RISKS AND COMPLICATIONS ASSOCIATED WITH OVARIAN STIMULATION FOR FERTILITY TREATMENT

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Declaration

I have commenced my pursuit of a higher research degree with a general question, "How can we make fertility treatment safer?" In this thesis, I aim to answer some of the uncertainties surrounding the risks involved with fertility treatment.

I am proud to be able to contribute to research surrounding the area of human reproduction, at the same time I recognize and hold in great regard my predecessors on whose research my findings are founded.

Except where acknowledgement is made by reference, the studies undertaken in this thesis were devised and conducted unaided by the author.

No part of this work has been previously accepted for, or is currently being submitted in candidature for, another degree.

M.N. Baumgarten

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Abstract

Almost all fertility treatment involves ovarian stimulation. This can be in the form of ovulation induction in women with anovulation or controlled ovarian stimulation for *in vitro* fertilisation treatment. The stepwise approach to fertility treatment in most cases it will be safe and successful. In certain cases, it might be however ineffective or related with adverse health outcomes for the woman causing significant distress for the couple and the treating clinicians.

Fertility treatment could be perceived as a paradox, as healthy women undergo treatment 'willingly', with often no cause identified of their failed conceptions. Exposure to assisted reproduction can therefore result in these healthy women being admitted to hospital suffering from complications of fertility treatment.

In this thesis of the genetic make-up of women, who had ovulation induction with or without success, is evaluated. Aiming for potential prediction of response for future patients. In order to gain more insight in the pathophysiology of ovarian overresponse, the ovarian renin angiotensin system was assessed during stimulation for IVF. The use of a dopamine agonist to alleviate or prevent OHSS was studied. And any assisted reproductive treatment adverse outcome rates after a salpingectomy prior to treatment were investigated.

The studies presented in this thesis utilized various research modalities, including systematic literature reviews, three-dimensional ultrasound, single nucleotide polymorphism analysis and polymerase chain reactions.

When OHSS develops dopamine agonists provide limited symptomatic relieve, but causes no harm. Salpingectomy prior to ovarian stimulation with potential harm to the ovarian blood supply does not influence the overall treatment outcome. Serum renin levels during ovarian stimulation might be able to differentiate which women become symptomatic of OHSS, but is a difficult test to implement in daily practice. For ovulation induction the use of genotyping of CYP450 might allow adjusting the dose needed to prevent unwanted response.

We need to remain critical of our practise. Registration and evaluation of complications will allow achievement of safer and better fertility treatment in the future. The overall conclusion is that the next trial should focus on the patient group found to be at risk of overresponse and randomise them to an antagonist protocol with agonist trigger and modified luteal support. Controlled ovarian stimulation should be in conjunction with a national complication registration to have a real picture of the risks associated with fertility treatment.

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Abbreviations

Abbreviation	Explanation
11β HSD	11-β Hydroxysteroid Dehydrogenase
ASO	allele specific oligonucleotide
AFLPD	amplified fragment length polymorphism detection
AMH	Anti-Müllerian Hormone
AFC	Antral Follicle Count
AUC	Area Under the ROC Curve
ART	Artificial Reproductive Techniques
BMI	Body Mass Index
CPR	clinical pregnancy rate
CC	Clomiphene Citrate
CI	Confidence Interval
COH	Controlled Ovarian Hyperstimulation
CYP	cytochrome P450
dH2O	distilled water
DCC-FCS	Dextran Coated Charcoal treated Foetal Calf Serum
DA	dopamine agonist
EDTA	Ethylene Diamine Tetra Acetic acid
FI	Flow Index
FSH	Follicle Stimulating Hormone
FER	Frozen Embryo Replacement
GR	Glucocorticoid Receptor
GnRH	Gonadotropin-releasing hormone
FSH-HP	highly purified FSH
hCG	Human Chorionic Gonadotropin
hMG	Human Menopausal Gonadotropin
HSG	Hysterosalpingography
IVF	In vitro fertilisation
IGF	Insulin-like Growth Factor
IU	international unit
ICC	Intra-Class Correlation Coefficient
ICSI	Intra-Cytoplasmic Sperm Injection
IUI	Intra-uterine Insemination
IVF	In-vitro Fertilization
JZ	Junctional Zone
LIMS	Laboratory Information Management System
LOD	laparoscopic ovarian drilling
LR	Likelihood Ratio
LOA	Limits of Agreement
LPS	luteal phase support
LH	Luteinising Hormone
MRI	Magnetic Resonance Imaging

MHC	Major Histocompatibility Complex
MG	Mean Grey Scale Value
mRNA	messenger RNA
ml	Millilitre
MR	Mineralocorticoid Receptor
MoM	Multiples of the Mean
NHS	National Health Service
NICE	National Institute for Health and Care Excellence
nIVF	natural cycle IVF
NK	Natural Killer (cells)
NPV	Negative Predictive Value
E2	oestradiol
OHSS	Ovarian Hyperstimulation Syndrome
PFA	Paraformaldehyde
PSV	Peak Systolic Velocity
PID	Pelvic Inflammatory Disease
PCO	Polycystic Ovaries
PCOS	Polycystic Ovary Syndrome
PCR	polymerase chain reaction
PPV	Positive Predictive Value
PD	Power Doppler
PDSTIC	Power Doppler Spatio-Temporal Image Correlation
PRL	Prolactin
PI	Pulsatility Index
PRF	Pulse Repetition Frequency
PWPD	Pulse Wave Power Doppler
FSH-P	purified FSH
RAPD	random amplified polymorphic detection
ROC	Receiver Operating Characteristics
r-LH	recombinant LH
r-FSH	recombined FSH
RIF	Recurrent Implantation Failure
RM	Recurrent Miscarriage
ROI	Region Of Interest
RAS	renin-angiotensin system
REC	Research Ethics Committee
RI	Resistance Index
RFLPI	restriction fragment length polymorphism identification
RNA	Ribonucleic Acid
RCOG	Royal College Obstetricians and Gynaecologists
SERM	selective oestrogen receptor modulator
SNP	single nucleotide polymorphism
SonoAVC	Sonography Based Automated Volume Calculation
STIC	Spatio-Temporal Image Correlation

SD	Standard Deviation
TDL	The Doctors laboratory
3D	Three-dimensional
3DPD	Three-dimensional Power Doppler
TVOR	transvaginal oocyte retrieval
2D	Two-dimensional
UK	United Kingdom
UA	Uterine Artery
VEGF	Vascular Endothelial Growth Factor
VFI	Vascularisation Flow Index
VI	Vascularisation Index
VOCAL	Virtual Organ Computer-aided AnaLysis ™
vWF	Von Willebrand factor
WBC	white blood count
WHO	World Health Organisation
ZIFT	Zygote Intra-fallopian Transfer

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Chapter 1: Introduction

1.1 Subfertility

Reproduction can be seen as the universal goal of all species. The desire to have children is natural feeling for most people. Unfortunately, not all couples are able to achieve a pregnancy.

There are several definitions for infertility. The World Health Organization (WHO) uses the clinical definition of a disease of the reproductive system defined by the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse (Mascarenhas, Flaxman et al. 2012). A couple is considered infertile if, after two years of regular sexual intercourse, without contraception, the woman has not become pregnant (and there is no other reason, such as breastfeeding or postpartum amenorrhoea). Infertility can be subdivided into primary and secondary infertility. Primary infertility is infertility in a couple who have never had a pregnancy and secondary infertility is failure to conceive following a previous pregnancy (Zegers-Hochschild, Adamson et al. 2009).

In the United Kingdom, the National Institute for Health and Care Excellence

(NICE) guidelines define infertility as failure to conceive after regular unprotected sexual intercourse for 2 years in the absence of known reproductive pathology (National Collaborating Centre for Women's and Children's 2004).

The government's National Health Service (NHS) website states that around one in six couples may have difficulty conceiving, accounting for approximately 3.5 million people in the UK. About 85% of couples will conceive naturally within one year if they have regular unprotected sex. However, the chances of becoming pregnant are much lower in older women. For couples who have been trying to conceive for more than three years without success, the likelihood of pregnancy occurring within the next year is 25% or less (NICE Feb 2013).

1.2 Causes of sub fertility and aetiology

There are many known causes for infertility, including some amenable to treatment (Makar and Toth 2002). Simplistically speaking, only one oocyte and one sperm cell are needed to form a pregnancy. The main causes of infertility can be divided into male or female, with further subdivision into ovulatory or tubal. Combination of causes is common and in approximately 25% of couple no cause can be found, as demonstrated in Figure 1-1. Treatment of all these causes will involve some form of stimulation of the ovary.

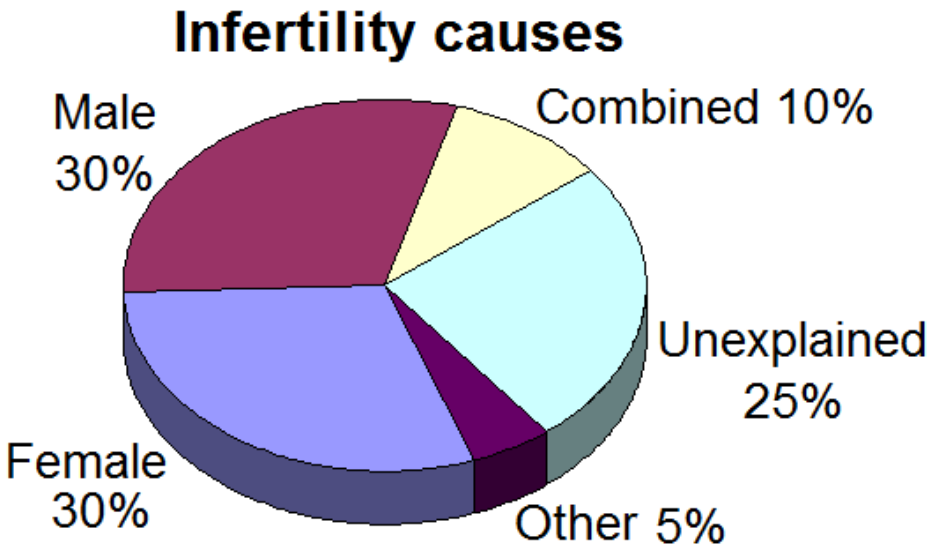


Figure 1-1 Division Causes of Infertility (http://www.dh.gov.uk/DH_101070 Regulated fertility)

1.3 The ovary

1.3.1 Anatomy, vascularisation and embryology

The follicular pool is established in early embryonic life, when nearly 1000-2000 germ cells are settled in the gonadal ridges after migrating from the yolk sac. They multiply and reach a maximum limit of around 7 million oogonia at 16-20 weeks of gestation. The follicle number decreases to reach 2 million prior to delivery and 300.000 at menarche (Madankumar, Cohen et al. 2003). With ovulation and apoptotic atresia the number of follicles declines with time, in association with a decrease in oocyte quality (Eichenlaub-Ritter 1996, te Velde, Scheffer et al. 1998, Allen, Freeman et al. 2009).

1.3.2 Ovarian Volume

In most of the organs in a human body, there is a correlation between the size of the organ and its function. Ovarian volume increases from 0.7ml in a 10 year old to 5.8ml in a 17 year old (Ivarsson, Nilsson et al. 1983). After 40 years of age there is a drop in ovarian volume which is unrelated to parity (Andolf, Jorgensen et al. 1987). The decrease of ovarian volume is an early sign of depletion of follicles (Lass and Brinsden 1999). During the menopause there is a further decline of ovarian volume (Kelsey, Dodwell et al. 2013). This decline in volume seems to be related to the onset of menopause and not age, as in women with oestrogen (E2) substitution the ovarian volume remained stable (Flaws, Rhodes et al. 2000).

Ovarian volume was one of the first makers for ovarian function which could be measured using ultrasound. With the improvement in quality of ultrasound machines, antral follicles became detectable and their count replaced ovarian volume measurements.

1.3.3 Antral follicles and Folliculogenesis

Ovarian follicles are created in the embryonic period as primordial follicles. At birth a woman has approximately 1 million of primordial follicles. Out of this pool a cohort will start growing. Until puberty this growth will only results in atresia. By menarche, approximately three quarters of the follicles are lost (Himelstein-Braw 1976). During the reproductive life, approximately 400 follicles will mature and produce an oocyte, with the remaining vast majority undergoing atretic changes (Macklon and Fauser 1998). This process of atresia will continue until all follicles are depleted.

Under the influence of follicle stimulating hormone (FSH) and luteinizing hormone (LH), the gonadotropins, which start being produced in puberty, the antral follicles can continue growing (recruitment). With increasing FSH levels over a threshold follicle selection will occur. The increasing oestrogen production by the selected follicle will suppress further FSH production ensuring the development of a single follicle, the dominant follicle. Early growth of the primordial follicles occurs continuously and at random. The whole process takes approximately 85 days (Gougeon 1986). Granulosa cells in the primordial follicle increase in numbers, the oocyte increases in size leading to formation of the primary follicle. Vascular development around the follicle precludes the formation of the theca layer of the follicle creating the secondary follicle (Channing and Kammerma.S 1973). Formation of a cavity within the follicle heralds the antral phase of follicular development. Up until this stage, the follicular growth has been gonadotropin-independent (Peters 1976). From the 1000 follicles commencing growth every month, by the time they become gonadotropin dependent, only few continue the growth, with one reaching maturity (Faddy, Gosden et al. 1992). Pre-antral follicles, due to the size cannot be visualized by means other than histological staining. Development of a fluid filled cavity within the antral follicle and its size (approximately 2 mm) allow for visualization of these structures using transvaginal ultrasound.

Reaching the FSH-level threshold allows for selection of a small pool of follicles, one of which will undergo final maturation. A subtle rise in the FSH level following achievement of the threshold, allows for further follicular development. The mid follicular-phase drop in FSH levels, leads to selection of a single dominant follicle that is most sensitive to FSH (Hsueh 1986).

Remaining follicles undergo atresia. Excessively high doses of FSH as administered in artificial reproduction cycles, lead to development of multiple follicles, which in cases of *in vitro* fertilisation (IVF) and intracytoplasmic sperm injection (ICSI), are harvested to obtain oocytes (Macklon and Fauser 1998).

Early in the menstrual cycle, between day 2 and 5, assessment of the antral follicle pool is used as a marker of ovarian reserve- the antral follicle count (AFC). The early antral phase consists of follicle of 2-5mm filled with antral fluid. They represent the actual functional reserve, as they are a reflection of the remaining primordial pool (Hansen, Hodnett et al. 2011).

1.3.4 Oogenesis

During the formation of oocytes, DNA is halved, leading to the formation of haploid cells. This process is called meiosis. For oocytes this process starts even before birth (the first meiotic division) and will only be completed during ovulation (Grinsted 1979). During the long prophase crossing over of maternal and paternal genes takes place. Half of the chromosomes get excluded in a small polar body. After the second division initiated by fertilisation a second polar body is extruded.

1.3.5 The hormones

Regulation of reproduction takes place on three levels. The primary level involves the gonads, in the women specifically the ovary. The ovary is responsible for the production of progesterone, testosterone and oestrogen. The two other levels are responsible for the regulation of the gonad. The pituitary is the master gland responsible for the function of all other glands. In relation to the ovary it produces FSH and LH. The pituitary is under the

influence of hypothalamus. Specialist neuroendocrine cells under influence of electrical stimuli, produce gonadotropin releasing hormone (GnRH). Both hypothalamus and pituitary process signals from the ovary, in combination with signals from other glands and the brain. This complex regulation is called feedback, see Figure 1-2. Low levels of oestradiol have a negative inhibitory effect on the secretion of FSH, at higher levels oestradiol combines with inhibine for a suppression of FSH. The influence of oestradiol on LH varies with concentration and duration of exposure. At low levels oestradiol has a negative feedback and at higher levels a positive stimulatory effect on LH release. The transition from suppression to stimulation of LH occurs as oestradiol rises. There are two critical features in this mechanism, first the concentration and second the length of oestradiol elevation. In women the oestradiol concentration needed to achieve positive feedback is more than 200 Pg/ml and this levsl must be sustained for approximately 50 hours (Young and Jaffe 1976).

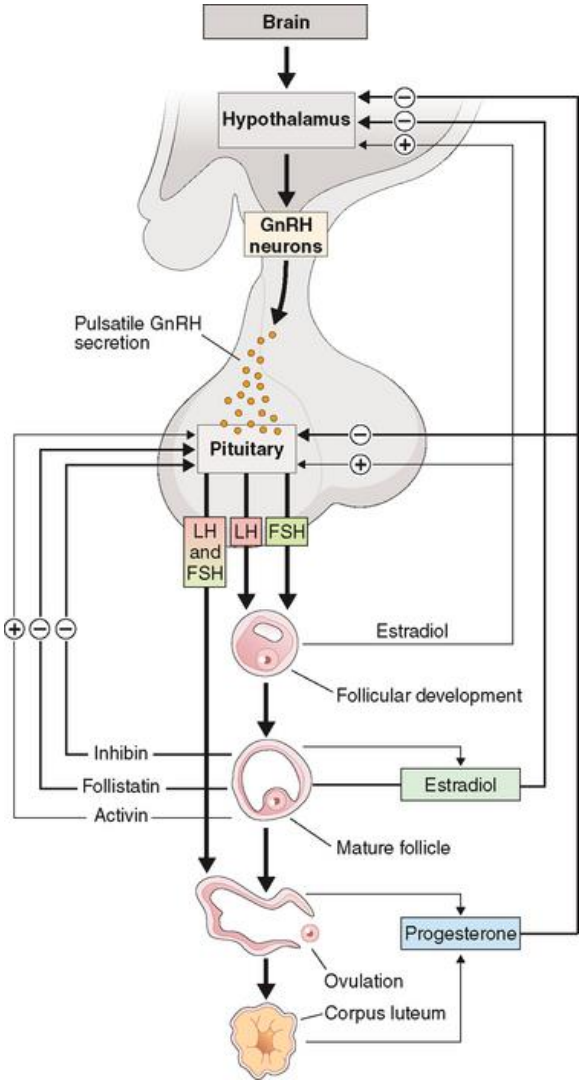


Figure 1-2: Hypothalamus-pituitary- ovary axis

(image from Manjula K. Gupta, SuYnn Chia)

GnRH is a protein hormone consisting of 10 amino acids (decapeptide), with a short half-life of several minutes. The secretion of GnRH is in pulses and will depend on the time point in the menstrual cycle. The sixth amino acid (glycine) is important for the breakdown of the hormone. Substitution of this amino acid can produce a hormone with a longer half-life. This is the basis for the development of GnRH-agonist.

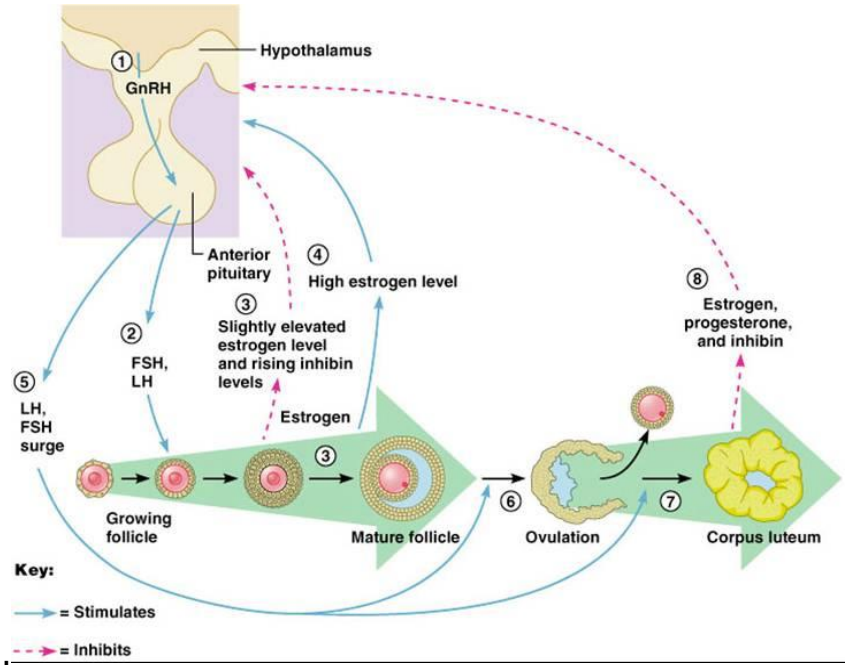


Figure 1-3: Ovarian feedback on the hypothalamus and pituitary

(image from Manjula K. Gupta, SuYnn Chia)

Both GnRH-agonist and antagonist have the potential to suppress the secretion of gonadotropins, but the mechanism is completely different. Agonists bind to the receptor and stimulate the production of LH and FSH (flare-up effect). Through continuous exposure, the pituitary becomes insensitive and FSH and LH-levels will fall (Dierschke, Bhattacharya et al. 1970, Conn and Crowley 1991, Silberstein and Merriam 2000). The GnRH agonists are more potent and have got a longer half-life than native GnRH. They produce an initial stimulation of pituitary gonadotrophins that results in secretion of FSH and LH and the expected gonadal response. This response is followed by down-regulation and inhibition of the pituitary-gonadal axis (Kumar and Sharma 2014).

The GnRH antagonist works through competition with endogenous GnRH by binding to the GnRH-receptor. However, they do not induce a biological effect. They have an acute suppression of FSH and LH secretion, but they

do not switch off the reaction potential of the pituitary (Backstrom, McNeilly et al. 1982, Filicori, Butler et al. 1984, Merriam GR. 1984).

LH and FSH are produced in gonadotropic cells. The half-life for LH is 60 minutes and for FSH 240 minutes. Target cells for FSH are the granulosa cells. FSH and oestrogens are complementary in their function on follicular growth and maturation. Oestrogens stimulate the proliferation of granulosa cells and increase the production of FSH receptors. Oestrogens are derived from androgens, a process which is stimulated by the effect of FSH on the aromatase enzyme (Silberstein and Merriam 2000). The ultimate sign of maturation is the induction of LH-receptors on the granulosa cells by FSH. Without this the future *corpus luteum* cannot function. FSH also promotes the secretion of inhibin which, together with oestrogen, suppresses FSH secretion by the pituitary (negative feedback loop) (Silberstein and Merriam 2000). Activin promotes FSH release by the pituitary and suppresses progesterone production by theca cells, preventing premature ovulation (Mishell DR. 1997). A slight mid-cycle rise in progesterone levels causes reversal of the inhibitory action of elevated oestrogen and inhibin levels on the pituitary, resulting in a surge of LH secretion (Laborde, Carril et al. 1976, Silberstein and Merriam 2000). The increasing level of oestrogen in the late follicular phase is responsible for a time and dose dependant positive feedback on the LH level (Marsh, Shaw et al. 2011).

The function of LH changes throughout the cycle. In the proliferative phase, LH stimulates the production of androgens by the *theca interna* (Ryan and Petro 1966, Ryan, Petro et al. 1968, McNatty, Makris et al. 1980). Once LH

receptors are formed LH plays a role in oocyte maturation, follicle rupture and the formation of the *corpus luteum* (Silberstein and Merriam 2000).

Rising levels of LH stimulate follicular progesterone secretion further (Silberstein and Merriam 2000). The granulosa cells under the influence of FSH produce plasminogen activator which converts plasminogen into plasmin. This in turn converts procollagenase into collagenase degrading the follicular basement membrane of the leading follicle (Smith, Roberts et al. 1996). Following formation of the follicular stigma as a result of follicular degradation, rupture of the leading follicle and ovulation occur. These processes are prostaglandin dependant (Phi, Moon et al. 1977). The rise in LH causes the resumption of meiotic division in the oocyte and causes luteinisation of the granulosa cells (Smith, Roberts et al. 1996) see Figure 1-3.

1.4 Fertility treatment options

Infertility has an increasing impact on health services worldwide due to the rising rate of fertility impairment among the general population, which often requires artificial reproductive techniques (ART) (Sharpe and Franks 2002). These techniques are expensive and frequently require multiple attempts to prove successful. Medical treatment options are available to induce ovulation or spermatogenesis, in cases the condition is based on specific hormonal problems.

Assisted conception (where the intervention requires human gamete manipulation) can be achieved by either intra uterine insemination (IUI) or

in vitro fertilisation (IVF). With intrauterine insemination the partner's sperm is washed and prepared prior to its introduction into the uterine cavity. The latter is undertaken by the medical staff who uses a fine catheter that is gently passed through the cervix and then perform the IUI by depressing the syringe plunger containing the prepared sperm that is attached to the device. It is a simple, routine procedure and not associated with pain or discomfort in the majority of patients. It can be combined with medical ovulation induction. IVF on the contrary is an ART procedure that involves extracorporeal fertilisation of the ovum.

With the birth of the first IVF baby in 1978, it became possible to give hope of a biologically own child to couples with various causes of infertility that otherwise would remain childless. Although throughout the years these techniques have been used more commonly, there is still limited data on the, especially long-term, health effect for the women.

In the 1970's during the beginnings of IVF treatment, oocyte retrieval was performed via the laparoscopic route in a natural cycle. Embryo and gametes were replaced into the Fallopian tubes (Toner 2002). Enormous progress has been made aiming to improve the efficacy of all components of the IVF treatment process. Natural cycle IVF with laparoscopic oocyte retrievals and laparoscopic embryo transfers have been replaced by controlled ovarian stimulation (COS) with transvaginal oocyte retrieval (TVOR) and ultrasound guided intra uterine embryo transfers. All these steps aim to make the treatment as patient-friendly and minimally invasive as possible. Although current IVF practice is generally safe, it is still not without associated complications.

1.5 Ovulation induction: Stimulation with Clomiphene citrate

In couples with an identifiable cause of subfertility, one of the most common problems is anovulation, which is often associated with polycystic ovarian syndrome (PCOS). There are several treatment options for these couples, but one of the most commonly used approaches is ovulation induction with clomiphene citrate (CC). Clomiphene citrate (marketed under several trade names, most commonly Clomid®) is used as first line treatment of anovulation.

CC was one of the first selective oestrogen receptor modulators (SERMs) and has partial agonist and antagonist activities. It induces ovulation through its antagonist effect on the oestrogen receptor, which leads to an increase in the secretion of GnRH from the hypothalamus. This induces the anterior pituitary to secrete FSH, which induces folliculogenesis. Once initiated these follicles continue to grow and develop as in a normal spontaneous menstrual cycle. Ovulation occurs following the natural late follicular surge of luteinising hormone (LH) or can be induced through the administration of human chorionic gonadotropin (hCG), which acts like LH. Patients are informed of this and advised as regards to the timing of intercourse. Most often couples try 3-6 cycles of CC (Nasseri and Ledger 2001). If this is unsuccessful the treatment may be combined with IUI, to optimise timing (Guzick, Sullivan et al. 1998).

CC is given orally once a day. Current practice is to start with 50mg of CC from the second day of the cycle for 5 days until day 6. The first cycle is monitored by ultrasound, which is conducted on a regular basis from the 8th

day of the cycle. If follicle growth occurs, the scans are repeated on a daily or alternate day basis until ovulation occurs or is induced as outlined above. Serum progesterone levels are measured in the mid-luteal phase of the cycle, approximately day 21, to confirm ovulation has occurred. If the cycle does not result in pregnancy, the couple continue treatment with the very next period in an identical fashion. Treatment can be continued for a maximum of12 cycles depending on the indication for treatment, concurrent fertility problems, the woman's age, the response to treatment including side effects, and the patient's / couple's attitude, but the manufacturer advises a maximum of 6 cycles (Nasseri and Ledger 2001).

If no follicular growth is demonstrated, or ovulation fails to occur the dose of CC is increased to 100mg in the next cycle. Further increments of 50mg are used if the patient remains anovulatory. The maximum recommended dose is 250mg but the majority of patients respond to 150mg or are offered alternative therapy (Homburg 2005). Other adjuncts are available including the addition of metformin for women with a BMI above 25 and laparoscopic ovarian drilling (LOD) for those with a normal BMI. There is no national or local consensus as to when these additional treatments are offered. the woman is said to be 'clomiphene-resistant' is she fails to ovulate after receiving 150 mg of CC daily for 5 days per cycle, for at least three cycles.(Brown, Farquhar et al. 2009)

In contrast, some patients demonstrate an exaggerated response to treatment. Ovulation induction aims to induce the growth of one follicle (monofollicular development), but in some cases two or more dominant (>14mm) follicles develop. Treatment is usually continued if there are just

2 dominant follicles, but the patient / couple is warned about the risk of multiple pregnancy, which affects 5% of CC cycles. Treatment is cancelled, and the couple advised to avoid intercourse, if more than 3 dominant follicles develop due to the increased risk of higher order multiple pregnancies. In some cases, many more follicles develop and rarely the patient may show signs ovarian hyperstimulation syndrome (OHSS). OHSS is typically seen in patients undergoing ovarian stimulation as part of IVF, but is occasionally seen in association with CC use.

Some patients are unable to tolerate the side effects of treatment. Adverse effects of CC include hot flushes, ovarian hyperstimulation, abdominal discomfort, and multiple pregnancy (Royal Pharmaceutical Society of Great Britain 2003). In these circumstances, alternative treatments are offered or ovulation induction may be induced by swapping CC for another SERM like tamoxifen. Tamoxifen is equally effective and has fewer side effects (Steiner, Terplan et al. 2005). It is, however, not the first line drug for ovulation induction as more data are available on the use of CC.

1.6 Stimulation for IVF

Various protocols of controlled ovarian hyperstimulation (COH) have been developed. All with the goal to increase live birth rate. The protocols attempt to improve the quality of the oocyte and simultaneously enhance the response in poor responders and minimise the risk in hyper responders. Different regimens use different gonadotropin preparations with or without pituitary down-regulation using GnRH agonist or antagonist. There is no one protocol that fits all and treatment should be individualised to take into

account woman's age, ovarian reserve, endocrine status and any other associated conditions such as endometriosis, polycystic ovary syndrome (PCOS) and ovarian cysts (Nardo, Fleming et al. 2011).

1.6.1 Choice of gonadotropins

The first generation gonadotropin used in the 1970s, was human menopausal gonadotropin (hMG, a combination of FSH and LH in a 1:1 ratio) produced from the urine of menopausal women. Since the early 1980s, progress in protein purification technology facilitated the production of a variety of urinary gonadotropins with lower concentrations of contaminating proteins, such as purified FSH (FSH-P) which contains less than one international unit (IU) of LH per 75 IU of FSH and highly purified FSH (FSH-HP) with less than 0.1 IU of LH per 75 IU of FSH. FSH, and hMG were equally effective in achieving ovarian stimulation, and there were no differences in the clinical pregnancy rates per cycle of IVF. However, in protocols where no pituitary desensitization was used, FSH alone was more efficacious (Agrawal, Holmes et al. 2000). A rising concern emerged regarding the limited batch-to-batch consistency and possibilities of urine contaminants (Balen, Hayden et al. 1999). Advancement in recombinant DNA technology allowed for the large scale production of human recombinant FSH (r-FSH), which is free from LH activity (Keene, Matzuk et al. 1989).

A recent Cochrane review (van Wely, Kwan et al. 2012) compared the effectiveness of rFSH with the three main types of urinary gonadotropins (hMG, FSH-P and FSH-HP) for ovarian stimulation in women undergoing IVF

or ICSI treatment cycles. Overall, there was no difference in pregnancy outcomes or the incidence of OHSS when r-FSH was compared to all urinary gonadotropins. There was also no difference in cumulative live birth and clinical pregnancy rates following frozen-thawed embryo transfer cycles for r-FSH versus urinary gonadotropins. Comparison of r-FSH with hMG or highly purified hMG (12 of the 42 trials) resulted in a lower live birth rate in the r-FSH group, though differences were small. The authors' conclusion was that all available gonadotropins appear equally effective and safe. The choice of gonadotropin for COH will depend upon the availability of the product, the convenience of its use and the associated costs. Collectively, there is not sufficient evidence to recommend the use of one type of gonadotropins over the other for women undergoing IVF or ICSI.

1.6.2 LH supplementation

According to the two-cell/two-gonadotropin hypothesis, both LH and FSH are essential for ovarian steroidogenesis. LH supplementation during the follicular phase is needed for ensuring an appropriate follicular oestradiol (E_2) production, complete oocyte maturation and endometrial development. The requirements of LH for ovarian stimulation in normogonadotrophic women have been widely debated. A Cochrane review (Mochtar, Van der et al. 2007) included 14 RCTs that evaluated the effectiveness of a combination of r-LH and r-FSH versus r-FSH alone in COH for IVF or ICSI. Of the 14 included trials, 10 trials involved women using long GnRH agonist protocol, one trial used a short GnRH agonist protocol and 3 trials used GnRH antagonist. Regarding GnRH antagonist protocol, there was no significant difference in pregnancy outcomes, OHSS incidence, r-FSH dose used or number of oocytes retrieved when r-LH was co-administered with

r-FSH. However, the data were far too small to affirm any conclusion. Considering the GnRH agonist studies, the two studies that reported on live birth rate found remarkably different results. One study found a beneficial effect in favour of r-LH co-treatment and the other was in favour of treatment with r-FSH alone. The pooled data showed no evidence of a significant difference in live birth rate and significant statistical heterogeneity was found. However, the pooled pregnancy estimates of trials including only poor responders showed significant increase in pregnancy rate, in favour of co-administration of r-LH.

It is widely accepted that there is no benefit of LH supplementation in the unselected population. The review of current evidence suggests that there is a potential benefit of r-LH administration in patients with poor response to ovarian stimulation and in those older than 35 years.

1.6.3 GnRH agonist versus GnRH antagonist

Continuous administration of GnRH agonist causes desensitisation after an initial short period of gonadotropin hyper-secretion, resulting in a reversible state of chemical menopause. GnRH antagonists cause an immediate and rapid, reversible suppression of gonadotropin secretion, by competitive occupancy of the GnRH receptor (1.3.5).

Different treatment regimens utilising GnRH agonists in COH have been developed. In the long protocol, GnRH agonist is commenced in the follicular or luteal phase of the preceding cycle and is continued until pituitary and ovarian suppression is achieved before stimulation with gonadotropins is started. In the short, or flare-up, protocol GnRH agonist therapy is started on cycle day 2, with gonadotropins initiated a day later. The ultra-short

protocol consists of daily subcutaneous administration of GnRH agonist during the first 3 days of ovarian stimulation.

A recent Cochrane review (Maheshwari, Gibreel et al. 2011) comparing ultra-short, short and long IVF protocols showed a higher number of oocytes retrieved and higher clinical pregnancy rates with the long protocol, at cost of more ampoules of gonadotropins. There was no difference in the outcomes amongst various long protocols such as luteal versus follicular start. Similarly, no difference in cycle outcome was found in cycles stimulated with long protocols when GnRH agonist is stopped or reduced at the start of gonadotropin stimulation.

GnRH antagonists have been introduced in clinical practice for COH in IVF/ICSI cycles to prevent premature luteinisation. Because of the acute gonadotropin suppressive activity of these agents, GnRH antagonists may be administered at any time during the follicular phase of a treatment cycle to prevent a premature LH surge (Kumar and Sharma 2014). The principal approach is the multiple dose antagonist protocol with cetrorelix (Cetrotide® by Merck Serono) or ganirelix (Orgalutran® by MSD) 0.25mg given daily subcutaneously from day 6 of gonadotropin stimulation onward or from when the leading follicle is ≥ 14 mm in diameter.

A recent systematic review addressing interventions for poor responders included an analysis of a variety of GnRH analogue schedules, concluded there is insufficient evidence to support the routine use of any particular intervention for pituitary down regulation in the management of poor responders in IVF cycles (Pandian, McTavish et al. 2010). Regarding

overresponders, the recent Cochrane meta-analysis showed significantly lower incidence of OHSS after GnRH antagonists than after GnRH agonist. The evidence suggested that if the risk of OHSS following GnRH agonist is assumed to be 11%, the risk following GnRH antagonist would be between 6% and 9% (Al-Inany, Youssef et al. 2016).

1.6.4 Conclusions

When planning a new IVF or ICSI treatment cycle a thorough assessment of the woman's hormone profile and ovarian reserve is essential before decisions are made regarding the appropriate COH protocol. There is no evidence to recommend the use of one type of gonadotropins over the other. In the unselected population, there is no benefit of LH supplementation in cycles stimulated with FSH only. There is some evidence to suggest a potential benefit for LH supplementation in patients with poor response to ovarian stimulation and in those older than 35 years. The long GnRH agonist protocol is the most widely used and the preferred protocol for normal responder women undergoing COH for IVF or ICSI. The GnRH antagonist protocol is best used for known or suspected high responders including women with PCOS, as it reduces the risk of OHSS. There is lack of robust evidence to suggest that the GnRH agonist protocol is better than the antagonist protocol in poor responders.

1.7 Complications in relation to IVF

In approximately 2% of ART cycles, various complications occur. Some of these can be prevented, some cannot. Complications of the ART process can be stimulation related, procedure related or pregnancy related. Ovarian hyperstimulation syndrome (OHSS) is responsible for the majority of cycle

related complications, bleeding and infection secondary to oocyte retrieval are less common. In the next sections, these complications will be discussed in more detail.

1.8 Stimulation complications

Controlled ovarian hyperstimulation (COH) entails hormonal treatment which was developed to allow multifollicular growth. By creating the possibility to obtain multiple oocytes for treatment the chance of a pregnancy is enhanced. There are various protocols of controlled ovarian hyperstimulation. Most used regimes establish pituitary down regulation by using gonadotropin releasing hormone (GnRH) agonists. After establishing down regulation follicular stimulating hormone (FSH) will be added to induce follicular growth. As with any medical treatment the envisioned result does not always occur.

1.8.1 Ovarian hyperstimulation syndrome

Ovarian HyperStimulation Syndrome (OHSS) is a condition inherently related with controlled ovarian stimulation. In extremely rare occasions it can occur in a spontaneous ovulatory cycle. This exceptional event is more common in the case of multiple gestation, molar pregnancy, hypothyroidism or polycystic ovary syndrome. The underlying mechanism is believed to be related to a mutation in the FSH receptor (Dieterich, Bolz et al. 2010).

OHSS is a clinical diagnosis based on clinical symptoms, with laboratory results and ultrasound assessment as complementary measures. OHSS symptoms are the result of increased vascular permeability of the gonadotropin primed ovaries. The syndrome can be best characterized by bilaterally enlarged ovaries due to multiple follicular and thecal lutein ovarian cysts. In addition to the physical enlargement of ovaries, an acute shift of intravascular fluid redistribution occurs, causing clinical ascites.

OHSS occurs typically as an iatrogenic complication in women undergoing IVF/ICSI, due to the supraphysiological doses of gonadotropins which are used to induce multi-follicular growth. Some patients respond excessively to the administration of exogenous FSH. When a large number of follicles are recruited and start to grow, serum oestrogen levels rise. In combination with the increased size of the ovaries, this can cause serious complications and ultimately death (Mozes, Bogokowsky et al. 1965, Cluroe and Synek 1995, Beerendonk, van Dop et al. 1998, Semba, Moriya et al. 2000).

1.8.1.1 Definition and Classification

The diagnosis of OHSS is usually made after a history of ovarian stimulation, either by gonadotropins or anti-oestrogens, followed by typical symptoms of abdominal discomfort, bloating, nausea and vomiting. Any other reason for these symptoms should be excluded. The full range of the syndrome includes severe ascites requiring drainage, adult respiratory distress syndrome, hydrothorax and trombo-embolisms.

The accurate incidence of OHSS is difficult to estimate, mainly due to the different classifications used in literature. The reported incidence varies between patient groups and treatments used. In IVF, up to 33% of cycles develop mild OHSS. Although they are considered clinically less significant, they still have the potential to develop into the severe form, which is estimated to occur in 3.1-8.0% of cycles (Delvigne and Rozenberg 2002).

The objective of having a classification of OHSS is to make it possible to compare the incidence and evaluate the efficacy of the different approaches for prevention and treatment of the syndrome. Until now, no single uniformly accepted classification of OHSS has been devised, making it difficult to compare literature on the subject.

The Royal College of Obstetricians and Gynaecologists (RCOG), through its Guideline and Audit Committee and Professional Standards Committee, produces Clinical Governance guidance documents for gynaecologists practising in the UK. This provides us with a classification and advice on appropriate treatment, with the reflection on mild and moderate OHSS classification (Mathur R 2005). The current UK classification according to severity is presented in Table 1-1. With the footnote that ovarian size may

not correlate with severity of OHSS in cases of assisted reproduction because of the effect of follicular aspiration.

Grade	Symptoms
Mild OHSS	Abdominal bloating Mild abdominal pain Ovarian size usually less than 8cm
Moderate OHSS	Moderate abdominal pain Nausea with or without vomiting Ultrasound evidence of ascites Ovarian size usually 8-12cm
Severe OHSS	Clinical ascites (occasionally hydrothorax) Oliguria Haemoconcentration with a haematocrit >45% Hypoproteinaemia Ovarian size usually more than 12cm
Critical OHSS	Tense ascites or large hydrothorax Haematocrit >55% White cell count > 25 000/ml Oliguria or anuria Thromboembolism Acute respiratory syndrome

Table 1-1: Classification of severity of OHSS from RCOG guideline No. 5

Sterility.94(2):389-400

1.8.1.2 Pathophysiology

When affected by OHSS, ovaries show a significant degree of stromal oedema, multiple haemorrhagic follicular and theca-lutein cysts, in combination with areas of cortical necrosis and neovascularisation. Most investigators believe that the fluid shift from the intravascular to the third space, is responsible for the majority of the clinical symptoms, and that this is the result of increased vascular permeability (Rizk and Aboulghar 1991, Elchalal and Schenker 1997). This theory has been supported in animal models of hyperstimulated rats (Gomez, Simon et al. 2002).

1.8.1.3 Hormones

The association between severe OHSS and high oestradiol levels is well established (Asch, Li et al. 1991). Considering the overall incidence of OHSS in all women undergoing IVF treatment is 1%, an increase to an incidence as high as 38% can be observed in women with oestradiol concentration on the day of hCG administration of more than 20000pmol/l. Oestrogens were shown to induce capillary permeability in uterine and ovarian circulation (Pappan 1965). Because of the high levels of this hormone in the sera of OHSS patients, it was assumed to be correlated. Contradicting this statement, animal studies on rabbits did not lead to reproduction of OHSS symptoms following administration of large doses of oestrogens (Yarali 1993). Human cases of low oestradiol levels and severe OHSS have also been observed, casting doubt on the role of oestrogens as the sole mediator of vessel permeability in the pathophysiology of OHSS (Levy, Orvieto et al. 1996). To speculate, oestradiol seems to be a by-product of multifollicular development and might not contribute to the development of OHSS. It can

however be used as a marker to identify women at risk of developing the syndrome.

In the context of OHSS, the role of progesterone itself is still unclear. The use of progesterone supplementation is preferable over hCG for the luteal phase support in women with overresponse. OHSS is caused by increased vascular permeability through ovarian hypersecretion of vascular endothelial growth factor (VEGF)-activating VEGF receptor 2 (VEGFR-2). Dopamine receptor 2 (Dp-r2) agonists, used in the treatment of human hyperprolactinemia including pregnancy, inhibit VEGFR-2-dependent VP and angiogenesis when administered at high doses in animal cancer models. Gomez et al used a well-established OHSS rat model to test whether VEGFR-2-dependent VP and angiogenesis could be segregated in a dose-dependent fashion with the Dp-r2 agonist Cabergoline. A 100 microg/kg low-dose Dp-r2 agonist Cabergoline reversed VEGFR-2-dependent VP without affecting luteal angiogenesis through partial inhibition of ovarian VEGFR-2 phosphorylation levels. No luteolytic effects (serum progesterone levels and luteal apoptosis unaffected) were observed. Cabergoline administration also did not affect VEGF/VEGFR-2 ovarian mRNA levels. Results in the animal model and the safe clinical profile of Dp-r2 agonists encouraged the researchers to administer Cabergoline to oocyte donors at high risk for developing the syndrome. Prophylactic administration of Cabergoline (5-10 microg/kg x d) decreased the occurrence of OHSS from 65% (controls) to 25% (treatment) (Gomez, Gonzalez-Izquierdo et al. 2006). In a study of similar design, rats were given progesterone obtaining a normal progesterone level. No change in vascular permeability or VEGF levels was noted. The authors concluded that progesterone neither effects

ovarian VEGF expression or vascular permeability (Ujioka, Matsuura et al. 1997).

1.8.1.4 Renin-angiotensin system

The role of the ovarian renin-angiotensin system (RAS) in OHSS has been extensively investigated (Delbaere, Bergmann et al. 1994). In humans, 90% of renin exists in the form of prorenin. The major source of plasma renin and prorenin is the kidney. However, the ovary is also a source of circulating prorenin, as has been demonstrated in a bilateral nephrectomised woman. Prorenin is synthetized in the theca cells and in a natural menstrual cycle its expression peaks in response to the LH surge. The basis for the hypothesis that RAS is part of the OHSS pathogenesis is due to the fact that levels of prorenin are higher in follicular fluid than in the plasma levels (Yoshimura, Koyama et al. 1994). A direct correlation between plasma renin levels and severity of OHSS was established and in cycles where a pregnancy occurred, elevated plasma aldosterone levels were observed. Another indication of the role of the RAS system in the development and symptomatology of OHSS is the presence of high levels of renin in ascites of patients with OHSS compared with controls (Navot, Margalioth et al. 1987, Rosenberg, McKenzie et al. 1994). It is therefore likely that, in women with OHSS the high local levels of renin reflect a peripheral activation of RAS, probably of both ovarian and renal origin (Delbaere, Bergmann et al. 1997, Delbaere, Bergmann et al. 1997).

Angiotensin and its receptors have also been investigated in the context of OHSS. Data on the effect of angiotensin-converting enzyme (ACE) inhibitors on OHSS in animal models is at best conflicting (Morris, Wong et al. 1995,

Sahin, Kontas et al. 1997, Teruel, Carbonell et al. 2001, Teruel, Carbonell et al. 2002). In humans, the possible teratogenicity of ACE inhibitors limits the potential use of these medications or the use of angiotensin receptor blockers, in the prevention or treatment of OHSS.

