertility has not been convincingly established. However, the impact of obesity on ovulation rates was previously mentioned by several authors (Hamilton-Fairley *et al.*, 1992; Yarali *et al.*, 1999; Vicino *et al.*, 2000). The present meta-analysis explicitly shows that obese women are less likely to ovulate following gonadotropin ovulation induction and therefore suggests that ovarian dysfunction in these women is more severe. However, differences in absorption and distribution of exogenous FSH may also be involved (Mannaerts *et al.*, 1993). Weight reduction may normalize insulin resistance and androgen metabolism (Kiddy *et al.*, 1992; Holte *et al.*, 1995) and may significantly improve menstrual abnormalities, ovulation, and fertility rates (Norman *et al.*, 2002).

Obesity does not seem to be associated with decreased pregnancy rates, as previously reported (Hamilton-Fairley *et al.*, 1992; Dale *et al.*, 1993; White *et al.*, 1996; Strowitzki *et al.*, 1998; Vicino *et al.*, 2000). It should be noted that some of these conclusions were drawn based on studies of a selected group of non-obese women (i.e. BMI < 27 kg/m^2). The current analysis, however, shows an increased incidence of spontaneous miscarriage with increasing BMI in women with PCOS. This finding has been reported before (Hamilton-Fairley *et al.*, 1992). The present analysis, though, shows that all other studies are in line with this observation. This result again stresses the importance of weight reduction. Likewise, it has been

described that the incidence of spontaneous miscarriage increases with decreasing insulin sensitivity (Dale *et al.*, 1993). However, the small number of miscarriages precludes definitive conclusions in this regard. Along these lines, it has been suggested that insulin-sensitizing agents also reduce miscarriage rates (Glueck *et al.*, 2001).

Hyperandrogenism is considered to be a key feature in PCOS and constitutes a hallmark for the diagnosis (Dunaif *et al.*, 1992). Intra-ovarian inhibitors of FSH action (such as the IGF system) (Schipper *et al.*, 1997; van Dessel *et al.*, 1999) might possibly promote follicle maturation arrest and concomitantly ovarian hyperandrogenism (Giudice, 1999). Hyperandrogenism has proven to be a powerful predictor for the response to ovulation induction, emphasizing its significance for ovarian dysfunction in these women (Imani *et al.*, 1998; Imani *et al.*, 1999; Imani *et al.*, 2000; Imani *et al.*, 2002a; Imani *et al.*, 2002b; Mulders *et al.*, 2003a). The impact of these biologically plausible factors involved in ovarian dysfunction in normogonadotropic anovulation, such as serum androgens and free IGF-I, unfortunately could not be scrutinized in the current analysis, because of lack of data.

Elevated LH levels are frequently encountered in PCOS, but this is not a mandatory diagnostic of PCOS (Laven *et al.*, 2002). Although it was previously reported that elevated serum LH concentrations were associated with increased miscarriage rates on the basis of retrospective studies (Howles *et al.*, 1986; Balen *et al.*, 1993b; Watson *et al.*, 1993), prospective data do not support the concept that elevated LH is implicated in ovarian dysfunction and ovulation induction outcome (Imani *et al.*, 2002b). The present analysis, however, shows a small but significant association of elevated serum LH with increased pregnancy rates.

Upon pelvic ultrasound, ovaries of women with normogonadotropic anovulation might be enlarged (Puzigaca *et al.*, 1991; Pache *et al.*, 1992a), contain an increased number of follicles (Obhrai *et al.*, 1990; Jonard *et al.*, 2003), and exhibit an increased density of ovarian stroma (Dewailly, 1997). It has been shown that the value of these sonographic parameters as a screening test to predict endocrine abnormalities characteristic of PCOS is limited (van Santbrink *et al.*, 1997). In addition, sonographic parameters are predictive of patients remaining anovulatory following CC (Imani *et al.*, 1998). Others recently described a correlation between initial ovarian volume or mean follicle number and subsequent response applying gonadotropin induction of ovulation (van der Meer *et al.*, 1998; Lass *et al.*, 2002; Mulders *et al.*, 2003a). These findings could not be reconfirmed in the current analysis since none of the included studies reported sufficient data to perform the analysis.

The association of advanced age with reduced treatment outcome following CC- or FSH-induced cycles, as previously reported (McClure *et al.*, 1993; Imani *et*

al., 2002b; Mulders *et al.*, 2003a), could not be confirmed by the present meta-analysis because of lack of data.

In summary, the current results are perhaps somewhat disappointing. However, this should not be too surprising as most studies did not intend to predict treatment outcome by patient characteristics. The possibility that some conclusions from this analysis may be affected by the repetitive inclusion of data cannot be completely discarded. However, we believe that such effects, if they exist, are minor. In addition, pooling of the original data files rather than the published data might also have resulted in slightly different outcomes. Principally, the association between initial clinical screening parameters (reflecting the extent of ovarian dysfunction in normogonadotropic anovulatory infertility) and treatment outcome deserves further attention. In addition, more individualized ovulation induction treatment algorithms may subsequently be developed. For the future, there is a need to standardize the definitions of ovulation induction treatment outcome in women with normogonadotropic anovulatory infertility (including PCOS). Live birth from a singleton pregnancy following gonadotropin induction of ovulation could then be more effectively achieved by treatment strategies individually tailored on the basis of initial screening characteristics.

Acknowledgement

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5.3 IVF outcome in WHO II anovulatory infertility (including polycystic ovary syndrome) following previous unsuccessful ovulation induction

Abstract

This follow-up study represents in-vitro fertilization (IVF) treatment characteristics and outcomes in women with World Health Organization (WHO) group II anovulatory infertility after previous unsuccessful ovulation induction compared to controls. Furthermore, the possibility of initial screening parameters of these anovulatory women to predict IVF outcome was examined.

Twenty six patients with WHO II anovulatory infertility who failed to establish a live birth following previous induction of ovulation (using clomiphene citrate as first line and follicle-stimulating hormone (FSH) as second line) were compared with 26 IVF patients with tubal infertility matched for age, treatment period and treatment regimen. The WHO II patients underwent 49 IVF cycles, whereas the normo-ovulatory controls underwent 46 cycles. In WHO II patients 15 cycles were cancelled compared to 6 cycles in controls ($P = 0.04$). Cycles were predominantly cancelled due to insufficient response ($P = 0.04$). In case the cycle was cancelled, body mass index (BMI) was significantly higher ($P < 0.001$) in WHO 2 women compared to controls. Overall live birth rates were comparable ($P = 0.9$).

Obese women suffering from WHO II anovulatory infertility are at an increased risk to have their IVF cycle cancelled due to insufficient response. Once oocyte retrieval is achieved live birth rates are comparable to controls.

5.3.1 Introduction

Chronic anovulation is a common cause of infertility. Most anovulatory women have irregular menstrual cycles and normal serum FSH concentrations (World Health Organization (WHO) group II) (The ESHRE Capri Workshop Group, 1995; Rowe *et al.*, 2000). Depending on the criteria used, polycystic ovary syndrome (PCOS) is diagnosed in approximately 60-70% of these women (van Santbrink *et al.*, 1997; Laven *et al.*, 2002).

When classical induction of ovulation (including clomiphene citrate as first line and exogenous gonadotropins as second line treatment) fails to result in pregnancy, in-vitro fertilization (IVF) has proven to be a feasible therapeutic option (Shulman and Dor, 1997). Previous studies comparing IVF treatment outcome in PCOS versus controls have shown that more oocytes could be retrieved, but with a reduced proportion of oocytes fertilized. These observations suggest that an increased number of immature oocytes are recruited (Dor *et al.*, 1990; Urman *et*

al., 1992; Homburg *et al.*, 1993). Moreover, both mature and immature oocytes of PCOS patients show reduced fertilization rates, presumably due to endogenous hormonal imbalance (Dor *et al.*, 1990; Urman *et al.*, 1992). Despite reduced overall fertilization, IVF pregnancy rates in PCOS patients appear to be comparable to normo-ovulatory women (Dor *et al.*, 1990; Urman *et al.*, 1992; Homburg *et al.*, 1993).

These findings should be interpreted with caution, however, because of possible confounding factors. First of all, pituitary down-regulation by gonadotropin-releasing hormone (GnRH) agonist co-treatment, associated with overall increased pregnancy rates following IVF (Hughes *et al.*, 1992; Homburg *et al.*, 1993), was not uniformly applied (Dor *et al.*, 1990; Urman *et al.*, 1992). Furthermore, some reports compared IVF treatment outcome in patients with and without polycystic ovaries (PCO) (Owen *et al.*, 1991; MacDougall *et al.*, 1993). Subjects involved did not necessarily suffer from anovulatory infertility, and therefore it does not seem appropriate to extrapolate these findings to women who suffer from PCOS. Other potential confounders might constitute the inclusion of patients in whom, besides PCOS, additional factors such as tubal infertility or oligospermia (Salat-Baroux *et al.*, 1988; Dale *et al.*, 1991) were involved, or there were uncertainties regarding previous ovulation induction strategies (Dor *et al.*, 1990; Tanbo *et al.*, 1990; Homburg *et al.*, 1993). These above mentioned factors, along with the limited patient numbers and the absence of proper controls (Tanbo *et al.*, 1990; Dor *et al.*, 1992; Urman *et al.*, 1992) seriously hamper interpretation of these studies.

The present study was designed to compare IVF treatment characteristics (such as amount of FSH administered, number of oocytes retrieved, cancellation rates and fertilization rates) along with clinical outcomes in women suffering from normogonadotropic anovulatory infertility versus controls. Women with WHO II infertility, originating from a large series of consecutive women all prospectively followed from the initiation of fertility work-up, were matched with a control group consisting of women with tubal infertility. Distinct variability in individual patient characteristics might be indicative for IVF outcome in women with normogonadotropic anovulatory infertility, as was previously shown for ovulation induction strategies using either clomiphene citrate (Imani *et al.*, 1998; Imani *et al.*, 1999; Imani *et al.*, 2000; Imani *et al.*, 2002b) or gonadotropins (Imani *et al.*, 2002a). Therefore, clinical, sonographic and endocrine screening characteristics possibly predicting stimulation characteristics or IVF outcome were also examined.

