# ASSESSMENT OF OVARIAN RESERVE IN WOMEN UNDERGOING CYSTECTOMY FOR BENIGN OVARIAN DISEASE

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## Declaration

I commenced my pursuit of a higher research degree with a question,

"Does an ovarian cystectomy affect ovarian reserve and if it does, how do I assess it?" This thesis attempts to answer this question and many other related questions that arose during my research in this area. Whilst, I am proud to contribute towards our understanding of human reproductive patho-physiology and the assessment of ovarian reserve, I am humbled by the great minds and the relentless researchers in this field of medicine that have informed this piece of work".

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.

**S Deb**

**May 2012**

## Acknowledgements

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**Dedicated to**

**The women who volunteered to participate unconditionally
in my research studies**

**For**

**Rahul and Arush Deb**

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# Abbreviations

| | |
|---|---|
| 2D | Two-dimensional |
| 3D | Three-dimensional |
| AFC | Antral follicle count |
| AMH | Anti-Müllerian hormone |
| ART | Assisted Reproduction Treatment |
| AUC | Area under the ROC curve |
| BMI | Body mass index |
| CI | Confidence Interval |
| CCCT | Clomiphene citrate challenge test |
| COS | Controlled ovarian stimulation |
| EFORT | Exogenous FSH ovarian reserve test |
| FI | Flow Index |
| FSH | Follicle stimulating hormone |
| GAST | GnRH agonist stimulation test |
| GnRH | Gonadotrophin stimulating hormone |
| HCG | Human chorionic gonadotrophin |
| ICC | Intra-class correlation coefficient |
| ICSI | Intracytoplasmic sperm injection |
| IGF | Insulin like growth factor |
| IUI | Intrauterine insemination |
| IVF | *In vitro* fertilisation |
| LH | Luteinising hormone |
| LLOA | Lower limit of agreement |
| LOA | Limits of agreement |

| | |
|---|---|
| LR | Likelihood ratio |
| MEIA | Microparticle Enzyme Immunoassay |
| MG | Mean Grey Value |
| MoM | Multiples of the mean |
| NPV | Negative predictive value |
| OHSS | Ovarian hyperstimulation syndrome |
| PDA | Power Doppler angiography |
| PI | Pulsatility Index |
| PPV | Positive predictive value |
| PRF | Pulse repetition frequency |
| PSV | Peak systolic velocity |
| rFSH | Recombinant follicular stimulating hormone |
| RI | Resistance Index |
| ROC | Receiver operating characteristic |
| SD | Standard Deviation |
| SonoAVC | Sonography based automated volume calculation |
| ULOA | Upper limit of agreement |
| VEGF | Vascular Endothelial Growth Factor |
| VFI | Vascularisation Flow Index |
| VI | Vascularisation Index |
| VOCAL | Virtual Organ Computer-aided AnaLysis™ |

# Abstract

Ovarian cystectomy is commonly performed to treat benign ovarian cysts, but might cause inadvertent damage to normal ovarian tissue, thereby influencing a woman's ovarian reserve. Ovarian reserve is defined as the existent quantitative and qualitative supply of follicles which are found in the ovaries that can potentially develop into mature follicles which in effect determine a woman's reproductive potential.  It is commonly quantified by the levels of serum FSH and recently by total antral follicle count (2.0-10.0 mm follicles in both ovaries) and AMH levels. These tests however have inherent biological variation in relation to menstrual cycle and ageing; and are also influenced by the intra- and inter-observer variations. The aim of this thesis was to develop a reliable method of examining the effect of ovarian cystectomy on ovarian reserve.

 I began by examining the ultrasound markers of ovarian reserve. AFC is measured using 2D ultrasound and there is some evidence that 3D ultrasound can make more reliable counts than 2D. I examined the reliability of these two methods and compared them to a new 3D assisted method, SonoAVC which is designed to make automated AFC. I found that the intra- and inter-observer reliability of SonoAVC in counting the number of antral follicles was superior to 2D and 3D manual methods. It however required post-processing of the counts by manually clicking on the antral follicles initially missed in the automated version, thereby making it a semi-automated method.

I then compared 2D ultrasound to SonoAVC in measuring the size of antral follicles as there is increasing evidence that the small antral follicles might be more predictive of ovarian reserve. I found that SonoAVC measured the size of antral follicles significantly quicker than 2D and also that the number of small follicles measured by 2D were more

than SonoAVC, thereby raising the possibility that 2D might overestimate the number of small antral follicles.

I then studied the ability of antral follicle counts stratified by size in prediction of ovarian response and pregnancy. I found that the small antral follicles measuring between 2.0-4.0 mm were independent predictors of clinical pregnancy and ovarian response to assisted reproduction treatment.

I then examined the AFCs of different sizes made by SonoAVC and 2D in bovine ovaries and compared to the follicles obtained by manually dissecting the follicles. I found that SonoAVC with post-processing significantly underestimated and 2D overestimated the number of antral follicles measuring 4.0mm or less, but both made comparable counts of follicles measuring more than 4.0mm when compared with the antral follicles dissected manually. However, the agreement with SonoAVC with post-processing was more than that with 2D.

Having established that SonoAVC albeit with post-processing was the most reliable method in measuring the size of antral follicles, I began to examine the intra- and inter-cycle variation and compared to AMH. I found that the small antral follicle measuring 2.0-6.0 mm showed least intra-and inter-cycle variation and that it was comparable to AMH. The larger antral follicles showed significant intra-cycle variation but a non-significant inter-cycle variation in the early follicular phase of menstrual cycle.