1.8.1.5 Endothelial function: prostaglandins, Von Willebrand factor, endothelin-1

Prostaglandins have been investigated as possible mediators of increased capillary permeability. Early experiments demonstrated that indomethacin, a blocker of prostaglandin synthesis, could prevent the fluid shift associated with ascites, pleural effusion and hypovolaemia as seen in patients with OHSS (Schenker and Polishuk 1976). Unfortunately, the desired clinical improvement could not be demonstrated (Katz, Lancet et al. 1984). Caution is advised when using prostaglandin synthetase inhibitors, as they might induce renal failure, which might further compromise an already affected renal function.

von Willebrand factor (vWF) is a blood glycoprotein that is required for normal haemostasis and its level can be increase by endothelial cell damage. This makes it a suitable marker of endothelial dysfunction (Horvath, Hegedus et al. 2004). Its levels are diminished in von Willebrand syndrome and increased in clinical conditions characterised by endothelial cell dysfunction such as pre-eclampsia and thrombocytopenic purpura. Plasma levels of vWF are increased with the use of desmopressin due to a selective effect on endothelial permeability. This effect is used in the treatment of patients with von Willebrand syndrome. Tomorrow et al. were the first to demonstrate elevated vWF in patient with severe OHSS, which returned to normal when the clinical syndrome improved (Todorow,

Schricker et al. 1993). In a retrospective study by Ogawa and colleagues, elevated serum levels of vWF were found before the clinical manifestation of the severe form of OHSS, but not the mild form (Ogawa, Minakami et al. 2001).

Endothelin-1 is produced by vascular endothelial cells and is a potent vasoconstrictor, which increases vascular permeability. Its presence has been demonstrated to be higher in follicular fluid than in blood plasma, however there is no correlation with the severity of OHSS, suggesting a homeostatic response rather than an initiating function (Balasch, Arroyo et al. 1994).

1.8.1.6 Vascular Endothelial Growth Factor (VEGF)

VEGF is a signal protein that promotes the growth of new blood vessels. It forms part of the system that restores the blood supply to cells and tissues when they are deprived of oxygenated blood due to compromised blood circulation. The most important member of the VEGF family is VEGF-A. The different roles of the various VEGF proteins are described below:

- VEGF-A – VEGF-A is required for angiogenesis. Specifically for the creation of the blood vessel lumen and fenestrations. It promotes the migration and mitosis of endothelial cells, and stimulates the matrix metalloproteinase and αvβ3 activity. This protein is also chemotactic for granulocytes and macrophages and stimulates vasodilation through the release of nitric oxide.
- VEGF-B – This protein is required for blood vessel formation in the embryo (embryonic angiogenesis).
- VEGF-C – This protein is key to the formation of new lymphatic vessels from those already existing.

- VEGF-D – This form of VEGF is required for the development of lymphatic vessels that surround the bronchioles or air passages within the lungs.
- PlGF (placenta growth factor) – This form of VEGF is required for vasculogenesis and angiogenesis (Neufeld, Cohen et al. 1999, Ferrara, Gerber et al. 2003, Roskoski 2007).

In relationship to OHSS, the VEGF referred to is VEGF-A. The role of VEGF-A in OHSS is extensively investigated (Rizk, Aboulghar et al. 1997). Albert et al. suggested three important reasons supporting the role of VEGF as a potential mediator in the development of OHSS (Albert, Garrido et al. 2002).

First, VEGF and its isoforms have vasoactive properties; secondly VEGF-A has been identified in the follicular fluid; third reason mRNA transcripts and respective proteins have been detected in granulosa cells, in normal functioning ovaries. Finally, VEGF-A is noted to be increased in serum and peritoneal fluid of women who developed OHSS compared with healthy control patients (Abramov, Barak et al. 1997, Agrawal, Chimusoro et al. 1997, Agrawal, Conway et al. 1998, Artini, Fasciani et al. 1998, Artini, Monti et al. 2002, Peitsidis and Agrawal 2010).

A Lancet article by McClure et al. details the role of VEGF-A as a capillary permeability agent in the pathophysiology of OHSS (McClure, Healy et al. 1994). Molecular studies suggested a strong link between VEGF-A and hCG. Animal studies using rats and primates demonstrated that VEGF-A mRNA expression is abundant predominantly after the LH surge. In humans luteinized granulosa cells are found to produce VEGF-A (Balasch, Guimera et al. 2004). The same surge of LH is also an important part of the development of OHSS. In the IVF setting, luteal phase treatment (after

oocyte retrieval) with a GnRH antagonist supresses LH secretion and results also in a decrease in VEGF-A mRNA expression in follicular fluid, implying that VEGF levels are dependent on LH. This is consistent with the clinical observation suggesting that luteal phase support by hCG increases the likelihood of OHSS compared to the sole use of progesterone (Smitz, Camus et al. 1990, Rizk and Smitz 1992). It is known that hCG has a longer half-life that LH and a greater biological activity, therefore possibly being responsible for a higher incidence of OHSS in patients triggered or supported with hCG. In rats, LH, hCG, FSH were equally effective in triggering ovulation, but VEGF was significantly lower in the LH group. Gomez et al. treated immature female rats with 10 IU PMSG for 4 days, and ovulation was triggered with saline, 10 IU hCG, 10 IU FSH, 10 IU LH or 60 IU LH. The number of oocytes ovulated into the tubes, VP and mRNA VEGF expression were evaluated and compared. If the administrated LH concentration was increased by six fold it induced the same levels of VEGF and vascular permeability as hCG and FSH. This indicates that lower LH doses can induce similar ovulation rates then higher doses, with a lower risk of OHSS (Gomez, Lima et al. 2004). VEGF mRNA is present in human luteinized granulosa cells. This expression is enhanced by hCG, and is dose and time dependent, with a maximum enhancement three hours after a concentration of 1 IU of hCG/ml was reached (Neulen, Yan et al. 1995). The effect of hCG on the pathogenesis of OHSS was investigated by measuring oestradiol, VEGF and insulin-like growth factor II in serum and follicular fluid. Analysis of the mRNA expression of VEGF in luteinized granulosa cells was also performed. Women who developed OHSS were compared to women who were not affected by the syndrome. hCG up-regulates VEGF

expression of granulosa cells in the OHSS population and not in the control group. Follicular VEGF relays its effect through an autocrine mechanism using kinase insert on VEGF-R2. The authors corrected the VEGF concentration for the follicular volume. They showed that an increase in follicular VEGF accounted for an increased serum level and this was associated with the development of OHSS (Wang, Horng et al. 2002).

These results were responsible for subsequent research attempting to understand the role of VEGF in OHSS. While many studies have reported a causative relationship, others have reported contradictory results with no differences between OHSS and controls (Rizk, Aboulghar et al. 1997, D'Ambrogio, Fasciani et al. 1999, Geva, Amit et al. 1999, Enskog, Nilsson et al. 2001, Delvigne and Rozenberg 2002).

Some important conclusions may be drawn and should be taken into account when considering VEGF in the context of OHSS. VEGF levels can be measured in plasma or in serum, but the clotting process increases VEGF 8- to 10-fold in the serum. Degranulation or haemoconcentration in OHSS may cause a false reading of the real levels of free active VEGF. In women with OHSS, large amounts of VEGF could be trapped in the ascetic fluid, or in the large *corpora lutea* cysts of the ovary, underestimating its true levels when measured from peripheral blood. The biological, active isoform of VEGF could also vary from one patient to another, and immunoassays may not be able to differentiate between the four or more isoforms of VEGF. Soluble VEGF receptors may influence the biological activity of VEGF by rendering some of the free molecules inactive (Delvigne 2004).

Krasnow et al. measured VEGF in serum, peritoneal fluid and follicular fluid. Serum VEGF was significantly higher in the group who developed severe OHSS compared to those who did not. Follicular fluid VEGF level was approximately a 100-fold higher than serum or peritoneal fluid levels 36 hours after hCG administration, suggesting that the ovary is a source of VEGF (Krasnow, Berga et al. 1996).

In an unstimulated cycle there is only one *corpus luteum*, which is unlikely to be producing a sufficient amount of VEGF to induce OHSS. In cases of severe OHSS, there are 21 or more follicles present at the time of hCG administration, hence we can hypothesize that the hCG will rescue all *corpora lutea*, causing a significant increase in VEGF levels leading to the development of OHSS. No studies are available who have compared *corpora lutea* in a stimulated versus an unstimulated cycle in the same patient. There also is a debate regarding the effect of the actual follicle aspiration procedure on VEGF production. However, limited data on the matter prevents from drawing any conclusions (Neulen, Yan et al. 1995).

Abramov et al. followed the kinetics of VEGF in patients admitted to hospital with severe OHSS (Abramov, Schenker et al. 1996). All patients had elevated plasma VEGF levels. In the study population, a decrease of VEGF levels correlated with the clinical improvement of the syndrome. There was also a positive correlation between VEGF levels and haematocrit and white blood cell count (Aboulghar, Mansour et al. 1999). D'ambrogio at al. found that serum VEGF levels before starting stimulation in women who have developed moderate forms of OHSS did not show a significant difference when compared to a control group (D'Ambrogio, Fasciani et al. 1999).

Agrawal et al. suggested that serum VEGF levels predict the risk of development of OHSS. The increase in VEGF may be a better predictor than oestradiol concentration or the number of follicles during stimulation, or the number of oocytes collected, which individually predict 15-25% of cases of OHSS (Agrawal, Tan et al. 1999).

In a prospective cohort study Enskog et al. evaluated VEGF165 concentration during stimulation of 15 OHSS cases with 30 matched (age, follicle numbers, pregnancy) controls. The authors did not find a raised plasma concentration of the isoform of VEGF in patients with OHSS. This lack of correlation may suggest a disruption of the normal controls regulating follicular VEGF expression in patients that do develop OHSS (Enskog, Nilsson et al. 2001).

McElhinney et al studied the variations in serum VEGF-binding profiles. They observed that patients that do not develop OHSS appear to have a high-molecular-weight protein (α-2 macroglobulin) that binds VEGF to a greater degree, than what is observed in patients who do develop OHSS (McElhinney, Ardill et al. 2002). Gomez et al. reported that VEGF receptor-2 activation induces vascular permeability in hyperstimulated rats and this effect can be prevented by a receptor blocker (Gomez, Simon et al. 2002). VEGF plays an important role in the pathogenesis of OHSS. Levels of the growth factor on its own cannot be used as a predictive marker for the development of OHSS. However a rapid rise in VEGF levels does seem to indicate a high risk of development of OHSS. VEGF seems to be a good marker of resolution of the syndrome. The definitive diagnosis of OHSS

remains a clinical one and is related to signs and symptoms, but VEGF may play an important role as a therapeutic target.

1.8.1.7 Interleukins

There is a growing evidence for a role of the immune system in the pathogenesis of OHSS, with cytokines as mediators. Cytokines are a family of low-molecular-weight proteins that play an important role in the regulation of haemostatic responses. They exert their effect on cellular differentiation and activation. Interleukins are a subset of cytokines and have a variety of action on endothelium, fibroblasts and granulosa and theca cells (Mathur, Jenkins et al. 1997).

1.8.1.8 Genetics

Follicle stimulation hormone (FSH) is the central hormone of human reproduction. It is necessary for gonadal development and maturation at puberty and for gamete production during the reproductive phase of life. FSH, together with luteinizing hormone (LH), is produced and secreted by the pituitary gland as a highly heterogeneous glycoprotein. FSH, LH and hCG consist of a common alpha subunit and a receptor specific beta subunit. FSH acts by binding to specific receptors localized in the gonads. The FSH receptor is a glycoprotein belonging to the family of G-protein-coupled receptors.

FSH plays a central role in oogenesis. It triggers the maturation of follicles, proliferation of granulosa cells and aromatase induction. Its role is pivotal in the recruitment of the dominant follicle. FSH action is mediated by the FSH receptor. Screening for mutations of the FSH receptor has been performed in the search for causes of infertility (Aittomaki, Lucena et al.

1995, Gromoll, Simoni et al. 1996, Gromoll, Simoni et al. 1996, Simoni, Gromoll et al. 1997, Simoni, Nieschlag et al. 2002, Gromoll and Simoni 2005).

The chromosomal mapping of the FSH receptor was performed and the gene has been located on chromosome 2p21 in the human. The LH receptor gene can be mapped to the same chromosome 14q3. The FSH receptor gene is a single-copy gene and spans the region of 54 kbp in the human (Simoni, Nieschlag et al. 2002).

The few cases of spontaneous OHSS have been explained by mutations of the FSH receptor. The mutation is believed to make the receptor hypersensitive for hCG. Allelic variants of the FSH receptors have been associated with the response to FSH in stimulation procedures, as well as with the severity of OHSS when present (Rodien, Beau et al. 2010).

Polymorphisms of the FSH receptor have been investigated and seven hundred and forty four single nucleotide polymorphisms have been identified in the FSH receptor gene, of which only eight are located in the coding region. Ovarian response is dependent on FSH receptor genotype. Clinical studies on the p.N680S polymorphism of the FSHR gene have demonstrated the homozygous Ser/Ser variant to be less sensitive to endogenous or exogenous FSH in terms of oestradiol production. Polymorphism of the FSHR, N680S could be a predictor of the severity of symptoms in patients who develop OHSS (Rizk 2009). Activating mutations of the FSHR gene that cause ovarian hyper-responsiveness to circulating FSH or even cross-responsiveness of FSHR to hormones having a structure similar to FSH, such as hCG or TSH (Lussiana, Guani et al. 2009)

1.8.1.9 Risk factors associated with OHSS

1.8.1.9.1 Patient characteristics

There are some demographic and phenotypic characteristics related with a higher chance of developing OHSS. In brief, these are age, body mass index (BMI), cause of infertility, polycystic ovaries and immunology.

1.8.1.9.2 Age

OHSS does not exclude older women, but is more frequently observed in young women. This association with age was demonstrated by retrospective Belgian multi centre survey. Data was collected in 13 centres concerning 128 cases of OHSS. For each case 2 controls were sought, which had the same stimulation protocol, in the same centre, and oocyte collection just prior or just after the OHSS case. Patients who developed OHSS were significantly younger compared to controls (30.2±3.5 years versus 32.0±4.5 years, $P<0.002$). This association was confirmed in a prospective Swedish study following 428 cycles. Forty-nine patients developed OHSS (mild, moderate or severe) with a mean age of 31.04±0.46 years, of which eighteen patients developed the severe form as classified by Golan, being 30.22±0.68 years. Both demonstrated a significant difference with the group which did not develop any symptoms and was 32.61±0.18 years of age (Delvigne, Demoulin et al. 1993, Enskog, Henriksson et al. 1999).

No definite cut-off age value to predict high risk of OHSS can be found in the literature, but most the most often used is age less than 30 years old (Navot, Relou et al. 1988).

1.8.1.9.3 Body Mass Index (BMI)

It is a common belief that OHSS is more frequent in slim patients. Navot et al. described a positive correlation with lean BMI and OHSS in 1988 (Navot, Relou et al. 1988). Later studies have not confirmed this association (Delvigne, Demoulin et al. 1993, Enskog, Henriksson et al. 1999). Irrespective of the available contradictory literature, most centres consider patients with a BMI below 20kg/m^2 to be at increased risk of developing OHSS, especially in the presence of other risk factors.

1.8.1.9.4 Cause of infertility

Any attempts to identify an association between higher risk of developing OHSS and primary or secondary infertility did not result in any positive findings. The duration of infertility, irrespective of the cause, has not been shown to influence the risk of developing OHSS. On the other hand, women who had a previous cycle complicated by OHSS remain at an increased risk of developing this complication in other treatment cycles (Navot, Bergh et al. 1992).

Patients suffering from infertility caused by WHO 2 anovulation (normoestrogenic or hyperandrogenic; this group includes PCOS) have a significantly higher incidence of OHSS, which is repetitively shown. Patients suffering from WHO 1 primary amenorrhoea (hypogonadotropic hypogonadism) are not considered to be of higher risk.

1.8.1.9.5 Polycystic ovarian syndrome (PCOS)

Several studies have confirmed that patients with PCOS, defined using the Rotterdam consensus, are at increased risk of developing OHSS. During a

workshop held in Rotterdam in 2003, it was decided that the diagnosis of PCOS should be made if 2 out of the following 3 criteria were present:

- Oligoovulation and/or anovulation
- Excess androgen activity
- Polycystic ovaries by gynaecologic ultrasound. (either 12 or more follicles measuring 2-9mm, or increased ovarian volume of >10cm^3).

Approximately 40-60% of women with PCOS are overweight and 60% have insulin resistance. Hyperinsulinemia contributes to hyperandrogenism. Hyperinsulinemia has been shown to be more profound, but not exclusive, in obese patients compared to women with a BMI within the normal range. The former group of women has been reported as having a higher incidence of OHSS compared with PCOS patients with normal insulin levels. Granulosa cells play a major role in development of OHSS. The elevated levels of insulin increase the aromatase activity of granulosa cells, resulting in a higher ratio of follicular oestradiol to androstenedione (Fulghesu, Villa et al. 1997). Higher insulin levels alter the ovarian response to FSH and enhance the production of antral follicles. Studies comparing the association of hyperinsulinemia and IVF outcome as predictor for OHSS rates did not find any significant relationships (Delvigne, Kostyla et al. 2002).

1.8.1.9.6 Allergy

Interestingly, a study involving 420 patient having IVF treatment, observed an increased prevalence of allergies in patients who developed OHSS. This gave rise to the formulation of the hypothesis that differences in immunologic sensitivity of patients may be a predictor of OHSS. The authors

suggested that the changes which occur in an ovary resemble an overactive inflammatory response with oedema and increased vascular permeability. No other studies looked into this association (Enskog, Henriksson et al. 1999).

1.8.1.9.7 Other associations with OHSS

There is some observational mentioning of an association between OHSS and migraines (Rollene, Khan et al. 2011) and between OHSS and blood type A (Binder, Flegel et al. 2008, Binder, Flegel et al. 2008). This is not investigated any further in the current available literature.

1.8.1.10 Risk factors for prediction

Management of OHSS is important, but prevention of a disease from occurring in the first instance, is better than identifying the cure. To prevent, and evaluate if preventive measurements are useful, we need to establish which patients are at risk of development of the syndrome. All prevention starts with prediction.

In the whole of medicine an accurate and detailed history will provide a lot of information for primary prevention. For secondary prevention, the addition of expert ultrasound monitoring and endocrine evaluations is essential.

Rizk provided a table at the annual ASRM meeting for prediction of OHSS (Rizk 2002). There are 8 risk factors incorporated; OHSS in a previous cycle, PCOS, high serum oestradiol levels with a rapidly rising slope, ultrasound features of a PCO-pattern and a high number of follicles, low intra ovarian vascularity resistance Doppler, conception (especially multiple pregnancy), young age and a low BMI.

Asch et al. conducted one of the first studies trying to define patients at risk of developing OHSS (Asch, Li et al. 1991). They did so by retrospectively analysing 637 cycles of controlled ovarian stimulation with oocyte collection between 1987 and 1989. They used criteria by Schenker and Weinstein to define all six cases of severe OHSS. These criteria included ovarian enlargement, ascites, oliguria, haemoconcentration and electrolyte disturbances. All study patients were hospitalised. One of the six patients did not have an embryo transfer, and three of the five who did have a transfer, achieved a pregnancy (pregnancy rate of 60%). The patient who did not have a fresh transfer had a life birth after a frozen embryo replacement. Luteal support was provided by progesterone in all cases (Asch, Li et al. 1991). Looking at serum oestradiol level on the day of hCG administration, none of the patients with serum oestradiol below 3500pg/ml developed severe OHSS. Of 67 cases with oestradiol between 3500 and 5999 pg/ml, one patient developed severe OHSS (1.5%). Out of 13 patients with an oestradiol level above 6000pg/ml, all developed severe OHSS (38%) (Asch, Li et al. 1991). No cases of severe OHSS were demonstrated among patients with less than 20 oocytes collected. Only one severe OHSS occurred among patients having 20-29 oocytes retrieved (1.4%). Patients who had more than 30 oocytes collected showed significantly higher rate of severe OHSS (22.7%). Data derived from this study, demonstrates that there's an 80% chance of severe OHSS if the oestradiol levels are >6000 pg/ml and >30 oocytes are collected (Asch, Li et al. 1991).

Morris et al compared 72 donor oocytes cycles with 67 IVF patients, which either had an oestradiol >4000pg/ml, >25 oocytes collected or both. There were no cases of severe OHSS in the donating group. Six patients in the

IVF group developed severe OHSS. Among the 10 patients with an oestradiol concentration of more than 6000pg/ml, only one developed OHSS (Morris, Paulson et al. 1995). The prevalence is this study is less than the 80% calculated by Asch et al. (Asch, Li et al. 1991). The relative risk of OHSS with the establishment of pregnancy was 12 (95%CI 2.18-66.14) (Morris, Paulson et al. 1995).

1.8.1.10.1 AFC

Antral follicle count (AFC) is used to predict ovarian response to stimulation. Trying to optimise the accuracy of predictive multivariate models of ovarian response were constructed. When AFC alone is compared to other multivariate models it is found that the two measurements are comparable (Verhagen, Hendriks et al. 2008). Only limited data regarding the prediction of hyperresponse is available. Kwee et al conducted a prospective study in 110 women, performing basal AFC scans and a standardised ovarian stimulation protocol. Hyperresponse was defined as having more than 20 follicles developed or more than 20 oocytes collected. Univariate logistic regression showed that the best predictor was AFC [area under the curve (AUC)=0.92] with a sensitivity of 0.82 (rate of correct identification of cases with hyperresponse) and a specificity of 0.89 (rate of correct identifications of cases without hyper response). The cut-off level of an AFC of >14 gave the highest sensitivity and specificity with the highest accuracy in the population studied. With 15% of the population having had more than 20 oocytes collected, the accuracy was 88% (meaning that 88% of patients had a correct predictive test). A baseline scan with an AFC of more than 14 follicles correctly predicts (positive predictive value) overresponse to ovarian stimulation in 58% (Kwee, Elting et al. 2007).

AFC is an easy and commonly available test. Unfortunately, most studies focus on predicting poor response. Available literature consists mainly of small sample sizes in single centres. Large number studies and testing the predictive model in different centres are needed. Currently, AFC is probably the best available predictor of developing OHSS.

1.8.1.10.2 Anti-Müllerian hormone

Anti-Müllerian hormone (AMH) is expressed by the granulosa cells and is increasingly used as a predictor of ovarian reserve and to predict the response to ovarian stimulation. One study noticed that all cycle cancellations due to OHSS were in patients who fell into the highest AMH quartile (>7ng/ml) (La Marca, Giulini et al. 2007). These findings were verified by a recent cohort study of 262 IVF cycles with 21 cases (8%) of moderate or severe OHSS. This study found that baseline AMH levels were significantly correlated with the subsequent development of OHSS, OR 1.79; P=0.0004 (Lee, Liu et al. 2008). AMH values predicted OHSS better than age and BMI. An AMH cut-off value of 3.36 ng/ml gave a sensitivity of 90.5% and a specificity of 81.3%.

1.8.2 Prevention of OHSS

Prevention can be divided into primary or secondary prevention. When a specific stimulation protocol is chosen based on patient characteristics in order to minimise the risk of developing OHSS primary prevention is employed. Secondary prevention is when during stimulation alternations to the medication or treatment is made.

Humaidan et al. (2009) re-evaluated techniques for the prevention of OHSS(Humaidan, Quartarolo et al. 2010). The authors conducted a literature review, which included the widest understanding of current empirical and clinical experience. This consisted of randomised controlled trial, retrospective studies, pilot studies, case studies, meta-analysis and reviews. They summarized the most recent evidence for OHSS prevention strategies and graded the level of evidence. Giving the following results; there is a shortage of large, prospective RCTs reporting OHSS prediction and prevention strategies. Risk factors such as AFC and AMH may identify women at high OHSS risk. Preventative strategies that appear highly effective at reducing or preventing OHSS include GnRH antagonist protocols and the use of GnRH agonists to trigger final oocyte maturation. Moreover, alternative therapies, such as dopamine receptor agonists, have also emerged as potential new treatment modalities in the management of this disease.

1.8.2.1 Ovarian Stimulation

Ovarian stimulation is used to obtain a multi-follicular development in IVF or for mono follicular development in ovulation induction with or without the combination with IUI. For ovulation induction, decreasing the exposure to gonadotropins, low dose protocols or in limited ovarian stimulation (stopping when a follicle reaches 12mm), all reduces the incidence of OHSS. In IVF cycles mild stimulation (starting stimulation in the late follicular phase with addition of a GnRH antagonist) has significant lower OHSS rates (1.4%) than standard long protocol stimulation (3.7%) (Pelinck, Vogel et al. 2005). A remarkable observation is that the mild stimulation group had fewer multiple pregnancies, which might be a contributing factor to the

observed reduction in OHSS rates. It is expert's opinion not to give any FSH on the day of hCG. There have however not been any studies directly investigating this.

The latest review, which looked at the difference between agonist and antagonist protocols, concluded that the use of GnRH antagonist protocols in women with PCOS was associated with shorter duration of ovarian stimulation, lower total dose of gonadotropins used and comparable clinical pregnancy rate (CPR) and ongoing pregnancy rate (OPR). There was no significant difference found in the incidence of severe OHSS with the antagonist group as compared with the long agonist group. However, when combining the incidence of both moderate and severe OHSS reduction in occurrence was noted, amongst those in the GnRH antagonist protocol as compared to the GnRH long agonist protocol (Pundir, Sunkara et al.).

The first Cochrane review comparing the IVF outcome between GnRH agonist and GnRH antagonist cycles in 2002 showed no significant reduction in the incidence of severe OHSS (OR 0.51; 95% CI 0.22-1.18). However, there were significantly fewer clinical pregnancies in those treated with a GnRH antagonist (OR 0.79; 95% CI 0.63-0.99) (Al-Inany and Aboulghar 2002). In a subsequent meta-analysis by Kolibianakis et al. (Kolibianakis, Collins et al. 2006) the incidence of OHSS associated with hospital admissions was significantly lower for the antagonist arm (OR 0.46; 95% CI 0.26-0.8; $p<0.01$). Al-Inana et al. (Al-Inany, Abou-Setta et al. 2007) updated their Cochrane review and showed that there was a noticeable reduction in the incidence of severe OHSS using an antagonist protocol (OR 0.61; 95% CI 0.42-0.89; P=0.01). The on-going pregnancy/live-birth rate

showed significantly lower pregnancy rates in the antagonist group (P=0.03; OR 0.82, 95% CI 0.69-0.98). In a further Cochrane review (Al-Inany, Youssef et al. 2011) the difference was even more pronounced in demonstrating a lower incidence of OHSS with a GnRH-antagonist (29 trials, OR 0.43; 95% CI 0.33-0.57, p < 0.00001). There was no significant difference in rates of live births (OR 0.86, 95% CI 0.69-1.08) or on-going pregnancies (OR 0.87, 95% CI 0.7-1.00).

The above data suggests that the optimal stimulation protocol for patients at risk of developing OHSS would consist of the use of a GnRH antagonist. As with the initial use of GnRH agonists, there is a learning curve regarding the conduct of these protocols. An additional benefit of the use of an antagonist protocol is that it allows triggering final oocyte maturation with a GnRH agonist (GnRHa).

The notion that a GnRHa trigger eliminates OHSS in high-risk patients, was first introduced by Itskovitz et al. in 1988 (J Itskovitz 1988). Preliminary reports confirmed the ability of the GnRHa trigger to prevent OHSS in GnRH antagonist-based stimulation protocols, amongst those thought to be high-risk patients (Itskovitz-Eldor, Levron et al. 1993). Evidence from observational uncontrolled trials and randomized studies during the last decade, as shown in Figure 1-4, demonstrated absence of OHSS for GnRHa triggering in GnRH antagonist cycles (Humaidan, Quartarolo et al. 2010).

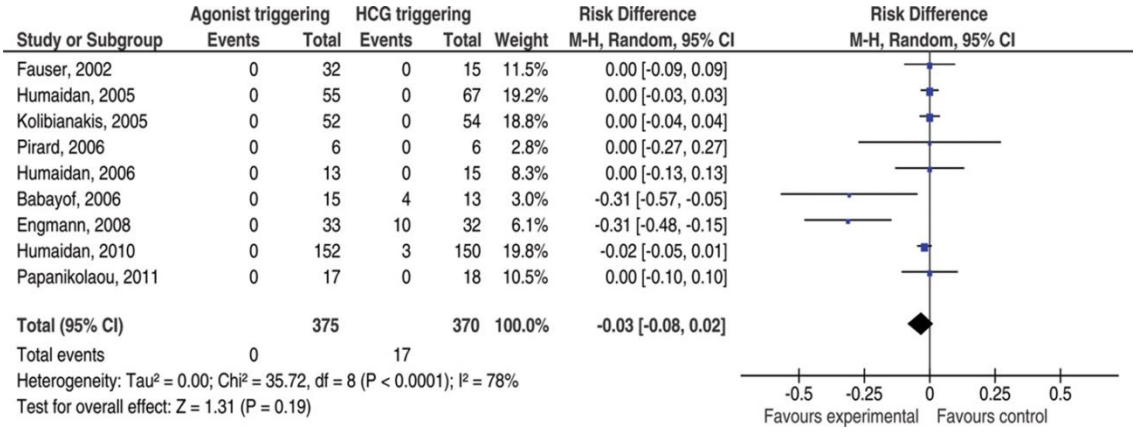


Figure 1-4: OHSS rate in fresh IVF cycles with embryo transfer (Humaidan, Quartarolo et al. 2010)

The GnRH-trigger for final oocyte maturation causes a direct pituitary down-regulation and therefore, the remaining *corpora lutea* lack LH-stimulus and are not capable to sustain progesterone production for the luteal phase (Beckers, Macklon et al. 2003). Additionally, LH secretion from the pituitary is suppressed via negative feedback by supraphysiologic levels of oestradiol and progesterone after ovarian stimulation, leading to severe luteal phase insufficiency (Fatemi, Polyzos et al. 2013). The profound luteolysis occurring after a GnRHa trigger necessitates modified luteal phase support (LPS) to secure the reproductive outcome. This can be provided by substitution of oestrogen and progesterone (Lawrenz, Garrido et al. 2017).

Two randomized prospective studies, Babayof et al. (2006) (Babayof, Margalioth et al. 2006)and Engmann et al. (2008), randomized patients at a high-risk of OHSS to either GnRHa or hCG triggering. Both studies reported 30% OHSS incidence in the hCG group versus 0% in the GnRHa triggering group. However, pregnancy rates were low in the study by Babayof et al., in contrast to the study by Engmann et al. although both

studies employed a very similar LPS (Babayof, Margalioth et al. 2006, Engmann, DiLuigi et al. 2008). Another option in OHSS high-risk patients is GnRHa triggering followed by cryopreservation of embryos and transfer in subsequent frozen thaw cycles. This approach has resulted in good reproductive outcome and may have several advantages: first, avoiding intensive LPS, second, avoiding an 'out of phase' endometrium, which is common in patients who are high responders and third, allowing embryo transfer in a natural cycle where applicable (Manzanares, Gomez-Palomares et al. 2010).

The oldest stimulation alteration is coasting. Coasting involves lowering or even stopping the FSH administration. There is insufficient evidence to determine if coasting is an effective strategy for the prevention of OHSS (D'Angelo, Brown et al. 2011).

1.8.2.2 Final maturation trigger

Reducing the dose of hCG from the standard dose of 10.000IU to 5.000IU has been successfully applied without impairing clinical outcome. Cycle cancelation and withholding the hCG is the only prevention of early OHSS. This is based on clinical experience and no studies have evaluated this strategy, as it seems extremely patient unfriendly.

Other possible trigger agents are GnRH agonist or recombinant LH. They both reduce the incidence of OHSS but outcomes might be poorer. For the GnRH agonist this might be solved with modified luteal support. The recombinant LH is not currently commercially available and has a high cost (Emperaire and Edwards 2004).

1.8.2.3 Luteal phase

After ovarian stimulation and oocyte retrieval a luteal phase defect is common. This is due to the mechanical disruption of the follicle. The high levels of oestrogen and progesterone after the ovarian stimulation cause a negative feedback resulting in low endogenous LH-levels. After withdrawal of the stimulation medication, sustained release of oestrogen and progesterone is impaired, leading to a luteal phase defect. This is the reason for luteal phase support. HCG can be used as LPS, but is a known contributor to OHSS. By using progesterone supplementation instead of hCG the risk of OHSS is halved with similar pregnancy rates (van der Linden, Buckingham et al. 2011).

1.8.2.4 Additional medication

Insulin sensitising agents, such as metformin can be used for ovulation induction in women with PCOS. A meta-analysis in 2006 of 6 trials of metformin during stimulation for ovulation induction or IVF did not see an improvement in outcome, but a significant decrease of OHSS (Tso, Costello et al. 2009).

Intravenous albumin use is only supported by limited evidence. The rationale for intravenous administration of albumin is replacing the low molecular weight proteins in order to maintain oncotic intravascular pressure and prevent the development of symptoms related to fluid extravasation associated with OHSS. Taking into account the potential side effects, of anaphylactic reaction this cannot be recommended as a first line plasma expander treatment. Hydroxyethyl starch (HES) is an alternative for albumin. There are only a small number of studies which seem to show a

reduction in the risk of moderate and severe OHSS (Konig, Bussen et al. 1998). Some units administer a HES infusion at the time of egg collection in an attempt to prevent OHSS developing.

1.8.2.5 Other preventive strategies for OHSS

In vitro maturation (IVM) is a promising new development, but no data on OHSS prevention is available. Cryo preservation of all embryo's will prevent late onset OHSS but not early onset, as hCG is still used as trigger for final maturation. The success rates of a subsequent frozen embryo replacement cycle are still lower than a fresh cycle. There is insufficient evidence to support the routine freezing of all embryos in order to reduce the rates of OHSS (D'Angelo and Amso 2007).

1.8.3 Management of OHSS

The clinical course of OHSS depends on its severity and the development of complications, as well as on the establishment of a pregnancy. It is considered a self-limiting disease and treatment is mainly based on symptom management (Delvigne and Rozenberg 2003). The critical cases remain a unique challenge for both fertility and intensive care specialists (Rizk and Aboulghar 1991). Medical management requires familiarity with the condition, and many of the problems that occur, happen as a result of lack of recognition by the intensive care and medical specialist. It is essential to realise that the syndrome is different from medical syndromes that present with similar symptoms.

1.8.3.1 Patient assessment

Careful assessment of the patient is needed to classify the severity of the disease. This should include a review of ovarian stimulation and a prediction of the underlying risk factors such as: patient age, onset of symptoms, as well as: number of follicles during stimulation, number of oocytes retrieved, peak oestradiol, and oestradiol levels at the time of trigger. The history should include an estimation of urine output and weight gain, and should seek to identify symptoms such as abdominal pain, bloating, shortness of breath and the ability to maintain oral hydration.

Physical examination should include the measurement of vital signs, body weight, and abdominal girth at the level of the umbilicus. Additional assessments for the presence of ascites, pleural effusion, and signs of venous thromboembolic disease, such as unilateral increase in calf diameter, should be conducted. Caution should be taken with pelvic examinations to minimize the risk of trauma to enlarged ovaries. Initial laboratory investigations should screen for haemoconcentration with a haematocrit and or haemoglobin measurements (Joint Society of, Gynaecologists of Canada-Canadian Fertility Andrology Society Clinical Practice Guidelines et al. 2011). Assessment of renal and liver function should also be undertaken.

1.8.3.2 Outpatient care

Mild and moderate OHSS can be monitored on an outpatient basis. Monitoring would consist of follow up by regular phone calls and patient visits to the unit. During these visits assessment by ultrasound and full blood count, liver function tests, urea and electrolytes, and coagulation

profile can be performed. Fluker et al. suggested that active outpatient intervention in the early stages of OHSS can prevent admission to hospital, and can minimize the progression and complications of OHSS. These interventions included paracentesis and albumin administration (Fluker, Copeland et al. 2000). Lincoln et al. assessed the effectiveness of outpatient treatment of 48 patients diagnosed with OHSS. The authors concluded that outpatient treatment, consisting of culdocentesis, intravenous rehydration and albumin, would minimize the need for hospitalisation. In this study no complications occurred and 91.6% of patients avoided hospitalisation (Lincoln, Opsahl et al. 2002). Clear instructions should be provided to the patient with regards to when to contact the unit or hospital in case of dyspnoea, diminishing urine output or any unusual symptoms, such as leg swelling, dizziness, numbness and neurological problems.

The question as to whether severe OHSS should or could be managed on an outpatient basis depends on compliance and reliability of the patient, and the comfort of the clinician. Shrivastav et al. reviewed their experience in the management of 18 patients who developed severe OHSS. The first group of 8 was managed conservatively in hospital, and the average duration of hospitalisation was seven days. The second 10 patients were treated on an outpatient basis, having paracentesis and contemporaneous intravenous rehydration. The time in hospital for these patients was on average 6-7 hours and no prolonged inpatient stay was required. Additionally, none of these patients required a second paracentesis. The authors suggested that day care management with abdominal paracentesis is a straightforward, safe and effective method, and is an acceptable method for treating patients with OHSS (Shrivastav, Nadkarni et al. 1994).

Gustofson et al. reported on 139 patients who developed severe OHSS during 8311 IVF cycles from 1998-2004. This study found that aggressive outpatient management of severe OHSS with early paracentesis, intra venous fluid hydration, antiemetics, and DVT prophylaxis minimized the necessity for hospital admission (Shrivastav, Nadkarni et al. 1994, Gustofson P 2005). The most recent study of Smith et al. (2009) was conducted from 1999 to 2007, and identified 183 patients with OHSS. They began performing outpatient transvaginal paracentesis to treat OHSS in 2002 and have performed 146 outpatient transvaginal paracentesis in 96 patients with no procedure-related complications. With this implementation of early, and aggressive, outpatient paracentesis, the number of patients requiring hospitalization for OHSS decreased. From 2006 to 2007, 29 patients were diagnosed with severe OHSS and 25 of these (86%) were managed as outpatients with transvaginal paracentesis with no complications (Smith, Hacker et al. 2009).

1.8.3.3 Hospitalization

In the situation when the patient requires hospitalisation, her condition should be monitored by regular vital signs, daily weight and girth measurement. Strict fluid balance is needed, particularly urine output monitoring. Minimal biochemical blood tests include electrolytes, kidney and liver functions test, coagulation and a full blood count. An ultrasound can be used to evaluate ovarian volumes and the presence of ascites. In addition, the diagnosis of a viable intra-uterine pregnancy can be made. Chest X-ray should only be performed in the suspicion of a hydrothorax or pulmonary embolus, as otherwise the woman is exposed unnecessarily to ionising radiation.

1.8.4 Treatment of OHSS

1.8.4.1 Pain relief

Abdominal discomfort can be treated with acetaminophen with or without narcotic agent. Non-steroidal anti-inflammatory drugs (NSAID) should not be used as in case of a fresh embryo transfer as they might interfere with implantation of the embryo. Also, in case of severe OHSS, NSAID can potentially compromise renal function (Navot, Bergh et al. 1992).

1.8.4.2 Paracentesis

Patients with tense ascites causing significant pain and/or respiratory compromise can benefit from paracentesis. The procedure can be carried out with ultrasound guidance with minimal risks. Paracentesis will also improve oliguria which is secondary to reduced renal perfusion due to ascites increasing intra-abdominal pressure and compromising the blood flow to the kidneys (Borenstein, Elhalah et al. 1989, Abramov, Elchalal et al. 1999).

1.8.4.3 Culdocentesis

Culdocentesis can be offered on an outpatient basis. In addition to alleviating discomfort, it should be considered for the prevention of disease progression in moderate or severe OHSS cases (Fluker, Copeland et al. 2000).

1.8.4.4 Fluids

Intravenous hydration should be initiated with a crystalloid solution to prevent haemoconcentration and provide adequate end-organ perfusion (Fluker, Copeland et al. 2000).

1.8.4.5 Prevention of thromboembolism

Hospitalized patients should be considered at risk of thrombosis secondary to haemoconcentration, especially in combination with immobilisation. Daily prophylactic doses of low-molecular-weight heparin and the use of thromboembolic deterrent stockings should be considered. However, there are currently no randomised controlled trials demonstrating that prophylactic anticoagulants prevent venous thromboembolism in cases of severe OHSS. In addition there have been several reports of thromboembolism in women with OHSS who were treated with thromboprophylaxis (Hignett, Spence et al. 1995, Todros, Carmazzi et al. 1999, Cil, Tummon et al. 2000).

1.8.4.6 Dopamine

Dopamine has been shown to be able to block VEGFR-2 phosphorylation. Cabergoline is one of the dopamine agonists and investigations have been carried out as to whether it has the potential to block the vascular permeability in OHSS (Gomez, Gonzalez-Izquierdo et al. 2006).

1.8.5 Ultrasound features of OHSS

According to the most recent classification of OHSS by the RCOG, ovarian size estimation is used as one of the factors differentiating the clinical categories of this condition. The authors of the guidance advise caution; as ovarian size may not directly correlate with the clinical severity of OHSS due to the effect of follicular aspiration in cases of assisted reproduction. Using ultrasound, mild OHSS usually presents with a maximal ovarian dimension of <8cm, moderate OHSS usually averages 8 to 12cm with

sonographic evidence of ascites, and in cases of severe OHSS the ovarian size usually exceeds 12cm (Navot, Bergh et al. 1992).

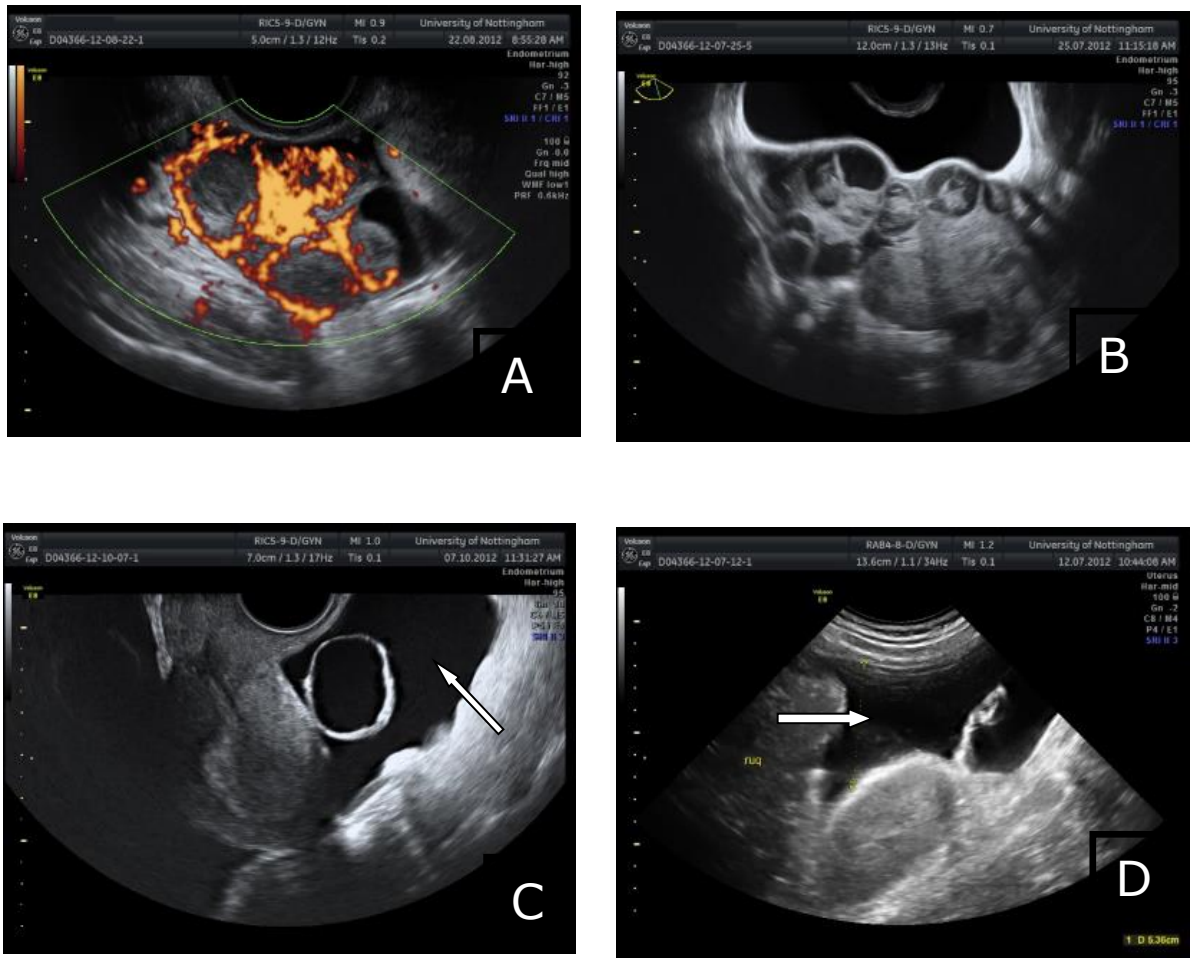


Figure 1-5: Ultrasound features of ovarian hyperstimulation syndrome

Transvaginal ultrasound images seen in Figure 1-5 demonstrate enlarged ovaries following transvaginal ultrasound guided oocyte retrieval in a patient that developed OHSS. Image A is a Power Doppler image demonstrating increased vascularity within the ovarian stroma of the post-oocyte collection ovary. Image B is a visualisation of 'kissing' ovaries. Image C shows a significant amount of free fluid in the pouch of Douglas (arrow) Image D is a transabdominal ultrasound image of the right upper quadrant. Significant amount of free fluid in Morrison's pouch (arrow).

1.9 Complication of Down Regulation: Ovarian cysts and torsion

1.9.1 Persistent follicle / *corpus luteum*

Although considered an adverse effect rather than a complication of ovarian stimulation, a cyst or a persistent follicle or *corpus luteum* can occur during pituitary down regulation with GnRH agonists (Jenkins, Anthony et al. 1993). These cysts rarely cause symptoms, but can prevent optimal ovarian stimulation and negatively influence treatment outcome. These cysts can be found during ultrasound assessment to confirm ovarian down regulation. Expectant or medical management can be attempted, however an ultrasound guided transvaginal cyst aspiration can be an option.

The incidence of functional cysts has been reported to be between 8% to 53% (Biljan, Lapensee et al. 2000). Qublan et al. (Qublan, Amarin et al. 2006) conducted a prospective study to assess the incidence and impact of ovarian cyst formation following administration of GnRH agonist on IVF cycle outcome. They concluded that the incidence of cyst formation during treatment is lower than previously reported. In their study the quality of oocytes and embryos was significantly compromised by the presence of a newly developed cyst, leading to a significant increase in cycle cancelation rates and a decrease in subsequent implantation and pregnancy rates. Neither conservative management nor cyst aspiration improved the IVF outcome.

1.9.2 Adnexal torsion

An adnexal torsion, when an ovary twists around its own pedicle, is an infrequent but serious complication of ovarian stimulation. It should be

suspected in every patient complaining of abdominal pain and nausea during, or after, ovarian stimulation. Ovarian torsion is mainly caused by ovarian cysts, either pre-existing or being a direct result of the stimulation protocols (Hibbard 1985).