5.3.2 Materials and methods

Study population

Between February 1993 and May 1999, a consecutive series of 240 women with WHO II anovulatory infertility visiting the fertility outpatient clinic were included in the present prospective follow-up study (Imani *et al.*, 1998; Imani *et al.*, 1999; Imani *et al.*, 2000; Imani *et al.*, 2002a; Imani *et al.*, 2002b). The local Medical Ethics Review Committee approved this study, and informed consent was obtained from all participants. All 240 women started with induction of ovulation using clomiphene citrate as a first line treatment. A total of 57 (24%) and 71 (30%) were found to suffer from CC resistant anovulation (CRA) and CC failure (CCF), respectively. Subsequently, a total of 84 women from the initial cohort underwent gonadotropin induction of ovulation as second line therapy. In 44 women, an ongoing pregnancy was achieved. Finally, a total of 26 women underwent IVF following unsuccessful classical ovulation induction and were included in the present analysis. Inclusion criteria were: [1] oligomenorrhea (bleeding intervals between 35 days and 6 months) or amenorrhea (bleeding interval > 6 months), [2] serum FSH and E_2 concentrations within normal limits (van Santbrink *et al.*, 1995a), [3] normal serum prolactin and thyroid-stimulating hormone concentrations, [4] spontaneous menses or positive bleeding response to progestagen withdrawal, [5] BMI (body mass index (kg/m^2)) > 18 kg/m^2, [6] age between 25 and 40 years, [7] total motile sperm count [TMC = ejaculate volume (mL) $\times$ sperm concentration (10^6/mL) $\times$ percentage of progressive motile spermatozoa] > 1 x 10^6, [8] a negative history for any tubal pathology along with negative antibody test for chlamydia or proven normal tubal patency upon hysterosalpingography or laparoscopic inspection, and [9] failure to establish either ovulation or live birth following ovulation during previous induction of ovulation.

For each subject undergoing IVF one normo-ovulatory control patient matched for age, period of IVF treatment (1995-2000) and a similar GnRH agonist long protocol as co-treatment for ovarian stimulation was included in the present study. All had a history of regular menstrual cycles (between 25 and 32 days). These matched controls suffered from tubal infertility (bilateral occluded tubes) as their indication for IVF.

Initial screening

Initial standardized clinical, sonographic and endocrine screening of women with oligo-amenorrhea was performed at intake as previously described (Imani *et al.*, 1998; Imani *et al.*, 1999; Imani *et al.*, 2000; Imani *et al.*, 2002a; Imani *et al.*, 2002b). Clinical screening parameters recorded were age, duration and type (primary versus secondary) of infertility, cycle history, BMI, waist-to-hip ratio (WHR). During subsequent transvaginal sonography (TVS) ovarian stroma echogenicity, ovarian

volume (mL) and total number of follicles, were assessed as previously described (Pache *et al.*, 1992a; van Santbrink *et al.*, 1997). Endocrine screening included the assessment of FSH, luteinizing hormone (LH), estradiol (E_2), androstenedione (AD), testosterone (T), sex hormone-binding globulin (SHBG), dehydroepiandrosterone-sulphate (DHEAS), fasting insulin and glucose, free and total insulin-like growth factor-I (IGF-I), IGF binding protein-I (IGFBP-I), IGFBP-III, inhibin B and leptin serum levels, as previously published (Imani *et al.*, 1998; Imani *et al.*, 1999; Imani *et al.*, 2000; Imani *et al.*, 2002b). The hormone assays used and the intra- and interassay coefficients of variation valid for this study have all been described previously (Imani *et al.*, 1998; Imani *et al.*, 1999; Imani *et al.*, 2000).

Preceding ovulation induction treatment regimen

The treatment protocol, assessment of ovarian response and conception have been described previously (Imani *et al.*, 1998; Imani *et al.*, 1999; Imani *et al.*, 2000; Imani *et al.*, 2002a; Imani *et al.*, 2002b). In brief, CC doses were 50 mg/day, starting on cycle day 3 for 5 subsequent days after a spontaneous or progestagen-induced withdrawal bleeding. In the case of absent ovarian response, daily doses were increased to 100 and 150 mg in subsequent cycles. All patients with normogonadotropic anovulation trying to conceive were said to be CRA if they remained anovulatory during at least 3 consecutive cycles. CCF was defined as failure to conceive despite having 6 ovulatory cycles during the preceding CC treatment. In case of CRA or CCF, gonadotropin induction of ovulation was applied as second line therapy. Exogenous FSH was initiated on days 3-5 after a spontaneous or an induced withdrawal bleeding. During the first cycle, a low-dose step-up regimen using either daily intramuscular (i.m.) injections of urinary (u)FSH (Metrodin HP®, Serono Benelux BV, The Hague, The Netherlands) or subcutaneous (s.c.) injections of recombinant (r)FSH (Gonal-F®, Serono Benelux BV) was used to assess the individual FSH response (threshold) dose (i.e. sonographic visualization of a follicle ≥ 10 mm) (Imani *et al.*, 2002a). During all subsequent cycles, a step-down protocol was performed. No significant differences in outcome between urinary and recombinant FSH were observed (data not shown).

IVF treatment regimen and monitoring of ovarian response

In those women suffering from amenorrhea or severe oligomenorrhea, pituitary down-regulation was initiated randomly. In the remaining patients and controls pituitary down-regulation was achieved by administering the GnRH agonist triptorelin (0.1 mg daily s.c., Decapeptyl®, Ferring Nederland BV, Hoofddorp, The Netherlands) starting one week before the expected menses (Beckers *et al.*, 2000). In all patients ovarian stimulation was commenced after two weeks of GnRH agonist use.

Different preparations of urinary FSH (i.m.) or recombinant FSH (s.c.) have been used (Humegon®, NV Organon, Oss, The Netherlands; Metrodin HP®, Serono Benelux BV; Puregon®, NV Organon). Multiple follicle development was induced with daily injections at an initial dose of 150 up to 450 IU (depending on previous response). The daily dose was adjusted according to the ovarian response as assessed by serial ultrasound scans. On the day ≥ 3 follicles > 15 mm were present and the leading follicle reached a diameter ≥ 18 mm, a single bolus of human chorionic gonadotropin (hCG) (Pregnyl®, NV Organon) (5,000 IU or 10,000 IU) was administered (Beckers *et al.*, 2000). Transvaginal ultrasound-guided oocyte retrieval was performed within 32-36 hours. After fertilization, a maximum of two embryos was transferred after 3-5 days of culture (Huisman *et al.*, 2000). Remaining embryos of sufficient quality were cryopreserved.

Luteal phase support, either hCG (Pregnyl®, NV Organon) (1,500 IU s.c. on the day of oocyte retrieval and 2, 4, and 6 days after oocyte retrieval) or micronized progesterone (Progestan®, NV Organon) (600 mg/day administered vaginally) was given from the day of oocyte retrieval (van der Gaast *et al.*, 2002).

Cycles were cancelled if there was insufficient response (the presence of no more than two dominant follicles) as assessed by ultrasound. Hyperresponse was defined as the presence of multiple dominant follicles (The ESHRE Capri Workshop Group, 1996). In the latter case, the cycle was cancelled because of the risk of ovarian hyperstimulation syndrome (OHSS). The severity of OHSS was assessed according to previously published guidelines (Navot *et al.*, 1992). A biochemical pregnancy was defined as a positive urinary pregnancy test (Clearview®: hCG > 25 IU/L, Unipath Limited, Bedford, Bedfordshire, United Kingdom) performed at least 17 days after oocyte retrieval. An ongoing pregnancy was defined as sonographic assessment of intrauterine gestational sac with positive heartbeat. Live birth rate was defined as the number of live born babies per 26 patients.

Data analysis

All patients were followed either until their first pregnancy or until discontinuation of therapy. Data of clinical and IVF treatment characteristics in both patient groups are presented as means ± SD if distributed normally. Otherwise data are presented as median and range. Comparisons between the groups were performed using a Student's *t* test, Mann-Whitney *U* test or χ^2-test. Cumulative conception rates were analysed with the Kaplan-Meier method, as were cumulative live birth rates. Censoring was defined either as definitive discontinuation of therapy without conception or end of follow-up period. The log rank test was used for statistical comparison in Kaplan-Meier analyses.

Student's *t* test and χ^2-test were used to compare initial screening parameters of cycles in WHO II women who respectively did and did not undergo

oocyte retrieval. Women with multiple treatment cycles will occur more than once in this analysis. When they have repetitive cancellation of cycles this may potentially lead to biased results. Association between screening characteristics and stimulation characteristics was assessed by Spearman's correlation. Multiple linear regression was used for multivariable analysis, with amount of FSH administered as a continuous outcome variable. Initial screening variables that were significant in univariable analyses were entered into the multiple regression model in a forward stepwise fashion.

Student's *t* test and χ^2-test were also used to compare initial screening parameters between the women with WHO II infertility included in the initial cohort and the WHO II women who finally underwent IVF.

Data were analysed using a commercially available software package (SPSS, Chicago, IL, USA). A P value of 0.05 (two-tailed) was considered as the threshold level for statistical significance.

5.3.3 Results

Twenty-six patients with anovulatory infertility (WHO II) fulfilled the inclusion criteria and subsequently underwent 49 IVF cycles. The control group consisted of 26 (46 cycles) normo-ovulatory women suffering from tubal infertility matched for age, treatment period, and treatment regimen. Clinical as well as IVF treatment characteristics of both groups are summarized in Table 1. In WHO II patients 15 cycles (31%) were cancelled, compared with only six cycles (13%) in the control group (P = 0.04). Cycles were cancelled either due to insufficient response (WHO II: 25% (n = 12), controls: 7% (n = 3); P = 0.04) or hyperresponse (WHO II: 4% (n = 2), controls: 4% (n = 2); NS). Only a few cycles were cancelled because of intercurrent disease (WHO II: 2% (n = 1), controls: 2% (n = 1); NS). In patients in whom the cycle was cancelled, BMI was significantly higher (P < 0.001) in WHO II women compared with controls (32.3 versus 22.7 kg/m^2).

TABLE 1 IVF characteristics (mean ± SD) of women with normogonadotropic anovulatory infertility (WHO II) after unsuccessful preceding classical ovulation induction versus matched control IVF patients.

	WHO II infertility	Tubal infertility	P value
	(N = 26 patients)	(N = 26 patients)	
Clinical characteristics			
Age (years)	34.9 ± 3.2	34.8 ± 3.5	NS
% Primary infertility (n)	62 (16)	69 (18)	NS
BMI (kg/m²)	26.5 ± 6.2	25.0 ± 5.2	NS
Total motile sperm count	183 (1 - 681)*	50 (1 - 4000)*	NS
	(N = 49 cycles)	(N = 46 cycles)	
Stimulation characteristics			
No. of cancelled cycles (%)	15 (31)	6 (13)	0.04
Duration of stimulation (days)[a]	11.4 ± 2.8	11.7 ± 2.5	NS
Total amount of FSH administered (IU)[a]	1946 ± 720	2100 ± 909	NS
Late follicular phase ovarian follicles[a,b]			
Total no. of follicles (≥ 10 mm)	15.6 ± 7.4	11.9 ± 5.6	0.02
No. of follicles 10-13 mm	11.4 ± 6.7	8.5 ± 5.4	0.04
No. of follicles 14-17 mm	3.8 ± 1.9	3.4 ± 1.4	NS
No. of follicles ≥ 18 mm	1.1 ± 1.1	1.2 ± 0.9	NS
Gamete and embryo characteristics			
No. of oocytes retrieved	11.8 ± 7.8	10.9 ± 5.9	NS
% normal fertilization	50 ± 27	62 ± 26	NS
No. of 2 PN embryos	5.8 ± 3.9	6.4 ± 3.7	NS
% embryo transfer with 1-2 good quality embryos (n)[c]	45 (13)	67 (25)	NS

* median (range). [a] Only for non-cancelled cycles. [b] Day of hCG. [c] Definition based upon morphology score (i.e. stage of development, amount of fragmentation, presence of degeneration and amount of granulation) as previously published (Huisman *et al.*, 2000).