I also examined the inter-ovarian variation in the AFC's and found that the small antral follicles measuring 2.0-6.0 mm again showed the least variation between ovaries within an individual. I was finally able to conclude that small antral follicles (≤6.0mm) measured using SonoAVC were the most reliable in prediction of ovarian reserve, and showed excellent correlation with AMH.

Finally, I examined the effect of laparoscopic ovarian cystectomy on the ovarian reserve for up to 6 months post-operatively using AMH and small AFC measured by SonoAVC. I found that ovarian cystectomy significantly reduces ovarian reserve and that this effect may be more pronounced with cysts of endometriotic nature, followed by dermoid cysts.

In summary, the effect of ovarian cystectomy on ovarian reserve is best quantified using AFC of small follicles measuring less than 6.0 mm as it provides reliable measures of ovarian reserve, has minimal biological variation and is comparable to AMH.

# Peer-reviewed publications

Deb S, Jayaprakasan K, Campbell BK, Clewes JS, Johnson IR, Raine-Fenning NJ. Intraobserver and interobserver reliability of automated antral follicle counts made using three-dimensional ultrasound and SonoAVC. *Ultrasound Obstet Gynecol* 2009;33(4):477-83.

Deb S, Campbell BK, Clewes JS, Raine-Fenning NJ. Quantitative analysis of antral follicle number and size: a comparison of two-dimensional and automated three-dimensional ultrasound techniques. *Ultrasound Obstet Gynecol* 2010;35(3):354-60.

Deb S, Batcha M, Campbell BK, Jayaprakasan K, Clewes JS, Hopkisson JF, et al. The predictive value of the automated quantification of the number and size of small antral follicles in women undergoing ART. *Hum Reprod* 2009;24(9):2124-32.

Deb S, Kannamannadiar J, Campbell BK, Clewes JS, Raine-Fenning NJ. The interovarian variation in three-dimensional ultrasound markers of ovarian reserve in women undergoing baseline investigation for subfertility. *Fertil Steril*;95(2):667-72.(Epub ahead of print)

Deb S, Campbell BK, Pincott-Allen C, Clewes JS, Cumberpatch G, Raine-Fenning NJ. Quantifying the effect of the combined oral contraceptive pill on the functional ovarian reserve as measured by serum anti-Müllerian hormone and the small antral follicle count made using three-dimensional ultrasound. *Ultrasound Obstet Gynecol;* 2011 Oct 13. doi: 10.1002/uog.10114. [Epub ahead of print]

# CHAPTER 1.  Background and Hypothesis

Benign ovarian cysts develop within the ovarian parenchyma and are often removed either using a minimally invasive procedure such as laparoscopy or an open abdominal procedure called the laparotomy. Both, the cyst in itself and the cystectomy i.e. excision and enucleating of cyst from the ovary can influence the ovarian reserve. Ovarian reserve is an important concept widely used in the field of reproductive medicine, especially when planning assisted reproduction treatments. It is defined as the existent quantitative and qualitative supply of follicles which are found in the ovaries that can potentially develop into mature follicles which in effect determine a woman's reproductive potential. It is also used as a term to determine the capacity of the ovary to develop oocytes capable of fertilisation, resulting in a healthy and successful pregnancy.

This chapter is an introduction to reproductive physiology, ovarian reserve and methods used for its evaluation; and management of benign ovarian cysts and its effect on ovarian reserve.

## 1.1    *Introduction to reproductive physiology*

### 1.1.1  Folliculogenesis

From birth, the ovaries of the human females contain primordial follicles with immature oocytes. In the adult ovary, a clutch of follicles leave the pool of resting follicles to begin folliculogenesis, entering the growth phase that will end in atresia or in ovulation (Gougeon 1998). The factors controlling this initial recruitment is still unclear but may be mediated by the counterbalance of various stimulatory and inhibitory hormones and locally expressed growth factors (Fortune, Cushman et al. 2000). The process of

folliculogenesis, from primordial to preovulatory follicle takes approximately 6 months. It is a continuous process, and therefore at any time, the ovary contains follicles in all stages of development.

## Morphology and function of follicles

During the basal follicular growth the mitotic activity of the granulosa cells progressively increases but is only slightly susceptible to gonadotrophins which are attributed to the number of receptors for gonadotrophins present on the follicle cells and the transducing mechanisms coupling with these receptors (Jonassen, Bose et al. 1982). In vitro studies have shown that the pre antral follicles produce very low amounts of progesterone and androstenedione and undetectable amounts of Oestradiol (Roy and Treacy 1993). Granulosa cells cultured from these follicles have low aromatizing capacity (McNatty, Makris et al. 1979; McNatty, Makris et al. 1979).

Once the primordial follicle is initiated, it is irreversibly committed to enter into the growing follicular pool and cannot return to the resting state (McNatty, Reader et al. 2007). The stromal cells further differentiate into an outer theca externa and an inner theca interna. Secondary follicles grow into preantral follicles when the theca interna commences its epithelioid differentiation, and constitutes the first category of growing follicles in a classification based on morphology and number of granulosa cells in each individual follicle. From the follicular diameter of 0.15mm, the preantral follicles increase in size to 0.18 – 0.25mm and are called early antral follicles. Certain local factors including GDF-9, BMP-15, basic fibroblast growth factor (bFGF) and EGF might be responsible for this initial initiation of primordial follicles (Webb and Campbell 2007). Activin is shown to

promote granulosa cell proliferation in the pre-antral follicles (Findlay 1993) and Inhibin A, expressed by the dominant follicles exhibits an inhibitory effect on the neighbouring early growing follicles thereby playing an important role in follicle selection (Vitale, Gonzalez et al. 2002). The role of Inhibin B is unclear on pre-antral follicle development (McNatty, Heath et al. 1999). These early pre-antral follicles are independent of gonadotrophins (Webb, Campbell et al. 1999), although FSH receptor mRNA is detected in secondary follicles and beyond (Webb and Campbell 2007). In vitro animal studies have shown that FSH alone may not be sufficient in the growth of primary and secondary follicles (Fortune, Kito et al. 1998; Liu, Andoh et al. 1998), and that addition of local factors such as IGF-1 and activin might assist the growth of follicles (Liu, Andoh et al. 1998; Webb and Campbell 2007).