The incidence of simple cyst formation and associated torsion during stimulation is not well registered. The estimation of the prevalence is difficult, as most of the cysts remain asymptomatic. Kemmann et al. (Kemmann, Ghazi et al. 1990) described four cases of adnexal torsion among 648 menotrophin-induced pregnancies. In a series of 154 women having 201 cycles complicated by ovarian hyperstimulation syndrome (OHSS) 75 got pregnant. Out of these 75 women with OHSS, twelve (16%) presented with torsion of hyperstimulated ovary. Only 3 out of 126 not pregnant patients (2.3%) had a torsion (Mashiach, Bider et al. 1990).

The diagnosis of adnexal torsion is usually difficult due to the non-specific presenting symptoms, often leading to a delayed surgical intervention and loss of the affected ovary. Combination of ovarian enlargement, abdominal pain, nausea, progressive leucocytosis, and anaemia might suggest adnexal torsion, especially given the history of previous ovarian cysts or ovarian hyperstimulation. On grey scale imaging, the ovary is cystic and enlarged with reduced heterogeneous echotexture. There may be small amount of free fluid in the pelvis. Doppler imaging may reveal reduced or absent Doppler signals with twisted ovarian pedicle (whirlpool sign). The only effective treatment of adnexal torsion can be achieved surgically. Although intraoperatively the adnexa appear dark, haemorrhagic and/or ischemic, it is most likely that circulation will be restored by simply unwinding the torted

pedicle in order to restore normal anatomy. This often is the only intervention required and allows for preservation of the ovary.

In eleven cases of ovarian torsion following IVF, intraoperative unwinding of the adnexa was performed resulting in return of spontaneous circulation in eight patients. In the mentioned case series, no postoperative complications were reported and when the ovary was spared, subsequent sonographic evaluation revealed its normal appearance. The authors concluded that torsion of hyperstimulated adnexa in patients that conceived after gonadotropin therapy is a special clinical entity requiring attention to achieve early diagnosis (Mashiach, Bider et al. 1990).

1.10 Complications of down regulation: low oestrogen level

At the opposite end of the spectrum from exaggerated ovarian response would be an ovary which would be profoundly down regulated. A way of measuring the level of down regulation is the serum oestrogen level, as this is a product of active follicles. In the literature there is no data available to evaluate the impact of this on treatment outcome.

1.11 Complications of oocyte retrieval

Transvaginal ultrasound guided oocyte retrieval (TVOR) during *in vitro* fertilization (IVF) treatment was first described in 1985 (El-Shawarby, Margara et al. 2004). By virtue of its simplicity and effectiveness, it gained widespread popularity and has now become the gold standard for IVF

therapy. Nevertheless, despite the obvious advantages, the aspiration needle may injure the adjacent pelvic organs and structures leading to serious complications. The most common complications are haemorrhage, trauma and injury to pelvic structures, and subsequent pelvic infection. Other complications described in association with ultrasound guided oocyte retrieval include adnexal torsion, rupture of endometriotic cysts, and even vertebral osteomyelitis. Puncturing the ovary under the guidance of ultrasound by introducing the needle into the field of vision manually is a difficult technique requiring a lot of experience. It becomes considerably simpler and safer with the use of fixed needle guides.

The transvaginal oocyte collection was studied in 1988 by Evers et al. (Evers, Larsen et al. 1988) describing complications and problems in the first 181 transvaginal scan-guided follicle aspirations. No major complications occurred. In 3% of patients it appeared impossible to aspirate the follicles because of a method-related problem such as localization of ovaries being too high above the vaginal fornix, or the ovaries being too mobile. The method was found to be easily mastered with short learning curve and the oocyte yield of this method was already high, even in the early learning phase of the procedure. The mean duration of the procedure decreased from more than 30 minutes to less than 20 minutes in the first 7 months after the introduction of this technique in the IVF program.

On rare occasions when the ovary is located high, as in certain cases of Rokitansky syndrome or due to other pelvic pathologies, or in case of cervical cancer, one may need to use laparoscopic guided oocyte retrieval. Laparoscopic oocyte collection has similar complications to those described

above, but the risk of injury to internal organs is higher when compared to ultrasound guidance. In addition, there is the risk of complications associated with the use of a general anaesthetic, although this is a rare event in healthy young women.

1.11.1 Incidence of oocyte retrieval complications

Bergh et al. (1992) conducted a survey of 12 large IVF clinics in Denmark, Finland, Norway and Sweden. The questionnaire sent to the Nordic centres asked mainly about the numbers of clinical complications. The number of cycles in the 12 clinics in total was 10 125 with 7 331 embryo transfers performed and 1 116 clinical pregnancies (Bergh and Lundkvist 1992). At the time of the article publication, most oocyte collections were done by transvaginal ultrasound guided aspirations, but less than 700 oocyte retrievals were still performed via the laparoscopic route. Of the commenced cycles, 0.7% were complicated by OHSS requiring hospitalization. In this group, one patient had an ovarian torsion requiring surgery. Complications during the laparoscopic oocyte collection occurred in 2 patients, both of whom had subsequent laparotomy. In one case, injured intestine necessitated repair. In the other case no intraperitoneal damage could be found. Complications were reported in the transvaginal ultrasound guided follicle aspirations in 35 cases. They all involved bleeding. Most of the bleeding was vaginal, which was successfully managed conservatively. In 2 cases, laparotomy was required to stop intraperitoneal haemorrhage from the ovarian veins. A retroperitoneal hematoma was suspected in 2 patients, but they were managed conservatively with full regression of symptoms. The overall complication rate of ultrasound guided puncture was 0.7% (35 complications out of 5122 embryo transfers). The

authors chose to calculate the complication rate per embryo transfer, because the collected data did not permit analysis per oocyte retrieval.

Deaths related to IVF were described by Braat et al (Braat, Schutte et al. 2010). Maternal mortality directly linked to IVF treatment in the Netherlands between 1984 and 2008 was investigated. Six deaths occurred in the period of 1990 to 1997. Three women suffered from severe OHSS, two died as a result of respiratory distress syndrome and multi organ failure. One woman died of cerebrovascular thrombosis 3 days after oocyte retrieval. Two other women died of sepsis 31 and 62 days after oocyte retrieval. The last fatal case was related to a dose error of local anaesthetic medication administered just before oocyte retrieval (Braat, Schutte et al. 2010).

Limited evidence suggests that TVOR risks might be related to the number of previous oocyte collections via the transvaginal route. A vaginal perforation occurred when pressure was applied to the ultrasound probe in a 41-year-old woman undergoing her sixth ovarian stimulation cycle. The authors of this case report suggested that difficult access on the side of the injury, multiple previous oocyte collections, advanced age and associated poor wound healing contributed to the complication (Cho, McGovern et al. 2004).

In the retrospective analysis of Roest et al. (Roest, Mous et al. 1996) 2495 consecutive cycles resulting in oocytes retrievals were assessed for major clinical complications. 0.7% of cycles were complicated by severe OHSS (grade 4 and 5) requiring hospitalisation. Adnexal torsion occurred in two cases. In one case, torsion and ovarian rupture were diagnosed requiring

an adnexectomy due to the inability to stop the bleeding following detorsion. One serious visceral injury was diagnosed when appendicitis developed a week after the IVF procedure. On day 8 after TVOR a laparoscopic appendectomy was performed and on histological examination multiple puncture wounds were demonstrated (Van Hoorde, Verhoeff et al. 1992). In the series of Roest et al. pelvic inflammatory disease was diagnosed in 0.24% of cases, with an increase in body temperature to more than 38°C and signs of pelvic peritonitis on physical examination, accompanied by elevated leukocyte count and erythrocyte sedimentation rate (Roest, Mous et al. 1996).

Overall, injuries to internal organs such as bowels, bladder or blood vessels during the transvaginal ultrasound guided oocyte retrieval are an extremely rare complication with a historically quoted estimated incidence of approximately 1 in 1000 cases (Bennett, Waterstone et al. 1993).

1.11.2 Bleeding

1.11.2.1 Background

Bleeding during or after oocyte collection from the ovary or from the vagina is usually minimal and is very rarely a significant problem, see Figure 1-6. The need for blood transfusion is rare and estimated to be around 1 in 500 (Mashiach, Stockheim et al. 2013).

1.11.2.2 Incidence

In a report concerning 600 ultrasound guided follicle aspirations, intra peritoneal bleeding was reported in 0.5% of cases, vaginal bleeding in 0.8%, and post-operative infection in 0.5% of the patients (Baber, Porter et al. 1988).

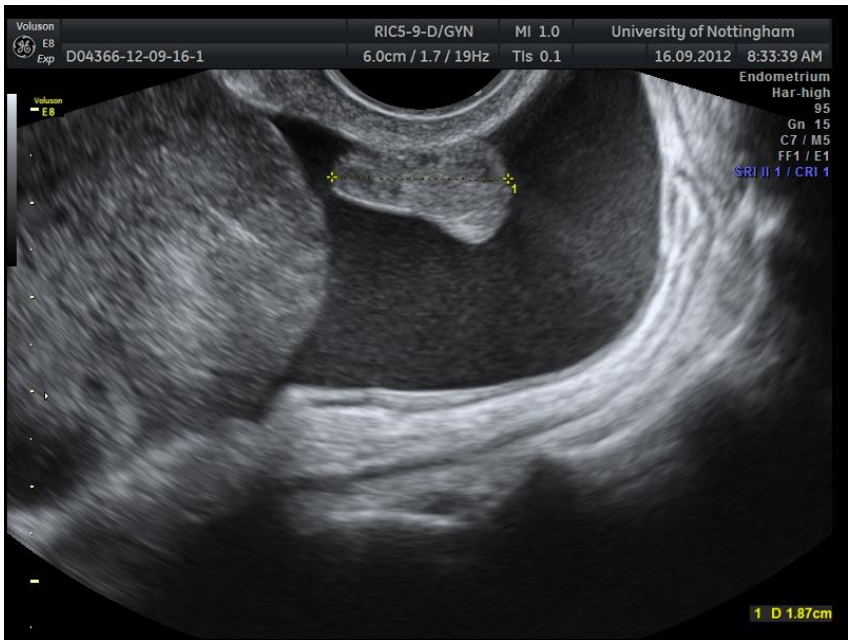


Figure 1-6 : Blood in the pouch of Douglas noted after oocyte collection

The callipers indicate the blood clot measuring almost 2cm at the site where the puncture wound through the vaginal wall has been. The ovary can be seen on the left.

1.11.3 Infection

Every invasive procedure caries the risk of introducing pathogens and subsequent development of localized or generalized infection. Infection following TVOR usually occurs within days after the intervention, although a case report by Sharpe et al. illustrated the possibility of infection as a complication of TVOR presenting months after the procedure. In the case report, the authors describe a patient who did not become acutely ill despite the formation of a pelvic abscess because of a spontaneous development of a vaginal fistula, which allowed drainage of the abscess (Sharpe, Karovitch et al. 2006).

1.11.3.1 Incidence

In the Nordic study, post-operative infections of clinical significance (pelvic abscesses) were reported in 20 patients, 0.3% per embryo transfer. Six of these patients had to be treated surgically. Two patients required drainage, two a bilateral salpingectomy and two a unilateral salpingo-oophorectomy. In the other 14 cases, antibiotic treatment gave adequate result. In the clinics that performed ultrasound guided aspiration this resulted in post-operative infection rate of 0.2% per embryo transfer (Bergh and Lundkvist 1992). In a cohort of 3656 patients, Dicker et al. (Dicker, Ashkenazi et al. 1993) reported 14 cases of acute abdomen (0.4%) following transvaginal oocyte retrievals due to pelvic infections or bleeding. Kelada et al. (Kelada and Ghani) performed a literature review stating incidences of pelvic infection between 0.4% (Kelada and Ghani) and 1.3% (Tureck, Garcia et al. 1993). They state that routine prophylactic administration of antibiotics remained controversial and of unproven benefit. It can be considered in patients with known adhesions, endometriosis and pseudocysts.

1.12 Hypothesis

1. A salpingectomy alters the ovarian blood supply and therefore negative influence the **response to ovarian stimulation**.
2. Symptoms of over **response to ovarian stimulation** can be prevented or reduced by the use of dopamine agonists.
3. The ovarian renin angiotensin system play a pivotal role in the development of OHSS, levels are expected to differ according to the **response to ovarian stimulation**
4.
5. Genotyping allows prediction of **response to ovarian stimulation** with commonly used ovulation induction agents.

Chapter 2: Factors influencing ovarian response to stimulation

2.1 Salpingectomy and ovarian reserve: a systematic review of literature and meta-analysis

2.1.1 Introduction

The surgical removal of a fallopian tube was performed for the first time by Lawson Tait in the last two decades of the 19th century (Glenn and Irvine 2011). Nowadays it is considered as one of several treatment procedures for tubal diseases before embarking on IVF treatment. Diagnosis of tubal disease with its subsequent distension prior to fertility treatment is one of the most common causes for salpingectomy, followed by ectopic tubal pregnancy (Murray, Baakdah et al. 2005). Tubal pathology is considered to be one of the most common causes of infertility, accounting for 14% of infertile couples (Evers 2002, Wigmore, Smythe et al. 2007). The severity of tubal disease can be assessed by laparoscopy. Peritubal adhesions can cause blockage with the formation of a hydrosalpinx considered to be the most severe form of tubal disease (Johnson, van Voorst et al. 2010). Tubal occlusion is irreversible and can be the results of an infectious event, *nodosa isthmica* salpingitis, or endosalpingiosis.

In vitro fertilization is the ultimate treatment option for women suffering from tubal infertility, but despite bypassing a hydrosalpinx with *ex vivo* gamete and embryo handling, the results of treatment are still

unsatisfactory. The fluid formed by the epithelial cells in the dilated fallopian tube may be toxic to embryos. It could interfere with the development and implantation of the embryo. The presence of a hydrosalpinx can decrease both conception and implantation rates, and increase the chances of subsequent pregnancy loss (Ajonuma, Chan et al. 2003). In spite of IVF being developed to overcome tubal infertility for which it has been shown to be effective (Steptoe and Edwards 1978), a paradoxical situation has arisen when it was discovered that IVF outcome in patients with tubal disease, was worse in terms of implantation rates and higher risk of early pregnancy loss compared to controls with no tubal distension (Andersen, Yue et al. 1994, Strandell, Waldenström et al. 1994, Camus, Poncelet et al. 1999). Salpingectomy via open or laparoscopic routes is considered a treatment option for women with a hydrosalpinx undergoing IVF treatment. Even in severe cases of hydrosalpinx, following salpingectomy increased chances of successful IVF have been observed (Strandell and Lindhard 2000).

Another major indication for salpingectomy is a tubal ectopic pregnancy. Options of treatment of tubal pregnancy include removal of the entire fallopian tube with an attempt to avoid damage to the blood supply of ovaries. The mesosalpinx can be electro-coagulated and cut from the fimbrial end toward the isthmus (Almog, Wagman et al. 2011). The consequences of a salpingectomy might include ipsilateral or bilateral damage to vessels and/ or nerves. This may affect the number of oocytes retrieved during IVF treatment as a result of decreased follicular development caused by decreased ovarian blood supply (Lass, Ellenbogen

et al. 1998, Chan, Ng et al. 2003). One of the apprehensions related to performing a salpingectomy is the potential impairment of the ovarian function after the procedure. The vital branch for the blood supply of the Fallopian tube is the median tubal artery. It derives from the same point of the median ovarian artery. The blood supply to the ovary may be disrupted if the procedure is not performed in a proper way (Chan, Ng et al. 2003). As a consequence, it may affect the ovarian reserve and the response to gonadotropins.

Two surgical procedures are available to manage an ectopic pregnancy. With a salpingostomy the tube is opened but not removed. However there is the risk of incomplete pregnancy tissue elimination, requiring extra treatment and the increased possibility of a future ectopic. The other surgical option is salpingectomy, or total removal of the affected tube. This procedure has none of the previously mentioned risks, but all potential reproductive functions of that fallopian tube are lost. In the situation of a ruptured ectopic, the surgical intervention is mandatory to save a patient's life. A ruptured ectopic is most common in women with no previous history of ectopic (Roussos, Panidis et al. 2000). This means that patients with an ectopic pregnancy have a high chance of having a salpingectomy either by elective or emergency surgical treatment, or after failed conservative or medical management (Dubuisson, Morice et al. 1996, Hajenius, Mol et al. 2003).

There are several other alternative procedures, aside from salpingectomy, used for the purpose of treating hydrosalpinx. Earlier mentioned salpingostomy being one of them, aspiration of the hydrosalpinx

(Aboulghar, Mansour et al. 1990, Russell, Rodriguez et al. 1991, Sowter, Akande et al. 1997), tubal ligation (Murray, Sagoskin et al. 1998), and tubal occlusion (Johnson, van Voorst et al. 2010) being the other methods, which may avoid vascular damage and hence have no negative impact on ovarian reserve.

A recent shift in the way tubal disease is managed in favour of surgical interventions prior to IVF treatment regardless of the type of indication can be observed. Notwithstanding the guidelines recommending salpingectomy, there is a wide variation in the methods used in the surgical management of hydrosalpinx. According to a number of surveys (Hammadieh, Afnan et al. 2004, Ducarme, Uzan et al. 2006) salpingectomy remains the most frequently used procedure. The efficacy of other surgical treatments has not been proven by sufficiently powered prospective randomised controlled trials (Kodaman, Arici et al. 2004, Johnson, van Voorst et al. 2010).

Ovarian reserve is defined as the number of follicles with the potential of producing an oocyte that is present in the ovaries at any given time (see section 1.3).

Different types of ovarian reserve tests exist. Initially, ovarian reserve was assessed dynamically by a clomiphene citrate challenge test (Navot, Rosenwaks et al. 1987). This test consists of 3 parts, part one an FSH level on day 3 of a menstrual cycle, followed by part two; 100mg clomiphene citrate day 5-9, and the last part another FSH levels on day ten. In normal functioning ovaries, the FSH level should be <10IU/L, in ovaries with reduced ovarian reserve the FSH level will remain higher. Other dynamic

tests involve administration of exogenous follicle stimulating hormone (FSH), or gonadotropin releasing agonist (GnRH) and subsequent measurement of serum hormone levels; FSH, inhibin-B and E2 (Gülekli, Bulbul et al. 1999, Meng, Henson et al. 2006, Sills, Alper et al. 2009). Static tests can be divided into two groups: biomarkers and sonographic markers. The former group includes levels of follicle stimulating hormone (FSH), inhibin-B, serum oestradiol (E2), serum progesterone (P4), and Anti-Müllerian hormone (AMH). The latter group of tests consists of sonographic markers such as antral follicle counts (AFC), ovarian vascularity and ovarian volume (Gougeon, Ecochard et al. 1994, Syrop, Willhoite et al. 1995).

Appraisal of ovarian reserve prior to ART allows for identification of women with potential low, optimal, or high response to controlled ovarian hyperstimulation (COH). All of the above mentioned tests can be used to evaluate ovarian reserve and quantify the ovarian follicular cohort. The ultrasound assessment of the AFC and ovarian volume give direct measures of defined gonadotropin-dependent follicles, while markers like inhibin-B, E2, FSH, and AMH reflect the ovarian reserve indirectly (te Velde and Pearson 2002). Both AMH and AFC are considered to be the best in predicting treatment outcome (van Rooij, Broekmans et al. 2002, Hendriks, Mol et al. 2005, Muttukrishna, McGarrigle et al. 2005). In women with early stages of diminished ovarian reserve plasma evaluation of AMH is more sensitive than the traditional FSH level or oestradiol (Tremellen, Kolo et al. 2005). AMH can detect the ovarian reserve with less variability between cycles and can compete with AFC in reliability (van Disseldorp, Lambalk et al. 2010).

Ovarian reserve is considered to be ideal, if stimulation with exogenous gonadotropins leads to the development of at least 8–10 follicles, or an equal number of oocytes to be retrieved at the time of follicular aspiration. If a woman produces a sufficient amount of oocytes, her chances of forming a pregnancy and achieving a life birth are optimal (Broekmans, Kwee et al. 2006). There is a striking decrease in pregnancy rates associated with diminished ovarian reserve with poor pregnancy rates independent of the woman's age (Scott, Opsahl et al. 1995). Ovarian reserve tests are considered the most useful tools allowing for adjustment of the IVF treatment protocol in order to optimize the outcome (Marca 2012).

The conclusions of studies assessing the effects of salpingectomy an ovarian reserve have been inconsistent (Almog, Wagman et al. 2011). In order to clarify the published evidence about the relationship of ovarian reserve and salpingectomy in an infertile population, a systematic review and meta-analysis of all the published literature was conducted.

2.1.2 Materials and methods

2.1.2.1 Protocol registration

The protocol of this review has been registered on the International Prospective Register Of Systematic Reviews website (PROSPERO, www.crd.york.ac.uk/prospero). The registration number is CRD42013006522

2.1.2.2 Eligibility criteria

Published journal articles and conference abstracts reporting on salpingectomy and ovarian reserve were sought. Study selection was restricted to human subjects only. No restriction was made on the publication language.

2.1.2.3 Information source and search strategy

The literature search was done through a search engine (OvidSP). The following databases were searched: MEDLINE, BIOSIS PREREVIEWS, EMBASE, Web of Knowledge (ISI), Web of Science (ISI), SCOPUS (Elsevier), Science Direct (Elsevier), and ZETOC from the databases inception to December 2014. The MeSH terms used included: salpingectomy, ovarian reserve, AMH, anti Müllerian hormone, AFC, antral follicle count, ovarian volume, ovarian stimulation, and ovarian response.

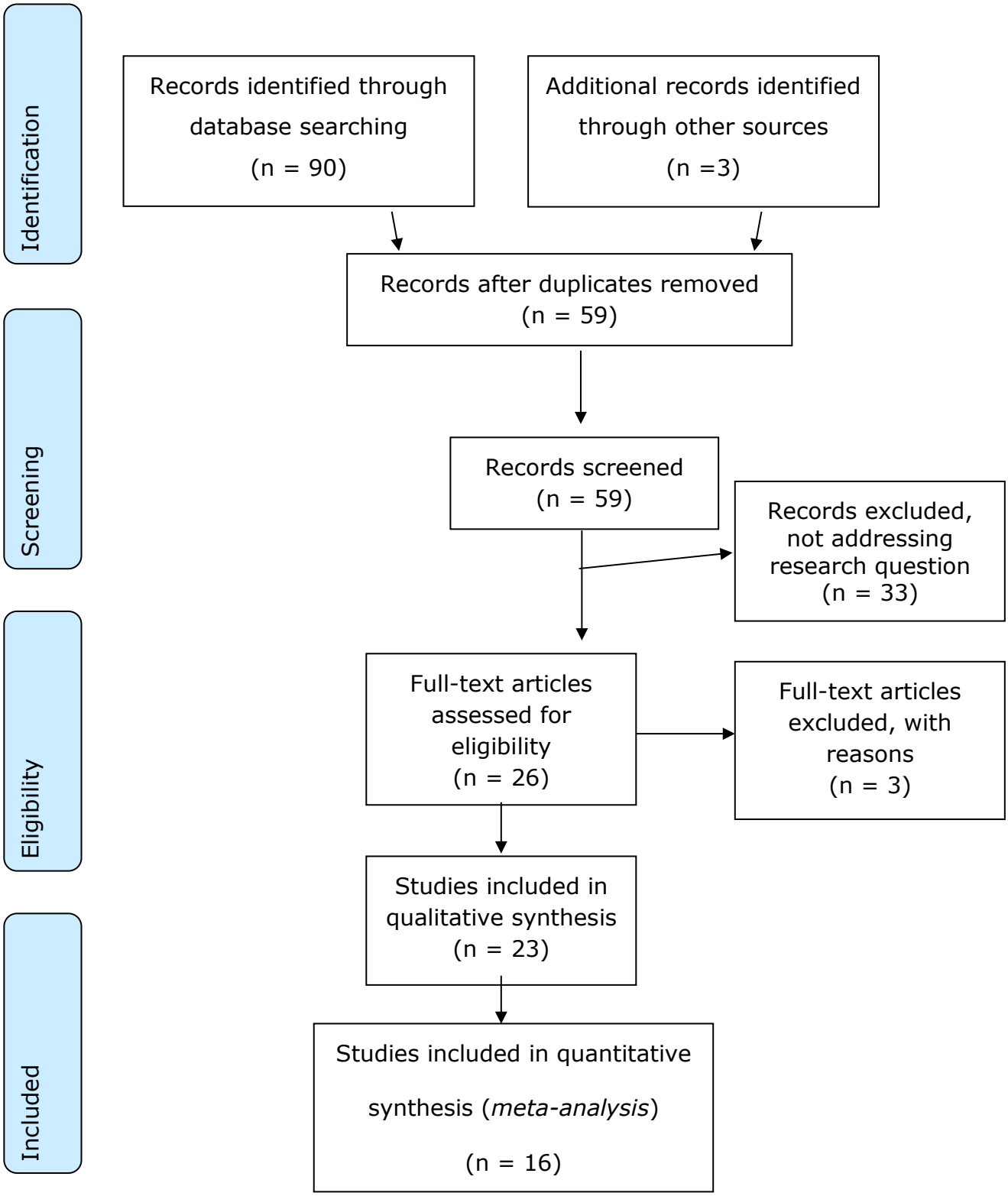


Figure 2-1 Flow chart study selection salpingectomy review

All searches and manuscript reviews were conducted independently by two reviewers (MB and ZF). Titles and abstracts were screened according to predefined criteria. Articles were obtained in full if a salpingectomy was performed and a comparison was made with a control group, and when IVF treatment outcomes were stated. The outcomes sought included: ovarian reserve and ovarian response. Any disagreement about inclusion or exclusion was resolved by consensus or by a third reviewer (LP).

2.1.2.4 Data extraction and collection process

Collected data included type of study, the type of salpingectomy, sample size, the cause for salpingectomy, the ovarian reserve markers used, the COH outcome including the number of follicles and oocytes collected, and the fertilisation rate.

2.1.2.5 Analysis

When appropriate, meta-analysis was performed using Review Manager (RevMan) version 5.2 (Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2011). Heterogenicity of data was assessed using I^2 statistics. In the presence of significant heterogeneity, random effects have been used to assess the relationship between the intervention and outcome.

2.1.3 Results

The literature search was performed on 26-11-2013 and resulted in 95 articles. Following removal of duplicates and limiting the search to human subjects, the number of citations was 56. Three additional articles were found through reference search, and the total of 59 records were screened. Of these, twenty-three articles were obtained in full and included in the

qualitative analysis. All articles commenting on salpingectomy and an ovarian reserve marker were included (see Figure 2-13).

2.1.4 Characteristics of excluded studies

One open communication regarding ovarian function after antegrade or retrograde salpingectomy was excluded. This retrospective analysis looked at FSH, E2 and AMH levels and no data was presented which would allow differentiating between the outcomes in the mentioned two study groups. No control population was presented (Mocciaro, Venturella et al. 2012). The study by Meng et al. (Meng and Zhu 2006) was not included in analysis as the original article was in Chinese. The English abstract reported a significant increase in duration of ovarian stimulation for unilateral and bilateral salpingectomy compared to the control group with tubal factor infertility. In the bilateral salpingectomy group and increase in FSH consumption and a lower oocyte yield was reported (Meng and Zhu 2006). It was not possible to obtain the full manuscript of another Chinese study on 134 IVF cycles including 26 bilateral salpingectomies and 34 unilateral salpingectomies. The authors found no difference in ovarian reserve (AMH) and ovarian responsiveness following salpingectomy (Ni, Sadiq et al. 2013). Shulman et al. (2002) compared a group of any form of tubal surgery to a control group (Shulman, Marom et al. 2002). As our study focuses on the effect of only a salpingectomy, data could not be included in analysis. The remaining studies were included in the meta-analysis.

Study design, sample sizes, inclusion criteria, control groups, and patient characteristics can be found in Table 2-1, Table 2-2, Table 2-3, and Table 2-4 respectively.

Study	Publication year	Study Design	Received procedure	Indication
Almog et al.	2011	Retrospective	US + BS	EP + HS
Bao et al.	2010	Prospective	Not stated	EP + HS
Chan et al.	2003	Retrospective	US	EP
Dar et al.	2000	Retrospective	US	EP
Gelbaya et al.	2006	Retrospective	US + BS	HS
Grynnerup et al.	2013	Prospective	US + BS	HS
Kamal et al.	2013	Prospective	US + BS	HS
Lass et al.	1998	Prospective	US	EP
Lin et al.	2013	Retrospective	Not stated	EP + HS
Meng et al.	2006	Not stated	US + BS	Not stated
Nakagawa et al.	2008	Prospective	BS	HS
Ni et al.	2013	Prospective	US + BS	EP + HS
Orvieto et al.	2011	Retrospective	US + BS	HS

Study	Publication year	Study Design	Received procedure	Indication
Shulman et al.	2002	Retrospective	US	EP + HS
Singer et al.	2011	Retrospective	Not stated	EP
Strandell et al.	1999	Prospective randomised	US + BS	HS
Surrey et al.	2001	Retrospective	Not stated	HS
Tal et al.	2002	Prospective	US	EP
Verhulst et al.	1994	Retrospective case-control	BS	EP + HS
Wiser et al.	2013	Retrospective	Not stated	EP
Wong et al.	2013	Retrospective	US	EP + HS
Xi et al.	2012	Retrospective	US + BS	EP
Yazbeck et al.	2010	Retrospective	US + BS	Not stated

Table 2-1: Salpingectomy review selected studies: study design, intervention and indication

US=unilateral salpingectomy, BS=bilateral salpingectomy, EP=ectopic pregnancy, HS=hydrosalpinx

Study	Number in salpingectomy group	Number control group	Age (years)	BMI (kg/m^2)	Control group definition
Almog et al. (2011)	36	36	34.2 ± 4.5	Not stated	a. Before and after surgery b. Non-operated ovary when applicable
Bao et al. (2010)	59	124	29.8±6.0	Not stated	IVF patients without HS
Chan et al. (2003)	32	32	34	Not stated	Non-operated ovary
Dar et al. (2000)	26	26	31.7±3.6	Not stated	a. Before and after surgery b. Non-operated ovary when applicable
Gelbaya et al. (2006)	40	103	32.8±3.57	Not stated	Tubal ligation or tubal factor, without HS

Study	Number in salpingectomy group	Number control group	Age (years)	BMI (kg/m2)	Control group definition
Grynnerup et al. (2013)	16	42	33	Not stated	Tubal factor
Kamal et al. (2013)	20	37	34.4±3.6	Not stated	a. Tubal factor without HS b. Before and after surgery
Lass et al. (1998)	29	73	33.1±4.9	Not stated	a. Unexpl/male factor infertility b. Non-operated ovary
Lin et al. (2013)	103	185	33.2±4.2	22.1±4.3	a. Tubal factor b. Non-operated ovary when applicable
Meng et al. (2006)	188	59	na	Not stated	Tubal factor

Study	Number in salpingectomy group	Number control group	Age (years)	BMI (kg/m2)	Control group definition
Nakagawa et al. (2008)	6	6	31.7±6.3	Not stated	Before and after surgery
Ni et al. (2013)	60	74	na	Not stated	Tubal factor
Orvieto et al. (2011)	15	15	32±4.4	24±5.5	a. Before and after surgery b. Non-operated ovary when applicable
Shulman et al. (2002)	33	12	na	Not stated	Salpingotomy
Singer et al. (2011)	6	29	35.67	23.09	a. MTX b. Before and after surgery
Strandell et al. (1999)	108	79	31.8±3.6	Not stated	Patients with HS without surgery

Study	Number in salpingectomy group	Number control group	Age (years)	BMI (kg/m^2)	Control group definition
Surrey et al. (2001)	32	35	35.1±0.7	Not stated	Tubal factor without HS
Tal et al. (2002)	26	52	na	Not stated	IVF patients
Verhulst et al. (1994)	26	52	30.5	Not stated	IVF patients
Wiser et al. (2013)	22	22	32.9±4.8	24.0±5.8	Before and after surgery
Wong et al. (2013)	80	80	33.6±3.5	Not stated	Non-operated ovary
Xi et al. (2012)	76	80	31.5±4.2	Not stated	a. IVF patients b. Before and after surgery c. Bilateral versus unilateral

Study	Number in salpingectomy group	Number control group	Age (years)	BMI (kg/m^2)	Control group definition
Yazbeck et al. (2010)	126 cycles	145 cycles	Na	Not stated	Tubal factor

Table 2-2: Study data presented as mean±SD and control groups

na=not applicable

Study	basal FSH level (IU/L)	AFC	AMH	Ovarian volume (cm3)
Almog et al. (2011)	Not stated	Not stated	Not stated	Not stated
Bao et al. (2010)	Non-significant	*Sign reduced*	Not stated	Non-significant
Chan et al. (2003)	Not stated	Non-significant	Not stated	Non-significant
Dar et al. (2000)	Not stated	Not stated	Not stated	Not stated
Gelbaya et al. (2006)	*Significant increased after surgery*	Not stated	Not stated	Not stated
Grynnerup et al. (2013)	Not stated	Not stated	*Significant reduced*	Not stated

Study	Basal FSH level (IU/L)	AFC	AMH	Ovarian volume (cm3)
Kamal et al. (2013)	*Significant increased after surgery*	Not stated	Not stated	Not stated
Lass et al. (1998)	Not stated	Non-significant	Not stated	Non-significant
Lin et al. (2013)	Not stated	Not stated	Not stated	Not stated
Meng et al. (2006)	Not stated	Not stated	Not stated	Not stated
Nakagawa et al.(2008)	Non-significant	Not stated	Not stated	Not stated
Ni et al. (2013)	Not stated	Not stated	Not stated	Not stated
Orvieto et al. (2011)	Not stated	Not stated	Not stated	Not stated

Study	Basal FSH level (IU/L)	AFC	AMH	Ovarian volume (cm3)
Shulman et al. (2002)	Not stated	Not stated	Not stated	Not stated
Singer et al. (2011)	Not stated	Not stated	Non-significant	Not stated
Strandell et al. (1999)	Not stated	Not stated	Not stated	Not stated
Surrey et al. (2001)	Non-significant	Not stated	Not stated	Not stated
Tal et al. (2002)	Not stated	Not stated	Not stated	Not stated
Verhulst et al. (1994)	Not stated	Not stated	Not stated	Not stated
Wiser et al. (2013)	Non-significant	Non-significant	Not stated	Not stated

Study	Basal FSH level (IU/L)	AFC	AMH	Ovarian volume (cm3)
Wong et al. (2013)	Not stated	Not stated	Not stated	Not stated
Xi et al. (2012)	Non-significant	Not stated	Not stated	Not stated
Yazbeck et al. (2010)	Not stated	Not stated	Not stated	Not stated

Table 2-3: Ovarian reserve parameters, study group compared versus (study specific) control group

Study	Total dose FSH (IU)	Duration stimulation (days)	Peak E2	Number of follicles	Number of oocytes	Fertilisation rate
Almog et al. (2011)	Non-significant	Non-significant	Non-significant	Non-significant	Non-significant	Not stated
Bao et al. (2010)	*Significant increased*	Not stated	*Significant reduced*	Not stated	*Significant reduced*	Not stated
Chan et al. (2003)	Non-significant	Not stated	Not stated	Not stated	Not stated	Not stated
Dar et al. (2000)	Non-significant	Non-significant	Non-significant	Not stated	Non-significant	Non-significant
Gelbaya et al. (2006)	Non-significant	Non-significant	*Significant reduced*	Non-significant	*Significant reduced*	Non-significant
Grynnerup et al. (2013)	Not stated	Not stated	Not stated	Not stated	*Sig reduced*	Not stated
Kamal et al. (2013)	Non-significant	Non-significant	Non-significant	Not stated	Non-significant	Non-significant

Study	Total dose FSH (IU)	Duration stimulation (days)	Peak E2	Number of follicles	Number of oocytes	Fertilisation rate
Lass et al. (1998)	Non-significant	Non-significant	Non-significant	Non-significant	Non-significant	Not stated
Lin et al. (2013)	Non-significant	Non-significant	Non-significant	Non-significant	Non-significant	Non-significant
Meng et al. (2006)	*Significant increase for BS, non-sign for US**	*Significant increased**	Not stated	*Significant decreased for BS, non-sign for US**	*Significant decreased for BS, non-significant fur US**	Not stated
Nakagawa et al.(2008)	Not stated	Not stated	Not stated	Not stated	Not stated	Not stated
Ni et al. (2013)	Not stated	Not stated	Not stated	Not stated	Not stated	Not stated
Orvieto et al. (2011)	Non-significant	Non-significant	Non-significant	Sig reduced	Non-significant	Not stated

Study	Total dose FSH (IU)	Duration stimulation (days)	Peak E2	Number of follicles	Number of oocytes	Fertilisation rate
Shulman et al. (2002)	Not stated	Not stated	Not stated	Not stated	Na	Na
Singer et al. (2011)	Not stated	Not stated	Not stated	Not stated	Non-significant	Na
Strandell et al. (1999)	Non-significant	Non-significant	Not stated	Not stated	Non-significant	Non-significant
Surrey et al. (2001)	Non-significant	Not stated	Non-significant	Not stated	Non-significant	Non-significant
Tal et al. (2002)	Non-significant	Non-significant	Non-significant	Non-significant	Non-significant	Not stated
Verhulst et al. (1994)	Non-significant	Not stated	Non-significant	Not stated	Non-significant	Not stated
Wiser et al. (2013)	Non-significant	Non-significant	Non-significant	Not stated	Non-significant	Non-significant
Wong et al. (2013)	Not stated	Not stated	Not stated	*Sign reduced*	*Sign reduced*	Not stated

Study	Total dose FSH (IU)	Duration stimulation	Peak E2	Number of follicles	Number of oocytes	Fertilisation rate
Xi et al. (2012)	*Sign increased*	Not stated	Not stated	Not stated	Not stated	Not stated
Yazbeck et al. (2010)	Not stated	Not stated	*Significant reduced**	Not stated	*Significant reduced**	*Significant reduced**

Table 2-4: Outcome parameters of ovarian stimulation in study group versus (study specific defined) control group

* No suitable data available for extraction for meta-analysis

2.1.4.1 Meta-analysis: Influence of salpingectomy on ovarian reserve markers

2.1.4.1.1 Effect of salpingectomy on basal serum FSH

Seven studies reported this outcome (Surrey and Schoolcraft 2001, Gelbaya, Nardo et al. 2006, Nakagawa, Ohgi et al. 2008, Bao, Hao et al. 2010, Xi, Gong et al. 2012, EM 2013, Kamal 2013, Wiser, Gilbert et al. 2013), study details can be found in Table 2-1. Based on the provided data, a meta-analysis assessing the effect of salpingectomy on basal serum FSH was performed. The combined data analysis shows no difference in baseline FSH levels in the salpingectomy versus the control population (P=0.41; see Figure 2-2 Serum FSH levels in early follicular phase (Figure 2-2). The heterogeneity of the data was significant (I^2=87%). Surrey at al. could not be included in the analysis as data was provided with SEM (standard error of the mean) (Surrey and Schoolcraft 2001).

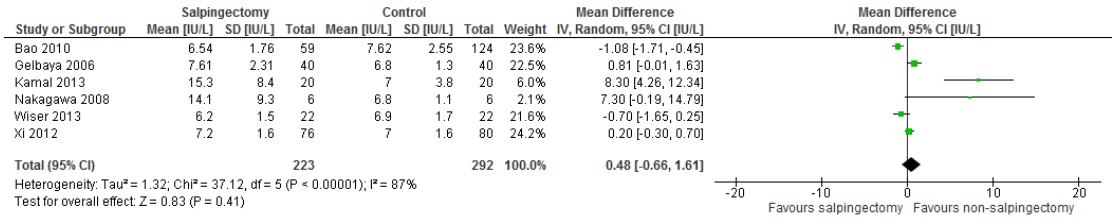


Figure 2-2 Serum FSH levels in early follicular phase

2.1.4.1.2 Effect of salpingectomy on antral follicle count

Two studies reported this outcome (Bao, Hao et al. 2010, Wiser, Gilbert et al. 2013); study details can be found in Table 2-1. The provided data could be used to perform a meta-analysis assessing the effect of salpingectomy on AFC. The combined data analysis shows no significant difference in AFC levels before and after salpingectomy (P=0.14; see Figure 2-3).

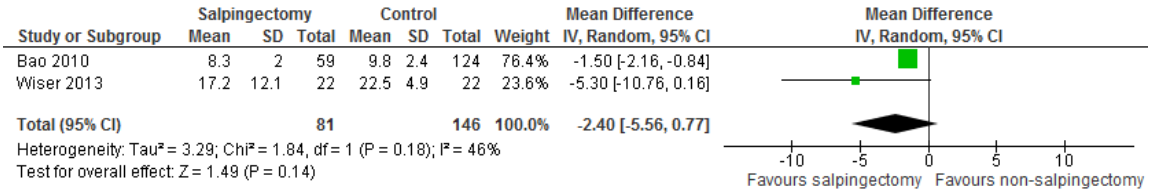


Figure 2-3 Antral follicle count

2.1.4.1.3 Effect of salpingectomy on ovarian volume

Two studies reported this outcome (Lass, Ellenbogen et al. 1998, Bao, Hao et al. 2010), study details can be found in Table 2-1. The provided data was used to perform a meta-analysis assessing the effect of salpingectomy on ovarian volume. The combined data analysis shows no significant difference in ovarian volume between the salpingectomy and the control group (P=0.87; see Figure 2-4). Chan et al. (Chan, Ng et al. 2003) has also shows a non-significant (P=0.349) difference in ovarian volume (cm^3) between operated and non-operated side after laparoscopic salpingectomy for ectopic pregnancy of 6.33 (4.07–6.90) versus 5.93 (4.57–8.24), respectively. As the data was not normally distributed it could not be included in the meta-analysis.

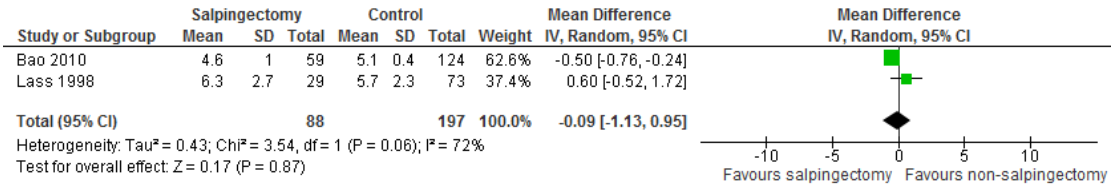


Figure 2-4 Ovarian volume

2.1.4.1.4 Effect of salpingectomy on AMH

A meta-analysis for AMH was not possible as the data was not normally distributed in one study (Grynnerup, Lindhard et al. 2013) and the other study was an open communication with 6 patients having AMH levels measured before and after surgery, no significant difference was reported (Singer, Kofinas et al. 2011).

2.1.4.2 Meta-analysis: Influence of salpingectomy on response to ovarian stimulation:

2.1.4.2.1 Effect of salpingectomy on total gonadotropins consumption

Fourteen studies reported this outcome (Verhulst, Vandersteen et al. 1994, Lass, Ellenbogen et al. 1998, Dar, Sachs et al. 2000, Strandell and Lindhard 2000, Surrey and Schoolcraft 2001, Tal, Paltieli et al. 2002, Gelbaya, Nardo et al. 2006, Bao, Hao et al. 2010, Orvieto, Saar-Ryss et al. 2011, Xi, Gong et al. 2012, Lin, Ou et al. 2013, Wiser, Gilbert et al. 2013), study details can be found in Table 2-1. The provided data of 12 studies could be used to perform a meta-analysis assessing the effect of salpingectomy on dose of FSH consumed for ovarian stimulation. The data analysis shows no significant difference (P=0.20; see Figure 2-5). The study by Almog et al. (Almog, Wagman et al. 2011) could not be included in the meta-analysis as only the daily dose was given and not the total dose of gonadotropins. The study by Surrey et al was excluded as date was provided in SEM (Surrey and Schoolcraft 2001).

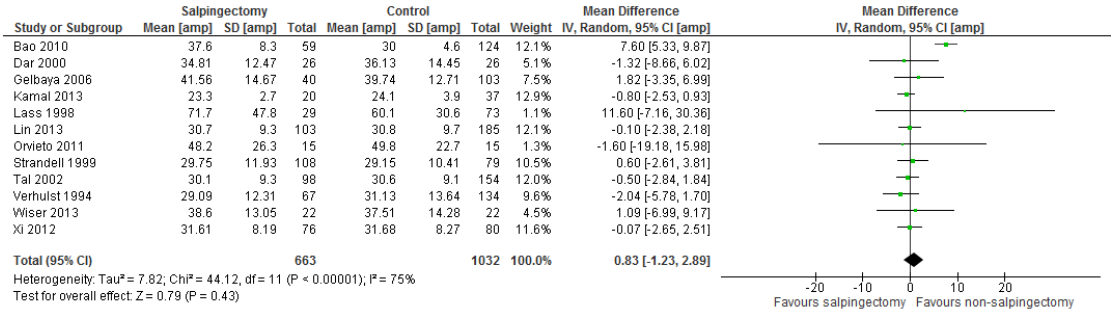


Figure 2-5 The total dose of gonadotropins in ampoules used for ovarian stimulation

2.1.4.2.2 Effect of salpingectomy on total days of ovarian stimulation

Elven studies reported this outcome (Lass, Ellenbogen et al. 1998, Dar, Sachs et al. 2000, Strandell and Lindhard 2000, Tal, Paltieli et al. 2002, Gelbaya, Nardo et al. 2006, Almog, Wagman et al. 2011, Orvieto, Saar-Ryss et al. 2011, Xi, Gong et al. 2012, Lin, Ou et al. 2013, Wiser, Gilbert et al. 2013), study details can be found in Table 2-1. The provided data could be used to perform a meta-analysis assessing the effect of salpingectomy on the duration of ovarian stimulation in days. The combined data analysis shows no significant difference increase (P=0.51; see Figure 2-6).