TABLE 2 Initial clinical, endocrine, and sonographic screening characteristics of women with normogonadotropic anovulatory infertility, separate for those who underwent oocyte retrieval and those who did not. Values are mean ± SD.

	Overall group N = 49 cycles	**Cycle cancelled** N = 15 cycles	**Oocyte retrieval** N = 34 cycles	**P value**
Clinical characteristics				
Age at onset IVF (years)	34.9 ± 3.3	34.1 ± 3.9	35.3 ± 3.0	NS
Primary infertility (%)	36 (74)	9 (60)	27 (79)	NS
Amenorrhea (%)	16 (33)	4 (27)	12 (35)	NS
Bleeding interval (days)	98.9 ± 72.8	87.7 ± 70.1	103.8 ± 74.5	NS
BMI (kg/m²)	27.8 ± 6.4	32.3 ± 6.2	25.8 ± 5.5	0.002
Endocrine characteristics				
FSH (IU/L)	4.9 ± 1.9	4.5 ± 2.0	5.4 ± 1.9	NS
LH (IU/L)	9.2 ± 4.4	8.6 ± 4.0	9.4 ± 4.6	NS
T (mmol/L)	2.9 ± 1.3	3.1 ± 1.6	2.7 ± 1.1	NS
SHBG (nmol/L)	51.4 ± 24.3	36.7 ± 15.4	57.8 ± 24.9	0.001
FAI (T x 100/SHBG)	7.5 ± 6.5	10.8 ± 8.1	6.0 ± 5.1	0.05
AD (nmol/L)	17.2 ± 10.3	18.6 ± 10.6	16.6± 10.6	NS
DHEAS (µmol/L)	8.3 ± 3.5	10.2 ± 3.7	7.4 ± 3.1	0.02
IGFBP-I (ng/mL)	23.1 ± 19.0	13.1 ± 10.6	27.9 ± 20.4	0.006
Ultrasound characteristics				
No. of patients with PCO (%)[a]	36 (74)	9 (60)	27 (79)	NS
Total stroma score (n)	3.4 ± 1.4	2.9 ± 1.2	3.7 ± 1.4	0.05

[a] Polycystic ovaries defined as mean ovarian volume ≥ 10.8 mL and/or mean follicle number per ovary ≥ 10 (van Santbrink *et al.*, 1997).

The differences found in initial parameters between WHO II patients who did and did not undergo oocyte retrieval are depicted in Table 2. Briefly, BMI was significantly different (P = 0.003) in those women that succeeded until oocyte retrieval and the ones who did not. The analysis on first IVF cycles gave similar results. BMI, SHBG and IGFBP-I were significantly different. The differences for FAI (free androgen index: T x 100/SHBG) and DHEAS were of the same magnitude, but not of statistical significance. Univariate analysis showed that the total amount of exogenous FSH (IU) administered correlated with BMI, serum levels of SHBG, FAI, DHEAS and IGFBP-I (Table 3).

TABLE 3 Spearman's correlation coefficients of initial clinical and endocrine screening characteristics of the overall group of anovulatory infertile patients (n = 26) with the total amount of FSH (IU) administered.

	Total amount of FSH (IU) administered	
	r[a]	No. of cycles[b]
BMI (kg/m²)	0.38[c]	48
SHBG (nmol/L)	- 0.47[c]	48
FAI (T x 100/SHBG)	0.34[d]	48
DHEAS (µmol/L)	0.56[c]	48
IGFBP-I (ng/mL)	- 0.50[c]	36

[a] Univariate analysis (step 0).
[b] Cancelled and non-cancelled combined.
[c] P < 0.01.
[d] P < 0.05.
Note: Only variables significant in univariate analysis were used in multivariate analysis. Forward stepwise multivariate analyses of initial screening characteristics for prediction of total amount of FSH (IU) administered. All analyses were corrected for BMI, to test whether an association exists between screening characteristics and total amount of FSH (IU), independent from BMI. In this analysis only DHEAS remained significant (P < 0.01). The adjusted R^2 of the final model (including BMI and DHEAS) = 0.54.

Subsequently, a multivariable analysis of initial screening characteristics for prediction of total amount of FSH (IU) administered in the overall group of anovulatory infertile patients was performed. All analyses were corrected for BMI, to test whether an association exists between screening characteristics and total

amount of FSH (IU), independent from BMI. In this analysis, only DHEAS remained significant ($P < 0.01$). The adjusted R^2 of the final model (including BMI and DHEAS) = 0.54.

Patients with anovulatory infertility achieving oocyte retrieval developed significantly more dominant follicles on the day of hCG administration compared with controls ($P = 0.02$). However, the oocyte recovery rates were comparable (Table 1). The correlations between the total amount of exogenous FSH (IU) administered versus the total number of follicles or BMI in 26 WHO II women and their controls are depicted in Figure 1. There were three cases of OHSS in the group with WHO II infertility whereas only one patient in the control group showed signs of OHSS (NS).

TABLE 4 Pregnancy and live birth rates as percentage (absolute numbers in parentheses) of women with normogonadotropic anovulatory infertility (WHO II) after unsuccessful preceding classical ovulation induction versus matched control IVF patients.

	WHO II infertility (26 patients)		**Tubal infertility** (26 patients)		**P value**
Clinical pregnancies/cycle	33	(16/49)	26	(12/46)	0.5
Clinical pregnancies/oocyte retrieval	47	(16/34)	30	(12/40)	0.1
Clinical pregnancies/embryo transfer	55	(16/29)	32	(12/38)	0.05
Implantation rate/embryo	36	(20/56)	19	(14/74)	0.03
Miscarriages	38	(6/16)	17	(2/12)	0.2
Live birth/cycle	16	(8/49)	15	(7/46)	0.9
Multiple pregnancies (twins)	50	(5/10)	30	(3/10)	0.4

IVF treatment outcome in both groups is summarized in Table 4. In WHO II patients, a total of 16 cycles (55%) resulted in a biochemical pregnancy compared with 12 (32%) cycles in the control group ($P = 0.05$). In the WHO II group, six biochemical pregnancies (38%) resulted in a miscarriage, compared with only two (17%) in the control group ($P = 0.2$). WHO II women with an ongoing pregnancy did not have lower levels of initial serum LH ($P = 0.6$) or seemed to be less obese ($P = 0.1$) (data not shown). Cumulative conception rates (after three cycles) in WHO II subjects were 68 and 54% for those with tubal infertility ($P = 0.5$). Similarly, the cumulative live birth rates were almost identical for both groups (38 versus 31%)($P = 0.9$) (Figure 2).

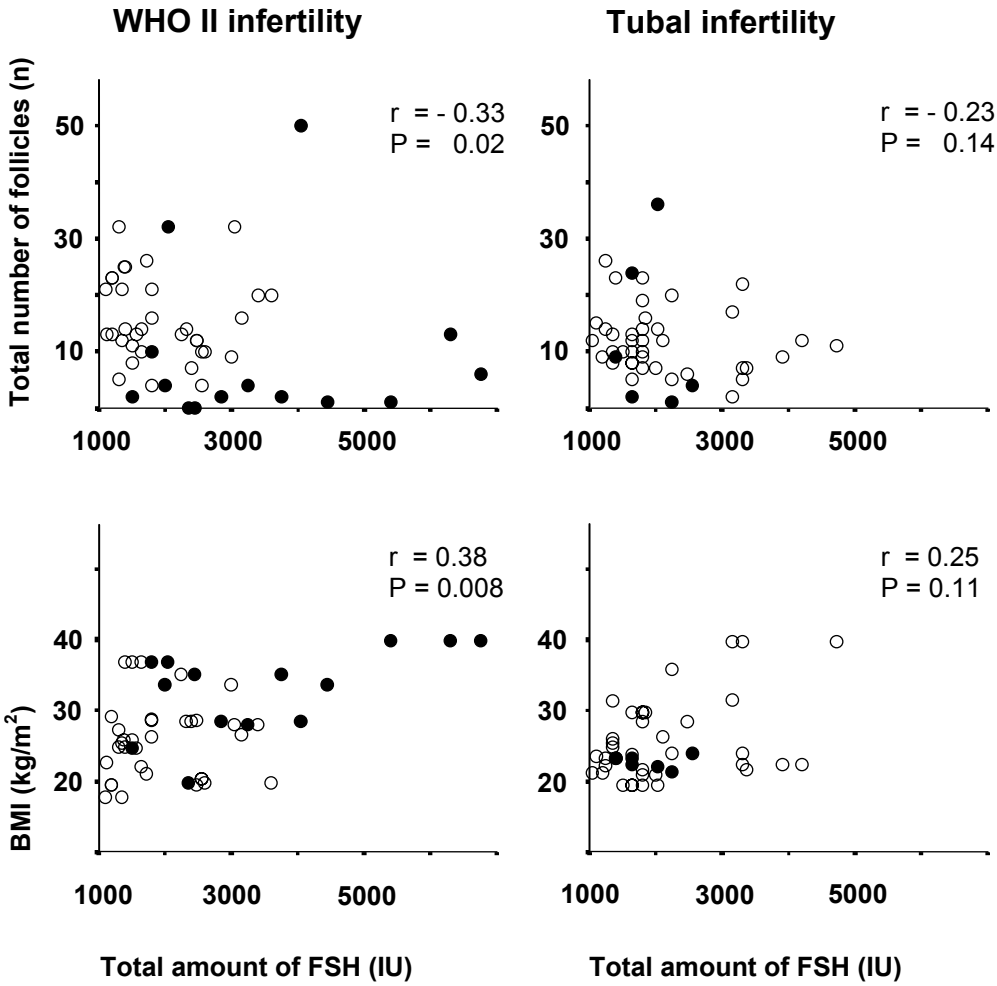

Figure 1 Scatter plots depicting the correlations between the total amount of exogenous FSH (IU) administered versus the total number of dominant (≥ 10 mm) follicles on the day of hCG or BMI in 26 women with WHO II infertility and tubal infertility. Open circles represent patients who underwent oocyte retrieval. Closed circles represent patients whose cycle was cancelled. Spearman's correlation coefficients and corresponding P values are depicted.