Through the accumulation of fluid in the antral cavity and proliferation of granulosa cells and theca interna cells, the follicle reaches a size of 2mm when it is called tertiary follicle or a Graffian follicle or an antral follicle. The stage of folliculogenesis when follicles grow from the pre antral stage to a size of 2mm is called basal follicular growth, which takes about 70 days, during which time they become gonadotrophin responsive (Gougeon 1996). During the follicular phase of the menstrual cycle, the final stages of follicular growth occur with the development of gonadotrophin dependent antral and preovulatory follicles. The selectable follicles constitute the pool of follicles from which the one destined to ovulate in the subsequent cycle will be selected (Gougeon and Lefevre 1983). Selectable follicles are present throughout the menstrual cycle. During the late luteal phase following the disintegration of corpus luteum and consequent rise in pituitary follicle stimulating hormone (FSH), the 2 – 5 mm follicles, which have entered the preantral stage 70 days earlier, are recruited into gonadotrophin dependent selectable

follicles, competing with each other for growth inducing FSH. They exhibit a significant increase in the rate of cell proliferation, becoming more dependent on FSH (Gougeon 1984). In vitro studies have shown that these follicles become responsive to gonadotrophins in terms of quality and growth rate but that their FSH-induced aromatase remains poorly expressed (McNatty, Makris et al. 1979; Sasano, Okamoto et al. 1989; Tamura, Kitawaki et al. 1992; Gougeon 1996). Once these follicles are selected for further growth, continued gonadotrophins support is essential for their survival and further development through the enlargement of the antrum to pre-ovulatory follicle stage (McGee and Hsueh 2000).

AMH expression begins in the primary follicles with highest levels present in secondary, pre-antral and small antral follicles measuring 4 mm or less in diameter (Weenen, Laven et al. 2004) and may have a role in regulation of follicular growth at this stage through an inhibitory effect on the sensitivity of follicles to FSH (Visser and Themmen 2005). They become responsive to gonadotrophins but are not completely dependent on them for their growth (McGee and Hsueh 2000). Animal studies have shown that follicles measuring >100 µm in diameter demonstrate significant steroid production in response to gonadotrophins in sheep (Scaramuzzi, Adams et al. 1993) and that FSH can accelerate the growth of pre-antral follicles through proliferation of granulosa cells (Campbell, Telfer et al. 2000; Campbell, Telfer et al. 2004). As they enlarge they become increasingly responsive to gonadotrophins due to acquisition of FSH and LH receptors (McGee and Hsueh 2000; Webb and Campbell 2007) progressing to a gonadotrophin-dependent phase. Transient increases in FSH levels that follows luteolysis in the late luteal phase of menstrual cycle after the onset of puberty is the key for cyclical recruitment of the follicle that express the FSH receptors maximally (McGee and Hsueh 2000).

An atretic follicle usually fails to develop beyond the size of 10mm (Gougeon and Lefevre 1983) (Figure 1.1). Only about 0.1% of follicles reach the mature pre-ovulatory stage and ovulate in a woman's lifetime and the remaining 99.9% of follicles undergo atresia (Markstrom, Svensson et al. 2002). Follicular atresia is a hormonally controlled apoptotic process through which the degenerating follicles are eliminated in a coordinated fashion. Although apoptosis can occur at all stages of follicle development, the most susceptible phase to atresia is the pre-antral to early antral transition and the subordinate follicles during selection for dominance (McGee and Hsueh 2000). The FSH requirement of the gonadotrophin dependent follicles is higher than that for the gonadotrophin responsive or ovulatory follicles and therefore, they are more vulnerable to atresia once the gonadotrophin level falls (Scaramuzzi, Adams et al. 1993). With increasing size of the follicles and the theca cells, the amount of estrogen increases sharply, mainly due to aromatization of theca derived androgen into estrogen by the granulosa cells. Generally single follicle gains dominance and this is invariably the one from the cohort with the largest number of granulosa cells as it demonstrates an increased sensitivity to FSH. Once the largest follicle reaches 10 mm or more in diameter, it starts to express LH receptors in the granulosa cells, a critical step in the selection of the dominant follicle as these receptors enable the follicle to switch its' gonadotrophin-dependence from FSH to LH and thereby reach dominance over the other FSH-dependent follicles (Webb and Campbell 2007). Oestradiol and later inhibin secreted by these follicles progressively begin to suppress FSH, causing the follicles with lower amount of FSH receptors to become atretic. As more Oestradiol is secreted, more LH receptors are made by the theca cells, inciting theca cells to create more androgen that will become oestrogen. This positive feedback

loop causes LH to spike sharply causing ovulation (Gougeon 1996; van den Hurk and Zhao 2005).