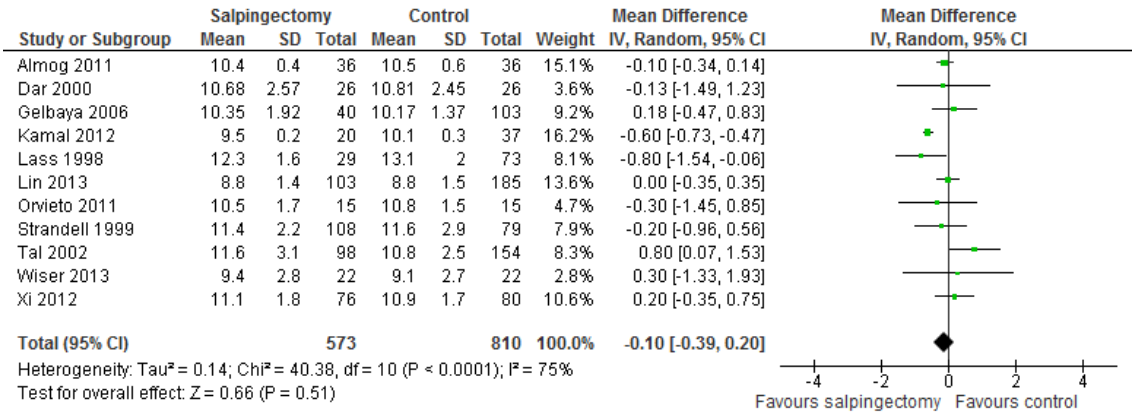


Figure 2-6 Total days of ovarian stimulation

2.1.4.2.3 Effect of salpingectomy on serum E2 on the day of final maturation trigger

Twelve studies reported this outcome (Lass, Ellenbogen et al. 1998, Dar, Sachs et al. 2000, Tal, Paltieli et al. 2002, Gelbaya, Nardo et al. 2006, Bao, Hao et al. 2010, Almog, Wagman et al. 2011, Orvieto, Saar-Ryss et al. 2011, Lin, Ou et al. 2013, Wiser, Gilbert et al. 2013), study details can be found in Table 2-1. The provided data could be used to perform a meta-analysis assessing the effect of salpingectomy serum E2 level on the day of trigger for final maturation, with exception of Surrey et al, Almog at al was contacted to confirm the format of their data (Surrey and Schoolcraft 2001, Almog, Wagman et al. 2011). The results do not demonstrate a significant decrease in peak E2 levels on the day of final maturation trigger in the group that underwent previous salpingectomy compared to the control group (P=0.061; see Figure 2-7). The data heterogeneity however was substantial (I^2=77%).

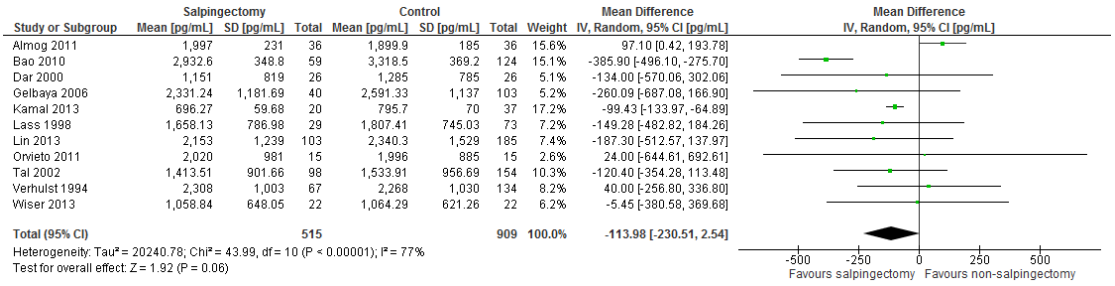


Figure 2-7 Peak E2 on the day of final maturation

2.1.4.2.4 Effect of salpingectomy on the number of recruited follicles during stimulation

Eight studies reported this outcome (Lass, Ellenbogen et al. 1998, Tal, Paltieli et al. 2002, Gelbaya, Nardo et al. 2006, Almog, Wagman et al. 2011, Orvieto, Saar-Ryss et al. 2011, Xi, Gong et al. 2012, Lin, Ou et al. 2013, Wong and Haines 2013), study details can be found in Table 2-1. The

provided data could be used to perform a meta-analysis assessing the effect of salpingectomy on the number of recruited follicles. This analysis included the dominant follicles as per definition used by the authors. The combined data analysis shows significant decrease in the number of recruited follicles in the salpingectomy group compared to the control group (P=0.02; see Figure 2-8).

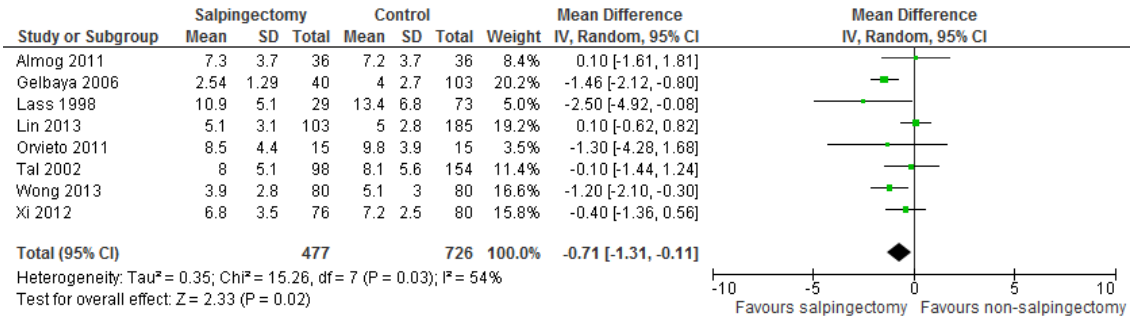


Figure 2-8 Total number of recruited follicles

A subgroup meta-analysis comparing the operated side versus the non-operated side and the number of follicles developing on each ovary was possible based on 3 studies (Almog, Wagman et al. 2011, Lin, Ou et al. 2013, Wong and Haines 2013). The combined data analysis shows no significant difference (P=0.18), see Figure 2-9. Orvieto et al. (Orvieto, Saar-Ryss et al. 2011) did comment on the number of follicles on the operated side but not on the number of follicles on the non-operated side, so data could not be included in the analysis.

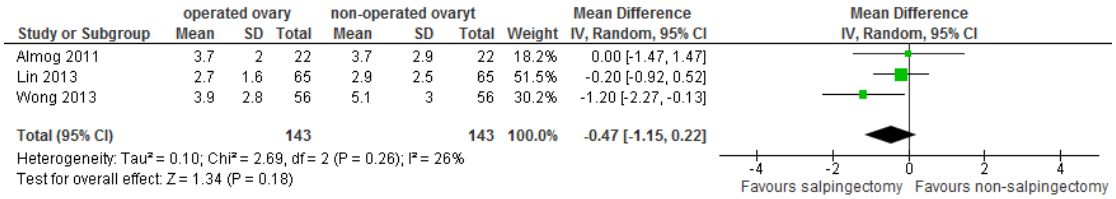


Figure 2-9: Number of recruited follicles per ovary in the same patient

2.1.4.2.5 Effect of salpingectomy on the number of collected oocytes

Sixteen studies reported this outcome (Verhulst, Vandersteen et al. 1994, Lass, Ellenbogen et al. 1998, Dar, Sachs et al. 2000, Tal, Paltieli et al. 2002, Gelbaya, Nardo et al. 2006, Bao, Hao et al. 2010, Almog, Wagman et al. 2011, Orvieto, Saar-Ryss et al. 2011, Xi, Gong et al. 2012, Kamal 2013, Lin, Ou et al. 2013, Wiser, Gilbert et al. 2013, Wong and Haines 2013), study details can be found in Table 2-1. The provided data could be used to perform a meta-analysis assessing the effect of salpingectomy serum number of oocytes collected during oocyte retrieval. The combined data analysis shows no significant difference (P=0.38; see Figure 2-10).

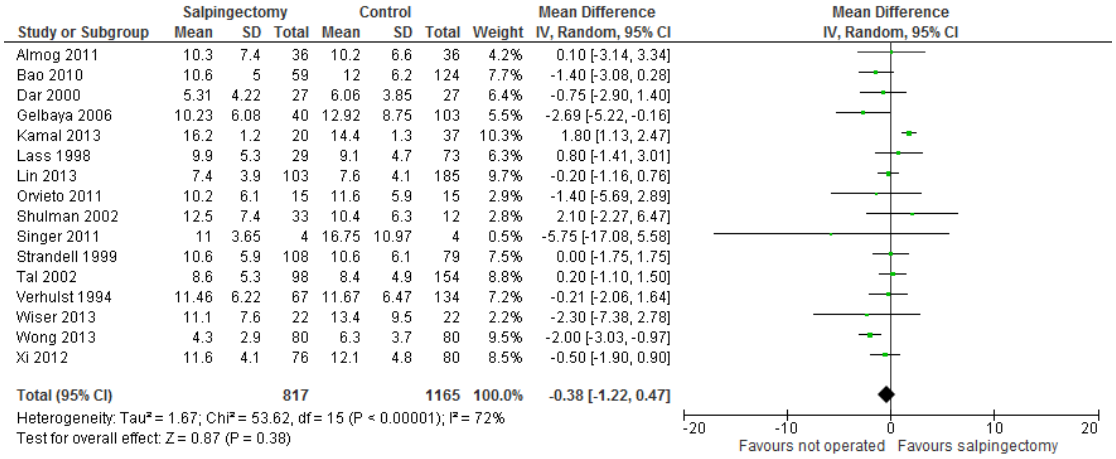


Figure 2-10 Number of oocytes collected after ovarian stimulation

A subgroup analysis where number of oocytes collected from the same patient before and after the surgery could be performed based on six studies (Dar, Sachs et al. 2000, Almog, Wagman et al. 2011, Orvieto, Saar-Ryss et al. 2011, Singer, Kofinas et al. 2011, Xi, Gong et al. 2012, Wiser, Gilbert et al. 2013).There was no difference in the number of oocytes collected before and after the intervention (P=0.76; see Figure 2-11).

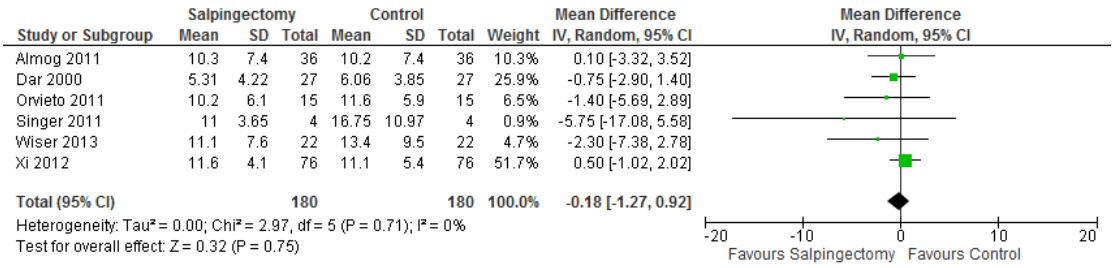


Figure 2-11 Number of oocytes retrieved in the same patient before and after salpingectomy

A second subgroup analysis comparing the operated side versus the non-operated side and the number of oocytes retrieved from each ovary was possible based on 4 studies (Lass, Ellenbogen et al. 1998, Dar, Sachs et al. 2000, Lin, Ou et al. 2013, Wong and Haines 2013). The combined data analysis shows no significant difference (P=0.12); see Figure 2-12).

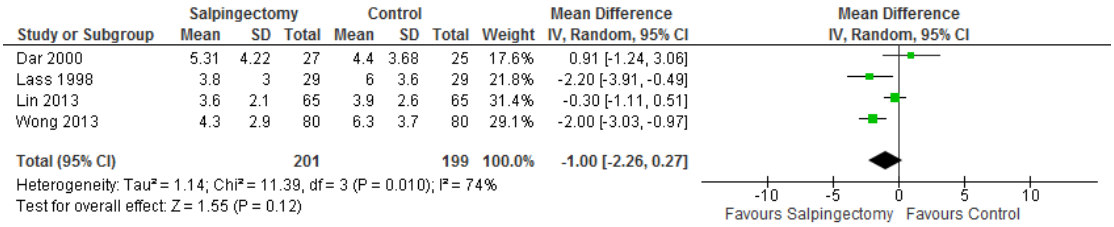


Figure 2-12: Number of oocytes retrieved per ovary in the same patient comparing operated versus non-operated side

2.1.4.2.6 Effect of salpingectomy on the number of fertilised oocytes

Eight studies reported this outcome, study details can be found in Table 2-1. The data of four studies (Strandell and Lindhard 2000, Gelbaya, Nardo et al. 2006, Xi, Gong et al. 2012, Kamal 2013) was sufficient to perform a meta-analysis assessing the effect of salpingectomy on the number of fertilised oocytes. The meta-analysis shows no significant difference between the salpingectomy and the control group (P=0.82), see Figure 2-13. Dar et al. (Dar, Sachs et al. 2000), Lin et al. (Lin, Ou et al. 2013), Surrey et al. (Surrey and Schoolcraft 2001) and Wiser et al. (Wiser, Gilbert et al. 2013) provided fertilization rates but no extractable data for inclusion in the meta-analysis.

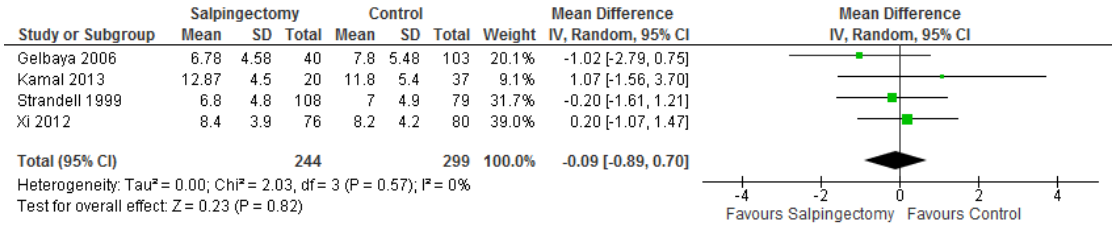


Figure 2-13 Total number of fertilised oocytes

Table 2-5: Quality assessment of included studies

Study	Newcastle-Ottawa scale		
	selection	comparability	outcome
Bao (2010)	*		**
Verhulst (1994)	*	*	**
Kamal (2013)	*	*	**
Lass (1998)	*	*	**
Dar (2000)	**	*	**
Strandell (1999)	na	na	na
Tal (2002)	*	*	**
Chan (2003)	**	*	**
Gelbaya (2006)	*	*	**
Almog (2011)	*	*	**
Orvieto (2011)	*	*	**
Ni (2013)	*	*	**
Nakagawa (2008)	*	*	**
Grynnerup (2013)	*	*	**
Lin (2013)	*	*	**
Meng (2006)	*	*	**
Singer (2011)	*	*	**
Shulman (2002)	*	*	**
Surrey (2001)	*	*	**
Wiser (2013)	*	*	**
Wong (2013)	*	*	**
Yazbeck (2010)	*	*	**
Xi (2012)	*	*	**

None of the studies in Table 2-5: Quality assessment of included studies had blinding or sample size calculation, apart from Strandell (Target sample size of 300, failed to achieve)

2.1.5 Discussion

This literature review demonstrates that a salpingectomy does not have a negative effect on ovarian reserve. The meta-analysis only demonstrated a significant decrease in number of follicles developing after ovarian stimulation. This is in agreement with a study by Lass et al. (Lass, Ellenbogen et al. 1998), who showed no significant difference in the total amount of oocytes collected in the salpingectomy group compared to the control group. However when looking at the patients with unilateral salpingectomy in their study group, significantly fewer follicles developed during COH and consequently fewer oocytes were retrieved from the ovary on the operated side (Lass, Ellenbogen et al. 1998). This can be proof towards the hypothesis that the contralateral (not operated) ovary has a compensatory mechanism.

Only one study by Orvieto et al. (2011) assessed the effect of a salpingectomy on ovarian reserve by comparing before and after and operated versus not-operated. The authors showed a significant decrease in the number of follicles on the operated side when that ovary was compared to itself before salpingectomy (Orvieto, Saar-Ryss et al. 2011).

Our meta-analysis shows no significant difference in antral follicle count between the groups with a salpingectomy and their controls. An interesting observation was made by Yazbeck et al. (2010). In their subgroup analysis, the number of small follicles (no definition stated) was significantly decreased on the operated side compared to the contralateral ovary. They

found this effect to be more pronounced in the prophylactic salpingectomy group than in salpingectomies for ectopic pregnancy. There was a significant decrease in the number of oocytes retrieved from the operated side.

AMH has taken over the role of AFC in most IVF clinics. It is surprising that no robust data is available in the literature regarding potential changes in its levels in relationship to a salpingectomy. Grynnerup et al. reported that AMH levels were significantly lower in the salpingectomy group than in the non-salpingectomy group (16.1 pmol/L [median age, 34 years] vs. 23.4 pmol/L [median age, 33 years], median; P = 0.04 (Grynnerup, Lindhard et al. 2013). Singer et al. reported no significant difference (Singer, Kofinas et al. 2011).

There are different ways of performing a salpingectomy. With the introduction of laparoscopic surgery, most salpingectomies nowadays are performed via this route. In the study by Chan et al. (Chan, Ng et al. 2003) no significant difference in AFC was observed for the total group of patients with salpingectomy when comparison was made between the operated with the non-operated side. However, in the subgroup analysis, the AFC was significantly reduced in the operated ovary when the salpingectomy was performed laparoscopically (Chan, Ng et al. 2003). The rationale being that open surgery is easier than laparoscopy. When placing the surgical clamps very close to the mesenteric margins of the affected fallopian tube any injury to blood vessels in the mesosalpinx may be minimized (Chan, Ng et al. 2003). Another explanation could be the use of diathermy instruments with minimal invasive surgery. Monopolar or bipolar instrument can cause

thermal spread further than their dissection plane, damaging ovarian vascularity. Most gynaecological surgeons prefer the minimally invasive approach to managing tubal disease, as this is related to shorter recovery time, duration of hospitalisation and treatment satisfaction. These issues make the widespread reintroduction of open surgery an unlikely option.

From the surgical perspective not all participants in the assessed studies had salpingectomy performed for the same indication. In most cases a mixture of hydrosalpinx or ectopic pregnancy was the indication for surgical (Verhulst, Vandersteen et al. 1994, Tal, Paltieli et al. 2002, Bao, Hao et al. 2010, Almog, Wagman et al. 2011, Singer, Kofinas et al. 2011). As a consequence, it is not possible to identify if the elective salpingectomy for a hydrosalpinx is associated with less negative implications than an emergency surgical intervention for an ectopic pregnancy, or vice versa.

Invasive surgery always entails the risk of complications. Some patients might not be ideal candidates for surgery, for example patients with extensive endometriosis or a frozen pelvis after pelvic inflammatory disease. The option of a hysteroscopic tubal occlusion might be worth taking into consideration in such cases (Legendre, Gallot et al. 2013, Matorras, Rabanal et al. 2013, Veersema, Mijatovic et al. 2013). The effect of this falls beyond the scope of this review, but hypothetically this would not interfere with the blood supply to the ovary, hence the potential negative effect on ovarian reserve could be removed. A comparison between ovarian stimulation and ovarian reserve markers before and after the procedure and to a control group with standard surgery might enable us to confirm if vascular injury leads to the negative effect on the ovary. For ectopic

pregnancies, the use of methotrexate is an option in selected cases (Singer, Kofinas et al. 2011, Uyar, Yucel et al. 2013, Wiser, Gilbert et al. 2013).

Overall, salpingectomy does not seem to negatively affect the outcome of ovarian stimulation in women undergoing IVF. This review reveals a significant reduction in recruited follicles and a trend toward reduction in peak E2 levels during stimulation. Although data still suggests a negative influence of salpingectomy on the assessed outcomes, patients and clinicians may be reassured as the negative impact of the hydrosalpinx on implantation rates outweighs the potential decrease in ovarian reserve markers. Presence of a tubal ectopic is beyond dispute an indication for surgery, either in the form of salpingectomy or salpingostomy. The current level of evidence seems to suggest surgical management of tubal disease is a safe option. In view of this, obtaining approval for a randomized clinical trial comparing surgery versus no intervention is unlikely; however comparison of surgery versus other effective methods of managing tubal disease seem like a viable option.

Strandell et al. was the only RCT identified, they failed to reach their target sample size due to time limitation (Strandell, Lindhard et al. 1999). There was no significant difference in primary outcome of pregnancy rate, but this might be caused by insufficient power due to the incomplete sample size.

This is the first systematic literature review trying to answer the question if salpingectomy has an effect on ovarian reserve. Systematic review methodology according to the PRISMA guidelines and Cochrane Collaboration was followed. The main limitation of the study was the quality

of the retrieved data, which was deemed to be poor with only one RCT meeting the inclusion criteria. There was also significant data heterogeneity between the study populations, study methodology and assessed outcomes. This is however a study collating all the data available to date on the effect of salpingectomy on ovarian reserve and subsequent ART-outcome. For evaluation of treatment RCT's are the golden standard, nevertheless cohort studies for meta-analysis for an observed effect or risk are appropriate.

Large studies with appropriate study methodology and clear inclusion criteria assessing the same outcomes are necessary to unequivocally describe the effect of salpingectomy on the outcome of women undergoing fertility treatment. The outcomes of such studies should also include pregnancy rates following ART.

Chapter 3: A dopamine agonist for the treatment and prevention of OHSS

3.1 Systematic literature review

Do dopamine agonists prevent or reduce the severity of ovarian hyperstimulation syndrome in women undergoing assisted reproduction? A systematic review and meta-analysis.

***Baumgarten M**, Polanski L, Campbell B, Raine-Fenning N.*

Human Fertility, September 2013, Vol. 16, No. 3 : Pages 168-174

3.2 Introduction

Ovarian hyperstimulation syndrome (OHSS), which could be defined as enlarged ovaries and symptoms of discomfort with or without the accompanying shift of intravascular fluid to the third space, is a common complication of assisted reproduction treatment (Rizk and Aboulghar 1991). A classification based on the severity of symptoms of OHSS can be made, classifying approximately 20-30% of patients undergoing ART as mild, 5% moderate and 1-2% severe (Navot, Bergh et al. 1992). Whilst the majority of cases are mild and can be managed conservatively, often on an out-patient basis, patients with moderate to severe disease invariably require admission (Gustofson, Browne et al. 2005) . The latter group comprise 1-2% of all patients undergoing controlled ovarian stimulation and, as such,

represent a considerable cost to the NHS. These women are at risk of thromboembolic disease, respiratory compromise and renal failure, all of which can ultimately result in death especially if not recognised early and treated actively ((CEMACH) 2007).

Clearly, the prevention of OHSS is preferable to its early detection and treatment, but ovarian stimulation is inherently associated with a risk of over response. Recent developments have seen the use of lower doses of gonadotropins and the introduction of gonadotropin-releasing hormone (GnRH) antagonist protocols (van der Linden, Buckingham et al. 2011), but OHSS can still occur despite these measures and treatments are therefore required. Once the syndrome has developed options include cancellation of the cycle prior to oocyte collection or continuation of treatment with the introduction of various measures to reduce its severity and, if these are ineffective, the subsequent freezing of all oocytes or embryos obtained and the postponement of the embryo transfer. These options represent a hierarchical approach with increasing effect predominantly related to the avoidance of endogenous or exogenous human chorionic gonadotropin and progesterone, both of which are known to exacerbate the condition (Kasum 2010). The indication for the use of these interventions is, however, unclear and dependent on both the definition used by the clinic and the threshold applied for cancellation and intervention.

OHSS characteristically occurs when ovaries primed with gonadotropins are subsequently exposed to human chorionic gonadotropin (hCG) either at the time of final follicular maturation (early onset OHSS) or during the luteal phase of a mostly conception cycle (late onset OHSS).

The ultimate pathophysiological step underlying the syndrome is one of increased vascular permeability. Human chorionic gonadotropin (hCG) increases the expression of vascular endothelial growth factor (VEGF) and VEGF receptor-2 (VEGFR-2) messenger RNA (mRNA) (4). Dopamine agonists, typically used for the treatment of hyperprolactinaemia, inhibit phosphorylation of the VEGFR-2 receptor and have the potential to ameliorate the symptoms associated with OHSS that are related to increased vascular permeability (Soares 2012). Several studies have been conducted to assess the efficacy of dopamine agonists in the prevention and treatment of OHSS but without any consensus on their use (Manno, Tomei et al. 2005, Alvarez, Marti-Bonmati et al. 2007, Aflatoonian, Gahndi et al. 2008, Shaltout, Shohayeb et al. 2009, Ahmadi, Rahman et al. 2010, Busso, Fernandez-Sanchez et al. 2010, Sherwal, Malik et al. 2010, Alhalabi, Samawi et al. 2011). Some studies show a reduction in the incidence of OHSS whilst others show no effect (Hwang, Lin et al. 2010). These differences may specifically relate to the dopamine agonist used or to the criteria applied for their administration, but this is difficult to clarify given the degree of variability in the protocols employed.

Despite the lack of evidence or clear guidance, dopamine agonists are being increasingly used in this context, and many clinics anecdotally report a beneficial effect. We decided, therefore, to systematically review the current literature to clarify the current evidence for the use of dopamine agonists according to the indication for their administration and on the basis of the drug regimen used. The plan was to conduct a meta-analysis of studies comparing a dopamine agonist to placebo or no intervention on the

incidence and severity of OHSS in women undergoing ovarian stimulation as part of assisted reproduction treatment.

3.3 Methods

3.3.1 Search strategy for identification of studies

EMBASE, MEDLINE and Cochrane electronic databases encompassing years 1948 until September 2011 were searched. A search strategy was carried out based on the following terms: ovarian hyperstimulation syndrome, controlled ovarian hyperstimulation, exaggerated response, dopamine, dopamine agonist(s), Cabergoline, quinagolide, bromocriptine, pergolide, talipexole, ropinirole, and pramipexole.

No limitations were applied to the definitions the authors used to define somebody at risk of developing OHSS or to the criteria used to diagnose OHSS. All searches and manuscript reviews were conducted independently by MB and LP and were not restricted by language. Relevant case reports were also included in the final analysis. Studies were selected if a dopamine agonist had been used in the context of fertility treatment. The final decision to include or exclude the study was only made after review of the full manuscript. Any disagreement about inclusion or exclusion was resolved by consensus or by a third reviewer (NRF).

3.3.2 Results

The search strategy and selection of manuscripts is shown in Figure 5-1. The initial search resulted in 388 citations. Following restriction to human subjects and the removal of duplicate citations, the number of articles for review reduced to 123. The titles and abstract were first reviewed and full manuscripts of all citations thought likely to meet the inclusion criteria were then obtained (n = 74). Two additional articles (Robert, Musatti et al. 1996, Ricci, Parazzini et al. 2002) were obtained from references in the retrieved

articles but subsequently excluded from the review after consideration of the full text. These were studies assessing the reproductive safety of Cabergoline when not in conjunction with ovarian stimulation.

Following review of these 76 manuscripts, 20 publications were considered to meet the inclusion criteria and describe the use of DA in any context. Only two of these described randomised, double-blind, controlled trials (Alvarez, Marti-Bonmati et al. 2007, Busso, Fernandez-Sanchez et al. 2010), the other 18 comprising seven randomised, controlled trials (Aflatoonian A 2008, Carizza, Abdelmassih et al. 2008, Edeen S 2009, Shaltout A 2009, Sohrabvand 2009, Ahmadi Sh 2010, Alhalabi M 2011), one non-randomised prospective study (Soliman 2011), three matched case controlled studies (Papaleo, Doldi et al. 2001, Alvarez, Alonso-Muriel et al. 2007, Sherwal, Malik et al. 2010), six case series (Ferraretti, Gianaroli et al. 1992, Tsunoda, Shibahara et al. 2003, Manno, Tomei et al. 2005, Rollene, Amols et al. 2009, Hwang, Lin et al. 2010, Spitzer, Wogatzky et al. 2011), and one case report (Ata, Seyhan et al. 2009). Only one of the studies was a multi-centre trial (Busso, Fernandez-Sanchez et al. 2010). Nineteen of the studies dealt with the development of OHSS in women undergoing controlled ovarian stimulation as part of *in vitro* fertilisation treatment. The other study (Papaleo, Doldi et al. 2001) involved patients undergoing ovarian stimulation as part of intrauterine insemination (IUI) treatment.

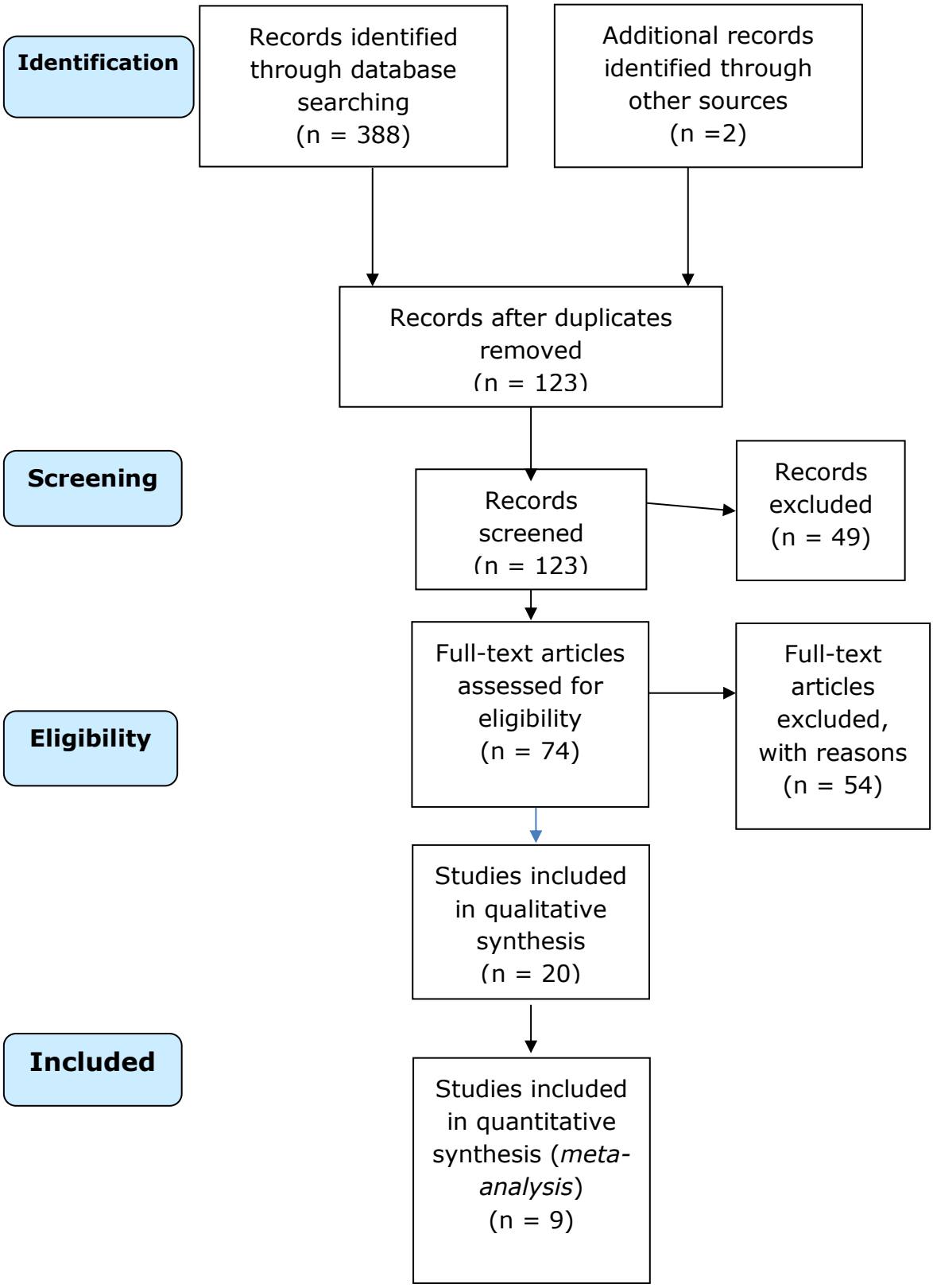


Figure 3-1: Flowchart of study selection for systematic review.

In total, 1646 women were included in these 20 studies of which 914 (55.5%) received a dopamine agonist, the remaining women receiving no treatment, placebo or 'standard practice' in the centre conducting the research. Fifteen of these 20 studies looked at the prevention of OHSS in 'high-risk' groups meeting their own criteria, whilst the other 5 studies (Ferraretti, Gianaroli et al. 1992, Tsunoda, Shibahara et al. 2003, Manno, Tomei et al. 2005, Ata, Seyhan et al. 2009, Rollene, Amols et al. 2009) focused on the treatment of clinically diagnosed OHSS and minimization of the severity of the condition.

In the group who received treatment as a *preventive measure*, 156 of 866 (18%) of women receiving dopamine agonists developed OHSS according to the definition used by the authors compared to 223 of the 732 (30.5%) controls (two-tailed p-value < 0.0001) (see Table 3-4). One other study also reported a significant reduction in OHSS in those receiving a dopamine agonist, but did not state the prevalence of OHSS in either group (Ahmadi, Rahman et al. 2010).

In the group who received a dopamine agonist as a *measure to reduce the severity* of OHSS, all patients were already affected by OHSS. No comparisons were possible as all patients were treated and nobody was denied treatment or received a placebo.

When *all of the studies* comparing the use of any dopamine agonist for the indication chosen by the authors are compared to those offering no treatment, including placebo, the total incidence of all types of OHSS was significantly less in the treated group (see Figure 5-2).

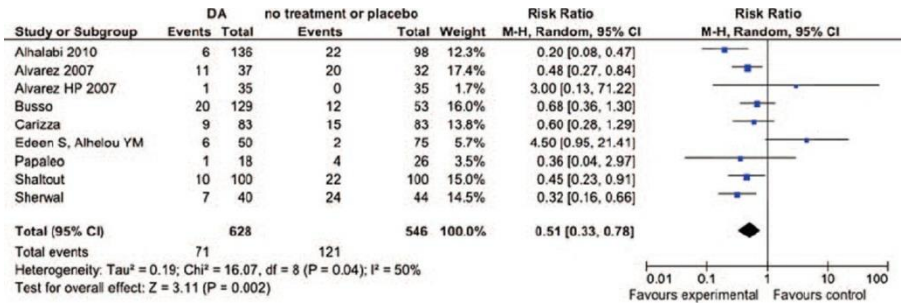


Figure 3-2: Meta-analysis of all studies comparing DA versus no-treatment/placebo for all forms of OHSS as prevention or treatment

There is a large variation in protocols described in the studies predominantly in the dopamine agonist used and in its method of administration such that no one protocol was comparable (see Table 3-4). In some studies, additional preventative or therapeutic treatments were also used concomitantly. The groups used as controls were also different; the use of a dopamine agonist being compared with placebo, no treatment, coasting, or the use of albumin (see Table 3-6). It is not possible to comment on the different dopamine agonists used, the optimal dose to be effective, or when to start and stop treatment. Side effects were not stated by all authors, but did appear to be dose-related (see Table 3-7).

3.4 Clinical experience with the use of Cabergoline

After performing the systematic review, a protocol for prescribing Cabergoline to women who were believed to be at increased risk of developing OHSS was devised. Whether or not this option was presented and discussed with the patient was up to the discretion of the treating clinician. As described in Table 3-1; 42 cycles received 7 days of Cabergoline 0.5mg once a day from the time of oocyte retrieval. Pregnancy rates can be found in Figure 3-3.

	Absolute number	% of OHSS
Total OHSS	54	100%
Freeze-all	21	38.9%
Cabergoline	42	77.8%
Hospital admission	10	18.5%
Late onset	5	9.3%

Table 3-1: overresponse cycles and their sequelae

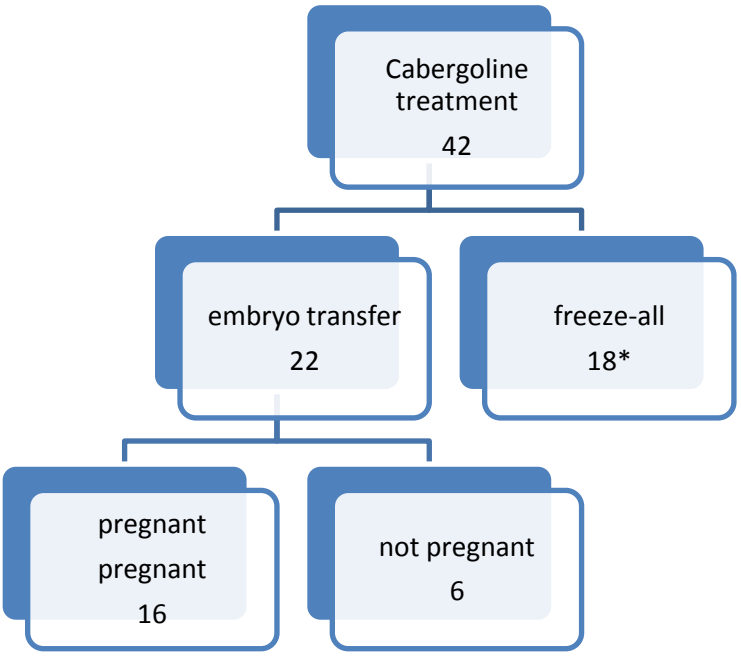


Figure 3-3: Cabergoline treatment and biochemical pregnancy

*oocytes donor and failed blastocyst formation account for the missing 2.

Of the 12 women not being prescribed Cabergoline, 3 had a freeze all (including the patient with agonist trigger). The 9 women who had an embryo transfer all got pregnant. Four developed late onset OHSS, of which 3 needed hospitalisation including a case of severe OHSS needing ascitic tap for 3 days.

Cabergoline appears to have no negative influence on implantation, p=0.08 chi2-test (see Table 3-2). Three of the twin pregnancies were the result of a double embryo transfer, as was the single triplet pregnancy.

Cabergoline	**Yes** **N=22**	**No** **N=9**
Biochemical	16(73%)	9 (100%)
Clinical	16	9
Twin pregnancy	4	0

Triplet pregnancy	1	0

Table 3-2: Cabergoline and implantation.

Data of pregnancy outcome is available for 4 pregnant women with Cabergoline and 4 pregnant women without Cabergoline (see Table 3-3).

	CBG yes	CBG no
Age (year)	34	32
Nr of oocytes collected	25	19
Female baby	2/4	1/4
Birth weight (grams)	3061	3261
Gestation (days)	273	270

Table 3-3: Comparison of women with known pregnancy outcome with and without Cabergoline (numbers too small for statistical analysis)

3.4.1 Discussion

This systematic review has clarified the current evidence-base for the use of dopamine agonists for the prevention and treatment of OHSS. The studies included show a high level of heterogeneity, which precludes a definitive conclusion, but on balance, dopamine agonists appear to reduce the incidence of OHSS compared to placebo or no treatment. Their exact role is still difficult to clarify given the inherent differences between their use for both the prevention and the treatment of OHSS. The findings are further compounded by the variable inclusion criteria which were expected, given there is no formal definition of women being at 'high risk' of developing OHSS. The definition and classification of OHSS also differs and was not always specifically stated.

Dopamine agonists appear to be more effective as a preventative measure than as a treatment strategy. Late onset OHSS remains the most difficult form of the disease to treat and does not appear to be prevented by prolonging the period of administration of the dopamine agonist (Carizza, Abdelmassih et al. 2008). The safety of this approach also remains to be determined as the only negative outcome of treatment was reported in the study by Hwang et al. (Hwang, Lin et al. 2010) who used Cabergoline after a period of coasting. If we accept that dopamine agonists are effective for the prevention of OHSS, then what effect there is, seems to be dose-related (Busso, Fernandez-Sanchez et al. 2010). This must be balanced against toxicity and drug intolerance, which accounted for more dropouts in groups assigned to relatively higher doses of an agonist. Side effects were only described in five studies (Manno, Tomei et al. 2005, Alvarez, Marti-Bonmati et al. 2007, Rollene, Amols et al. 2009, Busso, Fernandez-Sanchez et al.

2010, Spitzer, Wogatzky et al. 2011) with nausea, vomiting, somnolence, and dizziness being the most commonly reported. As nausea and vomiting are also symptoms of OHSS it difficult to categorically say whether these are due to the dopamine agonist or the underlying condition and disease process.

In the protocols starting treatment on the day of hCG administration, treatment was continued over a period of 8 days. This would mean that in case of a day 5 blastocyst transfer, no dopamine agonist would be taken after the transfer, trying to minimize any potential negative effect on the embryo or implantation.

The review is also limited by variations in the protocol used and the indication for treatment or randomisation. Despite identifying nine studies of level 1 evidence, the numbers involved are still relatively small given the incidence of severe OHSS, which rendered most, if not all of the studies, underpowered.

Compared to the current literature (Youssef, van Wely et al. 2010, Tang, Hunter et al. 2012), we agree that there is a reduction in OHSS with the use of Cabergoline. Our results also take the use of other dopamine agonist into account giving a more complete overview.

3.4.2 Conclusion:

Dopamine agonists appear to reduce the incidence of ovarian hyperstimulation syndrome (OHSS) in women considered at risk of developing the condition. The effectiveness of dopamine agonists in women who have developed the disease is less clear. The exact effect and the most appropriate drug regimen is impossible to clarify given the degree of

heterogeneity in the studies considered in this review, and there is a need for a standardized, randomized controlled trial, which must be appropriately powered and, given the incidence of moderate to severe disease, multicentre.

Table 3-4: Definition of patients considered at risk of developing OHSS and dopamine agonist regimen used

Author	Number of follicles (size not stipulated)	Total Number of oocytes retrieved	Serum E2 level on day trigger (pg/ml)	Other criteria	Dopamine agonist	dose	Regime	Duration	Start	Extra	OHSS incidence
Aflatoonian et al.	>20	-	2500	-	Cabergoline	0.5mg	1/day	8 days	Trigger		5/30
Ahmadi et al.	-	-	-	-	Cabergoline	0.5mg	1/day	12 days	Oocyte collection	-	Not stated
Alhalabi et al.	>20	-	4000	-	Quinagolide	150mg	1/day	15 days	Trigger		6/136
Busso et al.	>20, <30	-	-	-	Quinagolide	50mg	1/day	17 days	Trigger	-	8/51
						100mg	1/day	17 days	Trigger	-	10/52
Carriza et al.	-	-	4000	-	Cabergoline	0.5mg	1/day	21 days	Day after oocyte collection	Albumin during oocyte retrieval	9/83
Edeen et al.	-	-	-	PCOS	Cabergoline	0.5mg	1/day for 2 days, repeat after 1 week	1 week	Trigger		2/75

Author	Number of follicles (size not stipulated)	Total Number of oocytes retrieved	Serum E2 level on day trigger (pg/ml)	Other criteria	Dopamine agonist	dose	Regime	Duration	Start	Extra	OHSS incidence
Hwang et al.	-	-	-	-	Cabergoline	0.5mg	1/day	8 days	Trigger	After coasting	2/2
Papaleo et al.	-	-	-	PCOS with mild hyperprolactinaemia	Cabergoline	0.25mg	1/week	Not stated	Before and during stimulation		1/18
Shaltout et al.	>20	-	3500	Exclusion if E2>5000pg/ml	Cabergoline	0.25mg	1/day	8 days	Trigger	-	10/100
Sherwal et al.		>20	3000	Inclusion of PCOS and/or history of OHSS	Bromocriptine	2.5mg	1/day	16 days	Oocyte collection		22/24
						200mg	1/day	17 days	Trigger	-	2/26
Sohrabvand et al.	>20	-	3000	-	Cabergoline	0.5mg	1/day	7 days	Trigger	-	12/30
Soliman et al.	>20	-	3000	-	Cabergoline	0.25mg	Till trigger, thereafter 0.5mg/day	Till day 8	Not stated	-	1/15
						0.25mg	Till trigger, thereafter 0.5mg/day	Till day 8	Not stated	Albumin	1/15
Spitzer et al.	-	>20	4500	Symptoms on the day of oocyte retrieval	Bromocriptine	2.5mg	1/day	Until OHSS resolved	Oocyte collection	Some had coasting and/or IV-fluids	5/30

Table 3-5: Treatment with dopamine agonist following the diagnosis of OHSS

Author	Inclusion	DA	Dose	Regime	Length	Start	Extra	Study group
Ata et al.	Case report	Cabergoline	0.5mg	0.5mg/day for 5 days, then 1mg/day	22 days	Trigger	1 day of coasting, HES	1
Manno et al.	Severe hyperstimulation and pregnant	Cabergoline	1mg	Every 2 days	Not stated	Oocyte collection	After 24-48h dopamine-infusion	20
Rollene et al.	OHSS between TVOR and ET	Cabergoline	0.5mg	1/day	4 days	Oocyte collection	250 ganirlix	4
Tsunoda et al.	Early OHSS refractory to initial therapy with albumin	docarpamine	750mg	3/day	Not stated	Not stated	Albumin iv	27
Ferraretti et al.	Severe OHSS	dopamine iv	4,32mg/kg/24h	N/A	7-22days	Not stated	-	7

Table 3-6: Control groups and their definitions

Author	Control used
Aflatoonian et al., Sohrabvand et al.	Coasting (FSH withheld until serum E2 declined) / coasting (until E2 <3000pg/ml)
Ahmadi et al., Soliman et al.	20g albumin iv / Albumin
Alhalabi et al., Alvarez et al. (2007 HP), Carizza et al., Edeen et al., Papaleo et al., Shaltout et al., Sherwal et al.	No treatment
Alvarez et al., Busso et al.	Placebo

Table 3-7: Side effects and tolerance of dopamine agonists

Author	Medication	Side effects	Number of patients effected
Alvarez et al.	Cabergoline 0.5mg/day	None reported	0%
Busso et al.	Quinagolide 50-100-200mg/ day	Nausea	35% - 50% - 69%
		Dizziness	41% - 52% - 42%
		Somnolence	25% - 33% - 42%
		Diarrhoea	12% - 13% - 12%
		Vomiting 8% (4)	24% - 71% - 69%
		Headache (3)	10% - 10% - 12%
		Flatulence	8% - 2% - 4%
		Syncope	2% - 8% - 12%
Manno et al.	Cabergoline 1mg/2days	Postural hypotension	10%
Rollene et al.	Cabergoline 0.5/day	None reported	0
Spitzer et al.	Bromocriptine 2.5mg/day	Severe nausea and vomiting	5%

Chapter 4: Pharmacogenomics of Clomiphene response

Are we able to find genetic makers able to predict the ovarian response to and identify patients at risks of under or over response?

4.1 Introduction

As described in section 1.2, ovulation disorders account for approximately 20% in cases of subfertility. Clomiphene citrate (CC) has been the first line treatment of choice for anovulation for decades, due to the vast experience and the low costs. It has been 50 years since the first clinical trial demonstrated that administration of clomiphene citrate induces ovulation in more than 75% of amenorrheic women (Greenblatt 1961). CC can also be used for the treatment for women with unexplained infertility, although limited data is available to support this (Hughes, Brown et al. 2010).