No significant differences were found in initial screening characteristics between the WHO II women included in the initial cohort and those who finally underwent IVF (mean values for BMI, T, AD and mean ovarian volume are respectively 26.8 versus 27.3, 2.4 versus 2.6, 15.2 versus 16.9 and 9.8 versus 12.9).

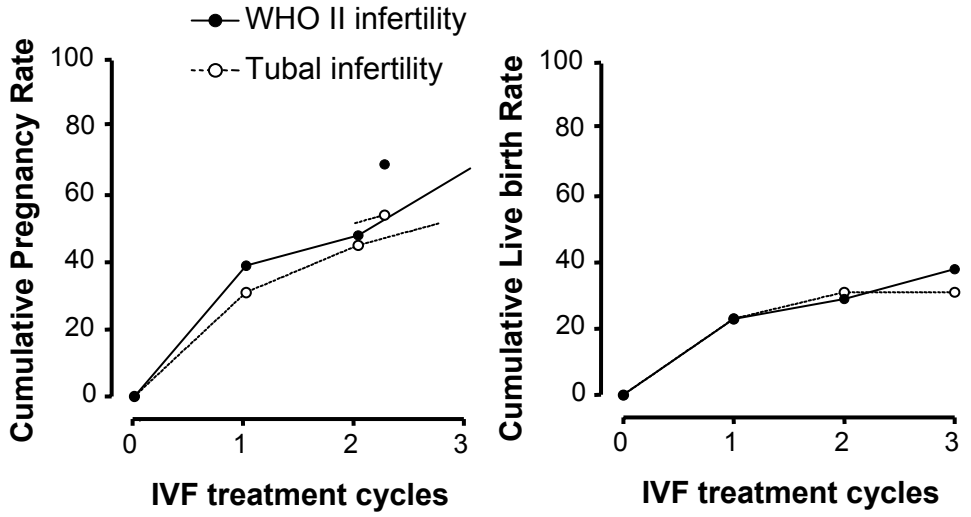

Figure 2 Life-table analysis of cumulative conception rates and cumulative live birth rates in 26 women with normogonadotropic anovulatory infertility and 26 controls with tubal infertility that underwent IVF.

5.3.4 Discussion

The present study demonstrates that cancellation rates during ovarian stimulation and the total number of late follicular phase follicles on the day of hCG (especially those of 10-13 mm in size) in the non-cancelled cycles were increased following IVF in women with WHO II anovulatory infertility after previous unsuccessful ovulation induction. In these women, obesity was correlated with an increased number of cancelled cycles (unexpectedly, mostly due to insufficient ovarian response) together with an increased amount of exogenous FSH administered. However, the numbers of retrieved - and normally fertilized - oocytes, as well as clinical and ongoing pregnancy rates, were not significantly different comparing women with WHO II anovulatory infertility and matched controls suffering from tubal infertility. Anovulatory women tended to have an increased miscarriage rate,

along with indications for slightly enhanced implantation. The observed incidence of ovarian hyperstimulation syndrome was comparable in both groups.

Patient numbers involved in the current study are relatively limited, arguing for some caution regarding conclusions drawn. Despite this limitation, it was not possible to identify major differences comparing IVF outcome in WHO II anovulatory infertility compared with matched control subjects. Beforehand, it would be reasonable to expect a difference of 10 - 15% in cancellation rates (starting from a mean cancellation rate of 22%). A much larger difference in cancellation rates was found, which was statistically significant. Still, this study does not exclude that differences are in fact in the order of 10% or lower (e.g. 95% CI of cancellation rates (%) = 1.4 - 33.7).

Dominant follicle selection is disturbed in polycystic ovaries, resulting in an increased number of follicles per ovary and presumably a variable number of healthy early antral follicles (Fauser and van Heusden, 1997). Women with PCOS seem to be at risk for multi-follicular development in response to gonadotropin stimulation (MacDougall *et al.*, 1992a). Others reported an increased number of follicles produced in PCO patients compared with normal controls (MacDougall *et al.*, 1993). The current study confirms that the number of small dominant follicles (10-13 mm) stimulated during ovarian stimulation in anovulatory patients is increased. Since the number and fertilization rates of retrieved oocytes were similar in patients and controls, the enhanced ovarian response may reflect stimulation of atretic follicles. This concept is in line with several previous observations reporting normal inhibin B concentrations in PCOS patients, suggesting a normal number of healthy early antral follicles despite increased overall follicle numbers in these women (Pigny *et al.*, 2000; Laven *et al.*, 2001a).

According to most authors, patients with PCOS are particularly prone to develop OHSS because of the presumed enhanced ovarian response to gonadotropins (MacDougall *et al.*, 1992b). Remarkably, the present data suggest that the incidence of OHSS may not be increased in women suffering from normogonadotropic anovulatory infertility. Cancellation was mostly due to insufficient response instead of hyperresponse. It cannot be excluded that the use of E_2 concentrations next to ultrasound for monitoring of ovarian response would have resulted in lower cancellation rates. Moreover, the addition of exogenous LH (Lisi *et al.*, 2001) next to FSH in GnRH antagonists cycles (Barri *et al.*, 2002) may possibly improve ovarian response. In the current study, women with WHO II infertility undergoing IVF originate from a well-defined group of patients for whom ovulation induction is a treatment with good prospects of singleton live birth (Eijkemans *et al.*, 2003). Therefore, differences in patient selection and preceding ovulation induction are the most likely explanation for this observed discrepancy. However, no discrepancy was observed in initial screening characteristics between the initial cohort and the women who finally underwent IVF. Nevertheless,

screening parameters indicative of ovulation induction outcome (Imani *et al.*, 1998; Imani *et al.*, 1999; Imani *et al.*, 2000; Imani *et al.*, 2002a; Imani *et al.*, 2002b) showed a trend toward reduced treatment outcome for those women that finally underwent IVF. Given the low power of the study, this trend is not statistically significant.

In the present analysis, a marked impact of obesity on the cycle cancellation rates was observed in women with anovulatory infertility. Since insufficient ovarian response following gonadotropin stimulation is related primarily to obesity, it might be speculated that in obese women exogenous FSH is diluted in a larger circulating volume. Indeed, after a fixed bolus injection of exogenous FSH, serum concentrations have been shown to be dependent on body weight (Mannaerts *et al.*, 1993). However, varying the gonadotropin dose on the basis of body weight has never been shown to result in improved IVF outcome. In case oocyte retrieval is accomplished, obesity no longer compromises IVF outcome. This suggests that the importance of body weight is only related to the extent of ovarian response following FSH administration. Previous findings indicating that BMI is the most prominent predictor of the individual FSH response (threshold) dose in normogonadotropic, anovulatory infertile women undergoing gonadotropin induction of ovulation (Imani *et al.*, 2002a) are in line with this observation.

Remaining biologically plausible factors involved in ovarian dysfunction in normogonadotropic anovulation, such as serum LH (Fauser *et al.*, 1991), inhibin B (Laven *et al.*, 2001a), free IGF-I (van Dessel *et al.*, 1999) and insulin/glucose ratios (Poretsky *et al.*, 1999), all failed to predict cancellation or total amount of FSH administered during ovulation induction (Imani *et al.*, 1998; Imani *et al.*, 1999; Imani *et al.*, 2000; Imani *et al.*, 2002a; Imani *et al.*, 2002b). Screening characteristics involved in the prediction of ovulation after CC are distinctly different from predictors of conception in ovulatory CC cycles (Imani *et al.*, 1999; Imani *et al.*, 2002b). This disparity suggests that the FSH threshold (magnitude of FSH required for stimulation of ongoing follicle growth and ovulation) and oocyte quality (chances for conception in ovulatory cycles) may be differentially regulated. The present analysis again stresses this concept, since no differences in initial screening characteristics in WHO II women were found once oocyte retrieval had been achieved.

Similar pregnancy rates have been reported following IVF in PCOS subjects and normo-ovulatory controls (Dor *et al.*, 1990; Urman *et al.*, 1992; Homburg *et al.*, 1993). According to these authors, these findings indicate no further impairment in treatment outcome once oocytes are fertilized. This is, however, in conflict with the present findings, where an increased number of clinical pregnancies in WHO II women resulted in a similar live birth rate because of an elevated number of miscarriages. Although the number of miscarriages was higher for women with anovulatory infertility, this finding was not statistically significant. It should be noted,

however, that the sample size of this group is limited. Hence, actual differences cannot be excluded.

Hypersecretion of LH has initially been implicated as the cause of reduced fertilization and high miscarriage rate in women with PCOS (Homburg *et al.*, 1988). However, more recently considerable disagreement has arisen with regard to the significance of elevated LH concentrations (Imani *et al.*, 1999). Likewise, it has been reported that the incidence of spontaneous miscarriage increases with increasing BMI both in women with (Hamilton-Fairley *et al.*, 1992) and without (Fedorcsak *et al.*, 2000) PCOS. In previous studies (Imani *et al.*, 1999; Imani *et al.*, 2002b), however, no influence of obesity on live birth rates was detected in patients who ovulated following CC therapy. Although the sample size was limited, the present analysis does not confirm a possible association of increased serum LH levels or BMI with pregnancy outcome.

In summary, it has become evident that obesity is associated with an increased risk for cycle cancellations and requirements for increased amounts of exogenous FSH for IVF in women with WHO II infertility following previous unsuccessful ovulation induction. Although the total number of follicles recruited during ovarian stimulation is increased, the number of healthy follicles appears similar. Once oocyte retrieval is performed, pregnancy rates for these women are comparable to controls suffering from tubal infertility. This observation suggests that factors involved in chances for conception in WHO II infertility are similar compared with controls. However, there seems to be tendency for increased early pregnancy loss in WHO II women resulting in a comparable overall live birth rate following IVF treatment.

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Chapter VI

General discussion and conclusions

Chapter VI: General discussion and conclusions

In every day clinical infertility practice all WHO II anovulatory patients will undergo the same treatment algorithm in case of a wish to conceive. Initial patient characteristics may help to discriminate patients with favourable from those with unfavourable chances to respond to fertility treatment strategies. By identifying high risk patients beforehand, these women could be monitored more closely or stimulation regimens could be individually adjusted, reducing chances for multiple gestations or other complications in a given patient. Hence, screening of anovulatory women should be recommended in order to counsel individual patients regarding treatment outcome.

Hyperandrogenism expressed as either an elevated FAI or elevated level of testosterone (T) or androstenedione (AD) is observed in about 67% of all WHO II patients (Laven *et al.*, 2002). Androgens have been identified as the most important endocrine feature predicting ovulation induction outcome. FAI has been recognized as the most significant parameter predicting patients remaining anovulatory following CC (Imani *et al.*, 1998). In addition, androgens (i.e T, AD, and FAI) have been associated with the amount of exogenous FSH required for ovarian response during a low-dose step-up gonadotropin regimen for induction of ovulation (Imani *et al.*, 2002a). Finally, it has been shown that women with increased serum levels of T and AD are at an increased risk of multiple follicle development during gonadotropin ovulation induction (Mulders *et al.*, 2003a).