**Figure 1.1 Process of folliculogenesis**

A fixed number of primordial follicles are present at birth and majority of them remain in the resting state. A proportion of these follicles undergo initiation of growth (initial recruitment) both before and during reproductive life and develop through primary, secondary and antral stages, at which majority of them undergo atretic changes. After puberty, a cohort of the antral follicles is rescued by an increase in gonadotrophin levels to develop into pre-ovulatory follicles (cyclic recruitment). However, only one of them emerges as the dominant follicle to grow into ovulatory stage and the remaining cohort of antral follicles subsequently undergoes atresia.
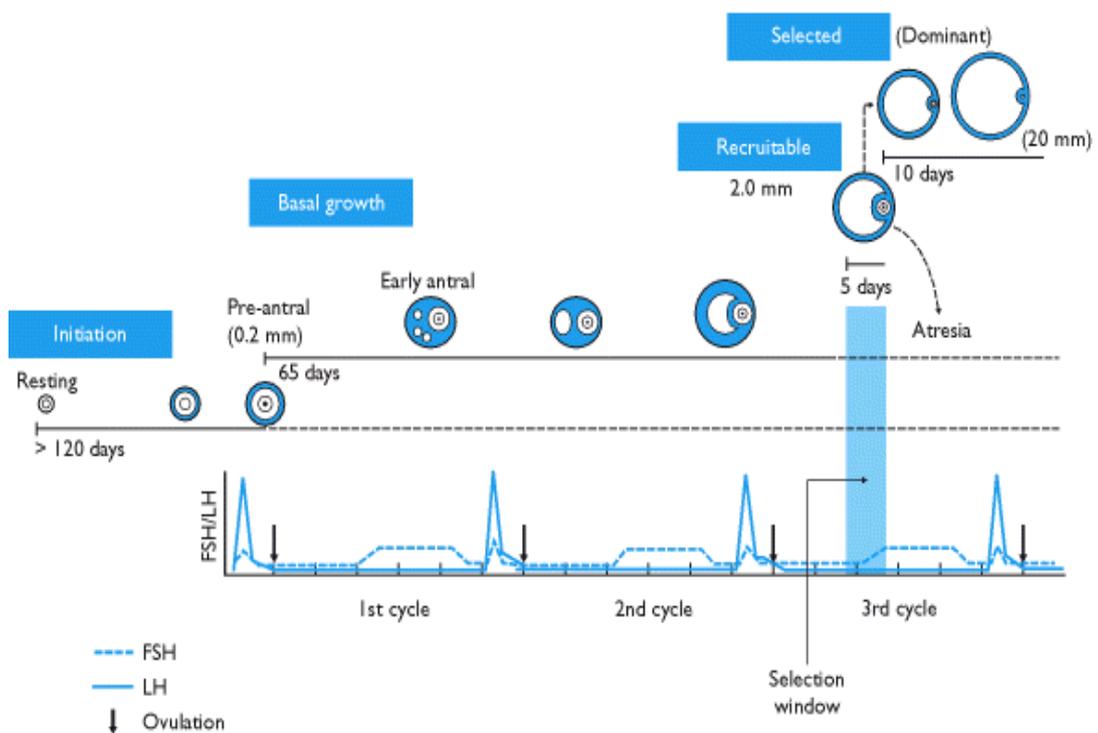
## 1.1.2   Bio-physiology of reproductive ageing

Reproductive ageing is thought to be due to a gradual decrease in both the quantity and quality of the oocytes contained within the follicles present in the ovarian cortex (te Velde and Pearson 2002). By the fourth month of foetal development, the ovaries contain 6-7 million oocytes within primordial follicles, which are surrounded by a layer of flat granulosa cells. This population reduces to 1-2 million primordial follicles by means of apoptosis at birth (Markstrom, Svensson et al. 2002). This process of apoptosis slows down till the age of menarche when approximately 300,000 to 400,000 primordial follicles remain. During the reproductive years, the decline in the number of primordial follicles continues, however at a steady rate of about 1000 follicles per month, accelerating further after the age of 37 years. With the onset of menopause, the population of primordial follicles drops below 1000 (Faddy and Gosden 1996). During the reproductive life, the number of all follicles in different stages of development decrease with an abrupt fall beyond the age of 40 years. This evolution parallels the number of resting follicles, suggesting that the number of growing follicles is positively correlated to the size of primordial follicles (Gougeon, Ecochard et al. 1994).

With the decline in the number of primordial follicles, oocyte quality also diminishes, especially after the age of 31 years, when fecundity gradually starts to decrease. The loss of oocyte quality is believed to be the result of an increase in meiotic nondisjunction, leading to an increasing rate of aneuploidy in the early embryo at higher female ages. Several factors might be responsible for this age related decline in the quality of oocytes. These may relate to differences between germ cells at the time they are formed during foetal life, damage to oocytes during the course of a woman's life or changes in the

quality of granulosa cells surrounding the primordial follicles (te Velde and Pearson 2002).

Oxidative stress associated with ageing could increase the meiotic nondisjunction  leading

to accumulated damage to oocytes (Tarin 1995), impaired perifollicular microcirculation

resulting in low oxygen levels and a  concomitant increase in anaerobic products in the

follicular fluid (Gaulden 1992). Also, endocrine imbalance caused due to increase in levels

of FSH and altered FSH: LH ratio is associated with decline in oocytes quality.

Natural population studies have shown that by a mean age of 41 years, the natural

fecundity has already reached its nadir (Broekmans, Faddy et al. 2004). A similar pattern

of decline in female fecundity has been reported from studies where coital behaviour and

male factor have been controlled for, suggesting that this decline in fecundity is mainly

accounted for by the ovarian factor (van Noord-Zaadstra, Looman et al. 1991). This age

related decline in fertility is also seen in couples undergoing assisted reproduction

treatments (2003 CDC ART report) (Figure 1.2).