Clomiphene citrate is a selective oestrogen receptor modulator (SERM). The activity of SERM is medicated by binding to the oestrogen receptor, leading to initiation of suppression of the transcription of target genes. SERM activity is unique for each oestrogen receptor ligand and has specific actions in the target cell, causing a variable tissue effect. Early SERMs such as CC used for ovulation induction have an estrogenic and anti-estrogenic effect (Shelly, Draper et al. 2008). With CC, its antagonistic effect predominates, leading to a depletion of oestrogen receptors at the level of the pituitary and hypothalamus. This interrupts the negative feedback of oestrogen and GnRH secretion will be increased, stimulating the release of FSH.

A Cochrane Review by Brown et al. of CC for ovulation induction in PCOS patients (Brown, Farquhar et al. 2009) included 15 randomized controlled trials. CC was shown to be more effective than placebo in increasing the pregnancy rate in anovulatory patients with possible PCOS (OR 5.8; 95%-CI; 1.6–21.5). An RCT by Legro et al. included 626 patients with PCOS, CC use resulted in a live-birth rate of 22.5% versus 7.2% with the use of metformin alone (P<0.001), with the consequence of an increased multiple pregnancy rate in the CC group (Legro, Barnhart et al. 2007).

Most studies have reported ovulation rates of 60%–85% and pregnancy rates of 30%–40% with CC (Palomba, Orio et al. 2005). There is a significant variation in the dose (50–150 mg) required to induce ovulation with CC, which is not predictable *a priori* (Dickey, Taylor et al. 1997). In properly selected women, approximately 46% will ovulate while using the 50-mg per day dosage, and approximately another 21% will ovulate if the dosage is increased to 100 mg (Rostami-Hodjegan, Lennard et al. 2004).

Several months of treatment may be required to achieve the optimal dose. A number of individual patient characteristics, such as age, body weight, BMI, free androgen index (FAI), and menstrual cycle history contribute to the success or failure of treatment with CC (Imani, Eijkemans et al. 1998, Imani, Eijkemans et al. 1999, Imani, Eijkemans et al. 2002). Until now, little attention has been paid to the interindividual variability in CC metabolism and its potential contribution to the therapeutic response. In theory, variation in biotransformation of CC will produce different plasma concentrations. CC is administered as a mixture of two geometric isomers, enclomiphene (En) and zuclomiphene (Zu) citrate in the ratio 62%:38%

(Ernst, Hite et al. 1976). The more bioactive isomer, E-clomiphene, is rapidly excreted so it will not accumulate from cycle to cycle. The less active isomer Z-clomiphene is excreted slowly so it will potentially accumulate over time if clomiphene is given month after month. The biological effect of this accumulated clomiphene is not known. The two isomers are metabolized by different enzymes, which include cytochrome P4502D6, 3A4/3A5, 2C19, 2C8 and 2B6. The main metabolic pathways for clomiphene are presented in Figure 4-1 (Murdter, Kerb et al. 2012).

Genotype is the genetic make-up of an individual. With genotyping, the individual's DNA sequence is compared to another individuals' DNA for the reference sequence using appropriate assays. A single nucleotide polymorphism (SNP) is a variation at a single site in DNA. It is the most frequent type of variation in the genome (Shelly, Draper et al. 2008). SNP array is a type of DNA microarray which is used to detect polymorphisms within a population. There are mandatory components of those assays: first that the array must contain immobilized nucleic acid sequences of the target; second, the existence of one or more labelled allele-specific oligonucleotide (ASO) probes is necessary; lastly, a detection system that records and interprets the hybridization signal must exist.

Current methods of genotyping include restriction fragment length polymorphism identification (RFLPI) of genomic DNA, random amplified polymorphic detection (RAPD) of genomic DNA, amplified fragment length polymorphism detection (AFLPD), polymerase chain reaction (PCR), DNA sequencing, allele specific oligonucleotide (ASO) probes, and hybridization to DNA microarrays or beads. Genotyping is important in genetic research

and analysis of gene variant association with disease. Due to current technological limitations, almost all genotyping is partial, only determining a small fraction of an individual's genotype.

SNP genotyping is the measurement of genetic variations of single nucleotide polymorphisms between members of a species. It is a form of genotyping, which is the measurement of more global genetic variation. SNPs are one of the most common types of genetic variation. A SNP is a single base pair mutation at a specific locus, usually consisting of two alleles (where the rare allele frequency is >1%). SNPs are found to be involved in the aetiology of many human diseases and are becoming of particular interest in pharmacokinetics. The most important applications of SNP arrays are the determination of disease susceptibility and for measuring the efficacy of drug. Each individual has many SNPs and this information can help design drugs that act on a group of individuals who share a common allele.

Pharmacogenomics (the combination of pharmacology and genomics) is the technology that analyses how genetic makeup affects an individual's response to drugs. It deals with the influence of genetic variation on drug response in patients by correlating gene expression or single-nucleotide polymorphisms with a drug's efficacy or toxicity (Wang 2010). Pharmacogenomics aim to develop scientific background to optimize drug therapy, by respecting the patients' genotype, trying to ensure maximum efficacy with minimal adverse effects (Becquemont 2009). Such approaches contains the promise of "personalized medicine" in which drugs and drug combinations are optimized for each individual's unique genetic makeup

(Squassina, Manchia et al. 2010). In order to provide pharmacogenomics based recommendations for a given drug, two possible types of input can be used: genotyping as described above, or whole genome sequencing (Huser and Cimino 2013).

The cytochrome P450 superfamily of monooxygenases (abbreviated as CYP) is a large and diverse group of enzymes that catalyse the oxidation of organic substances. The substrates of CYP enzymes include metabolic intermediates such as lipids and steroidal hormones, as well as xenobiotic substances such as drugs and other toxic chemicals. CYPs are the major enzymes involved in drug metabolism and bioactivation, accounting for about 75% of the total number of different metabolic reactions (Guengerich 2008). Cytochrome P450 genes, which encode enzymes, influence the metabolism of more than 80% of current prescription drugs (Hart, Wang et al. 2008, Gomes, Winter et al. 2009).

Most drugs undergo deactivation by CYPs, either directly or by facilitated excretion from the body. Many substances are bioactivated by CYPs to form their active compounds. Drugs may on their turn increase or decrease the activity of various CYP isozymes by inducing the biosynthesis of an isozyme (enzyme induction) or by directly inhibiting the activity of the CYP (enzyme inhibition). This is a major source of adverse drug interactions, since changes in CYP enzyme activity may affect the metabolism and clearance of various drugs.

There are several known genes which are largely responsible for variances in drug metabolism and response. Clomid is an example of a medication that follows the CYP metabolic pathway. Patient genotypes are usually

categorized into predicted phenotypes. For example, if a person receives one *1 allele each from mother and father to code for the CYP2D6 gene, then that person is considered to have an extensive metabolizer (EM) phenotype (Sim, Kacevska et al. 2013). An extensive metabolizer is considered normal. Other CYP metabolism phenotypes include: intermediate, ultra-rapid, and poor. In theory, each phenotype is based upon the allelic variation within the individual genotype. However, several genetic events can influence a same phenotypic trait, and establishing genotype-to-phenotype relationships can thus be far from consensual with many enzymatic patterns.

Murdter et al. (Murdter, Kerb et al. 2012) have shown that (E)-4-hydroclomiphene and (E)-4-hydroxy-N-desethylclomiphene have the strongest inhibition of the oestrogen receptor activity at 50%. CYP2D6 was identified as the major enzyme involved in their formation (Murdter, Kerb et al. 2012). CYP2D6 polymorphism substantially determines the formation of the active CC metabolites with an 8 to 12 times lower concentration in subjects with non-functional CYP2D6 alleles.

The CYP2D6 enzyme activity manifests in the population as ultrarapid (UM), extensive (EM), intermediate (IM) as well as poor metabolizer (PM) phenotypes with frequencies of 5–10%, 65–80%, 10–15% and 5–10% in the Caucasian population, respectively (Sachse, Brockmoller et al. 1997, Griese, Zanger et al. 1998). CYP2D6 genotypes of the human liver samples were classified on the basis of PM alleles (*3, *4, *5, *6, *7, *8), IM alleles (*9, *10, *41) and EM alleles (absence of any PM or IM allele), as PM/PM, PM/IM, IM/IM, PM/EM, IM/EM or EM/EM.

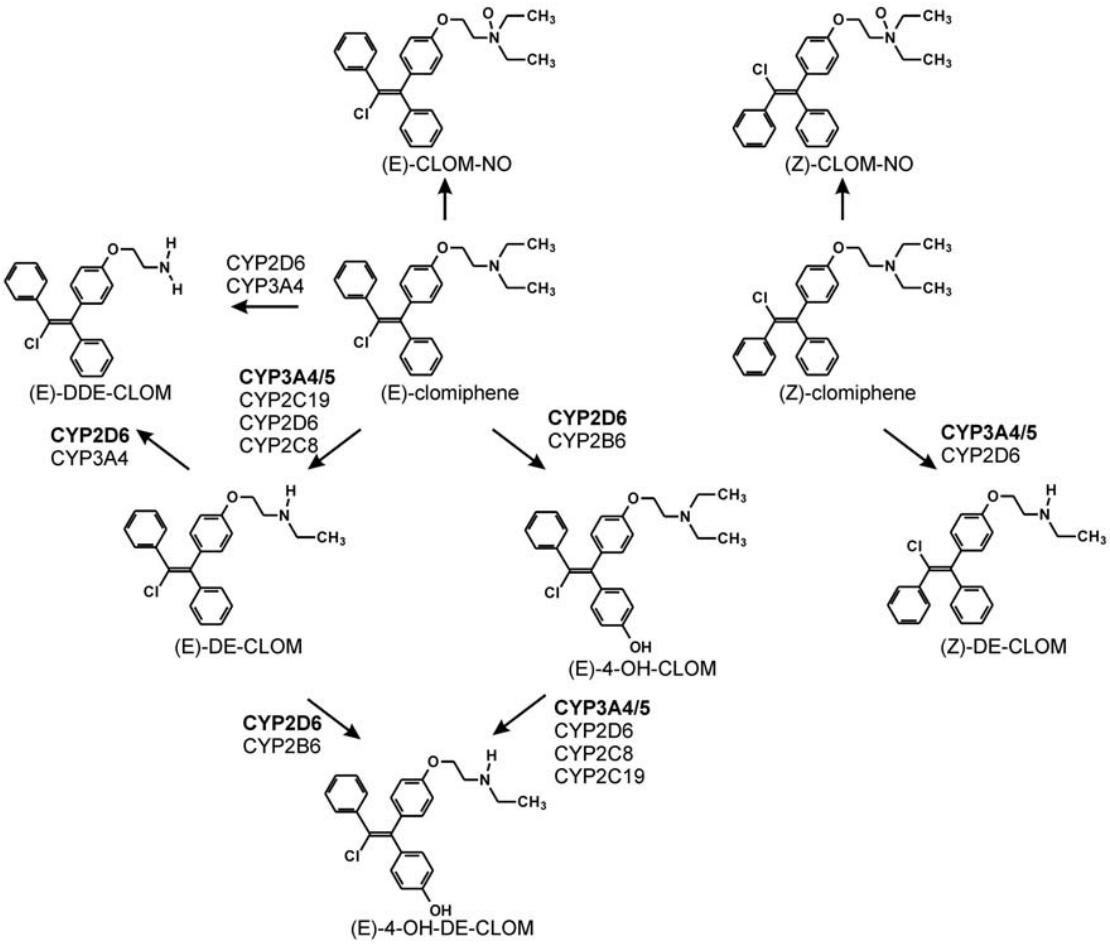


Figure 4-1: Human metabolism of (E)- and (Z)-clomiphene. Key CYPs presented in bold.

Image adapted from (Murdter, Kerb et al. 2012).

Four different phenotypes for CYP2D6 have been described, which are determined by several SNPs and SNP combinations. *In vitro* studies using human liver microsomes (Ghobadi, Gregory et al. 2008) have demonstrated that the extent of enclomiphene metabolism is correlated with the amount of CYP2D6. Formation of main clomiphene metabolites 4-hydroxyclomiphene and 4-hydroxy-desethyl-clomiphene is correlated with CYP2D6 genotype (Figure 4-2)(Murdter, Kerb et al. 2012).

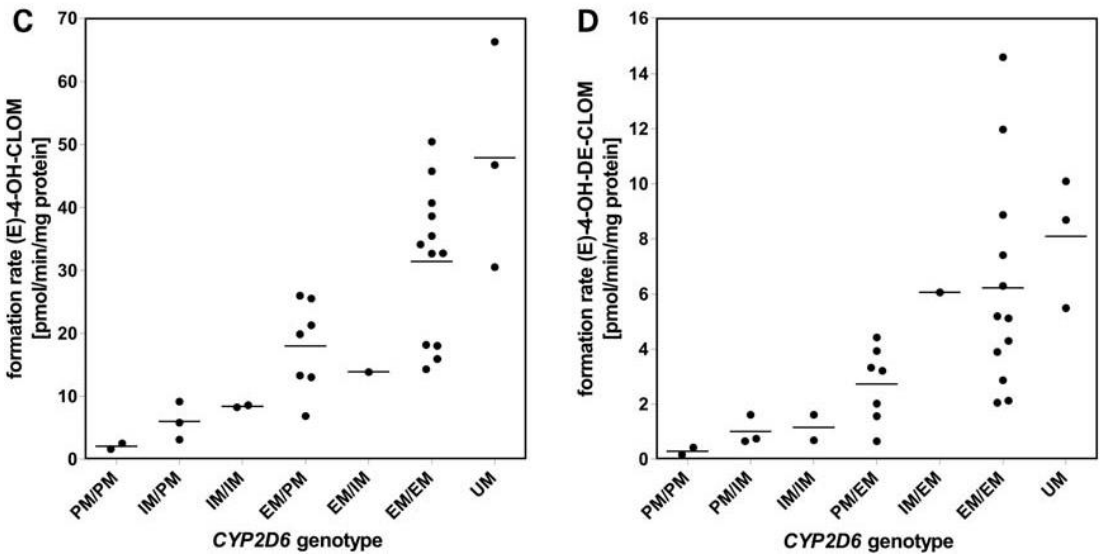


Figure 4-2: Formation of clomiphene metabolites by human liver microsomes is correlated to CYP2D6 genotype

Image adapted from (Murdter, Kerb et al. 2012).

4.2 Rationale for the study

Dosage of CC is mostly empirical, and treatment typically begins with the lowest dose. Some patients demonstrate an exaggerated response to this universal starting dose whilst others do not respond at all, even after increasing the dose. This is referred to as clomiphene resistance. Resistance to CC is associated with obesity, but the individual response to treatment remains unclear and many CC-resistant women are of normal weight. Resistance is more common in women with PCOS, but these patients also have a higher incidence of over-response and OHSS. Underlying genetic polymorphisms are thought to be responsible for the aberrant and often unpredicted response. These polymorphisms associated with various hepatic enzyme expression can modify the pharmacokinetics of CC. This study was designed to identify genetic markers related to prediction of over or under response to CC in women undergoing OI (subfertility based on anovulation with normal tubal patency and normal sperm parameters). A

matched group of women undergoing OI with a normal response were selected as controls.

Prior to conducting laboratory work, an extensive literature review was carried out aiming to identify evidence to support differential CC metabolism in correlation with genetic composition of an individual. The subsequent experimental study objective was to first create a DNA archive from women undergoing fertility treatment with CC and secondly investigate genetic polymorphisms in genes encoding metabolizing enzymes and correlate to clinical clomiphene response in a cohort of women undergoing fertility treatment.

4.3 Systematic literature review

4.3.1 Materials and methods

Published journal articles and conference abstracts reporting on clomiphene metabolism and associated genes were sought. No restrictions on the language of the publication were set.

The following databases were searched: MEDLINE, BIOSIS PREREVIEWS, EMBASE, Web of Knowledge (ISI), Web of Science (ISI), SCOPUS (Elsevier), Science Direct (Elsevier), Cochrane Central Register of Controlled Trials (CENTRAL); Cumulative Index to Nursing and Allied Health Literature (CINAHL) International Pharmaceutical Abstracts (IPA) and ZETOC from the databases' inception to present. The search terms used included: Clomid, Clomiphene, Androxal, Omifin, Serephene, enclomifene, zoclomifene, clomifene, enclomiphene, zoclomiphene and CYP2D6, CYP2C8, CYP3A4, genotype*, polymor*ism, adverse reaction, OHSS, response, gene, allele. Trials registers in Current Controlled Trials (www.controlled-trials.com), ClinicalTrials.gov (http://clinicaltrials.gov/ct2/search), and the World Health Organization International Trials Registry Platform search portal (http://apps.who.int/trialsearch/Default.aspx) have also been searched.

This review sought all articles reporting on genes associated with the metabolism, efficacy and toxicity of Clomiphene Citrate. Titles and abstracts were reviewed independently by two review authors (MNB, LP) checking for duplicates and using the pre-established criteria for inclusion. These authors retrieved the full-text manuscripts of studies considered to be potentially eligible for inclusion and independently evaluated eligibility of these studies. Disagreements were solved by consulting a third review

author (AA). There was no limitation on language, publication date or publication status. A hand search was conducted after reference review of the initial included studies.

The data was extracted from text, tables and graphs of included studies. A single author (MNB) retrieved the relevant data, which was subsequently verified by another author (LP).

The data extracted, comprised inclusion and exclusion criteria of the study, *in vitro* or *in vivo*, methods used for genetic analysis, outcome measurements.

Study quality was assessed using The Guidelines Manual 2009 published by National Institute for Health and Clinical Excellence (NICE). Risks of bias were assessed at the study design and outcome level. Possible sources of bias were considered and commented upon when performing data synthesis.

For every considered outcome, risk ratios (RR) were used with 95% confidence intervals (CI). P value of <0.05 was considered statistically significant.

When appropriate, meta-analysis was performed using Review Manager (RevMan) version 5.1 (Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2011). In the presence of significant heterogeneity, random effects have been used to pool the results.

Each analysed study was assessed for additional sources of bias (publication, reporting), which might influence the overall evidence. Each of these factors was considered when collating data.

The quality of the evidence is evaluated using the Newcastle-Ottawa Scale where studies are scored in 3 domains in which they can obtains stars; selection (4 stars), comparability (1 star) and outcome (3 stars). The higher number of stars the better the quality of the study.

4.3.2 Results

4.3.2.1 Study selection

The last database search was carried out on June 2014. The electronic database search revealed 26 citations. Excluding duplicate publications and irrelevant titles resulted in 18 titles and 2 additional titles were found thought hand search of references. Subsequently 17 abstracts were screened according to inclusion criteria and 14 articles were obtained in full and scrutinized (see Figure 6-3).

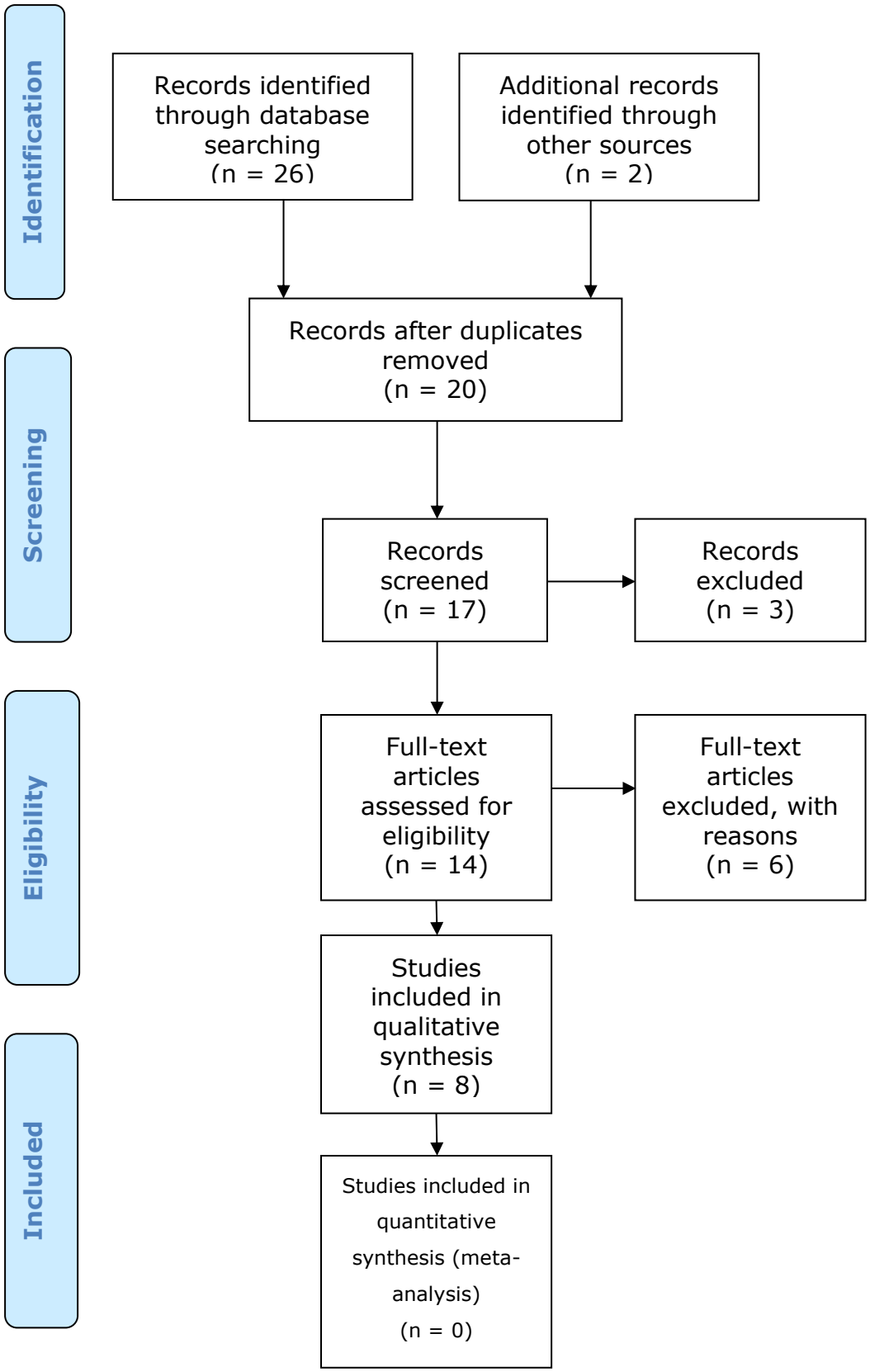


Figure 4-3: Systematic literature search for CC genes.

4.3.2.2 Characteristics of excluded studies

De Castro et al. (de Castro, Moron et al. 2005) and Overbeek et al. (Overbeek and Lambalk 2009) were excluded as they were review articles. Goldenberg (Goldenberg and Glueck 2008) was an appraisal article of the Legro (Legro, Barnhart et al. 2008) study. Mürdter et al. (2012) had a title referring to an abstract at a meeting, including the results of the study which has been included in this systematic review as a full article (Murdter, Kerb et al. 2012).

A study by Loutradis et al. (2012) was one of the titles found through hand search, but did not meet inclusion criteria as there was no available data regarding CC (Loutradis, Theofanakis et al. 2012). A study by Mohiyiddeen et al. (2012) was excluded as the data was regarding FSH receptor single gene polymorphism in relation to AMH and PCOS but not to CC (Mohiyiddeen, Salim et al. 2012).

4.3.2.3 Analysis of included studies

Four studies looking into the effect of CYP450 polymorphisms on CC response were identified (Ghobadi, Gregory et al. 2008, Legro, Barnhart et al. 2008, Murdter, Kerb et al. 2012, Ji, Jeong et al. 2013). Candidate genes and study design are presented in Table 4-1. Three studies investigated the role of the FSH receptor on CC response (Overbeek, Kuijper et al. 2009, Valkenburg, Van Santbrink et al. 2009, Valkenburg, Lambalk et al. 2011). Details of these studies can be found in Table 4-2.

Author	Gene	Method	Conclusion
Ghobadi et al (2008)	CYP2D6	*In vitro* incubation of enclomiphene with human liver microsomes (4 extensive and 1 poor metaboliser)	No metabolism in poor metaboliser. CYP2D6 is responsible for metabolism of CC.
Li et al. (2013)	CYP2D6	42 women treated with CC for anovulation, measuring CC metabolites day 3 and 10. Genotyping of 31 women.	32 responders, 10 non-responders. Lower metabolites concentrations in non-responders. No relationship proven between polymorphism and CC metabolites.
Legro et al. (2008)	CYP2D6 CYP2C9	RCT of women with PCOS -only CC (102) -only metformin -CC combined with metformin	In only CC group • CYP2D6 C/C 71.3% C/T 26.6% T/T 2.1% Difference in glucose change • CYP2C9 C/C 2.2% C/T 26.9% T/T 71% Difference in FAI and insulin change
Mürdter et al. (2012)	CYP2D6	*In vitro*: 30 liver microsomes classified on CYP2D6 genotypes and formation of CC metabolite In vivo: 4EM volunteers and 2 PM volunteers given 100mg CC, plasma levels of CC metabolites	Strong gene-dose correlation PM had a dramatic lack of clearance

Table 4-1: CYP450 polymorphisms studied in literature

FAI=free androgen index, PM=poor metaboliser

Author	Gene	Method	Conclusion
Overbeek et al. (2009)	FSH receptor (Ser680)	Retrospective, 193 PCOS treated with CC	FSHR-polymorphism and CC resistance: Asn/Asn 26% (15% resistance), Asn/Ser 50% (14% resistance), Ser/Ser 24% (28% resistance)
Valkenburg et al. (2009)	FSHRp.N680S	Prospective, 155 women with WHO2 anovulation treated with CC.	680S and ongoing pregnancy HR per allele 0.76[0.55-1.05], P=0.10
Valkenburg et al. (2011)	FSH receptor (rs6166, Asn680Ser)	Genotyping was performed in 159(first cohort) and 185 (second cohort) women. Outcome parameters were ovulation rate and ongoing pregnancy rate.	FSH receptor polymorphism (rs6166) was associated with clomiphene resistant anovulation. Pooled analysis showed 89% higher chance of anovulation (OR 1.9 [95%CI 1.1-3.3]) in homozygous carriers of the FSH receptor variant (Ser680). The pooled analysis did not show association of ongoing pregnancy rates with FSH receptor genotype.

Table 4-2: FSH receptor and CC response.

HR=hazard ratio

The previously unmentioned study by Costa et al. (Costa, Dornelles et al. 2012) investigated the *in vitro* antioxidant properties of CC. The hypothesis was that genetic alteration in antioxidant enzymes can lead to chronic oxidative stress which has been associated with poorer oocyte quality and ART outcome. CC has a potential anti-oxidative capacity. The authors genotyped 58 women for Val16AlaSOD2 polymorphism distributed as AA 25.9%, VV 27.6% and AV 46.6%. The AA genotype displayed a more responsive antioxidant effect than other genotypes.

Legro et al. (2008), apart from the genes mentioned in Table 6-1, also investigated ESR1 (C/T) and STK11(C/T, C/G). STK11 is a kinase gene expressed in the liver and implicated in metformin action. The authors demonstrated a negative trend for ovulation in C/C [OR 0.53 (90%CI 0.30-0.95) P=0.071] and C/G [OR 0.60 (90% CI 0.35-1.02) P=0.12] compared to G/G genotype. The ESR genotypes did not demonstrate any relationship with ovulation (Legro, Barnhart et al. 2008). None of the included studies had blinding or sample size calculation.

Study	Newcastle-Ottawa scale		
	selection	comparability	outcome
Costa et al. (2005)	**	**	**
Ghobadi et al (2008).	**	*	*
Ji et al. (2013)	*	**	**
Legro et al	**	*	**
Murdter et al. (2012)	**	**	**
Overbeek et al (2009)	**	**	**
Valkenburg et al. (2009)	**	**	**
Valkenburg et al. (2011)	**	**	**

Table 4-3: Newcastle Ottawa scale.

4.3.3 Discussion

The described studies give an insight to the underlying genetic factors to the response to CC. CYP450 and the FSHR seem to play a role. However, the ultimate outcome being; confirmed ovulation, or pregnancy rate, is still far from being correlated with specific genotypes.

CC response is a complex multifactorial process. CYP450 and FHSR are the most investigated targets. There is evidence that CC resistance is more prevalent in FSHR polymorphism, however CYP2D which is responsible for the metabolic pathway of CC seems to be of more importance. The antioxidant enzyme manganese superoxide dismutase (SOD2) is considered a key molecule involved in female reproductive metabolism. A functional SOD2 gene polymorphism (Val16Ala) could influence the *in-vitro* antioxidant clomiphene citrate response. Costa et al. (2012) speculate that alterations in SOD2 activity caused by the Val16Ala polymorphism can result in differential responses to drugs such as clomiphene citrate (Costa, Dornelles et al. 2012).

There are many other enzymes that may play a role in the outcome of ovulation induction of which one might be STK11 (Legro, Barnhart et al. 2008). *STK11* phosphorylates and activates AMP-activated protein kinase, the deletion of *STK11* in the liver of adult mice resulted in hyperglycaemia with increased gluconeogenic and lipogenic gene expression. Its presence was also required for metformin efficacy (Shaw, Lamia et al. 2005). Ghobadi et al. (2008) proves that CYP2D6 metabolises CC by performing a comparison of extensive and poor metabolisers. The genotype of these metabolisers is not described (Ghobadi, Gregory et al. 2008).

For CYP450 2D6 is the key player with alleles *3 *4 *5 *6 *7 *8 defined as poor metabolisers, alleles *9 *10 *41 as intermediate and carries of no variant as extensive and more than two extensive as ultra-rapid (Zanger, Raimundo et al. 2004). Regarding FSHR the SER/SER variant is the one associated with CC resistance (28%) and anovulation (OR 1.9 [95%CI 1.1-3.3]) (Overbeek, Kuijper et al. 2009, Valkenburg, Lambalk et al. 2011).

The type of identified studies varies, making direct comparison difficult. Six studies are conducted *in vivo* (Legro, Barnhart et al. 2008, Overbeek, Kuijper et al. 2009, Valkenburg, Van Santbrink et al. 2009, Valkenburg, Lambalk et al. 2011, Murdter, Kerb et al. 2012, Ji, Jeong et al. 2013) and 3 are *in vitro* (Ghobadi, Gregory et al. 2008, Costa, Dornelles et al. 2012, Murdter, Kerb et al. 2012), with one study (Murdter, Kerb et al. 2012) investigating both methods.

Outcomes in all studies are well defined, but not comparable. The two studies of Valkenburg et al. (Valkenburg, Van Santbrink et al. 2009, Valkenburg, Lambalk et al. 2011) were done in the same population which strengthens their results. It also highlights the need for replication of results before coming to a final conclusion as in the pooled data analysis the ongoing pregnancy rate was no longer significantly different as previously found in the first study group.

To be able to find significant differences between polymorphisms, sample sizes could have been calculated in advance. The numbers at present are too low to draw any definitive conclusion.

The pathway of CC involves the metabolism into the active components. CYP450 play a pivotal role in this process. The FSH receptor is the target

for these metabolites. Genetic research has targeted these 2 points for finding an explanation for unwanted responses.

Larger studies with prospective genotyping and follow up of CC metabolites and outcome will be needed to assess the clinical implications. Pharmacokinetics on its own will not be conclusive and will need to be combined with other confounding factors such as BMI, ethnicity, hormonal profile and combination therapy (e.g. metformin). Thus creating the possibility for individualised medicine preventing unwanted outcomes.

Based on the available literature presented in this systematic review CYP2D6 was deemed most promising to be further investigated.

4.4 Materials and methods

4.4.1 Experimental study design

The study was designed as a retrospective cohort study. Ethical approval for the study was obtained (13/EM/0148). All women on CC during years 2007-2012 at the Fertility Clinic, Queens Medical Centre, Nottingham were sent a letter inviting them to participate in the study. The recruitment to the lasted 12 months, starting in May 2013.

In accordance with the study protocol, postal addresses of all patients in the clinic's data base were obtained. An envelope containing an invitation letter, the patient information sheet, a participation response slip and a return envelope were sent to all patients. If a patient expressed interest in the study, they were contacted by phone or email, depending on their preference. An appointment for signing of the consent form and taking of the blood sample was made at a time convenient for the patient.
Patients that did not respond to the first invitation to participate were sent a second invite envelope containing the same content as the first. If a patient did not respond to the second invite, no further actions were undertaken. Patients having IVF treatment in a tertiary University affiliated fertility clinic who had Clomid in the past were also asked to participate.

Women who consented to take part in the study had a single blood sample (5ml) taken and their clinical records were examined. In case of logistic challenges, the patients were offered to provide a saliva sample.

The design of the study did not involve any additional visits to the clinic apart from the blood sample collection. If for whatever reason there was a

difficulty in withdrawing blood in the first attempt, no further attempts were made to include the participants into the study. Potential study subjects had the right and ability to decline to participate. It was made clear that the participant can withdraw from the study at any point before the samples were anonymised. After anonymisation of DNA samples, it was not possible to withdraw from the study as there was no mechanism to track the clinical data and biological samples to the demographic data of the patient.

4.4.2 Outcomes

Primary outcome measure was the response to clomiphene therapy defined as:

1) Normal- 1 to 2 follicles (>17mm) formed after CC treatment
2) No response- no follicles formed after CC treatment
3) Exaggerated response- formation of more than 3 follicles (>17mm) after CC treatment

Secondary outcomes consisted of determination of clomiphene response phenotype based on genetic data.

4.4.3 Tissue storage

Obtained blood was stored at the tissue bank in Nottingham and transported to the Wolfson Centre for Personalised Medicine Laboratory in Liverpool for analysis. Long term storage of DNA samples was provided by the University of Liverpool facility in the Wolfson Centre for Personalised Medicine in the Department of Molecular and Clinical Pharmacology. The samples were coded for the duration of the study, after that the samples were anonymised, no tracking to the individual participant demographic

information was possible. Personal patient data was not accessible to the laboratory research team.

4.4.4 Sample size calculation

The available database of the host Unit consisted of 700 women having had treatment with CC for ovulation induction. In the literature, it is estimated that approximately 30% of women do not ovulate after CC therapy. Those women would be considered as non-responders to the standard treatment protocol. With the assumption of a ratio of responder to non-responder to CC of 70:30, and an expected participation rate of 50%, 250 women in CC were responders and 100 non-responders. This number would provide 80% power with 5% α error to detect a small difference (OR 2-30) for higher frequency alleles (allelic frequency > 10%) or a larger difference (OR 4-5) for low frequency alleles (>5%).

4.4.5 Statistical analysis

Standard parametric (mean and standard deviation and t-test in normal distribution) or nonparametric methods (signed rank, Mann-Whitney) were used. For continuous data analysis, analysis of variance (ANOVA) and for case control analysis chi squared test and Fisher exact test was used. Correction for multiple comparisons was conducted using false discovery rate.

4.4.6 Genotyping

DNA was extracted from the whole blood samples using the Chemagic Magnetic Separation Module I protocol. In brief, the blood samples were first defrosted by leaving them at room temperature for 30mins to thaw. While the samples were defrosting, the racks were prepared.

- Position 1: Rack with disposable tips and place on the tracking system
- Position 2: Rack with 50 ml tubes and labelled clearly with study ID number. 7.5 ml Lysis Buffer 1 was dispensed into each tube
- Position 3: Rack with 50 ml tubes containing 10 ml Wash Buffer 3 in each tube
- Position 4: Rack with 50 ml tubes containing 10 ml Wash Buffer 4 in each tube
- Position 5: Rack with 50 ml tubes containing 10 ml Wash Buffer 5 in each tube
- Position 6: Rack with 50 ml tubes containing 2 ml Wash Buffer 6 in each tube
- Position 7: Rack with 50 ml tubes containing 10 ml Wash Buffer 6 in each tube
- Position 8: Rack with 50 ml tubes clearly labelled with study ID and 0.5 ml Elution Buffer 7 pipetted into each tube

Lyophilized Protease was dissolved in H_2O (according to volume given on the label). Residual protease solution was stored at 4 °C. The dissolved protease could be used for up to 4-6 weeks.

The blood lysate was prepared once the blood samples were completely thawed. 20 µl of protease was added into each tube containing the lysis buffer in position 2. This rack was then placed into the safety cabinet,

between 1-5ml of blood was pipetted into respective prelabelled tube containing the lysis buffer and protease. Each samples was treated individually (a separate pastette used for each sample). The sample and the aliquot tube held the study ID visible before separation. Once started, the procedure was completed without interruption until the tube was capped.

For blood samples collected in 9ml EDTA tubes, 4-5mls of blood was left in the original EDTA tubes as backup. If less than 5mls of whole blood was collected, at least 1ml of blood was left in a cryovial as backup.

Once all blood samples were added, the tubes were replaced in position 2 on the tracking system. Protocol A "Lysate mixing Blood5k V060330.txt" was selected and the automated lysate mixing was started by pressing the Start button; the lysate was mixed for 10 minutes.

After Protocol A was finished, all racks were placed with prefilled tubes in the given positions. Rack 2 was taken from the tracking system: 19.5 ml of Binding Buffer 2 and 500 µl Magnetic Beads (resuspending the beads carefully before adding to the lysate) were added to the lysate. The tubes were returned with the lysate to position 2 on the tracking system. This was done immediately as delay could results in low yield and purities.

Protocol B "chemagic DNA Blood5k elution in 50 ml tubes V080108.txt" was selected and the automated isolation was commenced.

Upon completion, any backup blood samples were stored in a -20ºC or -80ºC (if available) freezers. And the extracted DNA samples were stored in labelled eppendorfs in a cool room.

DNA was extracted from the saliva samples by putting the collection tubes in individual plastic bags and leaving them in a 50°C water bath for 2 hours. After this, the samples were pipetted into labelled 15ml tubes. Purification solution was added and the samples were placed on ice for 10 minutes. The samples were centrifuged for 10 minutes at 4600rpm at 21°C. The supernatant was decanted into a new labelled tube and 100% ethanol was added in the same volume as the samples (4-4.5ml). This allowed the DNA strings to become visible. The samples were gently shaken and left for 10 minutes. It was centrifuged once more for 10 minutes at 4600rpm at 21°C.

The samples were joined to the batch of samples retrieved from whole blood.

Initial quantifying measurements of the amount of nuclei acids per sample were done using Nanodrop® (Thermo Scientific). This spectrometer allowed 8 samples to be simultaneously be captured between an array of upper and lower optical surfaces. The software calculated the concentration in ng/µl .The extracted DNA was further quantified using Picogreen® reagent kit (Herefor Quant-iT™ PicoGreen® dsDNA Assay Kit; 2000 assays; 10 x 100 µL; supplied by Invitrogen; catalogue number P11496). A 1X TE working solution was prepared by diluting the concentrated buffer 20-fold with sterile, distilled, DNase-free water (10mM Tris-HCl, 1mM EDTA, pH7.5). For a complete 96 well plate 2.5ml of 20X TE was added to 47.5ml of DNase-

free water. TE buffer is also known as $T_{10}E_1$ buffer (1mlTris-HCL and 0.2ml EDTA with double distilled water up to 100ml) is has a pH of 8.0i, solubilizing DNA while protecting it from degradation.

The DNA standard curve was here after prepared: Diluting the lambda DNA standard (100µg/ml), provided in the Quant-iT™ 50-fold in 1X TE to make a 2 µg/ml working solution. 5µl of the 100 µg/ml dsDNA standard and 245µl of 1X TE was pipetted into a sterile Eppendorf tube. Briefly mixed by pipetting up and down. A five-point standard curve from 1 ng/ml to 1 µg/ml was prepared, diluting the 2 µg/ml DNA stock solution in sterile Eppendorf tubes.

Unused 1ng/ml standard and 2 µg/ml DNA stock was stored with the Quant-iT™ PicoGreen® kit for further assays. 100µl of each DNA standard was added to the wells of a 96-well plate according to the following wells:

[DNA Standard] (ng/ml)	Wells
2 µg/ml	A1 + A2
200 ng/ml	B1 + B2
20 ng/ml	C1 + C2
2 ng/ml	D1 + D2
0 ng/ml	E1 + E2

A plate plan was made allocating samples to a corresponding well on the plate. For samples containing under 200ng/µl of DNA by nanodrop, 1µl of samples was added to 99ul 1xTE (1/100 dilution) in the corresponding well. For samples containing over 200ng/µl of DNA by nanodrop, 1 to 9ul 1xTE (1/10 dilution) was added in addition to the 1µl diluted solution to 99µl 1xTE (total 1/1000 dilution).

On the first day of the experiment, Quant-iT™ PicoGreen® reagent was allowed to warm to room temperature before opening the vial. In a plastic container, a diluted working solution of the Quant-iT™ PicoGreen® reagent was created by making a 200-fold dilution of the concentrated solution in 1X TE. For a 96-well plate, enough diluted solution was prepared to assay 100 samples in a 100µl final volume per well, adding 50µl of Quant-iT™ PicoGreen® dsDNA reagent to 9.95ml of 1X TE.

The working solution was protected from light by covering it with foil or placing in the dark. The solution was used within a few hours of its preparation.

In reduced lighting, 100µl of the aqueous working solution of PicoGreen® reagent was added to each test sample and the DNA standard. The plate was then incubated for 2 to 5 minutes at room temperature protecting it from light. The sample fluorescence was measured using the Beckman Coulter DTX880. . The assay plate was placed on the equipment tray, ensuring that well A1 is in the left-rear position.

DNA concentration results were recorded in Excel (Microsoft Corp). The final concentration (ng/µL) of each well was calculated by using the constructed standard curve.

Once the concentrations of DNA were known, dilutions for PCR were made. The first dilution was 50µl of a 20ug/µl concentration in labelled Eppendorf safe-lock centrifuge tubes. Samples which were ≤ 20µg/µl were not further diluted. A 96 well plate was made containing 10% replicates and 2 blank wells. As this study had 203 samples, 3 plates were made; plate 1 and 2 with 84 samples, 10 replicates (same sample being analysed twice for quality control) and 2 blanks and a third plate with 35 samples and 3 replicates and 2 blanks. According the plate plan a 96 well plate was made containing 15µl DNA solution per well. These were the master plates from which the plates for PCR were pipetted. This was to assure no accidental sample order mistakes could be made. For the assay, 1µl using a multi-channel pipette was pipetted into the plate. The plate was centrifuged to make sure the drop was at the bottom of the well.

For genotyping, plates were spun in a dryer for 10 minutes. That plate was taken to the PCR free room. The assay for the chosen allelic discrimination was taken out of the freezer to defrost. It was shorty (approximately 1 second) spun to ensure it was mixed well. Distilled water was pipetted into an Eppendorf, the mastermix was added and lastly the assay was added (see below). The Eppendorf was closed and gently shaken. The mix was made according to the manufactures instruction (5µl 2x TaqMan Mastermix,

2.5µl TaqMan CN assay, 0.25µl TaqMan CN and 2.25µl dH2O) for the chosen SNP displayed in Table 4-4.

Assay	CYP2D6_rs1065852A	CYP2D6_rs28371725	CYP2D6_rs35742686	CYP2D6_rs3892097
Reference assay RNase	C_11484460_40	C_34816116_20	C_32407232_50	C_27102431_D0

Table 4-4: Four chosen assays for genotyping.

10µl of volume from the 20µg/µl concentration was transferred in a new 96 well plate. 10µl of dH_2O was added to get 10µg/µl. One µl was pipetted in the 384 well plates, duplicating each plate.

After the mastermix was made, using a multichannel pipette 5µl of the solution was distributed in all wells. An optical seal was placed over the 384 well plate, making sure that all wells were well sealed. The plated was centrifuged for 5 minutes.

Two independent PCR experiments were run using the same plate lay-out. This meant that some of the samples have been genotypes more than twice (as on the plate there were 10% duplicates already). This was to improve the quality of the obtained data.

Copy number variation by CopyCaller® software v2.0 from Life Technologies was obtained to ensure that deletions were real deletions and not absent DNA samples. The automatic real time PCR machine's software was used to select the appropriate detector for a 5µl reaction with labelling as shown in Table 4-5.

	CYP2D6_rs1065852 A	CYP2D6_rs28371725	CYP2D6_rs35742686	CYP2D6_rs33892097
Allele x (VIC)	A	C	T	C
Allele y (FAM)	G	T	deletion	T

Table 4-5: Corresponding base in assay.

4.5 Results

It was possible to identify 922 women who had Clomid ovulation induction through database search. 133 women responded after the first round of contact. An additional 70 responded to the second invitation letter. A total of 203 samples could be collected. DNA was extracted for analysis from 200 samples.

The demographics of the complete database of 922 can be found in Table 4-6; 25.2% had under response, 67.3% normal response and 7.5% overresponse. Study participants had 28.6% underresponse, 60.6% normal response and 10,9% overresponse.

	Under Responder N=232	Normo Responder N=621	Over Responder N=69
Baseline FSH (IU/L)	5.8±2.2	5.7±2.2	6.3±3.1
Baseline LH (IU/L)	9.0±7.1	8.8±11.2	8.7±10
BMI (kg/m^2)	25.6±3.9	24.8±4	23.7±4
Endometrial thickness (mm)	9.0±2.8	9.6±2.9	9.4±3.2
LH/FSH ratio	1.7±1.2	1.7±2.1	1.1±1

Table 4-6: Characteristics divided by clinical response whole data base (mean ± SD).

Demographics of the participants can be found in Table 4-7, with no significant differences between groups of interest.

	Under responder N=57	Normoresponder N=121	Overresponder N=22
Baseline FSH (IU/L)	5.8+/-±2.2	5.6±2.3	5.8±2.3
Baseline LH (IU/L)	7.0+/-±6.4	9.6±6.5	9.0±7.5
BMI (kg/m^2)	24.7+/-±3.7	26.1±4.3	22.6±3.3
Endometrial thickness (mm)	9.5+/-±2.9	8.4±3.1	9.5±3.3
LH/FSH ratio:	1.3+/-±1.2	1.8±1.1	1.4±0.9

Table 4-7 Characteristics divided by clinical response from included women.