Polycystic ovaries constitute yet another important feature of women presenting with WHO II anovulation. PCO morphology, as assesed by ultrasound, is also associated with ovulation induction treatment outcome (Imani *et al.*, 1998; Mulders *et al.*, 2003a). It has been shown that normogonadotropic anovulatory women who exhibit an augmented number of ovarian follicles, an important criterion for PCOS diagnosis, prior to ovarian stimulation are at an increased risk of multiple follicle development during gonadotropin ovulation induction (Mulders *et al.*, 2003a). Consequently, these patients exhibit higher chances for cancellation of the FSH-induced cycle. Due to disturbed dominant follicle selection the number of early antral follicles is usually increased in these women (Fauser, 1994). As a consequence, serum levels of AMH, predominantly produced by small follicles, are increased in patients with polycystic ovaries (Cook *et al.*, 2002; Pigny *et al.*, 2003; Laven *et al.*, 2004; Mulders *et al.*, 2004a). The subgroup of WHO II women exhibiting polycystic ovaries presented with the highest AMH serum levels (Laven *et al.*, 2004). Furthermore, it seems that AMH levels corrrelate with serum levels of LH and T, and follicle numbers and ovarian volume as established on ultrasound (Laven *et al.*, 2004). In other words, serum AMH levels are probably predictive for the extent of the disease since they correlate well with other parameters of ovarian dysfunction (Laven *et al.*, 2004).

Fertility treatment can be viewed as an extended ovarian challenge test. Patient characteristics being the most important predictors for ovarian response during ovulation induction treatment are perhaps the ones involved in the pathophysiology of ovarian dysfunction. Indeed, hyperandrogenism, together with polycystic ovary morphology, happen to be cardinal features of PCOS, a subgroup within the WHO II category, as recently determined by the 2003 Rotterdam consensus workshop (The Rotterdam ESHRE/ASRM sponsored PCOS consensus workshop group, 2004a; The Rotterdam ESHRE/ASRM sponsored PCOS consensus workshop group, 2004b). However, WHO II anovulatory women, as well as PCOS patients, known to suffer from ovarian dysfunction, present with variable clinical symptoms. Moreover, many WHO II patients also present with obesity and/or signs of insulin resistance. Hence, WHO II anovulatory infertility, including PCOS, does not seem to represent a clear-cut clinical disease but rather constitutes a syndrome. As a consequence, it is difficult to prove that clinical characteristics associated with fertility treatment outcome are indeed the causative factors involved in the etiology of this syndrome. Hence, women with WHO II anovulation (including PCOS) constitute an extremely heterogeneous patient group since various causative mechanisms might be responsible for the development of the same clinical entity.

Many studies have shown an increased prevalence among first degree relatives and hence a genetic basis for the development of WHO II anovulation/PCOS seems to exist (Ferriman and Purdie, 1979; Lunde *et al.*, 1989; Govind *et al.*, 1999). The heterogeneity of the clinical phenotype suggests that a complex pattern of inheritance is most probably involved (Franks *et al.*, 2001; Legro and Strauss, 2003). In complex disorders the relationship between genotype and phenotype is not straightforward, which may be explained by the interaction of a small number of key genes with environmental factors. Moreover, the observable phenotype is not a constant phenomenon for a given individual, but seems to be influenced by environmental features (i.e. BMI and OCP) (Norman *et al.*, 2002; Mulders *et al.*, 2004b).

It has been recognized that obesity, usually of central origin (also referred to as the visceral, android or abdominal type), frequently coincides with anovulatory infertility and especially with PCOS. In addition, insulin resistance and compensatory hyperinsulinemia have been documented in women with PCOS. Both insulin resistance and hyperinsulinemia are magnified in the presence of obesity (Dunaif, 1997). In addition, hyperandrogenism (associated with anovulation) could result from hyperinsulinism leading to increased adrenal and ovarian production and decreasing serum sex hormone-binding globulin levels (Legro *et al.*, 2004). When performing ovulation induction with CC in WHO II anovulatory women it has been shown that obesity is a strong independent predictor of patients remaining anovulatory (Imani *et al.*, 1998). BMI also predicted

an individual patient's exogenous FSH requirements for initation of ongoing follicle growth during gonadotropin induction of ovulation (Imani *et al.*, 2002a). Indeed, obesity and insulin resistance are associated with adverse treatment outcome during gonadotropin induction of ovulation (Mulders *et al.*, 2003c). Moreover, obesity seems to be associated with increased chances for cycle cancellation following IVF (Mulders *et al.*, 2003b). These results once more demonstrate that obese WHO II anovulatory patients constitute a subgroup for whom fertility treatment outcome is less favourable. Obesity seems to be an important modulator of the clinical phenotype and life style modification might correct this.

Switching from an anabolic to catabolic state of metabolism can improve insulin resistance and hyperandrogenism, and might eventually restore menstrual cyclicity and, in time, fertility. Moreover, it has been demonstrated that weight loss can improve the response to ovulation induction (Norman *et al.*, 2002). There is, however, limited understanding of the details of how these weight changes affect human reproductive function (Norman *et al.*, 2004). Dietary intervention and life style modification (through education and increased physical activity) remain the first line treatment strategies for overweight women suffering from PCOS (Norman *et al.*, 2004). Hence, a well-developed weight loss program as a first line therapeutic option should be offered to all women with obesity and anovulatory infertility.

Another environmental modulator of the clinical picture is the OCP which might change the clinical, endocrine and sonographic features characteristic for PCOS. When performing a study in a founder population (i.e. a genetically isolated population founded by < 200 ancestors), the genetic and environmental homogeneity of women provides a unique opportunity to asses the sole effect of OCP on the WHO II or PCOS phenotype. Indeed, it was confirmed that the PCOS phenotype is influenced by the use of OCP. However, despite taking OCP, women diagnosed as PCOS in the past currently still fulfilled PCOS criteria (Mulders *et al.*, 2004b). Hence, ovarian dysfunction in women with anovulatory infertility is modulated, but not completely corrected, by OCP use.

Overlooking the present findings the following conclusions can be drawn. WHO II anovulation, including PCOS, is a heterogeneous syndrome of ovarian dysfunction. Furthermore, the observable phenotype is not a constant phenomenon for a given individual, but seems to be influenced by environmental factors (i.e. BMI and OCP). The present data again stress the concept that women with PCOS constitute a subgroup of women with WHO II anovulation with a more severe form of the disease. Hyperandrogenism and polcycystic ovary (PCO) morphology seem to be its cardinal fairly constant features. These cardinal features have proven to be important predictors in terms of fertility treatment outcome. Hence, these parameters are perhaps involved in its pathophysiology. Accordingly genetic

research should focus on genes involved in androgen synthesis (including insulin action) and follicle development.

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Summary
Samenvatting

Summary

Chapter I:
Polycystic ovary syndrome (PCOS) is the most common endocrine disorder in reproductive age women and a major cause of subfertility. Women with PCOS might present with a number of various features and hence PCOS does not seem to be a clear-cut clinical phenomenon. In order to elucidate the background and pathophysiology of all symptoms displayed in PCOS it might be helpful to focus on these specific characteristics and features. In this chapter pathophysiology of ovarian dysfunction is highlighted. Additionally, several characteristic PCOS features used for classification and involved in phenotypical heterogeneity, will be briefly described. Furthermore, the complexity of research regarding the genetic component involved in the disease is discussed. Finally, the clinical implications of differences in phenotype expression are mentioned. In addition, the objectives of the present thesis are described in brief.

Chapter II:
Section 2.1: The present study was conducted to compare anti-Müllerian hormone (AMH) serum levels in 128 World Health Organization (WHO) group II women to those of 41 normo-ovulatory premenopausal women of similar age. Serum AMH concentrations are significantly ($P < 0.001$) elevated in WHO II patients (median: 7.6 µg/L (range: 0.1-40.0)) compared to controls (median: 2.1 µg/L (0.1-7.4)). In 106 patients presenting with polycystic ovaries (PCO) (≥ 12 follicles/ovary measuring 2-9 mm and/or an ovarian volume > 10 mL) AMH levels are elevated (9.3 µg/L (1.8-40.0)) compared to 22 patients without PCO (6.4 µg/L (0.1-22.1)) ($P < 0.0001$). In WHO II patients AMH concentrations correlate with features characteristic for polycystic ovary syndrome (PCOS) such as luteinizing hormone (LH) concentrations ($r = 0.331$; $P = 0.0001$), testosterone (T) levels ($r = 0.477$; $P = 0.0001$), mean ovarian volume ($r = 0.421$; $P = 0.0001$) and the number of ovarian follicles ($r = 0.308$; $P = 0.0001$). AMH levels correlated well with age in WHO II patients ($r = -0.248$; $P = 0.002$) as well as in controls ($r = -0.465$; $P = 0.005$). However, the relative decline in AMH with age is less pronounced in WHO II patients. In a subset of patients no significant correlation was found between AMH serum concentrations and the follicle-stimulating hormone (FSH) response dose, the duration of stimulation and the total number of ampoules of FSH used. In conclusion, serum AMH concentrations are elevated in WHO II women, especially in those patients exhibiting PCO. Since AMH concentrations correlated well with other clinical, endocrine and ultrasound markers associated with PCOS, AMH may be used as a marker for the extent of the disease. A less pronounced AMH decrease over time in these women may suggest retarded ovarian ageing.

Section 2.2: The current study was designed to investigate whether the decrease in AMH serum concentrations over time is different comparing women with normogonadotropic anovulation (WHO II) (including PCOS) and normo-ovulatory controls. AMH serum levels were assessed on 2 occasions in 98 patients suffering from WHO II anovulatory infertility as well as in 41 normo-ovulatory premenopausal women. Median time interval between both visits was 2.6 years (range: 0.3 - 9.0) for WHO II patients compared to 1.6 years (range: 1.0 - 7.3) in controls. Serum AMH concentrations were significantly ($P < 0.0001$) elevated at both occasions in WHO II patients (AMH_1: median = 7.5 µg/L (range: 0.1 - 35.8) and AMH_2: median = 6.7 µg/L (range: 0.0 - 30.6)) compared to controls (AMH_1: median = 2.1 µg/L (range: 0.1-7.4) and AMH_2: median = 1.3 µg/L (range: 0.0-5.0)). Regression analysis, corrected for age, indicated a significant relative decrease in serum AMH concentrations over time for both groups ($P < 0.001$). However, the decline in serum AMH in WHO II patients was significantly less compared to controls ($P = 0.03$). The present longitudinal study shows that serum AMH concentrations decrease over time both in women presenting with WHO II anovulatory infertility as well as in normo-ovulatory controls. The decrease in WHO II patients is less pronounced despite distinctly elevated concentrations. This observation may suggest retarded ovarian ageing and hence a sustained reproductive life-span in these patients.