A poor response to ovarian stimulation in such treatment programmes is a strong

predictor of poor prospects of becoming pregnant, and also of spontaneous fecundity and

early menopause (de Boer, den Tonkelaar et al. 2003; Lawson, El-Toukhy et al. 2003;

Klinkert, Broekmans et al. 2004)
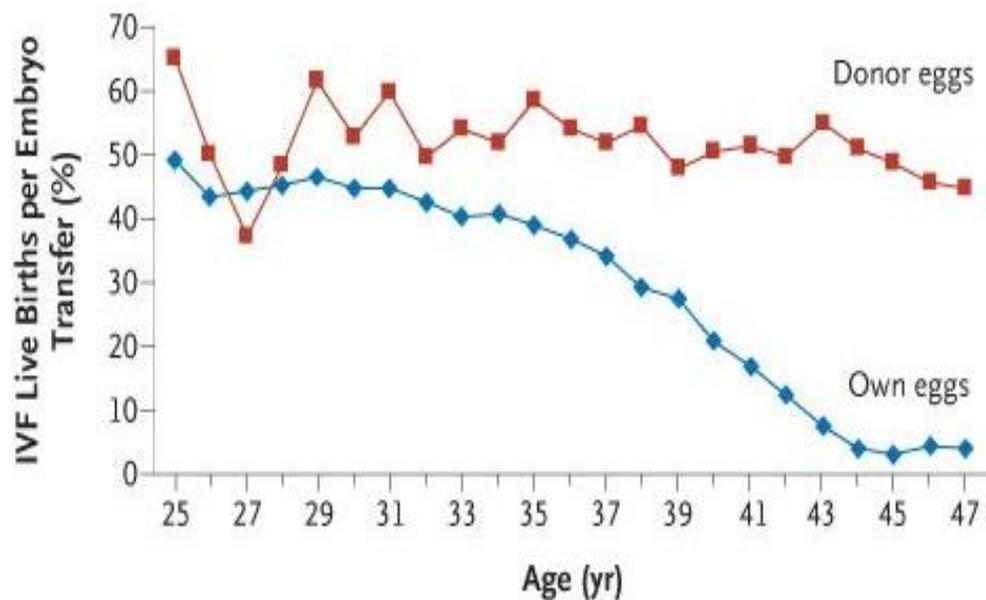
**Figure 1.2**          **Effect of women's age on live birth rates per embryo transfer.**

The live birth rate following IVF using own eggs is above 40% in women up to 34 years of age, then

shows a gradual drop with increasing age to below 5% in women aged ≥43 years. In contrast, the

live birth rate per embryo transfer in IVF cycles using donor eggs remains constant even up to the

age of 47 years. This indicates that the ovarian ageing and not the uterine function is primarily

responsible for the decline in live birth rates. (2003 Assisted reproductive technology report.

Atlanta: Centers for Disease Control and Prevention, 2003; http://www.cdc.gov).

## 1.2    Ovarian reserve and ovarian response

Ovarian reserve is defined as the existent quantitative and qualitative supply of follicles

which are found in the ovaries that can potentially develop into mature follicles which in

effect determines a woman's reproductive potential. It is also used as a term to

determine the capacity of the ovary to provide eggs that are capable of fertilisation

resulting in a healthy and successful pregnancy. Since true ovarian reserve cannot be

determined directly as it would entail performing ovarian biopsy to establish the number of primordial follicles, markers of ovarian reserve that can estimate the reserve have evolved.

Ovarian response is dependent on ovarian reserve thereby allowing the use of markers of ovarian reserve in prediction of ovarian response. It is clinically an important concept when evaluating the outcomes following assisted reproduction treatment (ART). Ovarian response to ART includes outcomes such as the number of stimulated follicles following controlled ovarian stimulation, number of oocytes retrieved, number of mature oocytes, fertilisation rate and formation of embryos.

Follicular exhaustion is a known fact in the fourth decade of reproductive life. With increasing levels of female education, their participation in the labour force, postponement of childbearing has lead to a so called sexual revolution causing an increasing incidence of subfertility due to ovarian ageing. This perhaps reflects on the increasing number of IVF cycles performed as reflected in the national U.K. statistics on live births following IVF and ICSI between 1992 and 2006.  The evaluations of ovarian reserve have therefore arisen to better counsel couples and guide the assisted reproduction protocols.

## 1.2.1  Evaluation of ovarian reserve

The commonly employed tests of ovarian reserve can be divided into static markers (oestradiol, FSH, inhibin-B, and AMH), dynamic markers (tests of stimulation with clomiphene citrate, gonadotrophins, and gonadotrophin releasing hormone analogue), and ultrasonographic markers (antral follicle count, ovarian volume and ovarian blood flow)

### 1.2.1.a  Static markers

## Oestradiol

Basal serum oestradiol is mainly produced by the ovulatory follicles and therefore can only be used in estimating the number of ovulatory follicles. The levels produced are also dependent on the LH levels in the cycle. It is an indirect marker of ovarian reserve as it is proposed to predict ovarian response in assisted reproduction cycles (Evers, Slaats et al. 1998).  In a few studies, a higher rate of cycle cancellation was seen among patients with basal levels of <20 or >80 pg/ml (Evers, Slaats et al. 1998; Frattarelli, Bergh et al. 2000). Other studies have failed to support its clinical application as they were unable to correlate it with follicular development (Frattarelli, Bergh et al. 2000) or predict pregnancy (Scott, Toner et al. 1989; Licciardi, Liu et al. 1995; Smotrich, Widra et al. 1995). Basal E2 levels do not differ significantly between poor and normal responders to assisted reproduction cycles (Lee, Lenton et al. 1988; Ficicioglu, Kutlu et al. 2006). The use of E2 as a marker of ovarian reserve therefore is no longer recommended (Broekmans, Kwee et al. 2006).