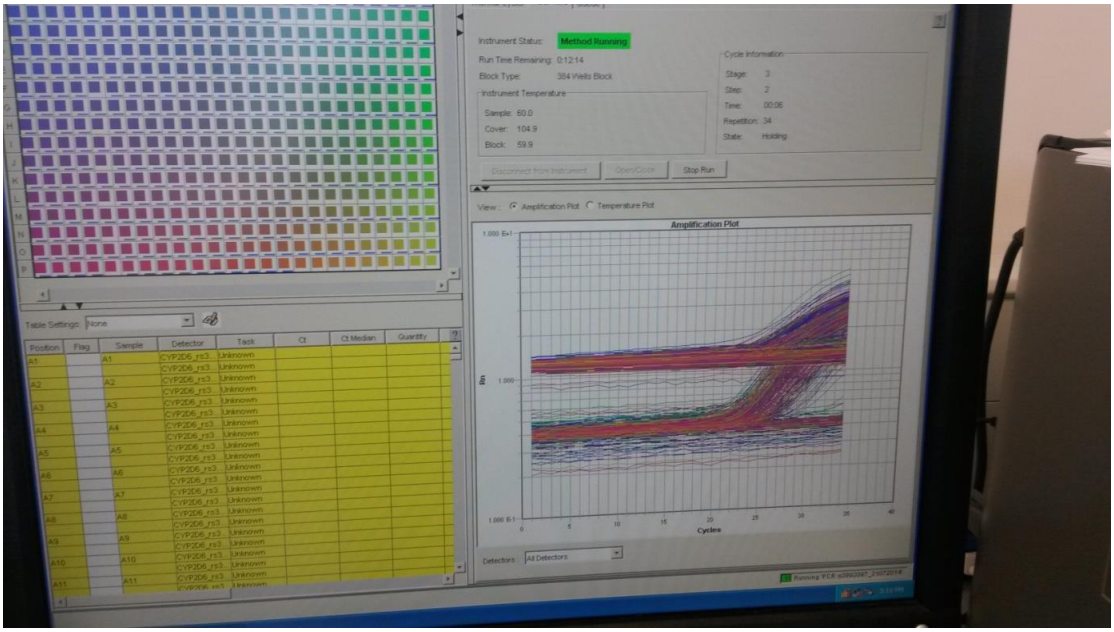


Figure 4-4: Screen shot of PCR output graphs.

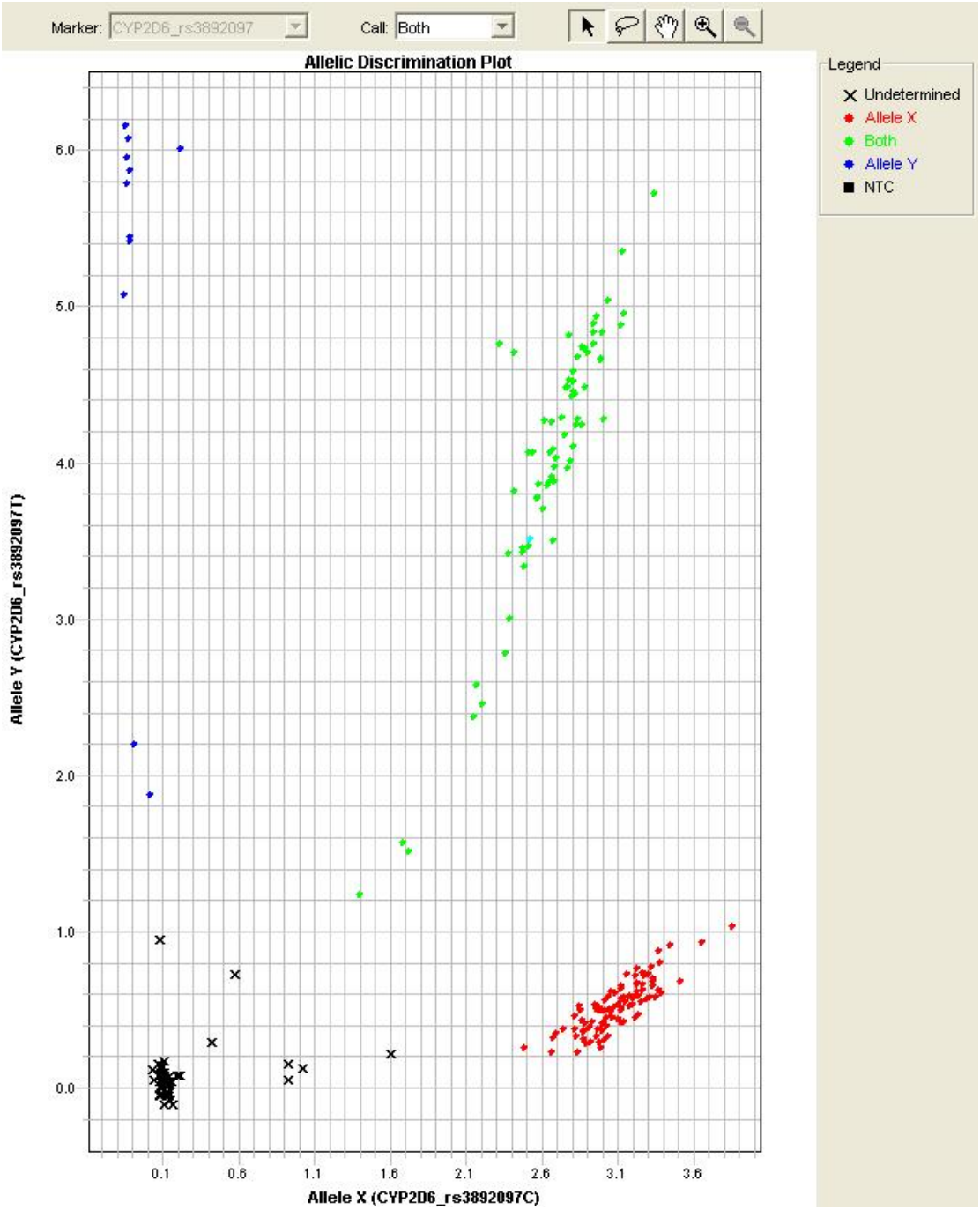


Figure 4-5: Allelic distribution plot

The cluster on the X axis (red) represent samples with 2 the same alleles, as does the cluster on the Y axis (blue). Samples with both alleles are found in the middle (green). The samples for which the PCR did not yield a result are in the left bottom corner (black) and are classified as undetermined.

The allelic distribution per SNP investigated can be found in Table 4-8, Table 4-9, Table 4-10 and Table 4-11 and in figure 6-12. It was not possible to classify 73 samples due to repeat failure in PCR.

	CYP2D6_rs1065852(A/G) Consensus
AA	13
AG	75
GG	107
Failed samples	5

Table 4-8: Allelic distribution observed for rs_1065852.

	CYP2D6_rs28371725(C/T) Consensus
CC	162
CT	26
TT	4
Failed samples	8

Table 4-9: Allelic distribution observed for rs_28371725.

	CYP2D6_rs35742686 (T/-) consensus
TT	147
T-	1
Deletion	0
Failed samples	52

Table 4-10: Allelic distribution observed for rs_35742686.

	CYP2D6_rs3892097(C/T) consensus
CC	114
CT	67
TT	11

Failed samples	8

Table 4-11: Allelic distribution observed for 10rs_3892097.

The Hardy-Weinberg equilibrium is a principle stating that the genetic variation in a population will remain constant from one generation to the next in the absence of disturbing factors. When mating is random in a large population with no disruptive circumstances, the law predicts that both genotype and allele frequencies will remain constant because they are in equilibrium. The Hardy-Weinberg equilibrium can be disturbed by a number of forces, including mutations, natural selection, non-random mating, genetic drift, and gene flow (Crow 1999). The Hardy/Weinberg proportions for our study subjects can be found in Table 4-12, Table 4-13, Table 4-14 and Table 4-15.

CYP2D6_rs1065852(A/G)	Frequency
Homozygote (GG)	0.55
Heterozygote (AG)	0.38
Homozygote (AA)	0.07
Allele G	0.74
Allele A	0.26
χ^2 test *P* value =	0.976719

Table 4-12: Allelic frequency and genotype (in samples with available results), consistent with expected frequencies.

CYP2D6_rs28371725(C/T)	frequency
Homozygote (CC)	0.84
Heterozygote (CT)	0.14
Homozygote (TT)	0.02
Allele C	0.91
Allele T	0.09

χ^2 test *P* value =	0.025681

Table 4-13: Allelic frequency and genotype (in samples with available results) the observed genotype is not consistent with the Hardy/Weinberg equilibrium.

CYP2D6_rs35742686 (T/-)	frequency
Homozygote (TT)	0.99
Heterozygote (T-)	0.01
Homozygote (--)	0.00
Allele T	0.997
deletion	0.003
χ^2 test *P* value =	0.967105

Table 4-14: Allelic frequency and genotype (in samples with available results), consistent with expected frequencies.

CYP2D6_rs3892097(C/T)	frequency
Homozygote (CC)	0.59
Heterozygote (CT)	0.35
Homozygote (TT)	0.06
Allele C	0.77
Allele T	0.23
χ^2 test *P* value =	0.780912

Table 4-15: Allelic frequency and genotype (in samples with available results), consistent with expected frequencies.

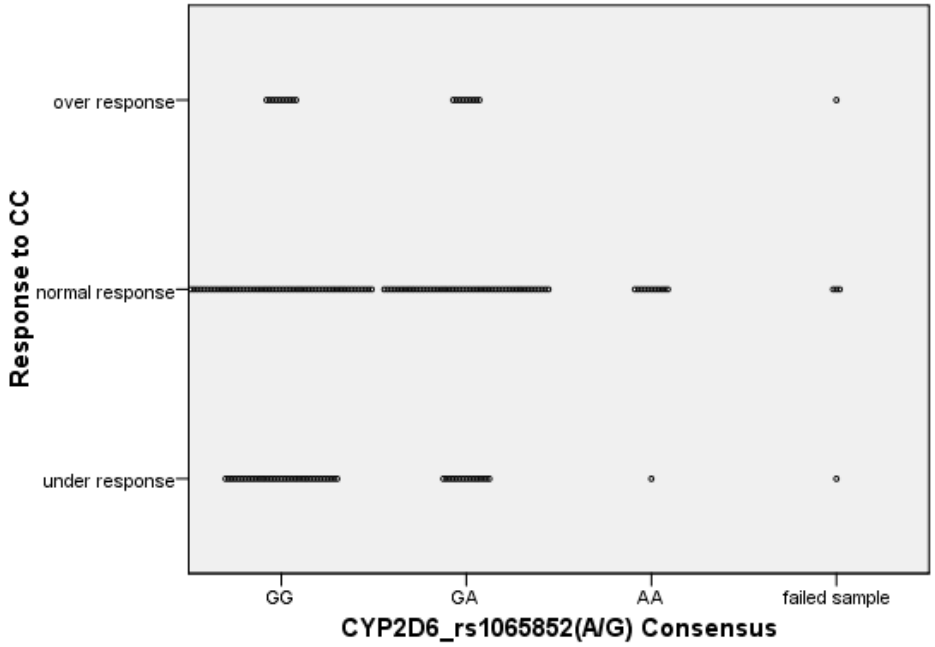
The frequency of genotype of each of the four studied SNPs and the corresponding response can be found in Table 4-16. The visual representation of the SNPs frequencies is presented in Figure 4-6. As

demonstrated, normal response to CC is more common in homozygotes alleles for all SNPs tested.

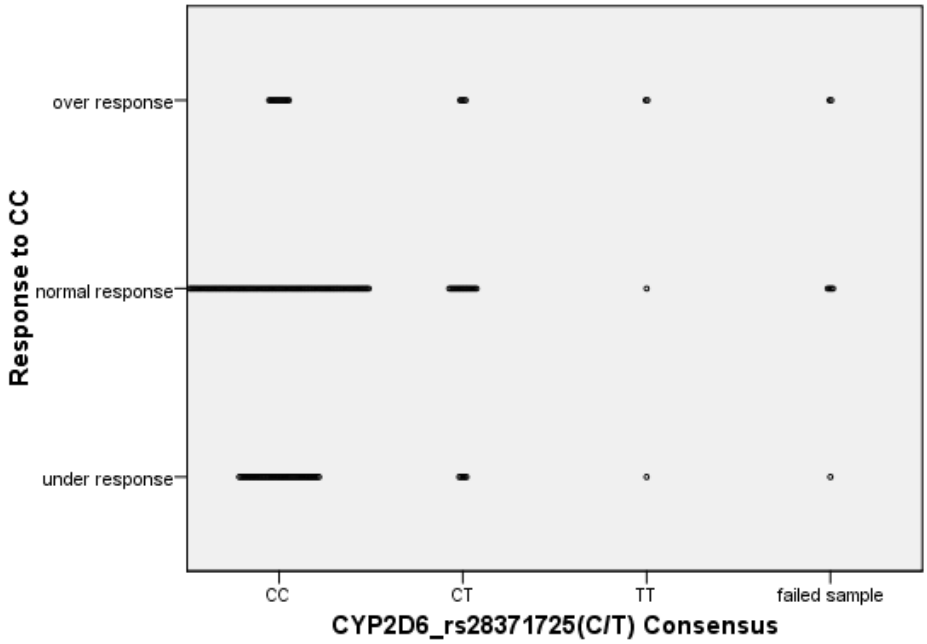
		Under response to CC	Normal response to CC	Overresponse to CC
CYP2D6_rs1065852(A/G) Consensus	GG	35	56	10
	GA	15	51	9
	AA	1	11	0
CYP2D6_rs28371725(C/T) Consensus	CC	45	100	12
	CT	5	16	4
	TT	1	1	2
CYP2D6_rs35742686 (T -) Consensus	TT	43	86	13
	T -	0	1	0
	deletion	0	0	0
CYP2D6_rs3892097(C/T) Consensus	CC	37	63	9
	CT	14	43	9
	TT	1	10	0

Table 4-16: SNP genotype frequency and associated CC response.

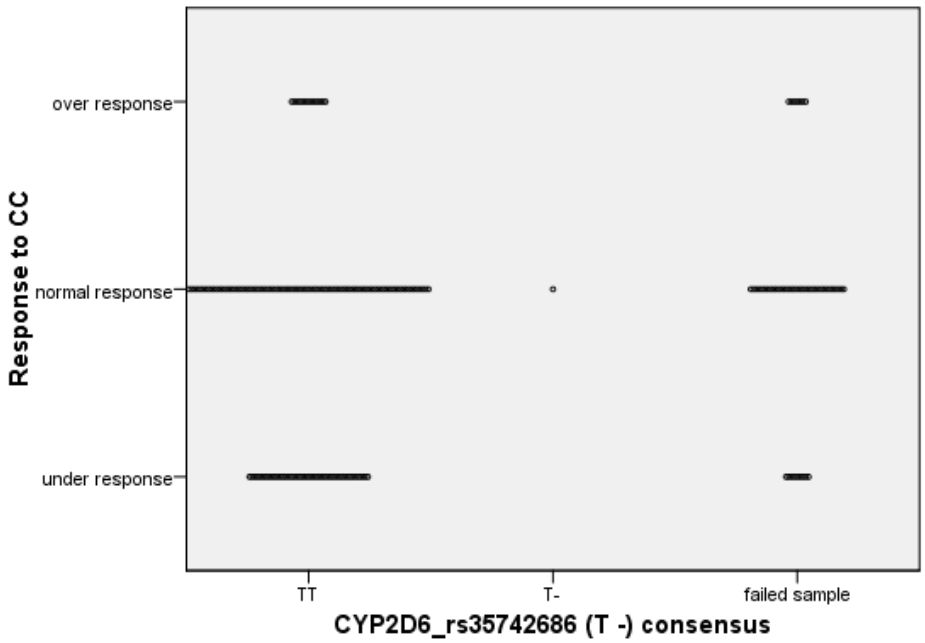
The below plot diagram gives a visual representation of majority of genotype being GG followed by GA alleles for CYP2D6_rs1065852. Most women in this group had a normal response to ovulation induction with CC. In the minority group with AA alleles, a normal response to CC is still most prevalent.



The below depicted plot diagram gives a visual representation showing majority of the study population to have the CC genotype for CYP2D6_rs28371725. Majority in this group had a normal response to CC, followed by under response and a small proportion of over response. In the smaller CT alleles group, again normal response is most prevalent, but under or over response are similar in numbers. The rare group having TT alleles have similar, but very small numbers in all response groups.



The following plot diagram is an illustration of the whole study population, excluding one sample, being TT genotype CYP2D6_rs35742686. Compared with the 3 other plot diagram, the high number of failed samples can be observed.



The last diagram for CYP2D6_rs3892097 shows the highest frequency to be CC alleles configuration followed by CT and only a small proportion of TT. Normal response to CC overruled in all groups, followed by under response and a similar number of over response in the first 2 groups. In the small third group there was only 1 under response and no over responses.

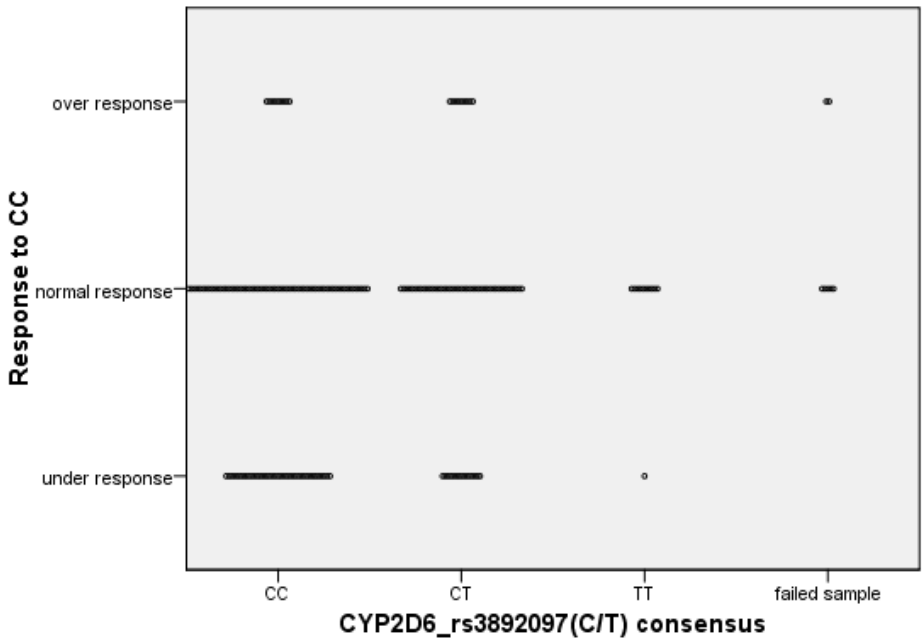


Figure 4-6: Dot plots of the 4 SNPs and CC response

4.6 Discussion

As most study designs in clinical pharmacogenetics, this study has been based on a candidate gene approach. There are only limited RCTs available in this field (Legro, Barnhart et al. 2008). The conducted systematic review of literature demonstrated a lack of available data for meta-analysis. And the descriptive analysis demonstrated that multiple genes are involved in the pathway for CC response.

The presented allelic frequencies in this study are consistent with the Hardy-Weinberg equilibrium, apart from CYP2D6_rs28371725(C/T). No correlations could be found with the ovulatory response. This is however the first study, with a sample size of 200 participants.

Limitations of this study include the response rate to the invitation being lower than expected at 22% (203/922). Data on cycle regularity or ultrasound appearance of the ovary was not available. The data base did not comment on the indication of treatment, being anovulation or unexplained infertility. The diagnosis of PCOS was not recorded in the database. However, some blood tests can be indicative for PCOS. The ratio of LH to FSH is elevated in half of women with PCOS (Banaszewska, Spaczynski et al. 2003). A higher LH level can negatively influence folliculogenesis (Wiser, Shehata et al. 2013), however the study data reported in this work did not demonstrate elevated LF/FSH ratios. Unfortunately, some SNPs could not be tested due to repeated failures of sample analysis.

Legro et al. (Legro, Barnhart et al. 2008) obtained CYP2C9 data on 93 PCOS patients who had clomiphene citrate: 2CC, 25CT, 66 TT, and about 94

CYP2D6 patients: 67CC, 25CT, 2TT. However, no data regarding response could be obtained from the original article. Ghobadi et al. (Ghobadi, Nguyen et al. 2007) tested the predictive model and a normogram constructed by Imani et al. (Imani, Eijkemans et al. 2002), which included free androgen index, menstrual pattern and BMI. However, this correlation could not predict the optimum dose required for an individual patient. Thus, the treatment with CC still requires empirical titration to establish the lowest effective dose and therefore has limited practical implications for clinical practice. This makes the use of phamacogenetics a more promising option.

Specific groups can be targeted, who may benefit from genotype-guided clomiphene dose adjustments based on the expected metabolism. The first group of women would be women who do not respond to therapy because they achieve a low plasma concentration of clomiphene after a standard dose due to an increased metabolism of the drug, they may benefit from dose increment. Women who are at risk of developing adverse effects due to slow metabolism of the drug, may benefit from a reduced starting dose. Women who do not respond to therapy despite achieving high plasma concentrations, they may benefit from changing to alternative therapy sooner to save time as the age of a woman is an important factor when trying to overcome infertility.

This retrospective study investigated an association between response to clomiphene measured by the number of follicles, and functional polymorphisms in genes encoding metabolising enzymes. As technologies for genome-wide association studies become more accessible, the

possibility to perform unbiased genome-wide screens to investigate genetic factors influencing drug-induced ovarian stimulation will arise.

A large number of genetic variants in both, pharmacokinetic and pharmacodynamic pathways of clomiphene exist and have been investigated in relation to many drugs. However, to our knowledge no studies have investigated treatment response to clomiphene and polymorphisms in metabolising enzymes in women undergoing ovulation induction for fertility treatment. Future studies should therefore be conducted to investigate this lack in knowledge.

The development of OHSS with ovulation induction using gonadotropins or clomiphene citrate is poorly studied. Anecdotal cases can be found in literature, but the true incidence is unknown (Thakur and El-Menabawey 1996, Abbas and Rouzi 2013). Recognized patient characteristics of women at risks for OHSS include previous OHSS, young, low BMI, PCO(S), high AMH and single nucleotide polymorphism for FSH-R, LH-R, E2-R, AMH-R (Humaidan, Quartarolo et al. 2010) as well as treatment characteristics, such as a rapid rise of oestradiol or a high serum level (4000pg/mL) (Aboulghar and Mansour 2003, Papanikolaou, Pozzobon et al. 2006). It is a safe assumption, that patients at risk of OHSS during COH are comparable to the ones at risk during ovulation induction, and *vice versa*. Checa et al. concluded in a small prospective study that an antral follicle count of 16 is predictive (sensitivity of 100%, specificity of 93%) for exacerbated response during ovulation induction leading to cycle cancellation, but not commenting on OHSS risk (Checa, Prat et al. 2010).Therefore, patients who hyperrespond to Clomiphene citrate are likely to overrespond to ovarian

stimulation and would therefore be classified as being at risk of OHSS, which is one of the groups of interest in this dissertation. Genotyping in this case might aid in primary prevention and personalized gonadotropin dose adjustments. Being cautious that the response to CC is reliant on its metabolism, and therefore the response to exogenous FSH may not be directly correlated. As such, the link between CC overresponse resistance and the response to gonadotropins should be further explored.

The understating of CC metabolism is important for the initial treatment of patients undergoing ovulation induction. Knowledge of CC pharmacogenetics could lead to the establishment of personalised approach to fertility treatment resulting in less adverse effects and better pregnancy outcomes. However, this knowledge can transcend the fertility population and be similarly extrapolated to cancer treatment, in which newer SERMs are being used. As our knowledge of pharmacogenetics is just being unravelled, there is still a great deal to be done before wide application of this approach. Larger studies assessing the genetics of CC metabolism are necessary to validate the lack of the associations found in this study. An analysis of association of aberrant CC response and response to gonadotropins would aid in prediction of response to IVF without undue risk of developing OHSS. Personalized medicine appears to be the future of medicine and CC pharmacogenetics can provide additional information for the infertile population.

Chapter 5: Ovarian Renin Angiotensin System and the relationship with ovarian follicles: a pilot study

5.1 Introduction

5.1.1 Renin Angiotensin System (RAS)

RAS may be involved in folliculogenesis and the development of OHSS (see section 1.8.1.4). Renin is an enzyme that is most known for its role in the regulation of blood pressure. It acts by releasing angiotensin I (AGT1) from angiotensinogen which is produced in the liver. AGT1 is in its turn converted to angiotensin 2 by a converting enzyme cleaving off two amino acids. The resultant octapeptide (previously called hypertensin, or angiotonin) causes vasoconstriction resulting in a rise in both systolic and diastolic blood pressure (Speroff 2005). The major source of plasma renin and prorenin is the kidney. However, the ovary is also a source of circulating prorenin, as has been demonstrated in a bilateral nephrectomised woman (Blankestijn, Derkx et al. 1990).

5.1.2 RAS and the pathophysiology of OHSS

The ovarian Renin Angiotensin System (RAS) is thought to be involved in folliculogenesis and the development of OHSS as previously mentioned in section 1.8.1.4. Most symptoms of OHSS are the effect of the cascade of fluid shift from intra to extra vascular spaces. It is widely believed that this is due to increased capillary permeability (Rizk, Aboulghar et al. 1997). Balasch et al. have adopted a different view in which they contribute the

changes in hemodynamics to arteriolar dilation instead of the reduction of circulating fluid (Balasch, Arroyo et al. 1994). However, most investigations concentrate on increased vascular capillary permeability. Many mediators play a role in follicular development and the question remains why in some patients a deregulation occurs, while in others it does not. RAS has been studied as being one of the responsible vasoactive systems contributing to OHSS. RAS has been investigated due to the fact that it has a proven effect on vascular permeability, hCG increases its expression and release, it is found in higher concentrations in cases of OHSS in serum, follicular fluid and ascites, and that inhibition improves OHSS symptoms (Palumbo, Avila et al. 2016).

RAS plays a role in angiogenesis, the formation of new blood vessels, and this increases the vascular permeability leading to extracellular fluid accumulation (Haning, Strawn et al. 1985). The ovary possesses an independent RAS (Blankestijn, Derkx et al. 1990). Prorerine is synthetized in the theca cells and in a natural menstrual cycle its expression peaks in response to the LH surge (Paulson, Do et al. 1989).

Angiotensin 2 plays a role in the autocrine and paracrine modulation of ovulation and is a major contributor to the neovascularisation required in luteinisation (Delvigne and Rozenberg 2002). Active renin levels in follicular fluid increase in follicular phase reaching their peak in the peri ovulation period after a LH surge or hCG trigger, this constitutes the activation of RAS (Itskovitz and Sealey 1987). There is a positive correlation between serum E2 and plasma renin-like activity in follicular fluid (Fernandez, Tarlatzis et al. 1985). In patients suffering from OHSS plasma levels of total rennin and

renin activity were increased even when extra vascular compartments were expanded, confirming the non-renal origin of the rennin (Ong, Eisen et al. 1991). In a study of 21 patients, a positive correlation of plasma renin activity and severity of OHSS was found (Navot, Margalioth et al. 1987). Angiotensin 2, total rennin and prorenin are higher in ascites than in plasma, suggesting an ovarian origin (Delbaere, Bergmann et al. 1994). Renin-like activity and AGT2 were highest in OHSS ascites as well as in peritoneal fluid of women with spontaneously conceived pregnancies, suggesting the stimulatory role of hCG (Delbaere, Bergmann et al. 1997).

Animal studies using rabbit models have demonstrated a reduction in severity of OHSS in the hyperstimulated rabbits by inhibition of RAS. Morris et al performed an experiment with 20 adult female New Zealand white rabbits (Morris, Wong et al. 1995). All rabbits were stimulated with hMG and triggered with hCG, with 2 groups receiving a different dose of enalapril and one placebo group. Total body weight and haematocrit was found to increase significantly in the placebo group. All ten rabbits in the placebo group met criteria for severe OHSS (ascites) whereas only six out of ten in the enalapril groups did. The authors concluded that ACE inhibition resulted in a 40% decrease in the incidence of OHSS in the rabbit model (Morris, Wong et al. 1995). Teruel et al used the same rabbit model with 16 stimulated rabbits and 6 unstimulated rabbits. Of the stimulated rabbits, 6 received additional treatment with captopril, and a reduction in incidence and volume of ascites was noted in 50% of cases. The authors also looked into hemodynamic changes, such as hypotension or decreased vascular resistance. There were no differences found, arguing against the arterial

vasodilation and for the vascular permeability (Teruel, Carbonell et al. 2002).

A case study of 4 women at high risk of developing OHSS described dual renin-angiotensin blockade therapy. The authors combined oral administration of angiotensin-converting enzyme inhibitor and angiotensin II receptor blocker with cryopreservation of the embryos. None of these women developed ascites or clinical OHSS, 7 days after oocyte collection (Ando, Furugori et al. 2003).

It remains controversial if activation of RAS causes OHSS or that OHSS causes activation of RAS. There is an evident contribution of ovarian and renal RAS to the process of OHSS. Ovarian RAS is probably not a *primum movens* in the pathogenesis of OHSS but may catalyse the hemodynamic degradation that occurs in OHSS. Alternatively, the activation of the renal RAS is a homeostatic response to hypovolaemia during OHSS (Manno and Tomei 2008). VEGF-A, the mediator most believed to be contributing to OHSS, is up regulated by ANG2 (Tamarat, Silvestre et al. 2002).

5.2 Study rationale

The aim of this study was to evaluate the correlation between RAS, ovarian volume and number of follicles in order to better understand the possible contribution to OHSS.

This study involves 4 aims.

1. To test if whole blood samples can be stored for RAS analysis at a later stage, in order to simplify the protocol for future research.

2. Identify the best dilution to measure plasma angiotensinogen concentration in samples of women with stimulated ovaries, as different protocol exists for placental or peripheral derived plasma, but none exists for target study group.
3. To assess if renin and AGT serum levels differ in the similar phase of subsequent cycle in the same patient.
4. To measure renin and AGT in the same women in a spontaneous ovulation cycle and in a controlled ovarian stimulation cycle, with or without OHSS, looking at number of follicles and ovarian volume.

5.3 Materials and methods

5.3.1 Study design

The study was designed as a single centre, prospective observational cohort study. Local research ethics committee approval was granted (12/EM/0345) and (13/EM/0277) and (13/EM/0148). The study involved women undergoing IVF or intra- cytoplasmic sperm injection (ICSI) treatment in a tertiary university affiliated fertility treatment centre. As part of the study participants were subjected to a midluteal phase ultrasound, as defined below, carried out using Voluson E8 Expert BT12 (GE Healthcare, Zipf, Austria) equipped with a trans-vaginal transducer (5-9MHz) and blood sampling. Timing of examination was determined by urinary luteinising hormone (LH) kit and scheduled for LH+7 to LH+9, or day 21 ± 2 of the menstrual cycle, in case of absent LH surge. Two experienced sonographers (LTP and MNB) conducted all ultrasound examinations. Recruitment to the

study was over a period of 12 months from February 2013 to February 2014 with no target number.

1. The first cohort of patients underwent standard IVF cycle with controlled ovarian stimulation, transvaginal oocyte retrieval and embryo transfer. The IVF/ICSI protocols were conducted as described in section5.3.2. Sonographic data at these time points was used.
2. The second cohort of patients had an endometrial biopsy in the midluteal phase of an unstimulated cycle and an endometrial biopsy at the same time in the subsequent cycle. During the study visits and the routine IVF procedures, blood samples were obtained for renin and angiotensinogen analysis. Collected data included participants age, duration and cause of infertility, previous obstetric history, and ovarian reserve markers.

5.3.2 Treatment Protocol for controlled ovarian stimulation

Most women (95%) underwent a long agonist protocol. This involved down-regulation with a GnRH agonist (500µg/day Buserelin; suprecur® Sanofi Aventis, or 800 µg/day of Naferelin; Synarel® Pfizer) beginning in the midluteal phase of the menstrual cycle, 7 days before the earliest expected date of menstruation. Successful ovarian suppression was confirmed 2 weeks later through ultrasound evidence of a thin endometrium, measuring <5 mm at the junction of the upper third and lower two thirds in the longitudinal plane, absent ovarian activity, and a serum E_2 level <200 pmol/L. Controlled ovarian stimulation was then begun using either recombinant FSH (Gonal-F; Serono Pharmaceuticals) or purified urinary

hMG (Menopur; Ferring Pharmaceuticals). The starting dose of gonadotropin used for ovarian stimulation was determined on the basis of subject's age (150 IU for women aged <30 years, 225 IU for women aged 30–38 years, and 300 IU for women aged ≥38 years), which was the unit's standard operating protocol at the time of the study; the dose was not modified according to the AFC. This dose was prescribed for 5 days and the patient then scanned to assess the initial response. The subsequent daily dose of FSH was only adjusted (up or down) at this stage if it was thought there was a risk of poor or exaggerated response. Subsequent response was assessed by daily serial transvaginal ultrasound and serum E_2 measurements from the seventh day of stimulation. Human chorionic gonadotropin (6,500 IU of Ovitrelle [Serono Pharmaceuticals] or 10,000 IU of Pregnyl [Organon Laboratories]) was administered when there were at least three follicles measuring ≥18 mm in diameter and oocyte retrieval was performed 36 hours later. The total number of oocytes retrieved was recorded, including the number of mature and immature oocytes. Subjects who did not develop at least three leading follicles measuring ≥14 mm in diameter after 12 days of gonadotropin treatment were advised to discontinue treatment or convert to IUI, depending on other clinical factors including their tubal status and their partner's semen quality. Such cycles were counted as cancelled cycles. One or two normally cleaved embryos, as set out by the Human Fertility Embryology Authority policy, were transferred into the uterus 2, 3, or 5 days after oocyte retrieval, and urine pregnancy test was performed 14 days later to determine the outcome. If the test was positive (biochemical pregnancy), a transvaginal ultrasound was arranged 3 weeks later to confirm a clinical pregnancy, defined as

presence of an intrauterine gestation with evidence of cardiac activity. All pregnancies were followed up to delivery and the outcome recorded.

5.3.3 Participants

In order to address aims 2 and 4, women undergoing ART were investigated (cohort 1). To address aim 3 women in spontaneous menstrual cycles were assessed (cohort 2). Healthy volunteers were used for the first aspect of the hypothesis (cohort 3). For the first cohort, women under the age of 49 undergoing IVF or ICSI treatment using own oocytes were considered eligible. Potential participants were identified during a pre-treatment scan carried out prior to commencement of treatment. If no major uterine anomaly was found, the patient information leaflet was given to the participant and at least 24 hours were given to the woman to familiarise herself with the study. Once the patient gave verbal and written informed consent, a urinary LH kit was supplied.

Cohort1:

The participants having IVF/ICSI treatment subsequently underwent ovarian stimulation with either a long agonist or an antagonist protocol with an adjusted dose of gonadotropins according to the patient's history and ovarian test results. Frozen embryo replacement (FER) cycles were carried out with the aid of down regulation and oestradiol supplementation protocols or in natural cycles. In controlled ovarian stimulation cycles, hCG was administered when at least 3 follicles were ≥18 mm in diameter, followed by transvaginal ultrasound guided oocyte retrieval 36 hours later. Embryo transfer of one or two embryos was conducted two or five days

after the oocyte collection depending on the number of oocytes collected and the fertilization rate. FER was planned when the ET was ≥ 8.0 mm. Luteal phase support was obtained with vaginal progesterone (Cyclogest ® 400mcg) pessaries twice a day starting on the second day after oocyte retrieval. Two weeks after the embryo transfer, the patients performed a home urinary pregnancy test.

Data from the same patient in the luteal phase proceeding the ovarian stimulation in case of a fresh IVF cycle or proceeding a frozen embryo replacement cycle was collected. In case of a stimulated cycle, ovarian volume and plasma were collected during transvaginal oocyte retrieval. During frozen embryo replacement cycles there is no oocyte collection and that data is therefore not available. In all cases ovarian volumes and plasma were collected during embryo transfer. When data sets were completed repeated measurements were available and analysed.

Cohort 2:

The second cohort was comprised of women who had 2 biopsies in subsequent cycles in order to evaluate natural killer cells in the endometrium, which results were used for a different research project. The inclusion and consent process followed the same route as outlined above.

Cohort 3:

In addition, for the third cohort, healthy volunteers were consented to have 2 bottles of blood taken.

5.3.4 Research Ethics Committee application

The research studies were conducted in accordance with the ethical principles originating from the Declaration of Helsinki on Ethical principles for Medical Research Involving Human Subjects, adopted by the General Assembly of the World Medical Association (1996), and the principles of the International Conference on Harmonization guideline on Good Clinical Practice.

The ethical application process was carried out through the Integrated Research Application System (IRAS) website (https://www.myresearchproject.org.uk/). All study templates for protocols, patients information leaflets, consent forms and posters were obtained from Nottingham University Hospitals Research and Development website (http://nuhrise.org/) or University of Nottingham Clinical Trials Unit website (http://ctu.nottingham.ac.uk/ctu/default.asp).

Nottingham Research Ethics Committee has granted ethical approval for the studies providing the participants for this study (Reference numbers: 12/EM/0345; 13/EM/0277; 13/EM/0148). Nottingham University Hospitals NHS Trust and The University of Nottingham, as study sponsors have granted approval for the studies conducted (Ref: 12GY005; 13GY009 and 13069 respectively).

Informed and written consent was obtained prior to enrolment of the study participants.

5.3.5 Ultrasound Technique

5.3.5.1 Image acquisition

All ultrasound investigations were carried out via the transvaginal route using Voluson E8 Expert BT12 (GE Healthcare, Zipf, Austria) equipped with trans-vaginal transducer (5-9MHz). Data identifiers were entered according to the study protocol. Patients were asked to empty their bladder before the procedure was carried out for comfort and best visualization of the pelvic organs. Before each scan, the ultrasound transducer was cleaned with ultrasound equipment sanitizer wipes, following which sonographic gel was applied to the transducer. Latex or non-latex probe covers were then used depending on the patients' sensitivity. All scans were conducted in the supine position, with hips abducted and knees flexed. The assessment consisted of conventional 2D ultrasound of the pelvis to identify the pelvic organs and exclude any pathology. A static 3D acquisition of the right and left ovary was also obtained.

Ultrasound settings were selected for their best depiction of two-dimensional and three-dimensional details allowing for accurate delineation of planes. These were as follows:

Two-dimensional ultrasound settings:

- Gain: adjusted to obtain the best image possible
- Tissue harmonics: On
- Power: 100%
- Focus: 5 cm deep (dependant on patient)

Static three-dimensional ultrasound settings:

- Quality: high

- Angle of acquisition: 120 degrees

5.3.5.2 Image display and analysis

All ultrasound images were analysed offline on a personal computer (PC) using 4D View™ software (version 10.5 BT12, GE Medical Systems). The 3D volumes were displayed using the multiplanar view which demonstrates three sectional planes (the A, B and C planes). All these planes are mutually related, so that manipulation of one plane produces equivalent movement of the other planes. This allows for standardisation of the analysed region of interest, namely, the ovary. Plane A displays the sagittal section of the ovary, plane B- the transverse section and plane C- the coronal reconstruction of the whole ovary. The magnification of the image was adjusted to allow for accurate delineation of the whole ovary. Volume of the ovary was measured using virtual organ computer aided analysis (VOCAL) on 4D View software (GE, Austria). This was done by delineating the outline of the ovary on the A plane as the volume was rotated through 180^{o} using 15^{o} rotational steps (see Figure 5-1). Only images where the ROI (region of interest) was no deeper than 5cm from the transducer and it was possible to delineate the outline of the ovary without doubt were included. If these factors were not met, the image was excluded from analysis.

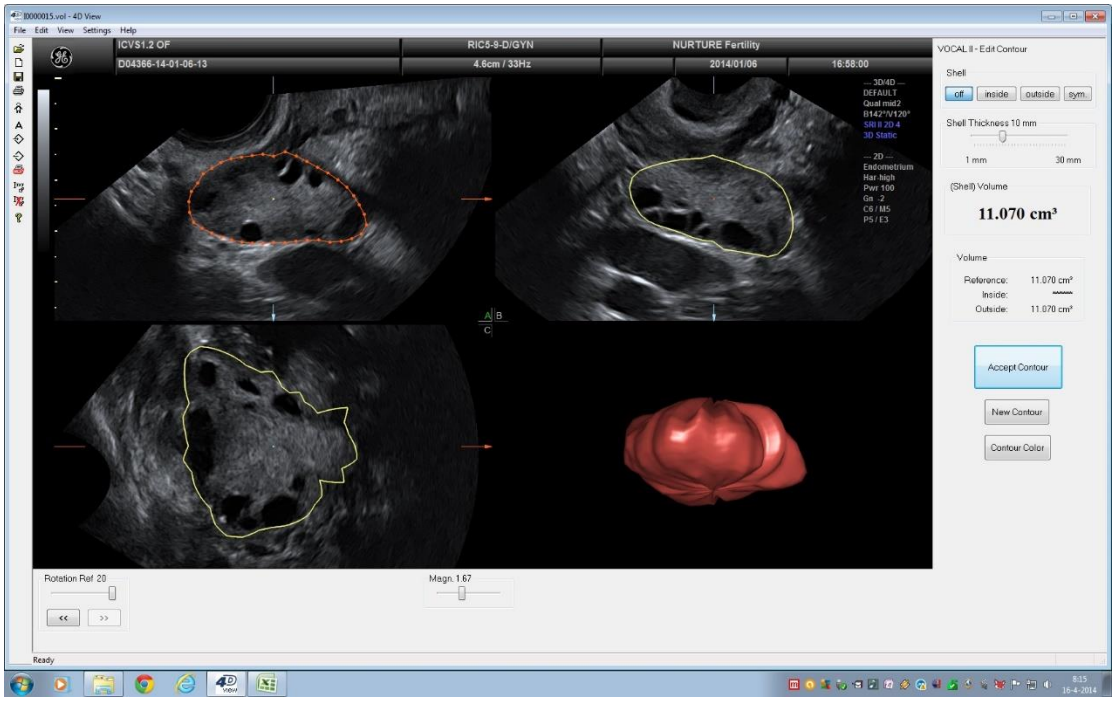


Figure 5-1: Multiplanar view of ovary.

Acquired datasets were analysed once by two observers (MNB and LTP) for the presence of free fluid at embryo transfer. All obtained volumes for endometrium or ovary were analysed by a single observer (MNB). The number of follicles was counted in two planes, A and B by scrolling through the ovary in the off-line modes until consensus was achieved.

5.3.6 Blood sample collections

Whole blood samples were collected by venipuncture into an EDTA tubes. Blood samples were collected after spontaneous ovulation, at time of egg collection and embryo transfer. Plasma samples were obtained by centrifuging whole blood in EDTA at 4000rpm for 20 minutes at 4°C straight after the collection. The supernatant plasma was decanted in labelled new tubes and stored at -20°C for later analysis. Samples were transported on dry ice from the hospital to the laboratory for analysis.

From the third cohort two bottles of blood were taken. One of the bottles was directly spun. The supernatant plasma was pipetted in clear tube. The whole blood sample in the EDTA tube and the plasma were stored at -20 degree. At a later time point the samples were thawed and the whole blood would be spun. Renin and angiotensin-1 were measured in both samples and compared to test the feasibility to store whole blood and perform analysis at a later time point.

5.3.7 Plasma Angiotensinogen Concentration (PAC) Assay

This assay was used for determination of endogenous (sample) angiotensinogen (AGT) in the presence of excess exogenous Renin using an indirect radioimmunoassay (RIA) for angiotensin-I (AGT1).

Molar excess of human renin is added to highly diluted plasma samples (containing AGT). Under these conditions, the amount of AGT substrate is the rate–limiting factor in the production of AGT1. Thus the amount of AGT1 produced is directly related to the starting amounts of AGT substrate.

5.3.7.1 Materials:

Acetone (Fisher Scientific – A/0600/PB17)

Acid washed activated charcoal (Sigma – C5510)

Angiotensin I^{125} (NEN, Perkin Elmer)

Anti-Angiotensin antibody (Rabbit polyclonal to Angiotensin)

Bovine Serum Albumin (BSA) (Sigma – A7030)

Dextran (Sigma – D4876)

Dimercaprol

EDTA (disodium, Sigma – E5134)

8-hydroxyquinoline (hemi) sulphate (8-HQS) (Sigma – H6752)

Disodium hydrogen (ortho) phosphate (Sigma – S3264)

Sodium dihydrogen (ortho) phosphate (Sigma – S3139)

Sodium Chloride (Fisher Scientific – S/3160/63)

5.3.7.2 Protocol for RAS assays

Samples were thawed and kept in ice boxes. All procedures were performed at 4°C or less. Two buffer solutions were made to maintain constant pH. Buffer A contains dibasic sodium phosphate (12.35g/L), monobasic sodium phosphate (1.90g/L) and sodium chloride (4.40g/L). Buffer B is the same as buffer A with the addition of BSA 3mg/ml. Samples were diluted according to their blood source. For whole blood from adult non-pregnant humans, this was 10µl in 2ml buffer A.

A solution of 5ml human renin and 5ml buffer A was made and stabilisers were added in the form of 0.1ml/ml EDTA, 0.05ml/ml 8-HQS and 0.01ml dimercaprol as angiotensinase inhibitors. To 400µl of the diluted samples 100µl of the renin solution was added and the sample was mixed well starting the enzymatic process. A 100 µl was taken out at time point 0 and the remaining 400µl were placed in a warm (37°C) water bath and incubated for 2 hours.

100 µl of ice cold acetone was added to the 100 µl of test sample to immediately stop the reaction. After mixing, this was centrifuged at 2500rpm for 10 minutes at 4°C. After spinning, 100µl of the supernatant was transferred into tubes containing 400µl of buffer B. The samples were

sealed and stored at 4°C until the remaining volumes of the test samples reached the 2hr time point.

After completion of the 2 hours of incubation at 37°C, 300µl of the incubated angiotensinogen samples were transferred to fresh tubes and an addition of equal volumes of ice-cold acetone was performed. The samples were mixed well and centrifuged at 2500rpm for 10 minutes at 4°C. 100 µl of the supernatant was transferred to 3 separate tubes containing 400µl of buffer B.

370 kBq of lyophilized Angiotensin I-^{125}I was reconstituted in 200µl buffer A. 10 µl of this solution was transferred into 5ml of buffer B. 50µl of the latter solution was added into every tube (the point 0 tube and the triple 2hr tubes from each sample).

Anti-Angiotensin I was reconstructed in 5ml of buffer A. The antiserum was diluted 1/3 in buffer B to form the working solution. 50µl of this freshly prepared antiserum was added to all tubes. The tubes were subsequently mixed and incubated at 4°C for 48 hours.

5.3.7.2.1 Standard curve preparation

Serial dilutions of AGT1 were made in triplicate by adding 10µl of 1mg/ml AGT1 to 50ml of 0.9% NaCl creating a solution of 200.0pg/µl. One ml of this dilution was added to 4ml of buffer B creating a dilution 40.0pg/µl. This dilution was further diluted by adding 1 ml to 1ml of buffer B. These steps are repeated 6 times creating standard solution A to G.