Chapter III:

The present observational study was performed to assess the sole effect of the oral contraceptive pill (OCP) on phenotype expression of PCOS in women descendant from a founder population. All patients with normogonadotropic anovulatory infertility (WHO II), descendant from a restricted area as identified by ZIP codes, underwent a standardised clinical, sonographic and endocrine screening. The previous diagnosis WHO II anovulation could be reconfirmed for a total number of 101 women. At present, a total of 81 (80%) women could be diagnosed as having PCOS (according to the revised 2003 criteria). From these women a total of 54 (67%) did not use the OCP, whereas 27 (33%) did. Corrected for age, the latter group presented with significantly increased serum concentrations of cortisol ($P < 0.001$) and sex-hormone binding globulin (SHBG) ($P < 0.001$). Serum concentrations of estradiol (E_2) ($P < 0.001$), progesterone (P) ($P = 0.02$), 17-hydroxyprogesterone (17-OH-P) ($P < 0.001$), T ($P = 0.04$) and androstenedione (AD) ($P = 0.01$) were significantly decreased in these women. These differences resulted in a significantly decreased free androgen index (FAI) (100 x T/ SHBG) for women presently on OCP ($P < 0.001$). Use of OCP influences phenotype expression (the observable trait) of individual women known to suffer from PCOS. Despite taking OCP, women diagnosed in the past as PCOS still fulfilled the revised 2003 criteria for the syndrome. Current data suggest that these women

may suffer from the most severe form of PCOS since ovarian dysfunction did not seem to be completely corrected. Moreover, the present study suggests that it is permitted to rely on historical data regarding diagnosis of PCOS for women presently on the OCP since its cardinal features appear to be relatively constant. In addition, OCP use does not appreciably affect the PCOS phenotype.

Chapter IV:
In the present cross-sectional study the incidence of different FSH receptor (FSHR) genotypes in normogonadotropic anovulatory infertile women (WHO II) and normo-ovulatory controls was assessed. In addition, these genotypes were correlated with baseline characteristics and ovarian responsiveness during ovulation induction for the WHO II women. Thirty normo-ovulatory controls were compared to 148 WHO II women. In WHO II patients and controls a standardized evaluation including cycle history, body mass index (BMI) and transvaginal ultrasound scannning of ovaries was performed. Fasting blood samples were obtained for endocrine evaluation. Ovarian responsiveness to FSH in WHO II women was assessed during ovulation induction and DNA was analyzed to determine the FSHR genotype. The Thr/Thr 307 genotype was significantly less (52% versus 23%; $P < 0.05$) and the Ser/Ser 680 polymorphism was significantly more (40% versus 16%; $P < 0.05$) prevalent in WHO II patients compared to controls. WHO II patients with Ser/Ser 680 polymorphism presented with higher median FSH serum levels (5.2 IU/L; range 2.4 - 9.7) compared to the Asn/Asn 680 (4.6 IU/L; range 1.4 - 5.8) and Asn/Ser 680 (4.5 IU/L; range 1.8 - 9.7) variants ($P < 0.05$). However, ovarian responsiveness to FSH was similar comparing different polymorphisms in anovulatory women. In conclusion, WHO II patients exhibit a different FSHR genotype compared to normo-ovulatory controls and although this is associated with increased baseline FSH serum levels, altered ovarian sensitivity to exogenous FSH during ovulation induction could not be established.

Chapter V:
Section 5.1: This follow-up study evaluated whether initial screening characteristics predict treatment outcome of gonadotropin induction of ovulation. One hundred and fifty four women with normogonadotropic anovulatory infertility for whom clomiphene citrate (CC) induction of ovulation was unsuccessful were included in the present study. Daily FSH injections were initiated on day 3-5 after a spontaneous or progestagen-induced withdrawal bleeding. In most patients a dose finding low-dose step-up regimen was applied during the first treatment cycle in order to identify the individual FSH reponse dose. In all subsequent cycles a step-down protocol was applied. The total number of first step-up cycles is 103. The total number of step-down cycles is 441, with 47% of these cycles presenting with mono-follicular development (1 follicle > 15 mm on the day of human chorionic

gonadotropin (hCG)). Initial serum levels of LH, T and AD were significant predictors in univariate logistic regression analysis for the probability of multi-follicular development. The area under the receiver operating characteristic (ROC) curve of the multivariate model (including AD and mean follicle number) to predict the chances of multiple dominant follicle development was 0.62. FSH treatment resulted in a total of 67 (44%) ongoing pregnancies. Comparing those women who did versus those who did not reach an ongoing pregnancy in a multi-variate Cox regression analysis, initial serum insulin-like growth factor-I (IGF-I), T and woman's age entered into the final model (AUC = 0.67). In conclusion, individual treatment outcome - multi-follicular growth and ongoing pregnancy - following gonadotropin induction of ovulation may be predicted by initial screening characteristics.

Section 5.2: A systematic review was conducted to determine if initial screening characteristics of women with normogonadotropic anovulatory infertility, predict clinically significant outcomes of ovulation induction with gonadotropins, and to obtain pooled estimates of their predictive value through meta-analysis. Relevant studies were identified by a search strategy which consisted of MESH headings and a check of bibliographies. Only those studies in which pre-treatment screening characteristics (such as BMI, serum LH and androgens, insulin sensitivity and ultrasound appearance of ovaries were related to outcome parameters (such as total amount of FSH administered, cancellation, ovulation, pregnancy and miscarriage), were included in this analysis. Studies reporting relationships between initial screening characteristics and outcome parameters of ovulation induction as measures of association (e.g. Odds ratios) were pooled if at least 2 studies reported an association. The measures of association were pooled using inverse variance weighting. Thirteen studies fulfilled the inclusion criteria. A positive association was seen in all studies between the level of obesity (definition applied as assessed by individual studies) and total amount of FSH administered (Weighted Mean Difference (WMD) of 771 IU (95% CI: 700 - 842)). Pooled Odds ratios of 1.86 (95% CI: 1.13 - 3.06) and 0.44 (95% CI: 0.31 - 0.61) were found between obesity with cancellation and ovulation, respectively. Pooled analysis did not show a significant association between obesity and pregnancy rate. The pooled Odds ratio for obese versus non-obese women and miscarriage was significant (3.05 (95% CI: 1.45 - 6.44)). Association measures between insulin resistance (definition applied as assessed by individual studies) and total amount of FSH administered produced a Weighted Mean Difference (WMD) of 351 (95% CI: 73 - 630) IU. A pooled Odds ratio of 0.29 (95% CI: 0.10 - 0.80) was found for insulin resistance with pregnancy rate. The pooled Odds ratio for insulin resistance (hyperinsulinemia versus normoinsulinemia) and miscarriage rate was not significant. A pooled Odds ratio of 1.04 (95% CI: 1.01 - 1.07) was found for LH (IU/L) with pregnancy rate. The pooled odds ratio for LH and miscarriage rate was not significant. Finally, pooled analysis did not find a significant association

between T and pregnancy rate. The included studies did not address possible links between initial sonographic parameters and treatment outcome. This analysis suggests that the most clinically useful predictors of gonadotropin ovulation induction outcome in normogonadotropic women are obesity and insulin resistance. These findings might be of clinical relevance since they discriminate between women with low or high chances for success. However, the best available evidence testing measures of association between screening characteristics and outcome is limited.

Section 5.3: This follow-up study represents in-vitro fertilization (IVF) treatment characteristics and outcomes in women with WHO group II anovulatory infertility after previous unsuccessful ovulation induction compared to controls. Furthermore, the possibility of initial screening parameters of these anovulatory women to predict IVF outcome was examined. Twenty six patients with WHO II anovulatory infertility who failed to establish a live birth following previous induction of ovulation (using clomiphene citrate as first line and FSH as second line) were compared with 26 IVF patients with tubal infertility matched for age, treatment period and treatment regimen. The WHO II patients underwent 49 IVF cycles, whereas the normo-ovulatory controls underwent 46 cycles. In WHO II patients 15 cycles were cancelled compared to 6 cycles in controls ($P = 0.04$). Cycles were predominantly cancelled due to insufficient response ($P = 0.04$). In case the cycle was cancelled, BMI was significantly higher ($P < 0.001$) in WHO II women compared to controls. Overall live birth rates were comparable ($P = 0.9$). Obese women suffering from WHO II anovulatory infertility are at an increased risk to have their IVF cycle cancelled due to insufficient response. Once oocyte retrieval is achieved live birth rates are comparable to controls.

Chapter VI:

This chapter summarises the conclusions which could be drawn from the work presented in the current thesis.

Samenvatting

Hoofdstuk I:
Het polycysteus ovarium syndroom (PCOS) is de meest voorkomende endocriene aandoening bij vrouwen in de reproductieve leeftijd en een belangrijke oorzaak van subfertiliteit. Vrouwen met PCOS presenteren zich mogelijk met een diversiteit aan klinische kenmerken met als gevolg dat PCOS niet lijkt samen te gaan met een eenduidig klinisch beeld. Ten einde de achtergrond en de pathofysiologie van alle symptomen die voorkomen bij PCOS duidelijk te begrijpen en te verhelderen kan het nuttig zijn om het onderzoek op deze specifieke patiëntkarakteristieken en kenmerken te richten. In dit hoofdstuk wordt de pathofysiologie van ovariumdysfunctie beschreven. Aanvullend volgt een korte beschrijving van een aantal karakteristieken gebruikt voor de classificatie van PCOS en tevens betrokken bij de heterogeniteit van het phenotype. Vervolgens wordt de complexiteit van onderzoek naar de genetische component betrokken bij de ziekte besproken. Tot slot, worden de klinische implicaties van verschillen in phenotype genoemd. Daarnaast worden de doelen van het huidige proefschrift kort beschreven.