## Follicle stimulating hormone (FSH)

Basal FSH is the most widely used test to assess ovarian reserve. It is secreted by the anterior pituitary and acts on the receptors expressed by the granulosa cells of gonadotrophin responsive antral follicles. Increasing levels of basal FSH is the earliest sign of human reproductive aging (Reame, Wyman et al. 1998; van Zonneveld, Scheffer et al. 2003). Inhibin B is assumed to suppress pituitary FSH secretion. Reduction in the production of inhibin B from a decreasing population of growing follicles may elevate FSH secretion (Klein, Battaglia et al. 1996). A poor outcome to assisted reproduction

treatment can be expected in women with high levels of basal FSH; however the cut-off levels reported differ in different studies, ranging from 8 to 15 IU/mL (Watt, Legedza et al. 2000; Ashrafi, Madani et al. 2005; Klinkert, Broekmans et al. 2005). Although, studies have reported lower chances of pregnancy with higher levels of basal FSH, there is no association established with lower levels of FSH (van der Steeg, Steures et al. 2007). Contrary to this study, Luna et al reported a higher pregnancy rate in women younger than 35 years and with high FSH levels high when compared to older women with normal levels (Luna, Grunfeld et al. 2007). In view of this, there is a search for more predictive markers of ovarian reserve.

**Inhibin-B**

Inhibin B is a glycoprotein hormone of the transforming growth factor β (TGF-β) family (Kingsley 1994). It is secreted by the granulosa cells of the growing follicles, and is selectively responsible for pituitary inhibition of FSH secretion (Klein, Illingworth et al. 1996; Hayes, Hall et al. 1998). In normal ovulatory cycles, serum concentration of Inhibin B is inversely correlated with FSH concentration and increases up to the mid follicular phase, when it reaches a maximum peak reflective of the mass of granulosa cells within recruited antral follicles, after which it progressively decreases (Groome, Illingworth et al. 1996). Studies have reported significant positive correlation between higher levels of Inhibin B and number of oocytes collected after stimulation (Fowler, Fahy et al. 1995; Seifer, Lambert-Messerlian et al. 1997). Tinkanen et al found a negative correlation between serum inhibin B and FSH levels, and a positive correlation between inhibin B levels and antral follicle counts (Tinkanen, Blauer et al. 2001). However, the predictive value of inhibin B in predicting ovarian response is only modest with high false positive rates (Broekmans, Kwee et al. 2006).

**Anti-Müllerian hormone (AMH)**

AMH is a glycoprotein hormone of TGF-β family. AMH also known as Müllerian inhibiting substance is one of the best markers of ovarian reserve (Dehghani-Firouzabadi, Tayebi et al. 2008; Nelson, Yates et al. 2009). AMH is thought to be expressed by the granulosa cells of pre-antral and small antral follicles measuring 6.0 mm or less (Modi, Bhartiya et al. 2006). The biological role of AMH is still unclear, but rodent data suggest that it acts as a modulator of follicle recruitment and ovarian steroidogenesis (Durlinger, Kramer et al. 1999; Durlinger, Gruijters et al. 2001; Fanchin, Schonauer et al. 2003). It is considered to be a marker that can estimate the quantity and activity of recruitable follicles in early stages of growth, thus being more reliable for the prediction of ovarian reserve (te Velde and Pearson 2002; van Rooij, Broekmans et al. 2002; Fanchin, Schonauer et al. 2003; Gruijters, Visser et al. 2003; Muttukrishna, McGarrigle et al. 2005; Visser and Themmen 2005).

Studies have shown reduced variability in the levels of AMH as compared to other endocrine markers of ovarian reserve (Fanchin, Taieb et al. 2005; La Marca, Stabile et al. 2006; Elgindy, El-Haieg et al. 2008). AMH has also been suggested as an important predictor of response to ovarian stimulation. Ficicioglu et al., for example, found that levels of AMH predict the number of oocytes retrieved with a positive predictive value of 96% (Ficicioglu, Kutlu et al. 2006) and other groups have reported a strong correlation between the number of oocytes retrieved and both AMH levels and the total number of antral follicles (de Vet, Laven et al. 2002; van Rooij, Broekmans et al. 2005; Fanchin, Mendez Lozano et al. 2007; Dehghani-Firouzabadi, Tayebi et al. 2008). AMH is expressed by the granulosa cells of the early growing, pre-antral and small antral follicles, which measure less than 6 mm,, but not by non-atretic, larger antral follicles or those that have

become atretic, and may reflect or represent the population of smaller antral follicles more than the overall number (Baarends, Hoogerbrugge et al. 1995; Gruijters, Visser et al. 2003; Weenen, Laven et al. 2004; Skalba, Cygal et al. 2008). Whilst serum levels of AMH correlate with the total number of antral follicles (de Vet, Laven et al. 2002; van Rooij, Broekmans et al. 2005; Fanchin, Mendez Lozano et al. 2007), it is likely that the smaller antral follicles more accurately reflect ovarian reserve than the total population of follicles.

### 1.2.1.b   Ultrasonographic markers

Transvaginal ultrasonographic assessment of the ovaries for ovarian volume, AFC and stromal blood flow has been described in relation to the assessment of ovarian reserve. 2D ultrasound is widely used for this application. 3D ultrasound has been more recently introduced for assessment of ovarian reserve and might have a role in improving the predictive accuracy of tests of ovarian reserve.  2D ultrasound can display images in two planes, the sagittal and the transverse and often make the imaginative 3D interpretation of objects difficult (Nelson and Pretorius 1998). 3D ultrasound overcomes this problem by displaying the object in the coronal plane as well and also when required create a 3D rendered image of the object (Merz 1999).