The first 3 tubes were the triplicate of "non-specific binding", this represents the background binding capacity. The next 3 tubes act as the nil standard.

The subsequent triplicates contain AGT1 concentrations of 0.3125 pg/µl, 0.625 pg/µl, 1.25 pg/µl, 2.5 pg/µl, 5.0 pg/µl, 10.0 pg/µl and 20.0 pg/µl. The total amount of AGT1 in each 100µl of standard ranges 0.00pg to 2000pg.

5 ml of ice-cold acetone was added to 5ml of buffer B and centrifuged for 10 minutes at 2500rpm at 4°C. 100 µl of this solution was added to the standard curve tubes. 50µl of freshly prepared Angiotensin I-^{125}I was added to every test tube. 50µl of this freshly prepared antiserum was added to all tubes except non-specific binding tubes. The samples were then incubated for 48 hours at 4°C.

5.3.7.2.2 After incubation

One part of dextran coated charcoal was diluted in 3 parts of buffer B. 500µl of this freshly prepared solution was added to all the tubes and centrifuged for 15 minutes at 4°C at a rate of 2500rpm. The supernatant was decanted into new tubes. The radioactivity was then counted using a Gamma counter.

The gamma counter software calculated the percentage of total sample radioactivity in each fraction and used this to derive from the standard curve the quantity of AGT1 in each of the fractions.

The mean amount of AGT

1 in the triplicates was calculated with subtraction of the zero time point amount. This gives the amount of AGT1 in pg for each sample. To correct for the dilution and to convert to µg/ml a multiplication by 5 and division by 1000 was performed.

5.3.7.3 Plasma renin concentration assay

Renin catalyses the formation of angiotensin I (AngI) by cleavage of the renin substrate called angiotensinogen. Plasma renin is therefore the initiator of the RAS. Circulating renin can be estimated by assays of plasma renin activity (PRA). PRA is measured by generating AngI from endogenous angiotensinogen, followed by measurement by RIA of the generated AngI. Determination of endogenous renin in the presence of excess exogenous Angiotensinogen substrate is performed using an indirect radioimmunoassay for Angiotensin-I production. Molar excess of sheep angiotensinogen substrate is added to undiluted plasma samples (containing renin). Under these conditions the amount of renin available is the rate limiting factor in the production of AGT1. Thus the rate of AGT1 production over a given period of time is directly related to the special activity of renin and this to the amount of active renin. Therefore, relative amounts of sample remaining (relative specific activity) are determined by comparing the rate of AngI production over a set period of time in each sample.

5ml of sheep substrate angiotensinogen was added to 5ml of buffer A. Subsequently, angiotensinase inhibitors, 0.1ml/ml EDTA, 0.05ml/ml 8-HQS and 0.01ml dimercaprol were added. To 200 µl of each undiluted sample, 400 µl of the prepared sheep angiotensinogen solution was added and mixed well. 100 µl of this solution was pipetted into a tube and immediately 100 µl of ice-cold acetone was added to stop the reaction. This acted as time point zero. The remaining 400µl of the solution was placed into a water bath at 37°C for 2 hours.

The time point zero samples were centrifuged for 10 minutes at 2500rpm at 4°C. After this, 100 µl of the supernatant was transferred into tubes containing 400µl of buffer B. The samples were sealed and stored at 4°C until the other time point samples reached the same stage. After 30 minutes, 1 hour and 2 hours 100 µl of the sample solution incubating in the water bath was pipetted into a fresh tube and 100 µl of ice-cold acetone was added. This was then centrifuged as above and 100µl of the supernatant was added to 400µl of buffer B and stored sealed at 4°C until all time points were collected.

Standard curve was prepared as described in section 5.3.7.2.1.

After 2 hours, 50µl of freshly prepared Angiotensin I-^{125}I were added to every tube. 50µl of freshly diluted antiserum was added to all tubes except the non-specific binding in complete analogy of the above described protocol. Samples were mixed well and incubated for 48 hours at 4°C.

5.3.7.3.1 After incubation

One part of dextran coated charcoal was diluted into 3 parts of buffer B. 500µl of this freshly prepared solution was added to all the tubes and centrifuged for 15 minutes at 4°C. The supernatant was decanted into new tubes. The radioactivity was counted using a Gamma counter.

The gamma counter software calculated the percentage of total sample radioactivity in each fraction. By using this in conjunction with the standard curve the quantity of AGT1 in each of the fractions was derived. This was reported in pg for each sample. Using excel and GraphPad Prism 6 the inclination of the slope was constructed as a line going through the 4 analysed time points (0, 30, 60, 120 minutes). A graphic example can be

seen in Figure 5-2. The intersection at 60 minutes gives the amount of AGT1 converted from angiotensinogen per hour (pg/hr). To correct for the sample volume this needs to be multiplied by 60. To obtain values in ng/hr the amount needs to be divided by a factor 1000. This produces the conversion rate to AGT1 in ng/ml/hr, which is the specific activity of renin.

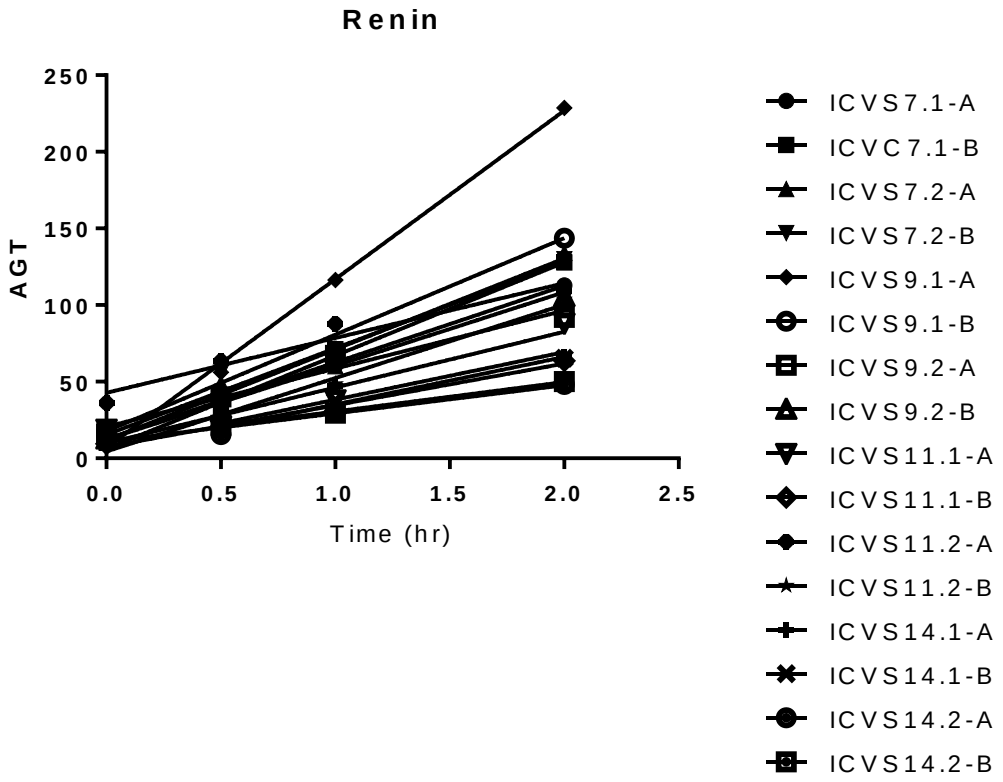


Figure 5-2 Example of linear regression analysis of duplicated samples

A and B in the above figure represent repeat analysis of the same sample. The interpretation of these results will be given in section 5.4.

5.3.8 Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (version 21; SPSS, Chicago, IL, USA). Distribution of data

was assessed using Kolmogorov-Smirnov test. For parametric un-paired data student's t test was used and for non-parametric data, Mann-Whitney U test was carried out. For paired samples, student's t test was used for parametric data and Wilcox signed rank test was used for non-parametric data. Repeated samples were analysed using a one-way repeated measures ANOVA. A P-value of <0.05 was considered as statistically significant. This is a proof of principle cohort study, and no target numbers was set.

5.4 Results

5.4.1 Data on freezing of whole blood versus plasma

In this part of the study, the feasibility of analysis of renin and angiotensin-1 from whole blood samples frozen straight after collection was investigated.

On 2 separate occasions a few randomly selected whole blood samples were thawed out. Samples were spun according to the protocol as described in section 5.3.6. After centrifuging for 20 minutes no separation of plasma occurred. As there was no plasma to be decanted, the samples were unsuitable for RAS analysis.

5.4.2 Participants

A total of 47 women provided samples for analysis. Ten women in the repeat natural cycle group and 37 in the IVF treatment group.

5.4.3 Relationship of renin and angiotensinogen with ovarian volume, number of follicles and steroids in the midluteal phase in spontaneous cycle.

All women who had a study appointment in a natural cycle were included (prior to IVF or the repeated biopsies). The participant characteristics can be found in Table 5-1.

	Mean ± SD (range)
Age (years)	33 ± 4
BMI (kg/m^2)	24.9 ±3
AMH (pmol/L)	22.4 ± 21.8
AFC value	16 ± 9

Table 5-1: Participant characteristics.

There was no significant correlation between renin and angiotensinogen concentration and any of the analysed variables as demonstrated in Table 7-2. As previously mentioned, age, BMI, and AMH have an association with ovarian response to stimulation (section 1.8.1.9). number of follicles, E2 levels and ovarian volume, would be able to influence ovarian RAS equilibrium.

		Renin concentration (ng/ml)	Serum angiotensinogen (ng/ml) concentration
Age (years)	Pearson Correlation	0,009	0,009
	Sig. (2-tailed)	0,959	0,959
	N	39	39
BMI (kg/m2)	Pearson Correlation	-0,264	-0,264
	Sig. (2-tailed)	0,213	0,213
	N	24	24
AMH (pg/mmol)	Pearson Correlation	-0,029	0,314
	Sig. (2-tailed)	0,872	0,075
	N	33	33
E2 (pmol/l) post-ovulation	Pearson Correlation	-0,024	-0,024
	Sig. (2-tailed)	0,886	0,886
	N	39	39
P (ng/ml) post-ovulation	Pearson Correlation	-0,026	-0,026
	Sig. (2-tailed)	0,874	0,874
	N	39	39
Total ovarian volume at ovulation	Pearson Correlation	-0,081	-0,157
	Sig. (2-tailed)	0,658	0,391
	N	32	32
Total nr follicles at ovulation	Pearson Correlation	-0,120	0,127
	Sig. (2-tailed)	0,520	0,498
	N	31	31
Log AMH	Pearson Correlation	-0,040	0,079
	Sig. (2-tailed)	0,824	0,661
	N	33	33
Log E2 at ovulation	Pearson Correlation	0,064	0,064
	Sig. (2-tailed)	0,698	0,698
	N	39	39
Log P at ovulation	Pearson Correlation	0,048	0,048
	Sig. (2-tailed)	0,770	0,770
	N	39	39

Table 5-2 : Correlation between renin and angiotensinogen concentration and outcomes (P=progesterone, E2= oestradiol, AMH=anti-Müllerian hormone, BMI=body mass index).

5.4.4 Data on the influence of an endometrial biopsy on renin and angiotensinogen levels

Women undergoing repeat endometrial biopsies in two consecutive menstrual cycles had blood samples obtained during study visits to assess R and AGT levels between cycles. This analysis was performed to exclude biopsy as a confounding factor in subsequent analysis.

Data was available for 6 women. Renin levels were not normally distributed and the distribution could not be normalised on a logarithmic scale. With the use of a related samples Wilcoxon Signed Rank test, the median of differences between renin at the time of the first biopsy and at time of the second biopsy was not significantly different (P=0.463).

As illustrated in Figure 5-3, 2 women had an increase in R levels and the remaining 4 patients had stable levels. Similarly, non-significant results were obtained for AGT levels between cycles (P=0.6; see Figure 5-4).

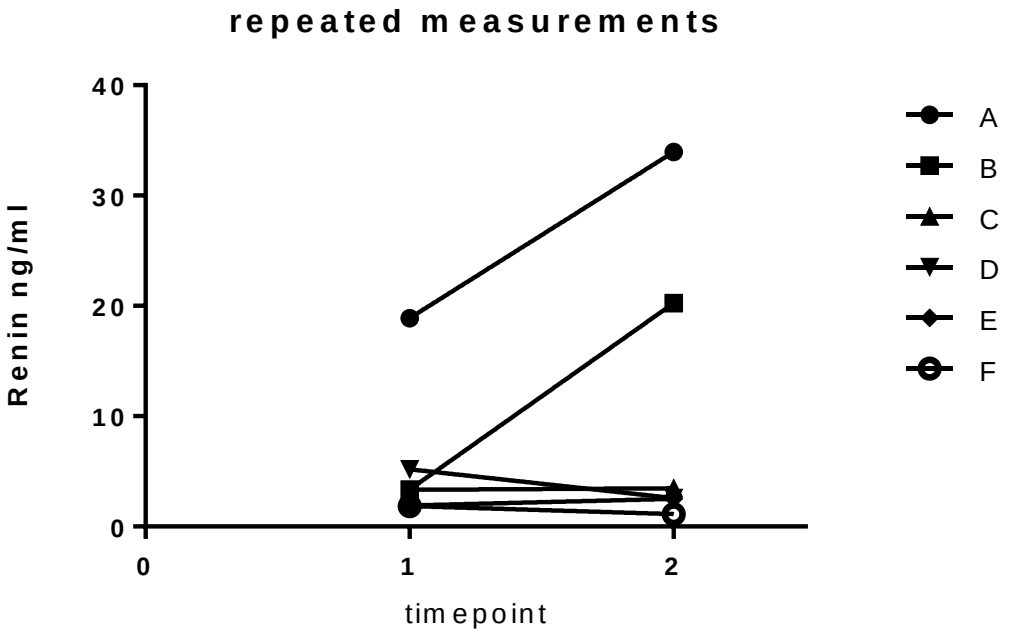


Figure 5-3 Same patient Renin levels in consecutive menstrual cycles

One of the patients in the intercycle group draws attention as she has presented with outlying RAS levels on both occasions. This was a 41 year old woman suffering from repeated miscarriages (G12P1A9+2; twelve pregnancies leading to 1 live birth and 9 miscarriages) with proven ovulation by as demonstrated by high progesterone levels.

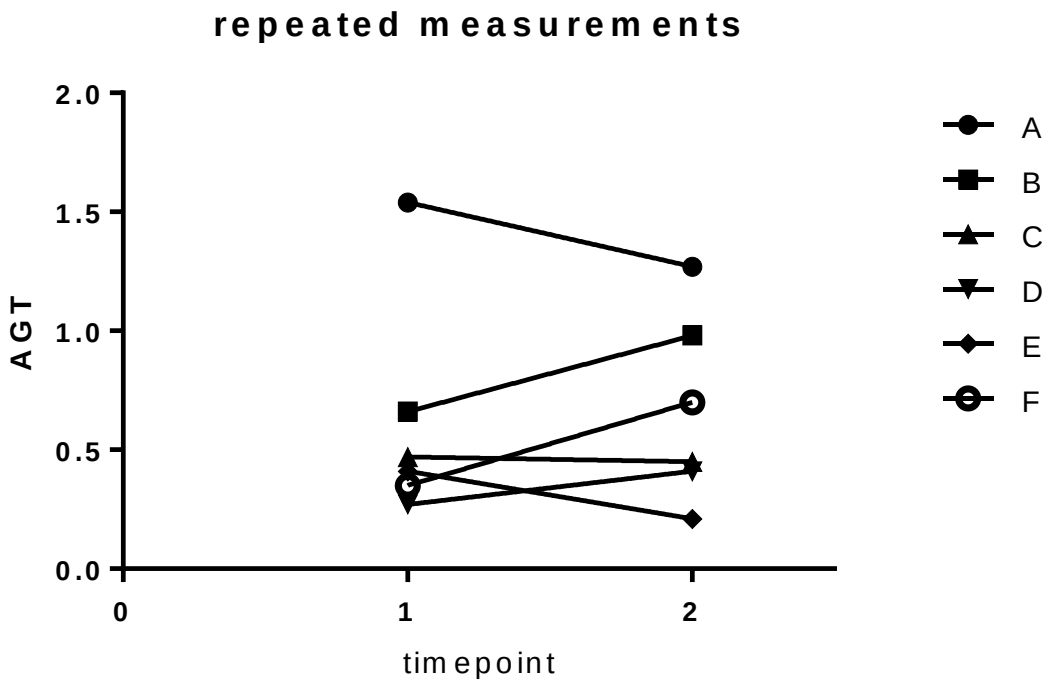


Figure 5-4 Same patient AGT levels in two consecutive menstrual cycles.

The median of renin and AGT were similar but the variance in the second cycle was increased, see Table 5-3 and Table 5-5. In the first table, repeated measurements in the same patient are compared, in the second table all available patients at that time point were independently compared.

	First month N=6	Second month N=6	P
Renin (ng/ml)	3.83 [6.7]	3.03 [21.5]	0.5
AGT	0.44 [0.6]	0.57 [0.4]	0.6

Table 5-3: Median and IQR in repeated measurement (Wilcoxon signed rank test).

	First month N=9	Second month N= 7	P
Renin	5.19[5.0]	3.5 [17.7]	0.79
AGT	0.59 [0.4]	0.45 [0.8]	0.63

Table 5-4 Median and IQR for independent samples (Mann-Whitney test).

5.4.5 Dilution of sample for AGT assay

Before commencing the analysis of samples collected during ovarian stimulation, identification of an optimum dilution to perform the assay was needed. It is known that samples need to be more diluted if the origin is from a pregnant woman or from umbilical cord blood. It was hypothesised that when RAS would be activated, it might be possible that levels in the samples are too high to get the best results, as the results may be outside of the detection range of the assay.

In order to find the optimal dilution, samples from 2 patients were used taken at spontaneous ovulation, oocyte collection and embryo transfer. The samples at oocyte collection and embryo transfer were diluted in 3 dilutions: 5, 10 or 25 fold.

After the assay was run results were compared. The standard 5 fold and the 25 fold dilution gave similar results. Whereas, the 10 fold gave unexplained (no protocol breaches, no identifiable quality control problems) lower results. The standard dilution was sufficient for analysis and in order to not deviate from standard practice, the decision was made to use the 5 fold dilution for all the AGT assays.

5.4.6 Ovarian stimulation renin and angiotensinogen levels

Complete data of 10 women including levels at a natural cycle, oocyte collection (if applicable; excluding frozen embryo transfer cycles)) and embryo transfer was available. The distribution of renin concentration at ovulation, oocyte collection and embryo transfer was significantly different (P=0.02; related samples Friedman's Two-Way analysis). The distribution of AGT at the 3 time points differed significantly (P=0.002). As data was not normally distributed, additional graphical representation of data is given in Figure 5-5 allowing the interpretation of the means, the individual points with variation, and the individual repeated measurements with the addition of the number of oocytes retrieved.

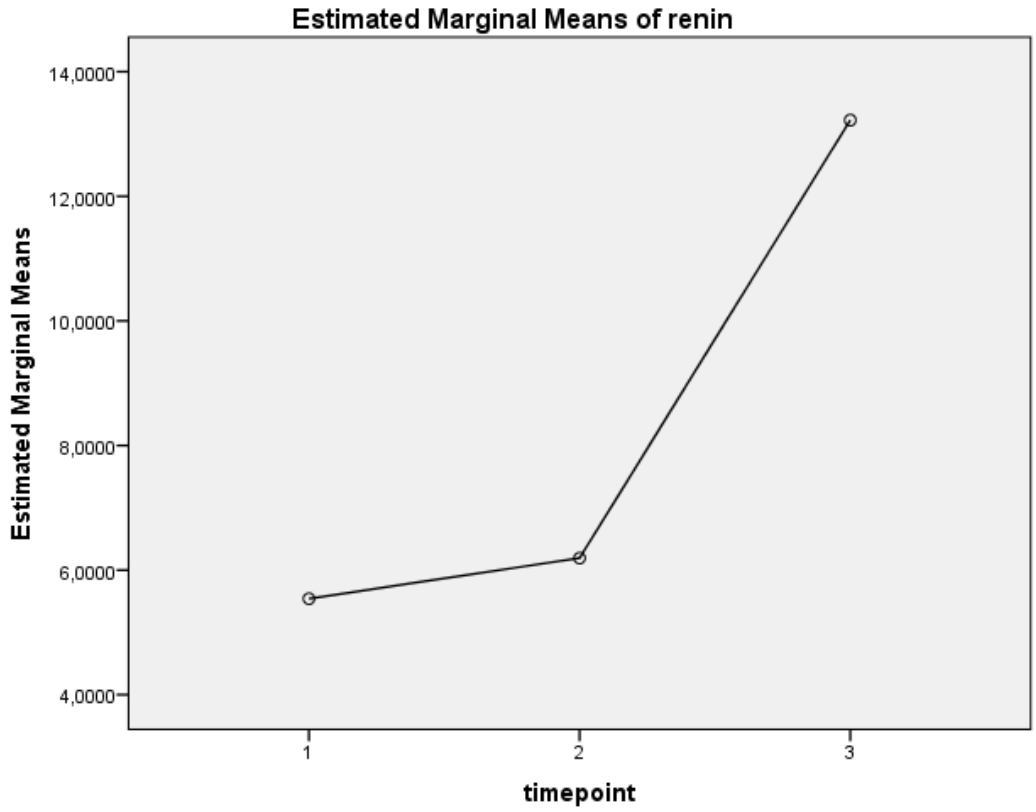


Figure 5-5: Estimated marginal means of renin levels at 3 time points (natural cycle, oocyte collection, embryo transfer).

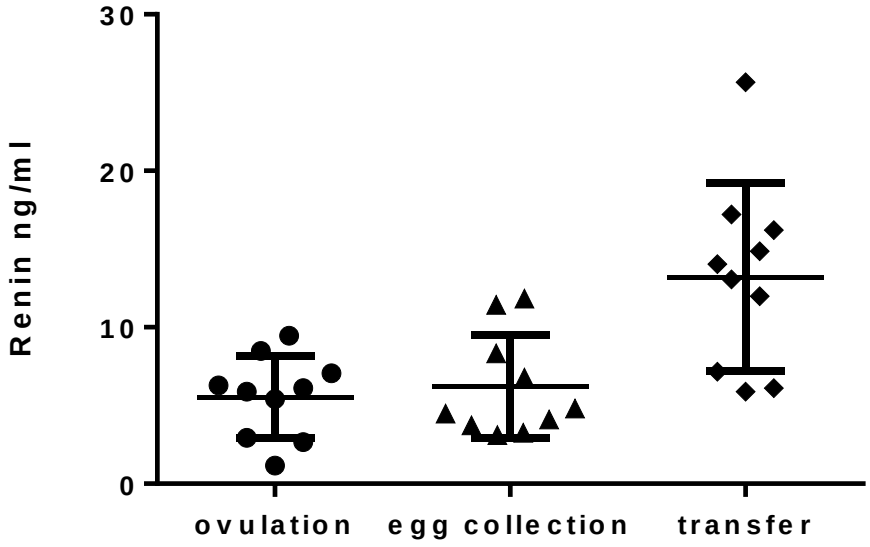


Figure 5-6: Scatter plot renin levels at ovulation, oocyte retrieval and embryo transfer.

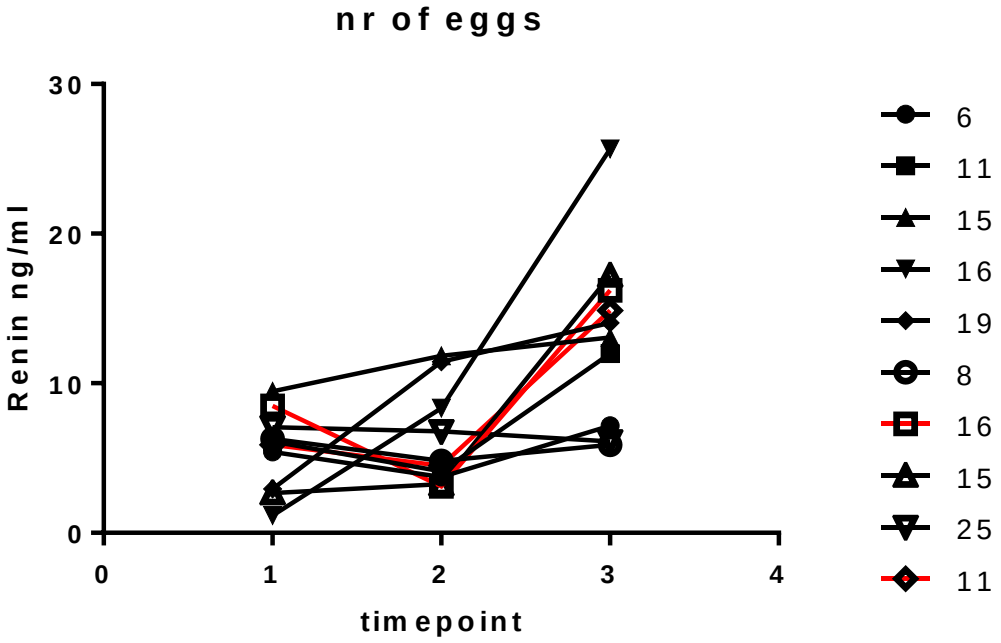


Figure 5-7: Renin levels at natural cycle, oocyte collection and embryo transfer and number of oocytes collected (red=non-conception).

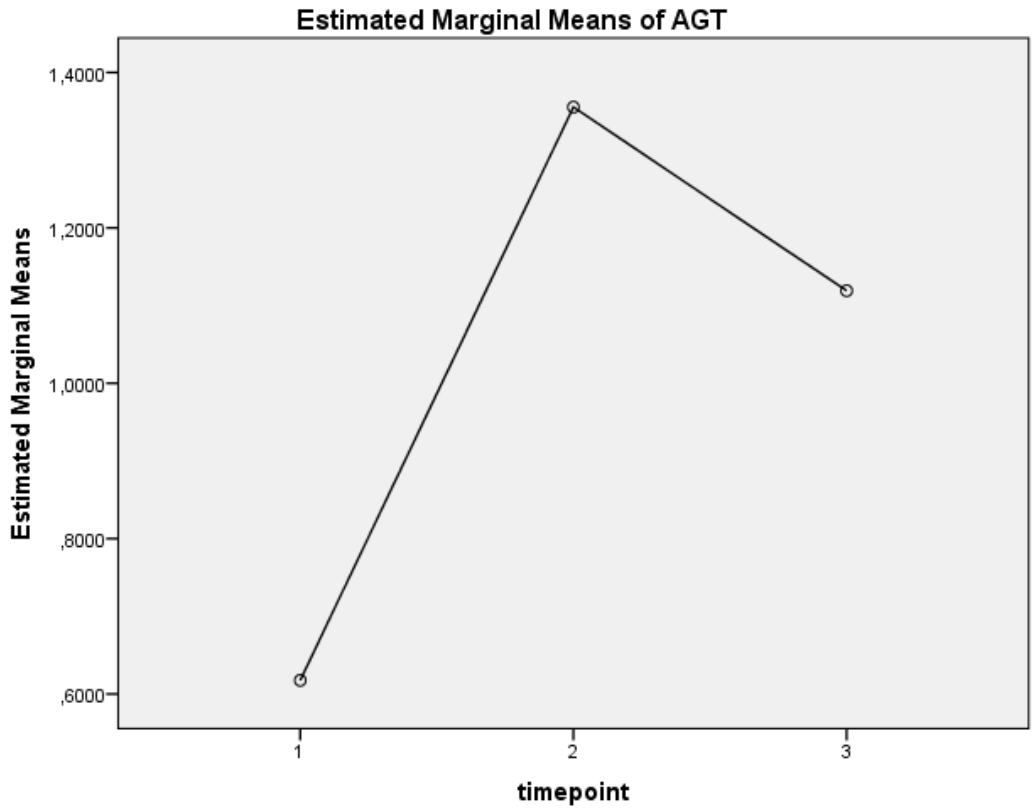


Figure 5-8: Estimated marginal means of AGT levels at 3 time points (natural cycle, oocyte collection, embryo transfer).

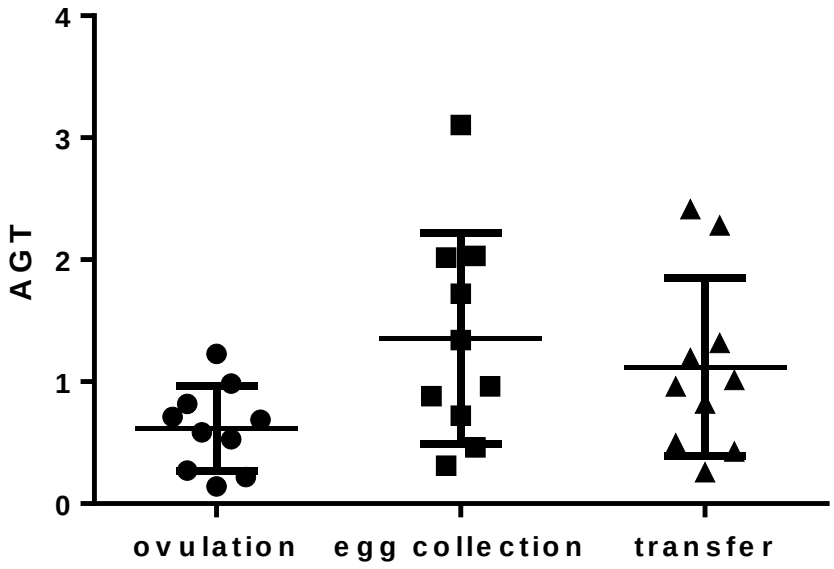


Figure 5-9: Scatter plots of AGT levels at ovulation, oocyte retrieval and embryo transfer.

Figure 5-10 illustrates each single patient at the 3 time points in addition to the number of oocytes which were collected. The 2 red lines represent the 2 patients who did not conceive during this treatment cycle.

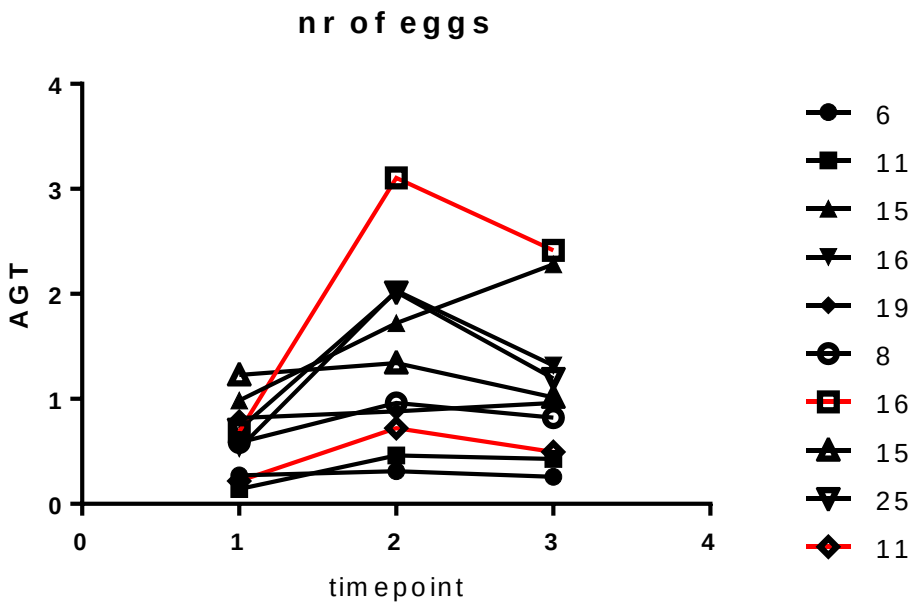
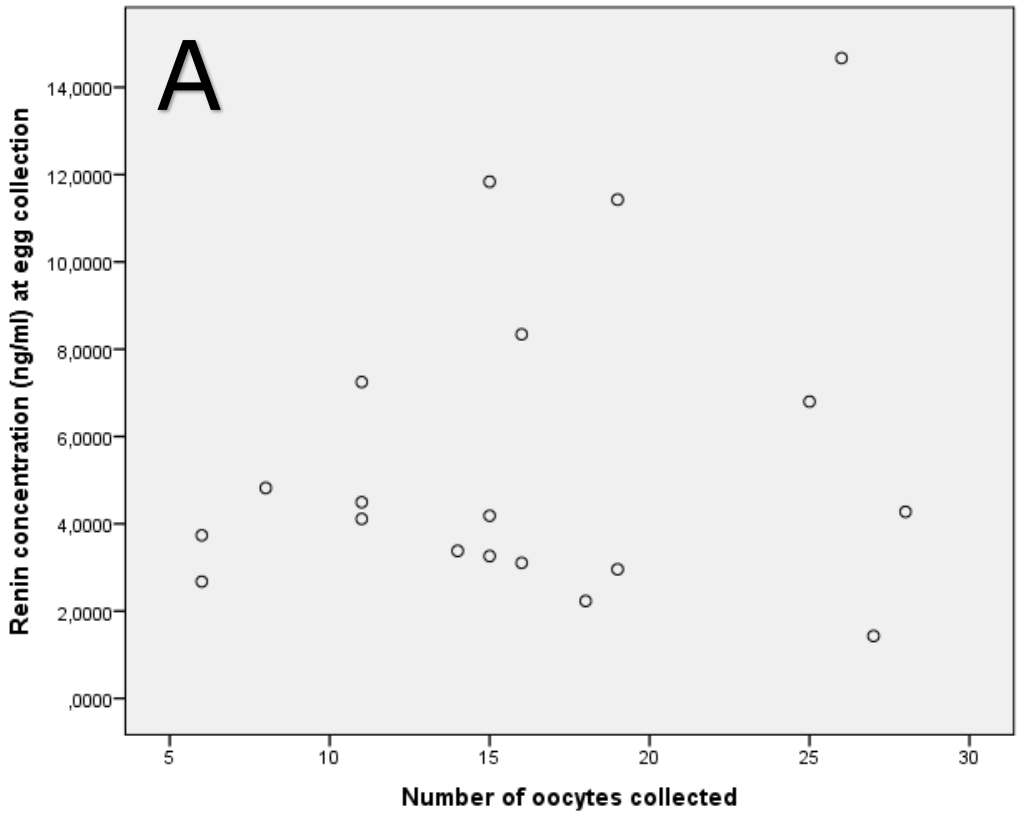


Figure 5-10: AGT levels at ovulation, oocyte collection, transfer, including number of oocytes retrieved (red=non-conception).

5.4.7 Data on number of oocytes collected, sonographic markers and RAS

As the hypothesis was that ovarian RAS is activated due to the multiple *corpora lutea*, the number of oocytes collected or the size of the ovary may be correlated to the RAS levels. Specifically, those women with high number of oocytes collected are at increased risk of developing symptoms of OHSS and that one of the diagnostic ultrasound features is enlarged ovaries.

Data on RAS and number of oocytes collected was available for 19 patients without demonstrating a correlation (Pearson's r 0.235; P=0.334; see Figure 5-11).



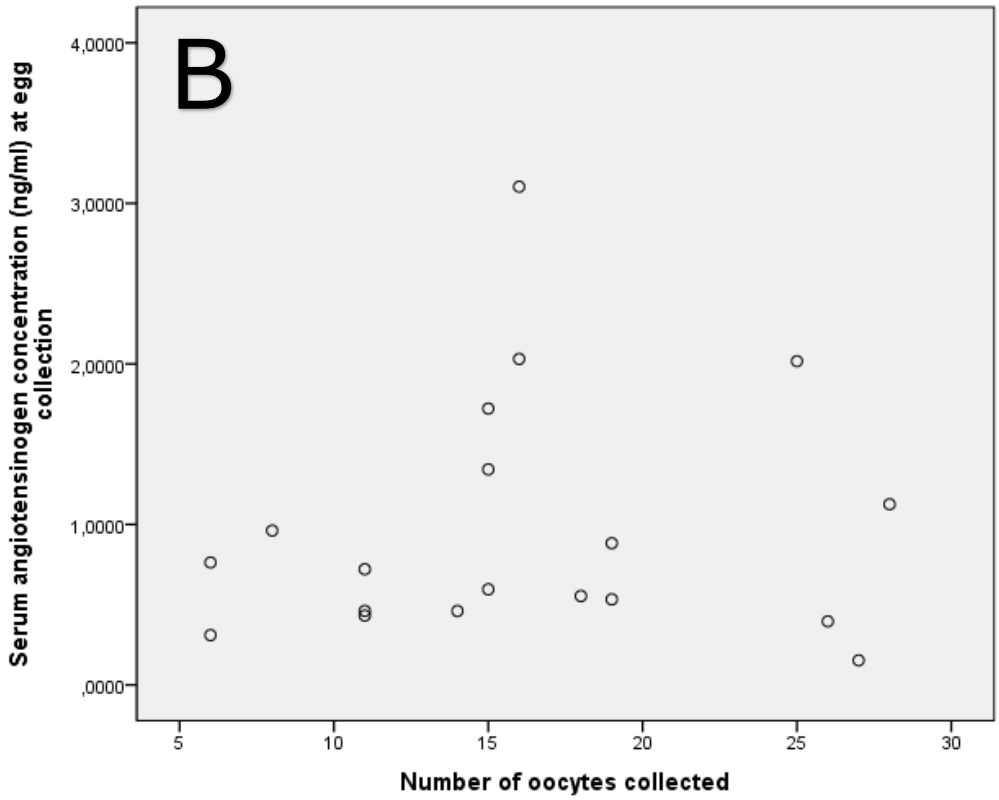


Figure 5-11: Renin (A) and AGT (B) levels per number of oocytes retrieved.

Renin in relation to the ultrasound data and the number of follicles was available for 16 women. The total ovarian volume was available for 18 women. For renin the Pearson's correlation was 0.375 (p=0.152) and 0.21 (P=0.403; see

Figure 5-12). The same analysis was repeated for AGT measurements, Pearson's correlation was 0.443 (P=0.233) and Pearson's correlation -0.096 (P=0.724), see Figure 5-12 and Figure 5-13.

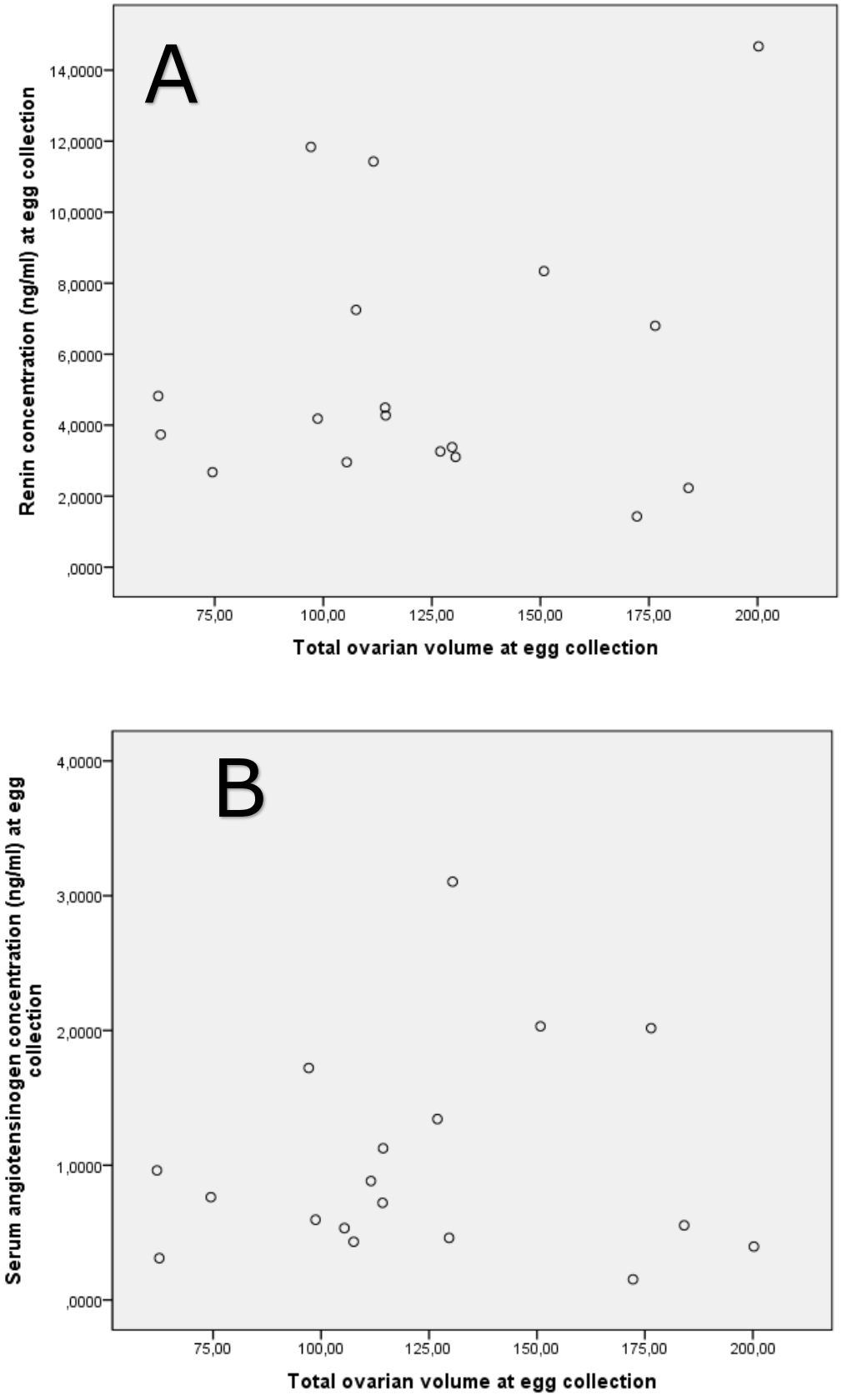


Figure 5-12: Renin (AI) and AGT (B) levels per ovarian volume at oocyte retrieval.

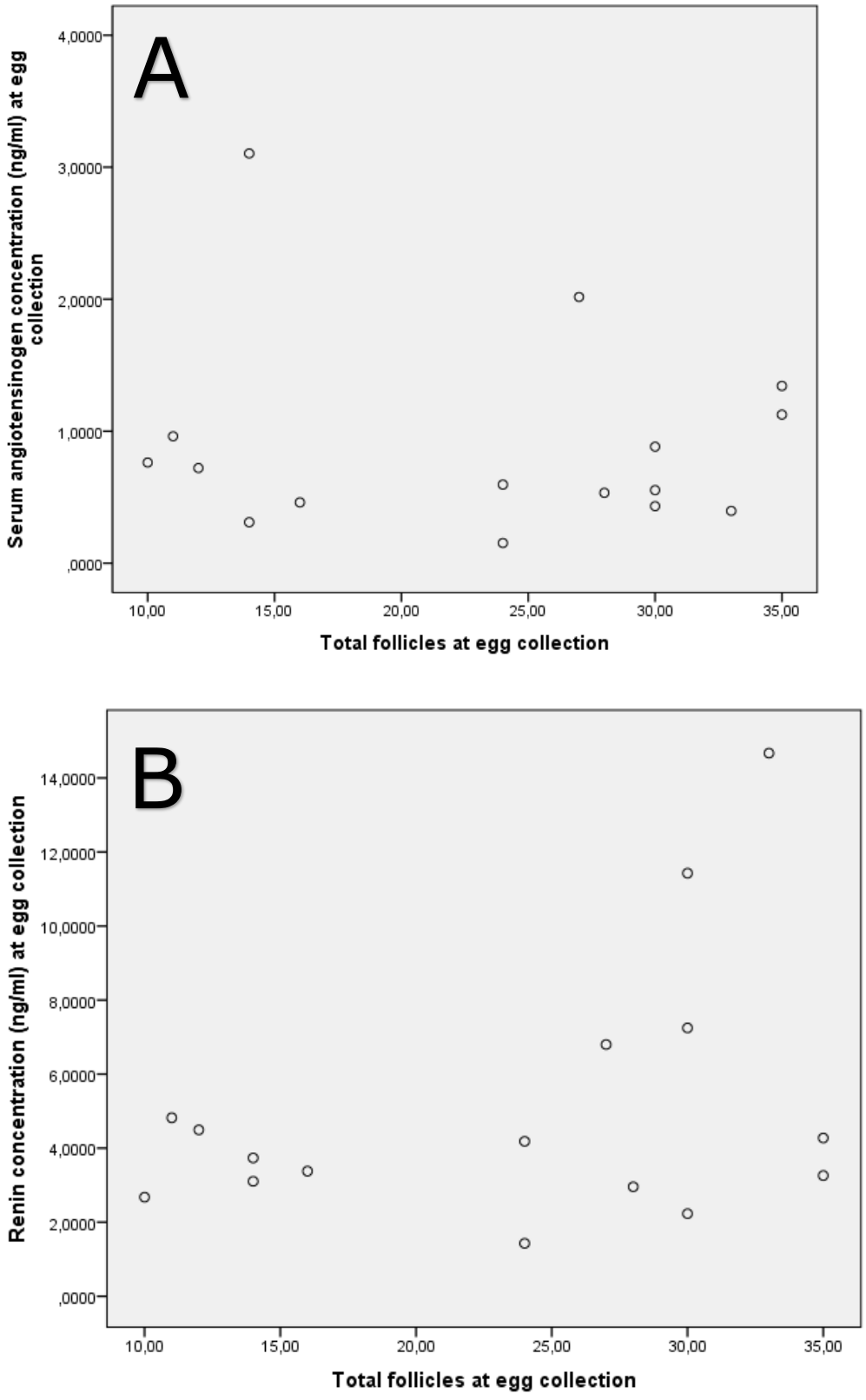


Figure 5-13: Renin (A) and AGT (B) levels per total follicles at oocyte collection.

5.4.8 Relationship between RAS and ultrasound makers at time of embryo transfer

In routine practise the number of follicles is not obtained at embryo transfer. The study protocol included this, as ovarian volume is part of the OHSS diagnosis, hence the number of present follicles will have an impact. At the time of embryo transfer there was a positive correlation between the number of follicles on scan and the renin level (Pearson's r=0.778; P=0.008). There was no correlation with ovarian volume (P=0.21), though ovarian volume was found to be correlated to the number of follicles (Pearson's r=0.678; P=0.005; see Figure 5-14). For AGT levels no correlation could be found for ovarian volume or number of follicles (see Figure 5-16).