Hoofdstuk II:
Paragraaf 2.1: Deze studie werd uitgevoerd om de serum waardes van anti-Müllerian hormoon (AMH) van 128 World Health Organization (WHO) groep II vrouwen te vergelijken met die van 41 normo-ovulatoire premenopauzale vrouwen van vergelijkbare leeftijd. De AMH serum concentraties waren significant ($P < 0,001$) verhoogd bij WHO II patiënten (mediaan: 7,6 µg/L (bereik: 0,1-40,0)) in vergelijking met de controles (mediaan: 2,1 µg/L (bereik: 0,1-7,4)). Bij 106 patiënten met polycysteuse ovaria (PCO) (≥ 12 follikels 2-9 mm/ovarium en/of een ovarieel volume > 10 mL) waren de AMH spiegels verhoogd (9,3 µg/L (1,8-40,0)) in vergelijking met 22 patiënten zonder PCO (6,4 µg/L (0,1-22,1)) ($P < 0,0001$). Bij WHO II patiënten correleerden de AMH spiegels met parameters karakteristiek voor PCOS zoals luteïniserend hormoon (LH) concentraties ($r = 0,331$; $P = 0,0001$), testosteron (T) spiegels ($r = 0,477$; $P = 0,0001$), gemiddeld ovarieel volume ($r = 0,421$; $P = 0,0001$) en het aantal ovariële follikels ($r = 0,308$; $P = 0,0001$). AMH spiegels correleerden met leeftijd zowel voor de WHO II patiënten ($r = -0,248$; $P = 0,002$) als voor de controles ($r = -0,465$; $P = 0,005$). Echter de relatieve afname van AMH met de leeftijd was minder uitgesproken voor de WHO II patiënten. Bij een deel van de patiënten werd er geen significante correlatie gevonden tussen AMH serum spiegels an de FSH respons dosis, de duur van stimulatie en het totale aantal gebruikte ampullen FSH. Concluderend, AMH serum concentraties zijn verhoogd bij WHO II vrouwen, in het bijzonder bij die patiënten met PCO. Omdat de AMH concentraties goed correleren met andere klinische,

endocriene en echografische markers geassocieerd met PCOS kan AMH gebruikt worden als een maat die de ernst van de ziekte weergeeft. Een minder uitgesproken afname van AMH over de tijd bij deze vrouwen is suggestief voor vertraagde ovariële veroudering.
Paragraaf 2.2: De huidige studie werd uitgevoerd om te onderzoeken of er een verschil bestaat in de afname van de AMH serum concentratie in de tijd tussen vrouwen met normogonadotrope anovulatie (WHO groep II) (inclusief PCOS) en normo-ovulatoire controles. De AMH serum spiegels werden bepaald zowel bij 98 patiënten met WHO II anovulatoire infertiliteit als bij 41 normo-ovulatoire premenopauzale controles. Het mediane tijdsinterval tussen beide bezoeken was 2,6 jaar (bereik: 0,3 - 9,0) voor de WHO II patiënten vergeleken met 1,6 jaar (bereik: 1,0 - 7,3) voor de controles. De serum AMH concentraties waren significant ($P < 0,0001$) verhoogd op beide momenten bij de WHO II patiënten (AMH_1: mediaan = 7,5 µg/L (bereik: 0,1 - 35,8) en AMH_2: mediaan = 6,7 µg/L (bereik: 0,0 - 30,6)) vergeleken met de controles (AMH_1: mediaan = 2,1 µg/L (bereik: 0,1 - 7,4) en AMH_2: mediaan = 1,3 µg/L (bereik: 0,0 - 5,0). Regressie analyse, gecorrigeerd voor leeftijd duidde op een significante afname in serum AMH concentraties over de tijd voor beide groepen ($P < 0,001$). Echter de afname in serum AMH spiegels bij de WHO II patiënten was significant kleiner in vergelijking met de controles ($P = 0,03$). De huidige longitudinale analyse toont aan dat serum AMH concentraties afnemen over de tijd zowel voor vrouwen met WHO II anovulatoire infertiliteit als voor normo-ovulatoire controles. De relatieve afname bij de WHO II patiënten is minder uitgesproken ondanks onmiskenbaar verhoogde concentraties. Deze observatie is suggestief voor vertraagde ovariële veroudering en gaat dus mogelijk gepaard met een verlengde reproductieve levensduur bij deze patiënten.

Hoofdstuk III:
De huidige observationele studie werd uitgevoerd om te onderzoeken wat het effect is van het gebruik van de orale anticonceptiepil op het PCOS fenotype bij vrouwen afkomstig uit een genetisch geïsoleerde populatie. Alle patiënten met anovulatoire infertiliteit (WHO groep II), afkomstig uit een beperkt gebied gedefinieerd met behulp van postcodes, ondergingen een random uitgevoerde klinische, endocriene en echografische screening. Voor een totaal van 101 vrouwen (118 vrouwen hebben uiteindelijk toegestemd in deelname) kon de diagnose WHO II anovulatie worden bevestigd. De diagnose PCOS (volgens de herziene 2003 criteria) gesteld in het verleden kon voor een totaal van 41 vrouwen bevestigd worden. Een totaal van 27 (73%) vrouwen van deze groep gebruikten ten tijde van de studie geen anticonceptiepil terwijl 10 (27%) van hen dit wel deden. De laatste groep had significant verhoogde serum concentraties van cortisol ($P = 0,003$) en sex-hormoon bindend globuline (SHBG) ($P < 0,001$). De serum

concentraties van LH (P = 0,030), estradiol (E_2) (P < 0,001), 17-hydroxyprogesteron (17-OH-P) (P = 0,012), de vrije androgenen index (FAI)(100 x T/SHBG) (P < 0,001) en de glucose:insuline (G:I) ratio (P = 0,035) waren significant verlaagd voor hen die de anticonceptiepil gebruikten. Bij een totaal van 81 (80%) vrouwen werd de diagnose PCOS gesteld. Van deze vrouwen gebruikte een totaal van 54 (67%) geen anticonceptiepil terwijl 27 (33%) vrouwen deze wel gebruikten. Gecorrigeerd voor leeftijd had de laatste groep significant verhoogde serum concentraties van cortisol (P< 0,001) en SHBG (P < 0,001) en verlaagde serum concentraties van E_2 (P < 0,001), P (P = 0,02), 17-OH-P (P < 0,001), T (P = 0,04) en androsteendion (AD) (P = 0,01). Deze verschillen resulteerden in een significant verlaagde FAI voor vrouwen die de anticonceptiepil gebruikten (P < 0,001). Samenvattend, het gebruik van de anticonceptiepil beïnvloedt expressie van het phenotype (de waarneembare kenmerken) van individuele vrouwen bekend met PCOS. Ondanks het gebruik van de anticonceptiepil voldoet een aantal vrouwen vanuit het verleden bekend met PCOS nog steeds aan de criteria voor de ziekte. Deze data suggereren dat deze groep mogelijk leidt aan de meest ernstige vorm van de ziekte aangezien de ovariumdysfunctie niet volledig is gecorrigeerd. Daarnaast staan de resultaten van deze studie toe om te vertrouwen op historische data aangaande de diagnose van PCOS voor vrouwen die nu de anticonceptiepil gebruiken. Aanvullend kan worden gezegd dat het gebruik van de anticonceptiepil het PCOS phenotype niet aanzienlijk lijkt te beïnvloeden.

Hoofdstuk IV:

In de huidige cross-sectionele studie werd de incidentie van verschillende FSH receptor (FSHR) genotypes bij vrouwen met normogonadotrope anovulatoire onvruchtbaarheid (WHO groep II) vergeleken met normo-ovulatoire controles. Voor de WHO II vrouwen werden de genotypes tevens gecorreleerd aan basiskarakteristieken en ovariumrespons tijdens ovulatie inductie. Dertig normo-ovulatoire controles werden vergeleken met 148 WHO II vrouwen. Bij WHO II patiënten en de controles werd een gestandaardiseerde evaluatie betreffende de cyclusanamnese, body mass index (BMI) en transvaginale echografie van de ovaria verricht. Nuchtere bloedafnames werden verkregen voor de endocriene evaluatie. De ovarium respons op FSH bij de WHO II vrouwen werd vastgesteld tijdens ovulatie inductie. Tevens werd DNA geanalyseerd om het FSHR genotype vast te stellen. Het Thr/Thr 307 genotype kwam significant minder (52% versus 23%; P < 0,05) en het Ser/Ser 680 genotype kwam significant vaker (40% versus 16%; P < 0,05) voor bij WHO II patiënten in vergelijking met de controlegroep. WHO II patiënten met het Ser/Ser 680 polymorfisme toonden hogere mediane FSH serum spiegels (5,2 IU/L; bereik 2,4 - 9,7) vergeleken met de Asn/Asn 680 (4,6 IU/L; bereik 1,4 - 5,8) en Asn/Ser 680 (4,5 IU/L; bereik: 1,8 - 9,7) variant (P < 0,05). Echter, de respons van het ovarium voor FSH was hetzelfde wanneer de

verschillende polymorfismen van anovulatoire vrouwen met elkaar werden vergeleken. Concluderend, WHO II patiënten tonen een verschillend FSHR genotype in vergelijking met normo-ovulatoire controles. Alhoewel, het FSHR genotype geassocieerd is met verhoogde basale FSH serum spiegels kan een veranderde ovariële gevoeligheid voor exogeen FSH tijdens ovulatie inductie niet worden vastgesteld.

Hoofdstuk V:

Paragraaf 5.1: In deze vervolgstudie werd onderzocht of het mogelijk was om met behulp van initiële screeningskarakteristieken de behandelingsuitkomst van gonadotrofinen ovulatie inductie te voorspellen. Honderdvierenvijftig vrouwen met normogonadotrope anovulatoire onvruchtbaarheid voor wie ovulatie inductie met clomifeen citraat (CC) niet succesvol was werden geïncludeerd. Dagelijkse injecties met FSH werden gestart op dag 3-5 na een spontane of progestageen geïnduceerde onttrekkingsbloeding. Bij de meeste patiënten werd tijdens de eerste behandelcyclus een low-dose step-up schema toegepast ten einde de individuele FSH respons dosis vast te stellen. In alle volgende cycli werd een step-down protocol toegepast. Het totale aantal eerste step-up cycli bedroeg 103. Het totale aantal step-down cycli bedroeg 441 waarbij er in 47% van deze cycli sprake was van mono-folliculaire ontwikkeling (1 follikel > 15 mm op de dag van humaan chorion gonadotrofine (hCG)). De initiële serum spiegels van LH, T en AD waren significante voorspellers in univariate logistische regressie analyse voor de waarschijnlijkheid van multi-folliculaire ontwikkeling. Het oppervlak onder de “receiver operating characteristic (ROC) curve” van het multivariate model (waarin opgenomen de serum spiegel AD en het gemiddelde aantal follikels) om de kans op multipele dominante follikelgroei te voorspellen was 0,62. De behandeling met FSH resulteerde in een totaal van 67 (44%) doorgaande zwangerschappen. Indien de vrouwen die wel en die geen doorgaande zwangerschap bereikten werden vergeleken in een multi-variate Cox regressie analyse kwamen de initiële serum spiegel van insuline-gelijkende groeifactor-I (IGF-I), T en de leeftijd van de vrouw in het uiteindelijke model (AUC = 0,67). Geconcludeerd kan worden dat de individuele behandeluitkomst - multi-folliculaire groei en doorgaande zwangerschap - volgend op gonadotrofinen ovulatie inductie kan worden voorspeld aan de hand van initiële screeningskarakteristieken.