**Ovarian volume**

Ovarian volume as measured by ultrasound has been assessed in studies as a potential marker of ovarian reserve. Syrop et al, in their study found a reduction in E2 levels, number of oocytes collected and pregnancy rates with decreasing volume of ovary in women undergoing assisted reproduction cycles (Syrop, Dawson et al. 1999). Bowen et al found a significant correlation between reduced ovarian volume, increasing age and FSH

levels (Bowen, Norian et al. 2007). Other studies did not detect a significant difference in ovarian volume between normal and poor responders in women aged less than 37 years (Elgindy, El-Haieg et al. 2008) and when evaluating women at high risk for cancellation of assisted reproduction cycles (McIlveen, Skull et al. 2007). Broekmans et al in their recent systematic review concluded that ovarian volume has little clinical application in prediction of poor pregnancy response (Broekmans, Kwee et al. 2006). However, de Carvalho et al in their recent review commented on the value of ovarian volume with regards to its easy execution, and therefore could be included in preparatory protocols providing data for continuity of research (de Carvalho, Rosa e Silva et al. 2008).

When using 2D ultrasound, the formula for volume of an ellipsoid, 'length × width × depth × $\pi$/6' is used to calculate the volume of ovary. When using 3D ultrasound, there are two basic methods employed to calculate volume from a 3D dataset: the conventional 'full planar' or 'contour' method and the recently introduced 'rotational' method possible through Virtual Organ Computer-aided AnaLysis (VOCAL™) which also generates a 3D model of the object of interest (Bordes, Bory et al. 2002). Both techniques involve manual delineation of the object of interest in the multiplanar display that shows the three perpendicular planes characteristic of 3D ultrasound. The reproducibility and validity of ovarian volume measurements made from 3D ultrasound data using the trapezoid formula were shown better than that those obtained by 2D ultrasound (Bonilla-Musoles, Raga et al. 1995; Kyei-Mensah, Maconochie et al. 1996). Whilst volume measurements using both the methods have been shown to be more reproducible than 2D measurements (Yaman, Sommergruber et al. 1999; Raine-Fenning, Campbell et al. 2003), the VOCAL technique is less time consuming (Bordes, Bory et al. 2002). VOCAL allows rotation of the 3D dataset about a central axis through a number of pre-defined rotation

steps (Bordes, Bory et al. 2002; Raine-Fenning, Campbell et al. 2002). The most appropriate rotation step for its use in a clinical or research setting recommended by the authors was 9° as it provided the best compromise between the reliability, validity and time taken for measurement (Raine-Fenning, Clewes et al. 2003). Another study by the same authors have shown that rotational measurement of ovarian volume from 3D data is significantly more reliable than volume estimation from 2D measurements using the ellipsoid formula (Raine-Fenning, Campbell et al. 2003). Ng et al. examined the predictive value of ovarian volume measured with the VOCAL imaging programme on datasets obtained from 3D ultrasound performed prior to ovarian stimulation but after down-regulation in 111 women undergoing their first cycle of IVF (Ng, Tang et al. 2006). Multiple regression analysis was applied to evaluate the predictive value of age, basal FSH, BMI, and various ultrasound markers including ovarian volume, AFC and ovarian stromal vascularity, with the number of oocytes as the dependant variable. AFC achieved the best predictive value with the largest $R^2$ change of 0.17 (P<0.001). Ovarian volume was excluded from the equation indicating that it was not a significant predictor. Furthermore, there was no statistically significant difference in the mean ovarian volume between pregnant and non-pregnant women. Recent meta-analyses did not find a significant impact on the predictive performance of ovarian volume regardless of whether it was measured by 2D or 3D ultrasonography. As an individual marker of ovarian reserve, the performance of ovarian volume in the prediction of non-conception is poor (Hendriks, Kwee et al. 2007; Verhagen, Hendriks et al. 2008).

**Ovarian stromal blood flow using Doppler ultrasound**

Of the four types of Doppler ultrasound described (continuous wave, spectral  also known as pulsed wave Doppler, colour Doppler and power Doppler, pulsed wave and power Doppler are commonly used when assessing ovarian blood flow (Whittingham 2007).

With pulsed wave Doppler the Doppler signals from a specific location can be isolated and assessed by placing a 'range gate' corresponding to the limits of sample volume.  Pulses rather than continuous waves are transmitted successively and it allows the use of a single transducer for both transmission and reception of the signals. A wave form is displayed against time as the Doppler frequency shifts are electronically converted by a mathematical technique, fast Fourier transformation. These waveforms can be analysed and quantified to generate indices of blood flow in terms of the absolute flow velocities during peak systole and during diastole as well as providing an indication of the resistance to flow over the whole cardiac cycle. Two formulae are used to calculate the degree of resistance to flow: the Resistance Index (RI) and the Pulsatility Index (PI) (Gosling and King 1974).  The resistance index is calculated by dividing the systolic peak velocity minus the end-diastolic velocity by the peak systolic velocity (Pourcelot, Arbeille et al. 1985). The index varies from zero to 1.00 with the latter indicating absence of end-diastolic flow. The resistance index is easily measured by defining the maximal systolic flow velocity and the end-diastolic flow velocity as two separate points whilst the pulsatility index is calculated by the on-board computer following manual delineation of the complete waveform over three to four consecutive and comparable cardiac cycles. The pulsatility index has the advantage therefore of taking into consideration more of the frequencies within any given waveform and is calculated by dividing the systolic peak velocity minus the end-diastolic velocity by the mean velocity. The pulsatility index has a distinct advantage in that it can produce variable values even when end-diastolic flow is reversed or absent, conditions

where the resistance index only records a negative value or a value of one respectively. The two main limitations of pulsed wave Doppler are that it is dependent on the angle of insonation (Rubin, Bude et al. 1994) and that it cannot differentiate between background noise and true flow (Jaffe 1992).