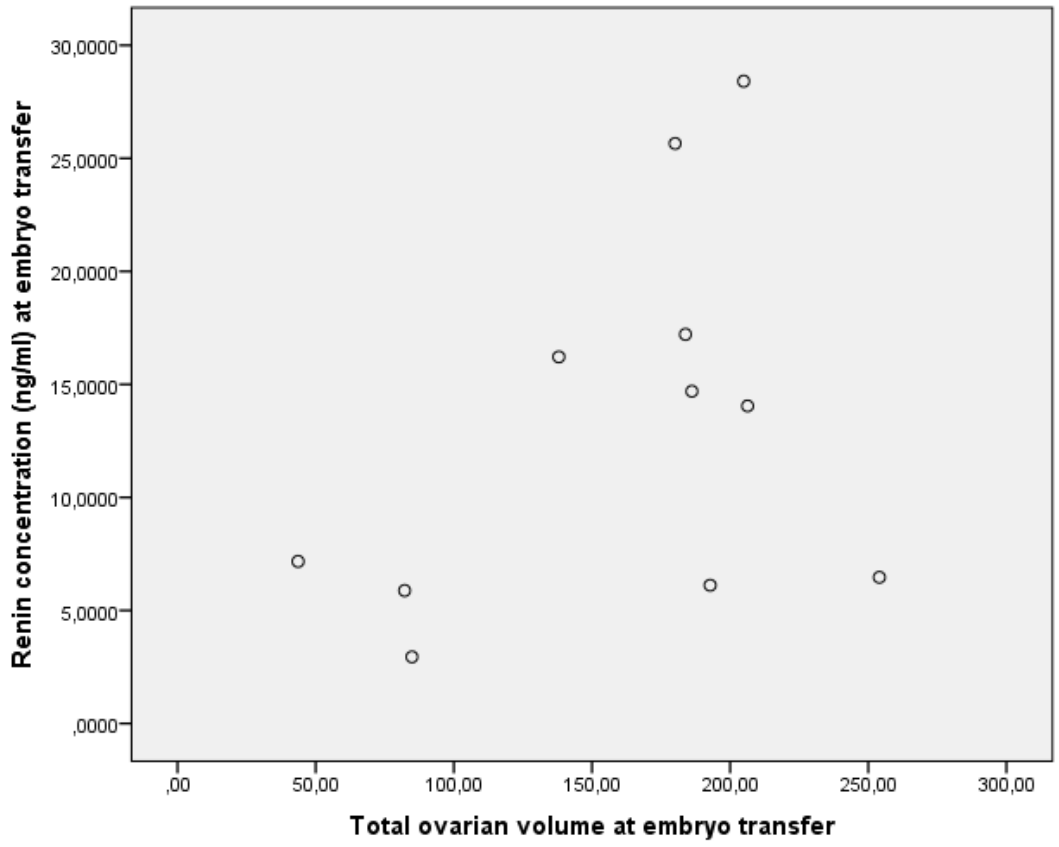


Figure 5-14: Renin levels and ovarian volume at embryo transfer.

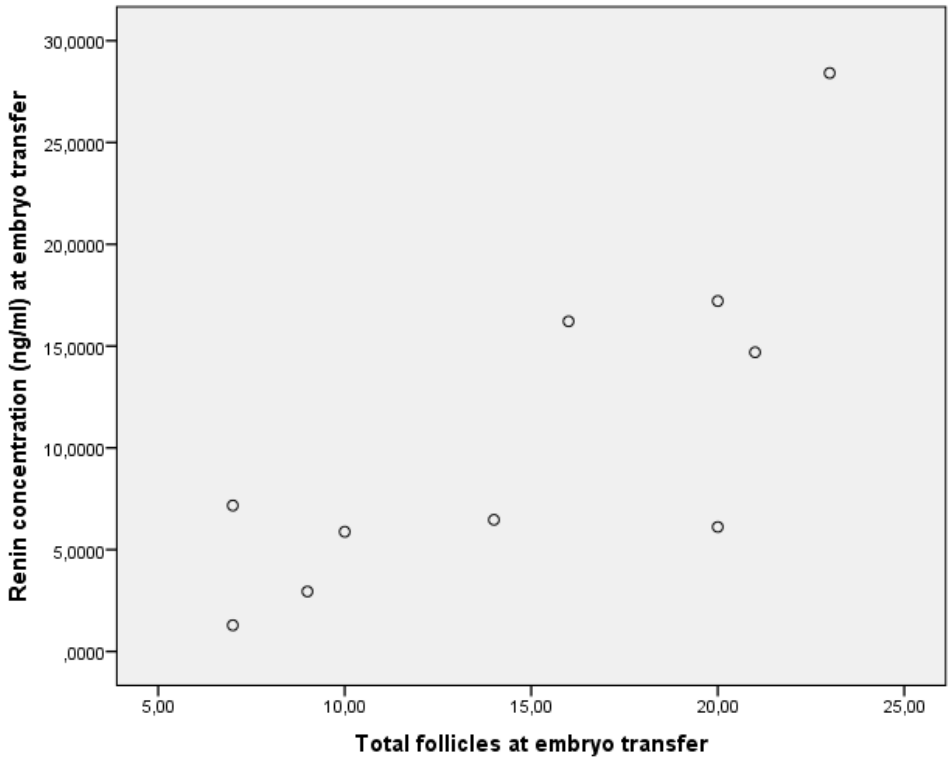


Figure 5-15: Renin levels per number of follicles at embryo transfer.

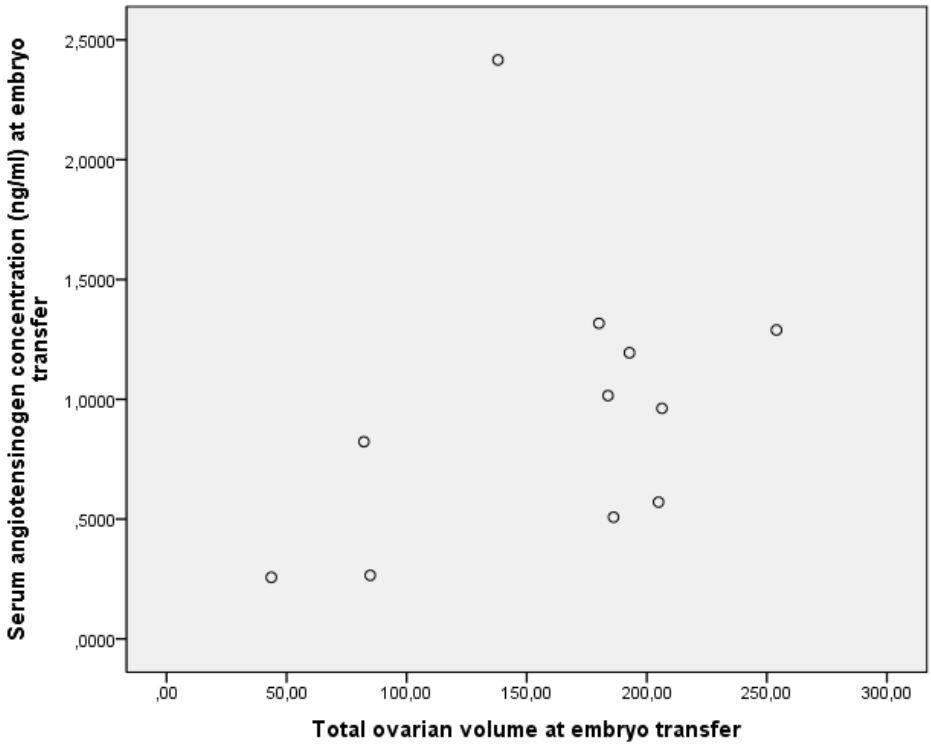


Figure 5-16: AGT levels and total ovarian volume at embryo transfer.

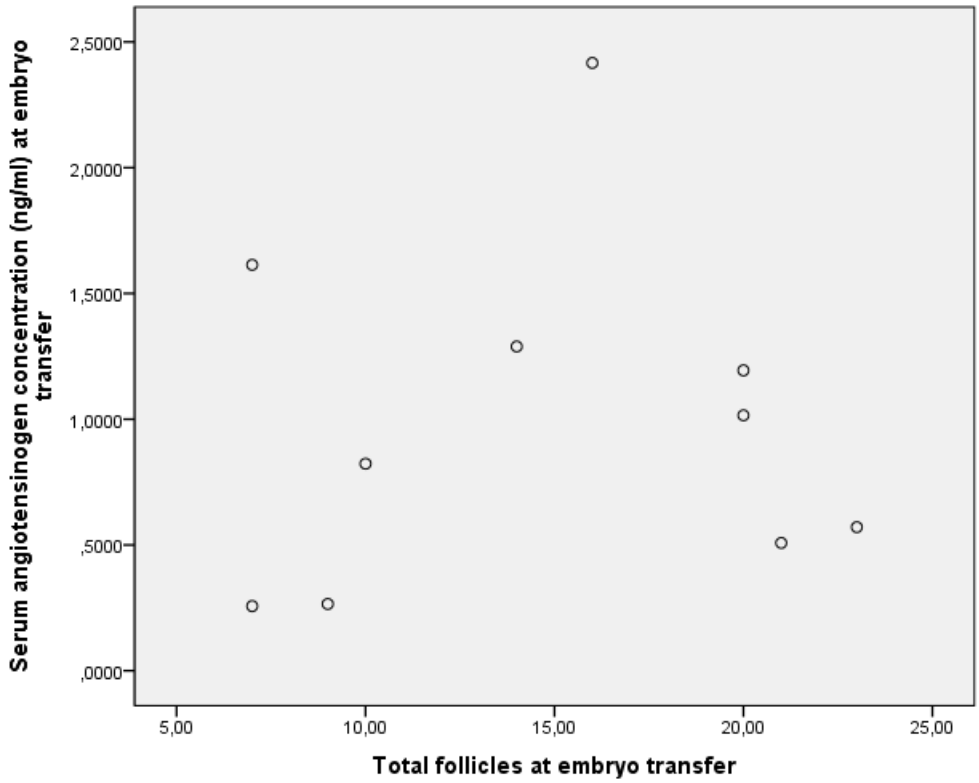


Figure 5-17: AGT levels per total follicles at embryo transfer.

Analysis of women with only data available on oocyte collection and embryo transfer, demonstrated a significant increase in renin levels ($P<0.001$). However, the difference in renin level is not correlated to the number of oocytes retrieved ($P>0.05$). When analysing AGT levels in repeated measurements between oocyte collection and embryo transfer, a non-significant decrease was demonstrated ($P=0.06$).

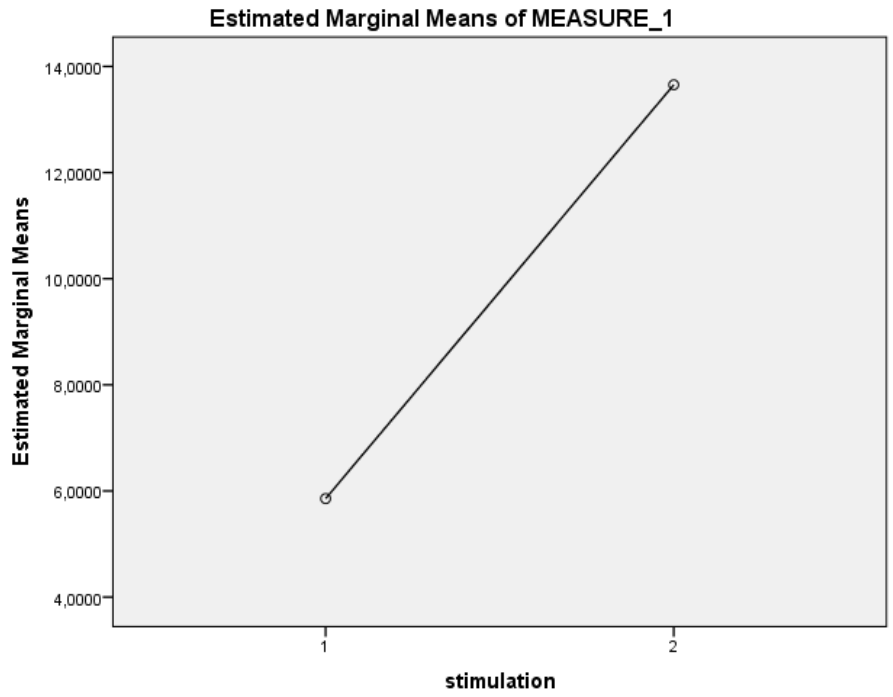


Figure 5-18: Mean Renin levels increase from natural cycle luteal phase to luteal phase after ovarian stimulation.

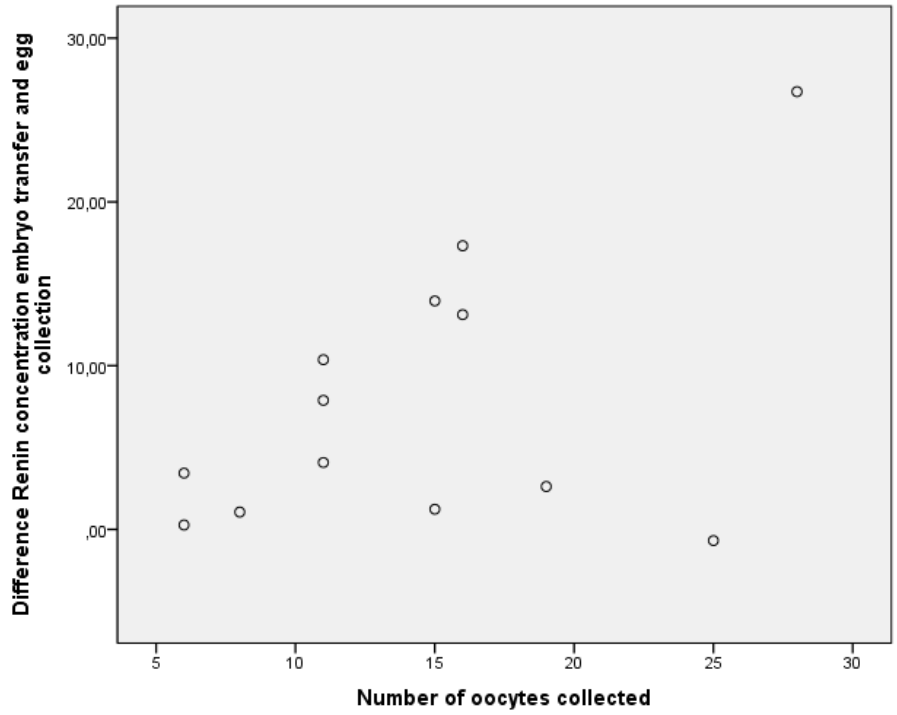


Figure 5-19: The difference between renin activity at oocyte retrieval and embryo transfer seems to increase with the number of oocytes collected.

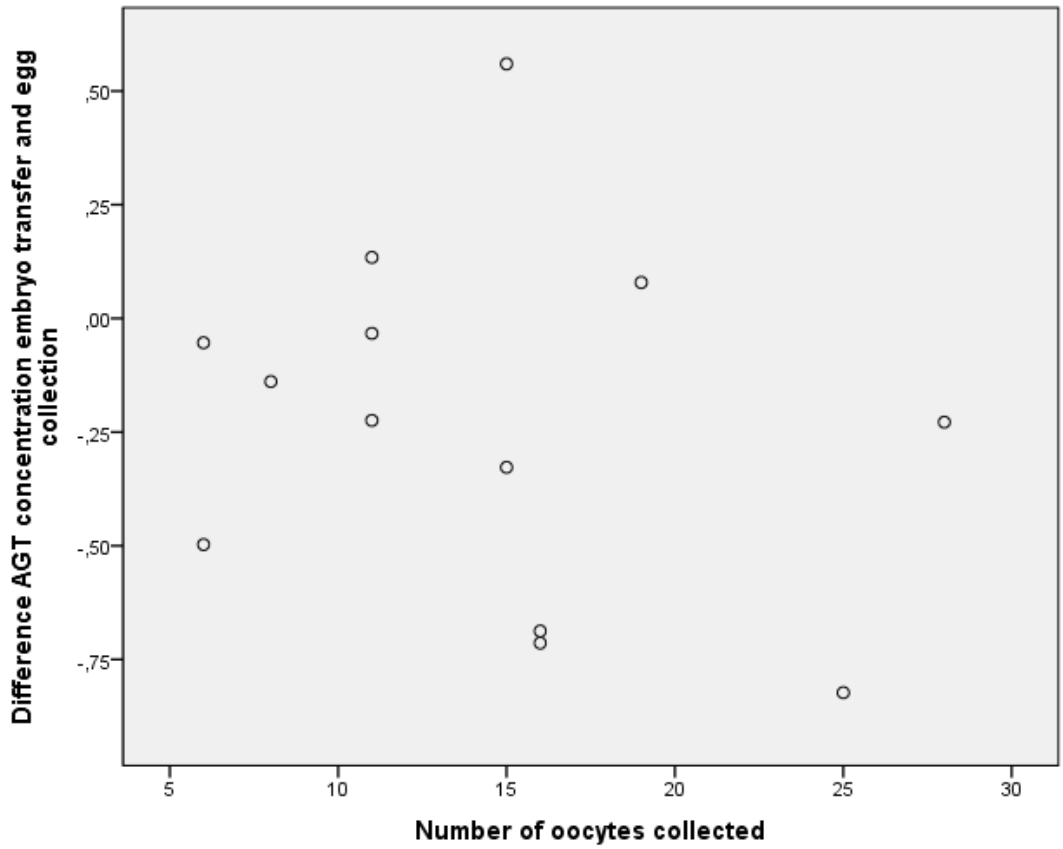


Figure 5-20: AGT at embryo tranfer deducted trom the AGT at oocyte retrieval, the diffrence (decrease on increase) seems unrelated witht the number of oocytes retrieved.

5.4.9 RAS at ovulation and embryo transfer

Of the 47 women at the first time point, 23 had a positive urine LH-kit result highly suggestive for ovulation (Behre, Kuhlage et al. 2000). The blood sampling and scans were performed on day 6 to day 9 after the positive test with, majority (87%) having the tests performed on day 7 or 8 after the LH surge. Patients who did not have a documented LH surge had their procedures carried out on cycle day 21, starting counting from menstruation. This is similar to the timing of procedures in women with a recorded LH surge.

In a stimulated cycle during IVF, hCG is given to mimic the LH surge which occurs in a natural cycle. The oocyte retrieval is a day and a half later and the embryo transfer ideally 5 days after the oocyte collection. Due to this similarity, time point one (luteal phase natural cycle) and two (embryo transfer) are deemed comparable. No correlation with the numbers of days after the LH surge and the RAS levels was found. Table 5-5 demonstrates the results. No significant differences for either renin or AGT were shown (P>0.05; Mann Whitney U test).

	Positive LH surge N=18	No LH surge N=20	P
Renin	5.35 [4.46]	4.24 [3.13]	0.38
AGT	0.38 [0.41]	0.56 [0.47]	0.11

Table 5-5: Median and IQR of renin and AGT in proven and non-=proven ovulation

As no differences in RAS levels were demonstrated between the proven LH surge and absent LH surge, it was deemed appropriate to compare the RAS levels of ovulation with the RAS levels at embryo transfer to see if RAS was activated by ovarian stimulation. Data of 13 women was available for this comparison (see Table 5-6). A significant increase in both Renin (P=0.003) and AGT (P=0.004) is observed. With the assumption that this is a similar time point in a menstrual cycle, the ovarian stimulation contributes to the activation of RAS.

	Ovulation	Embryo transfer	Wilcoxon Rank Test
Renin	5.42 [3.4]	14.69[11.9]	P=0.003
AGT	0.53 [0.6]	0.96 [0.8]	P=0.004

Table 5-6 Activated RAS at ET compared to natural cycle luteal phase (median and IQR)

5.4.10 RAS and patients with OHSS

There were 4 women who had a cancelled embryo transfer due to a high risk of developing OHSS. Their embryos were cryopreserved. Two of these women came for blood sampling and ultrasound scan on the day of the planned embryo transfer. Demographic details of these participants are presented in Table 5-7. None of the four patients had renin and AGT levels on all 3 time points, due to the fact that there was no sample collected during the oocyte retrieval by the anaesthetist or the refusal by the patient for additional blood tests beyond normal treatment protocol.

Patients with high numbers of oocytes collected (19 and 25) with data available from all three time points are of particular interest (N=2). Both women were prescribed Cabergoline from the day of oocyte collection (0.5mg daily), both had free fluid at time of embryo transfer and they also both became pregnant. A noteworthy observation is that the woman with 19 oocytes collected became symptomatic and needed to be hospitalized for symptom relief. Data in Figure 5-21 and Figure 5-22 demonstrates that the woman with more oocytes (25) who did not become symptomatic

seemed to not have an activation of RAS. No conclusion may be drawn as this observation is about 2 women.

	Patient A	Patient B	Patient C	Patient D
Nr of oocytes collected	32	37	26	27
Peak E2	10874	3407	6421	18350
E2 at egg collection	9028	3649	4611	5406
P at egg collection	221.18	11.21	14.62	13.7
R at egg collection	Not available	Not available	14.67	1.43
AGT at egg collection	Not available	Not available	0.396	0.153
Total ovarian volume at egg collection	Not available	227	200	172
Follicles at egg collection	Not available A	40	33	24
R day at embryo transfer	112.74	40.63	NA	NA
AGT at embryo transfer	1.343	0.496	NA	NA
OHSS symptoms	yes	No	No	yes

Table 5-7: RAS in patients with OHSS.

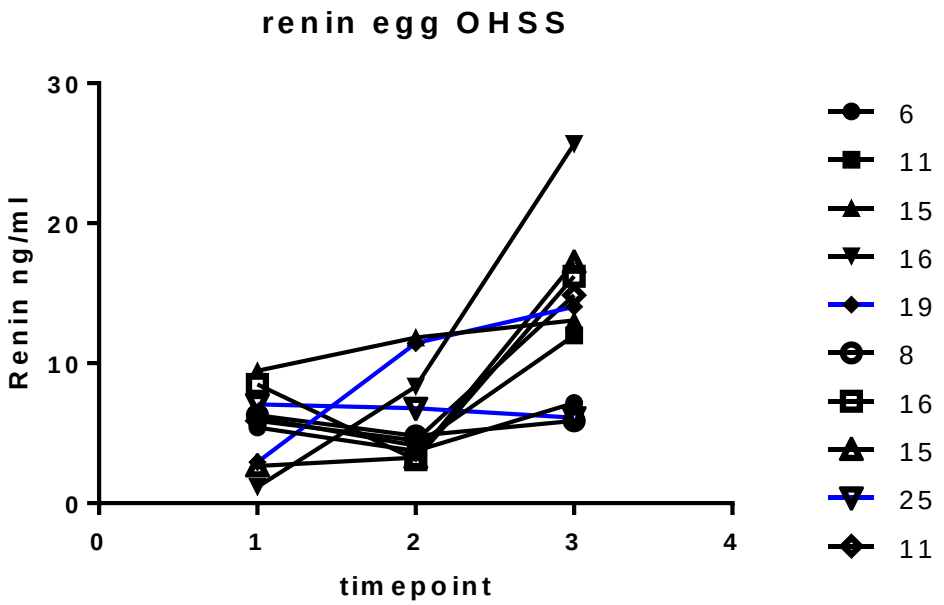


Figure 5-21: Renin levels at 1 (natural cycle), 2 (oocyte collection) and 3 (embryo transfer) time points with the number of oocytes retrieved, with blue representing the 2 OHSS cases..

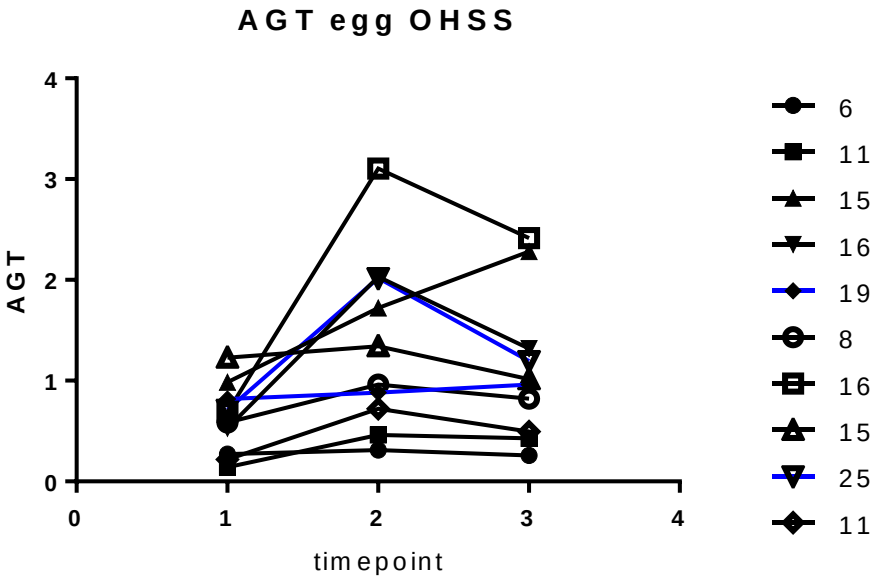


Figure 5-22: AGT levels levels at 1 (natural cycle), 2 (oocyte collection) and 3 (embryo transfer) time points with the number of oocytes retrieved, with blue representing the 2 OHSS cases..

5.4.11 RAS in patients with pregnancy after treatment

When comparing RAS levels between women who became pregnant and the ones that did not, with the exclusion of the patients with cancelled embryo transfer, only renin levels at embryo transfer were significantly different between participants (P=0.04; Mann Whitney U; see Figure 5-23).

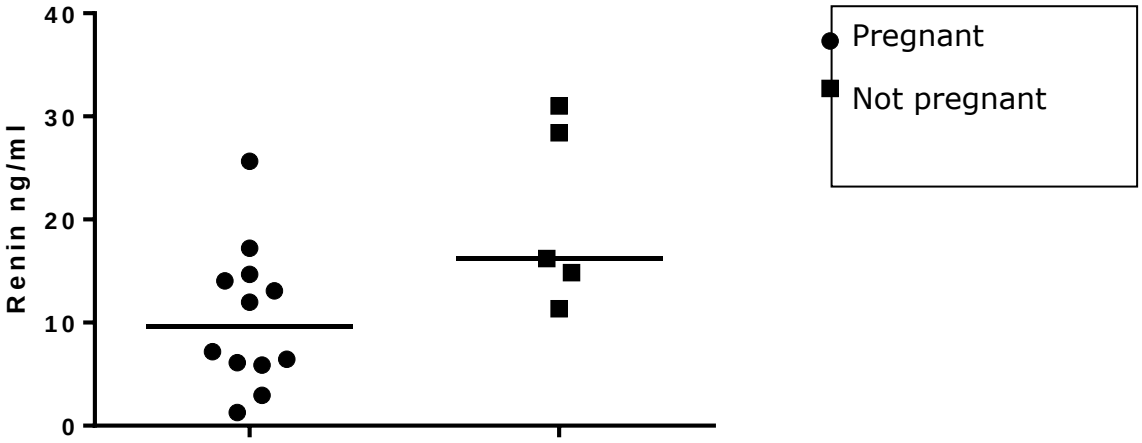


Figure 5-23 Renin levels at transfer in patients with an embryo transfer per outcome group (horisontal line representing median).

5.5 Discussion

The main findings for the four aims of this study are firstly, that whole blood cannot be frozen and stored for subsequent RAS analysis. Secondly, the dilution which is adequate to use for RAS analysis in patient with ovarian stimulation does not require any adjustment. Thirdly in natural cycles, RAS analysis at similar time points are consistent. And lastly in patient with OHSS do not demonstrate a difference in serum renin and angiotensin levels compared to other subject with ovarian stimulation.

Whole bloods samples collected in EDTA tubes and directly frozen and stored were found to be unsuitable for RAS analysis after thawing and spinning at a later date. The describe method for analysis for R and AGT is a cumbersome. With one of the complication factors being that the blood sample obtained needs to be spun down as soon as possible. The result that the samples cannot be stored, but need to be immediately handled with each individual patient, makes clinical implementation difficult.

The natural cycles having R and AGT measured at similar time points, suggests that one analysis should be sufficient as there is no intercycle variation demonstrated. The biopsy did not seem to have any influence on the result, although there was no group with inter cycles R and AGT without biopsy. In the population having IVF, some patient will have had an endometrial biopsy. This is unlikely to have had an influence on the results.

In our populations, in the spontaneous cycle, there was no relationship between renin and angiotensinogen levels and some of the markers of OHSS such as number of follicles, ovarian volume, AMH and oestrogen and progesterone levels. This was expected, as homeostasis should be

maintained throughout the menstrual cycle irrespective of ovarian reserve markers. With this proof of concept demonstrated we have looked in greater detail at how RAS reacts during ovarian stimulation.

It was believed that the hCG from pregnancy was responsible for the activation of RAS. In this small study, it has been demonstrated, that the rise of renin occurs even before the embryo has made contact with the endometrium and can start implanting. This was observed in the one patient with baseline high renin levels in the intercycle study, which suffered from recurrent miscarriages.

Although the number of participant having RAS levels measured before and after an endometrial biopsy is low, the differences observed are not significantly different. When following women through the stimulated cycle it is remarkable that renin at the time of oocyte collection is not elevated while AGT is. At the time of embryo transfer the AGT is at the same level as at oocyte collection, while the renin has made a significant rise.

This is the first study performing a longitudinal evaluation of R and AGT during ovarian stimulation. The combination with ovarian volume and the number of follicles is also a new aspect aiming to improve our understanding of ovarian RAS, folliculogenesis and OHSS. This is a pilot study and no power calculations were performed prior to recruitment mainly due to the unknown effect size that would be observed between various time points and populations.

Not all ultrasound acquisitions were suitable for analysis due to motion artefacts or inability to obtain a volume of the entire ovary because of its enlargement following controlled ovarian hyperstimulation. This makes counting ovarian follicles challenging, as some follicles will press on others, making it difficult to unequivocally ascertain the actual numbers. After oocyte collection the follicles fill with blood making a differentiation between a follicle and ovarian stroma not reliable.

RAS analysis is a very cumbersome process. It would however be of great interest to repeat our study in a prospective ovarian stimulation population. It might create a possibility to distinguish patients who have a lot of oocytes collected but remain asymptomatic (do not develop OHSS) and patients who develop extravasation and become symptomatic.

Future research should focus on ascertaining the relationship of RAS and pregnancy outcomes and the relationship with OHSS. The IVF setting is ideal because changes might be observed prior to the embryo reaching the cavity and starting implantation.

Chapter 6: Discussion

Risk is the potential of losing something of value, weighed against the potential to gain something of value. Values such as physical health or emotional wellbeing can be gained or lost by taking a risk. Risks in personal health may be reduced by primary prevention actions that decrease early causes of illness or by secondary prevention actions after a person has clearly measured clinical signs or symptoms recognized as risk factors. Tertiary prevention reduces the negative impact of an already established disease by restoring function and reducing disease-related complications (Kungwani 2014).

6.1 Overall conclusion

The aim of this thesis was to understand the risks involved with ovarian stimulation when treating couples with difficulty to conceive.

In the introductory chapter the background of the risks and complications of ovarian stimulation are given. The most significant complications are related to ovarian stimulation, a smaller portion are procedure related complications. The limited availability of evidence on how to correctly identify women who would be at risk of these complication is highlighted.

Performing a salpingectomy prior to ovarian stimulation does not influence the response. When low oestradiol levels are obtained as a result of the treatment, the pregnancy outcome is negatively influenced. Patient related factors are important in order to recognise the women who could benefit from adjustment of standard treatment. It is however equally important to

make sure no iatrogenic complications, such as tubal surgery for tubal pathology, are inflicted.

If during treatment an overresponse occurs, it is important to try to prevent the development or aggravation of symptoms. For this secondary form of prevention dopamine agonists can be used. The systematic literature review in Chapter 3: demonstrates that dopamine agonist can be used for prevention, but not for treatment (Baumgarten, Polanski et al. 2013). The clinical observations presented in that chapter support the literature findings. The numbers however are too small to draw any conclusions that the use is safe without further implications. A national registration (for example all dopamine agonist use should be registered with the HFEA) would allow long term follow-up of the offspring.

Data presented in Chapter 4: reports a candidate gene study aiming to predict ovarian response to ovulation induction with Clomiphene Citrate. After evaluation of the allelic distribution, no correlation could be found with the phenotypes. Due to the sample size it might be too early to construct a definitive conclusion. Systematic review of the literature did not find any suitable data for comparison in a meta-analysis.

The renin-angiotensin system is related to follicular development and potentially plays a role in OHSS. The small feasibility study did not find any significant correlation with ovarian volume and stimulation outcomes. Future basic science research might be better focussed on different factors of the follicular pathway with the aim to understand pathophysiology of OHSS.

In the future, the use of antagonist ovarian stimulation protocols combined with agonist triggers and modified luteal support in case of fresh embryo transfer will need to be explored.

Humaidan et al. (Humaidan, Kol et al. 2011) on behalf of the 'The Copenhagen GnRH Agonist Triggering Workshop Group' of the 'The European Society of Human Reproduction and Embryology' conducted several systematic reviews showing that in fresh IVF cycles with embryo transfer (9 RCTs) no OHSS was reported after GnRH agonist trigger [0% incidence in the GnRHa group: risk difference 5% (with 95% CI: 20.07 to 0.02)]. Importantly, the delivery rate improved significantly after modified luteal support [6% risk difference in favour of the HCG group (95% CI: 20.14 to 0.2)] when compared with initial studies with conventional luteal support [18% risk difference (95% CI: 20.36 to 0.01)]. In oocyte donation cycles (4 RCTs) the OHSS incidence is 0% [10% risk difference (95% CI: 0.02–0.40)].

The overall conclusion of this thesis is that future research should be done to prevent OHSS, as this is the most severe complication of ovarian stimulation.

6.2 Future research proposal

Women at risk of developing over response, which has been defined by expert group, clinicians survey and own data as the presence of more than 22 antral follicles during pre-treatment ultrasound, will be asked to participate in the study. They will receive written and oral information

related to the study. If they express the wish to participate, they will sign the consent form before embarking on treatment. The participants will undergo a standard GnRH-antagonist ovarian stimulation protocol, but they will be randomised for final follicular maturation with hCG (Pregnyl® 1000IU) or an GnRH-agonist (Suprecur®, buserelin, 0.5ml) and modified luteal support (Cyclogest®, progesterone, 400mcg twice a day, and Progynova®, oestradiol, 2mg three times a day).

Subjects will be randomised to one of the two treatment arms based on a computer generated pseudorandom code using random permuted blocks of randomly varying size, created by the Clinical Trials Unit (CTU).

The main objective of the study is to assess which final oocyte maturation protocol can prove to be most beneficial in primary prevention of ovarian hyperstimulation syndrome for women at risk of over response to ovarian stimulation. We will compare the new protocol (GnRHa) to the currently used protocol (hCG), to assess the prevalence of OHSS.

The primary objective of this study is to investigate whether standard treatment (hCG final maturation protocol) leads to a greater prevalence of OHSS than the alternative treatment under investigation (GnRHa final maturation protocol).

To assess the biochemical markers of folliculogenesis and clinical pregnancy rates in women undergoing a fresh embryo transfer IVF/ICSI per protocol.

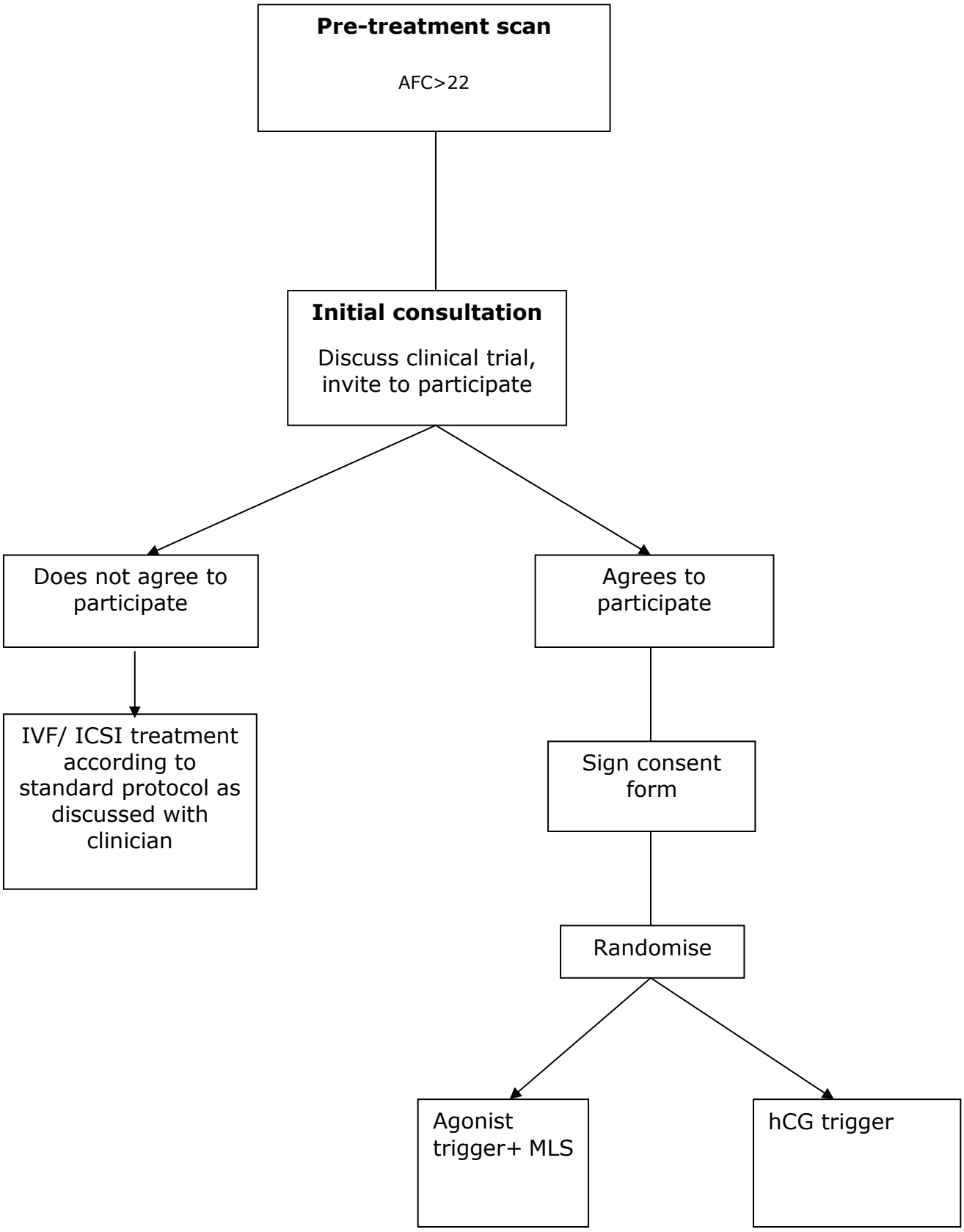
6.2.1 Summary of Trial Design

This study is designed as a randomised controlled trial between two triggers for final maturation in a GnRH-antagonist treatment protocol.

Treatment A entails an antagonist protocol with hCG as final maturation trigger and progesterone used for luteal support. Treatment B entails an antagonist protocol with agonist trigger as final maturation medication, with hCG, progesterone and oestrogen used as luteal support.

No extra visits to the unit will be required for this study.

The time period related to the IVF or ICSI treatment is, on average, 2 weeks. Embryo transfer will take place 2, 3, or 5 days after oocyte collection, depending on embryo quality. This is judged by the duty embryologist. After embryo transfer, a urinary pregnancy test is performed 14- 16 days later by the patient. A routine early ultrasound scan is performed at 6 weeks of pregnancy to assess the development of the embryo. A potential pregnancy will be followed up until the outcome of the pregnancy is known. The units' routine practice involves a phone call to the patient after the expected delivery date to enquire about the final outcome and any complications that might have arisen during the pregnancy.


Pre-treatment scan
AFC>22
Initial consultation
Discuss clinical trial, invite to participate
Does not agree to participate
Agrees to participate
IVF/ ICSI treatment according to standard protocol as discussed with clinician
Sign consent form
Randomise
Agonist trigger+ MLS
hCG trigger

6.2.2 Primary and Secondary Endpoints/Outcome Measures

Prevalence of OHSS in the two treatment groups comprises the primary outcome.

The secondary endpoints of the study entail:

- The number of recruited follicles (>10 mm)
- The number of mature follicles (>14 mm)
- The total number of oocytes collected
- The number of mature oocytes
- E2 levels before final oocyte maturation injection
- Fertilisation rate
- Embryo development
- Embryo quality
- Pregnancy rate
- Life birth rate
- Serum E2 levels during stimulation and oocyte retrieval
- Serum Progesterone levels during stimulation and oocyte retrieval
- Serum FSH and LH levels during stimulation and oocyte retrieval
- Serum VEGF and AMH levels during stimulation and oocyte retrieval
- Serum prolactin levels during stimulation and oocyte retrieval
- Serum androgen levels pre stimulation
- Serum testosterone levels during stimulation and oocyte retrieval
- Total renin levels during stimulation and oocyte retrieval
- Active renin levels during stimulation and oocyte retrieval

- Prorenin levels during stimulation and oocyte retrieval
- Aldosterone levels during stimulation and oocyte retrieval
- Endothelin-1 levels during stimulation and oocyte retrieval
- IGF-2 and IGF-1 levels during stimulation and oocyte retrieval
- AMH single nucleotide polymorphisms
- AMH receptor type 2 single nucleotide polymorphisms
- Androgen receptor single nucleotide polymorphisms
- Follicle-stimulation hormone receptor single nucleotide polymorphisms
- Cytochrome P450 aromatase single nucleotide polymorphisms
- VEGF type 1 receptor single nucleotide polymorphisms
- VEGF type 2 receptor single nucleotide polymorphisms
- Von Willebrand factor level/activity
- Follicular VEGF and AMH levels
- Micro arrays of follicular fluid
- Ovarian volumes measured by ultrasound during oocyte retrieval
- Evaluation of endometrial response to stimulation by obtaining an endometrial biopsy if no fresh transfer can be performed
- Multiple pregnancy rates
- Development of side effects
- Cycle cancelation rate
- Treatment cancelation rate
- Freeze all embryos rates

- Hospitalisation

Overall Description of Trial Participants:

All women eligible to undergo controlled ovarian stimulation for fertility treatment will have a pre-treatment ultrasound scan and an AMH level preformed as their routine work-up.

If they meet the criteria (established by an expert group and a clinicians survey) of being at risk of over response, they will be asked to participate in the study. They will receive written and oral information. They can be identified during the initial ultrasound scan used to assess ovarian reserve, or at the initial consultation.

If they want to participate, they will sign the consent form before embarking on treatment. This can be done at initial consultation with the consultant, or any time after, if the patient would prefer more time to consider participating in the study. Following consent, the patient will be randomized to one of the two study protocols, and afterwards they will commence IVF or ICSI treatment according to their allocated treatment plan.

A patient will contact the unit on day one of her period. She will start the allocated protocol the day after. They will follow the protocol as described in the unit's standard operating protocol (SOP):

Treatment A: GnRH ANTAGONIST (Cetrotide®) with hCG-trigger

- Commence gonadotropin (FSH) of choice on day 2 of menstruation
- Day 5 of stimulation add Cetrotide® 0.25 mg injection SC
- Commence monitoring follicular development with regular ultrasound scans, oestrogen, and LH-levels from day 5.
- FSH plus antagonist are continued until 3 follicles or more are >18mm
- A single dose of hCG (Pregnyl® 10.000IU) is administered 36 hours prior to scheduled oocyte recovery and FSH and antagonist are discontinued.
- Oocyte recovery and embryo transfer as per protocol
- Start luteal support from second day after oocyte retrieval: cyclogest® 400mg vaginally twice a day
- Pregnancy test 16 days post embryo transfer
- In case of a positive pregnancy test, luteal support is continued until 7 weeks after oocyte recovery, and an ultrasound scan is arranged 5 weeks after embryo transfer

Treatment B: GnRH ANTAGONIST (Cetrotide®) with Buserelin trigger

- Commence gonadotropin (FSH) of choice on day 2 of menstruation
- Day 5 of stimulation add Cetrotide® 0.25 mg injection SC
- Commence monitoring follicular development with regular ultrasound scans, oestrogen, and LH-levels, from day 5.
- FSH plus antagonist are continued until 3 follicles or more are >18mm
- A single dose of 0.5mg buserelin is administered 36 hours prior to scheduled oocyte recovery and FSH and antagonist are discontinued.
- Oocyte recovery and embryo transfer as per protocol
- Start modified luteal support: one bolus of hCG (Pregnyl®) on day of oocyte recovery and blastocyst transfer, Progynova 2mg twice a day after oocyte recovery, cyclogest to be started 2 days after oocyte collection 400mg vaginally twice a day
- Pregnancy test 16 days post embryo transfer
- In case of positive pregnancy test, luteal support is continued until 7 weeks after oocyte recovery and an ultrasound scan is arranged 5 weeks after embryo transfer

6.2.3 Inclusion Criteria

- Women with infertility requiring IVF/ICSI treatment, at risk of developing over response (AFC > 23 and/or AMH>35)

6.2.4 Exclusion Criteria

- Contra-indications for antagonist treatment (know allergies for components of the medication used)
- Age < 18 years
- Women who are unable to give informed consent

6.2.5 Screening and Eligibility Assessment

Potential participants will be identified and approached at the standard pre-treatment scan prior to commencement of IVF or ICSI treatment.

Recruitment will take place at initial consultation and will be conducted by the consulting clinician. There will be at least 24 hours between those two appointments, to allow sufficient time for the participant to familiarise with the study information.

6.2.6 Subsequent assessments

All patients will attend the unit according to the units' protocols for each treatment. In brief this includes informing the unit of the first day of the menstrual cycle and commencing stimulation on day 2, an ultrasound scan will be performed after 5 days of stimulation to assess the response to the stimulation medication. Subsequent visits are scheduled according to the results of this scan and decided upon by the duty clinician. Final oocyte maturation trigger will be administered when at least three follicles will be 18 mm in diameter. As in standard practice, in all patients who have more than 20 oocytes collected at oocyte recovery procedure, a day 2 assessment of eligibility for embryo transfer will be made. This will entail an anamneses

and physical examination including an ultrasound scan. A blood analysis to assess liver function, kidney function and clotting will be performed.

The unit's consultant will be available during out of office hours for any questions or concerns as is standard practice.

In case of clinically significant early or late onset OHSS, the patient will be admitted to the gynaecology ward for appropriate inpatient management. In case of clinically significant early onset OHSS when an embryo transfer is contraindicated, the woman will be asked to undergo an endometrial biopsy to assess the cellular and molecular response of the endometrium to ovarian stimulation medications.

Chapter 7: References

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