Paragraaf V.2: Een systematische literatuurstudie werd verricht om vast te stellen of initiële screeningskenmerken van vrouwen met normogonadotrope anovulatoire infertiliteit klinisch significante uitkomsten van ovulatie inductie met gonadotrofinen voorspellen. Tevens werd gezocht naar samengevoegde schattingen van de voorspellende waarde van screeningsparameters met behulp van meta-analyse. De relevante studies werden geïdentificeerd met behulp van een zoekstrategie die bestond uit MESH termen en een controle van de referentielijsten. Alleen die

studies waarin vooraf bekende screeningskarakteristieken (zoals BMI, serum LH en androgenen, insulinegevoeligheid en het echoscopisch beeld van de ovaria) werden gerelateerd aan uitkomstparameters (zoals totale hoeveelheid toegediend FSH, het staken van de behandelcyclus, ovulatie, zwangerschap en miskraam), werden geïncludeerd in deze analyse. Studies die rapporteerden over relaties tussen initiële screeningskenmerken en uitkomstparameters als maten van associatie (bv. Odds ratio's) werden samengevoegd als tenminste 2 studies over deze associatie rapporteerden. De maten van associatie werden samengevoegd door gebruik te maken van het omgekeerde gewicht. Dertien studies voldeden aan de inclusiecriteria. Een positieve associatie werd gezien in alle studies tussen het niveau van overgewicht (de toegepaste definitie werd door de individuele studies vastgesteld) en de totale hoeveelheid toegediend FSH (gewogen gemiddelde verschil (WMD) van 771 IU (95% betrouwbaarheidsinterval: 700 - 842)). De samengevoegde Odds ratio's van 1,86 (95% betrouwbaarheidsinterval: 1,13 - 3,06) en 0,44 (95% betrouwbaarheidsinterval: 0,31 - 0,61) werden gevonden tussen overgewicht met staken van de behandelcyclus en ovulatie, respectievelijk. De samengevoegde analyse liet geen significante associatie zien tussen overgewicht en het zwangerschapscijfer. De samengevoegde Odds ratio voor vrouwen met versus vrouwen zonder overgewicht en miskraam was significant (3,05 (95% betrouwbaarheidsinterval: 1,45 - 6,44)). De associatiematen tussen insulineresistentie (de toegepaste definitie is vastgesteld door de individuele studies) en de totale hoeveelheid toegediend FSH leverde een gewogen gemiddelde verschil (WMD) van 351 IU (95% betrouwbaarheidsinterval: 73 - 630) op. Een samengevoegde Odds ratio van 0,29 (95% betrouwbaarheidsinterval: 0,10 - 0,80) werd gevonden voor insulineresistentie met zwangerschapscijfer. De samengevoegde Odds ratio voor insulineresistentie (hyperinsulinaemie versus normoinsulinaemie) en het aantal miskramen was niet significant. Een samengevoegde Odds ratio van 1,04 (95% betrouwbaarheidsinterval: 1,01 - 1,07) werd gevonden voor LH (IU/L) met het zwangerschapscijfer. De samengevoegde Odds ratio voor LH met miskramen was niet significant. Tot slot, vond de samengevoegde analyse geen significante associatie tussen T en het zwangerschapscijfer. De geïncludeerde studies hebben de mogelijke verbanden tussen initiële echografische parameters en behandeluitkomst niet onderzocht. Deze analyse suggereert dat overgewicht en insulineresistentie bij normogonadotrope vrouwen de klinisch bruikbare voorspellers van gonadotrofinen ovulatie inductie zijn. Deze bevindingen kunnen van klinische relevantie zijn omdat zij een onderscheid maken tussen vrouwen met een hoge en een lage kans op succes. Echter, de hoeveelheid bewijs beschikbaar voor het onderzoek naar maten van associatie tussen screeningskarakteristieken en uitkomst is beperkt.

Paragraaf 5.3: In-vitro fertilisatie (IVF) behandelingskarakteristieken en uitkomsten van vrouwen met WHO groep II anovulatoire infertiliteit werden in deze

vervolgstudie vergeleken met controles. Daarnaast werd onderzocht of initiële screeningsparameters de uitkomst van de IVF behandeling voor anovulatoire vrouwen konden voorspellen. Zesentwintig patiënten met WHO II anovulatoire infertiliteit bij wie geen kind is geboren na voorafgaande ovulatie inductie (gebruikmakend van CC als 1e lijns en FSH als 2e lijns therapie) werden vergeleken met 26 IVF patiënten met tubaire infertiliteit met gelijke leeftijd, behandelperiode en behandelregiem. De WHO II patiënten ondergingen 49 IVF cycli, terwijl de normo-ovulatoire controlegroep 46 cycli ondergingen. Bij de WHO II patiënten werden 15 cycli gestaakt in vergelijking met 6 cycli bij de controles (P = 0,04). De cycli werden voornamelijk gestaakt wegens onvoldoende ovariële respons (P = 0,04). In het geval dat de behandelcyclus werd gestaakt was de BMI significant (P < 0,001) verhoogd voor de WHO II vrouwen in vergelijking met de controles. Het totale aantal levendgeboren kinderen was vergelijkbaar (P = 0,9). Vrouwen met overgewicht die lijden aan WHO II anovulatoire onvruchtbaarheid lopen een grotere kans op het staken van de gestarte IVF cyclus als gevolg van onvoldoende ovariële respons. Na het verrichten van de punctie zijn de aantallen levend geborenen vergelijkbaar met de controlegroep.

Hoofdstuk VI:
Dit hoofdstuk bevat de conclusies die kunnen worden gehaald uit de hoofdstukken opgenomen in dit proefschrift.

Publications

Publications

Publications included in the present thesis

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Dankwoord

Dankwoord

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Ter afsluiting….zullen we de komende zomer weer eens flink veel gaan skaten?

Ja, en dan inmiddels traditiegetrouw een woord van dank voor het follikelteam: allereerst de girls (gezeteld op de kopkamer onder het toeziende oog van de baas):
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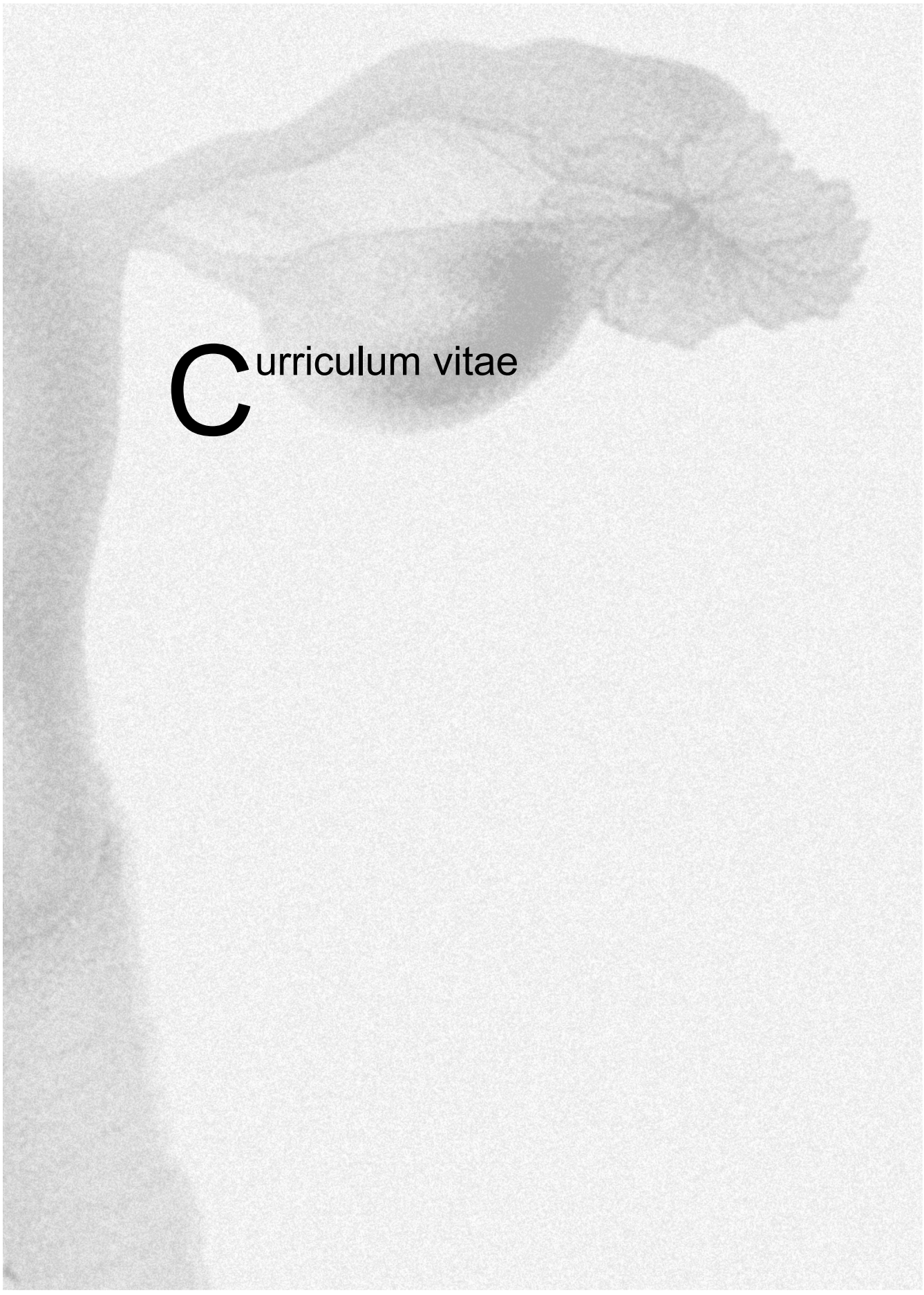

Curriculum vitae

Curriculum vitae

Annemarie Geerdina Maria Geertruida Johanna Mulders was born on the 29th of April 1972 in Waalwijk, The Netherlands. She passed secondary school at the Dr. Mollercollege in Waalwijk. She attended Medical School at the Erasmus University in Rotterdam from 1992 - 1999 from which she graduated cum laude. From December 1999 - August 2000 she worked as a resident at the department of Obstetrics and Gynaecology of the Sint Lucas Andreas Hospital (head: Dr. J.Th.M. van der Schoot) in Amsterdam. From August 2000 - December 2003 she worked as a PhD student at the division of Reproductive Medicine (head: Prof. Dr. B.C.J.M. Fauser) of the department of Obstetrics and Gynaecology of the Erasmus MC in Rotterdam performing the studies described in this thesis. In January 2004 she started her training in Obstetrics and Gynaecology at the Amphia Hospital in Breda (head: Dr. M. ten Kate - Booij).