Power Doppler is a variant of colour Doppler that utilises the amplitude or power of the Doppler signal to display the total integrated Doppler power from a Doppler shifted ultrasound signal in colour rather than the mean Doppler frequency shift (Rubin, Adler et al. 1995). Power Doppler signal depends on the density as opposed to the velocity of scatterers with the signal amplitude determined by the total energy or power within the returning signal. This allows a much greater sensitivity in detecting small vessels and slow-moving blood. The number of colour-containing pixels and their hue depend approximately on the log of reflector concentration rather than on velocity (Adler, Rubin et al. 1995). The colour scale range from black for zero power to orange for high power. This range of colours depends on the density of blood cells (scatterers) within the area being examined by Doppler and represents a complex interaction between the haematocrit, shear stress, vascular diameter and flow velocity (Martinoli, Derchi et al. 1998). However, the exact way in which blood and ultrasound interact to produce the Doppler signal is still not clearly understood (Paeng, Cao et al. 2001). The main advantages of it are that its acquisition is not angle dependent and that true flow is not affected by the background noise. Use of 3D ultrasound with power Doppler facilitates the acquisition of power Doppler data from a structure of interest as a whole. It is an appropriate method for the semi-quantification of the blood supply of an organ particularly ovarian blood flow because of the presence of small blood vessels with low velocity flow within an ovary within the pelvis (Raine-Fenning, Campbell et al. 2004). Furthermore, reliable acquisition

and measurement using VOCAL programme of 3D power Doppler information has been reported (Raine-Fenning, Campbell et al. 2003; Raine-Fenning, Campbell et al. 2004).

There are only a few studies on the predictive capacity of ovarian vascular flow parameters for ovarian response or the occurrence of pregnancy using very different flow-derived predictors. Few studies have evaluated 2D pulsed wave Doppler in assessment of ovarian response (Zaidi, Barber et al. 1996; Zaidi, Collins et al. 1996; Engmann, Sladkevicius et al. 1999; Kim, Ku et al. 2002; Kupesic and Kurjak 2002; Kupesic, Kurjak et al. 2003). Peak systolic velocity (PSV) and resistance index (RI) were shown to be of some value in predicting ovarian response. One study has used 2D power Doppler in prediction of ovarian response  (Popovic-Todorovic, Loft et al. 2003). Few studies have used 3D power Doppler (Ng, Tang et al. 2006) in the assessment of ovarian reserve. All these studies have reported AFC as a superior test of ovarian reserve.

**Antral follicle count**

Ultrasonographic assessment of the total number of antral follicles measuring 2-10 mm is generally considered a reliable determinant of ovarian reserve (Scheffer, Broekmans et al. 2003; Ng, Chan et al. 2005; Jayaprakasan, Campbell et al. 2008). The pool of antral follicles comprises of pre-antral and early antral follicles (0.2 – 2.0 mm) that are largely gonadotrophin-independent, small antral follicles (2.0-4.0 mm) that are gonadotrophin responsive, and larger antral follicles (>4.0 mm) that are gonadotrophin-dependent (Gougeon 1989). The follicle destined to become dominant is selected from the pool during the early follicular phase. With the exception of this dominant follicle, all other healthy follicles showing evidence of granulosa cell activity do not exceed 6 mm throughout the cycle (Gougeon 1998). Larger follicles are invariably atretic (Gougeon and Lefevre 1983). At any given timepoint during the menstrual cycle, the ovaries contain

follicles at different developmental stages and accordingly antral follicle counts are commonly made during the early follicular phase (Gougeon 1998). The total antral follicle count (tAFC) is made by counting the number of antral follicles measuring 2 to 10 mm in both ovaries and can be estimated using two-dimensional (Scheffer, Broekmans et al. 2002) or three-dimensional ultrasound (Jayaprakasan, Campbell et al. 2008). It is now accepted that antral follicle count shows less inter-cycle variation when compared to ovarian volume and FSH (Jayaprakasan, Campbell et al. 2008; van Disseldorp, Lambalk et al. 2010). AFC made using 3D ultrasound might provide more reproducible counts than 2D ultrasound (Jayaprakasan, Hilwah et al. 2007; Jayaprakasan, Walker et al. 2007; Jayaprakasan, Campbell et al. 2008). Studies have shown significant correlations with the other serum markers (Haadsma, Bukman et al. 2007) and with serum AMH (Fanchin, Schonauer et al. 2003; Visser and Themmen 2005).

The number of small antral follicles is also strongly correlated with other ovarian reserve tests, such as AMH, supporting the concept that these smaller follicles represent the functional ovarian reserve (Haadsma, Bukman et al. 2007). There is a linear decline in the number of antral follicles with age (Gougeon 1994) and this is more apparent in the smaller antral follicles (<6.0 mm) than the larger ones (>6.0 mm) (Scheffer, Broekmans et al. 2003; Haadsma, Bukman et al. 2007) and their total number is, therefore, more reflective of the primordial follicle pool. Very few studies have considered the size of antral follicles other than to define the limits of follicular diameter above and below which follicles should not be included in the final count (Chang, Chiang et al. 1998; Pellicer, Gaitan et al. 1998; Weenen, Laven et al. 2004). Studies that have considered the size of the follicles between and above these extremes suggest that follicles measuring 2-5mm correlate most strongly with assisted reproduction treatment outcomes (Chang,

Chiang et al. 1998). Pellicer et al. used 3D ultrasound to assess the population of these 'selectable' follicles in young, low responders and found that the number of antral follicles measuring 2-5 mm in diameter was significantly less age-matched normal responders (Pellicer, Ardiles et al. 1998).

The standard technique used to the quantify the size of an antral follicle involves calculation of the mean diameter from two measurements of the follicular diameter (Haadsma, Bukman et al. 2007). No studies have reported on the intra- or inter-observer reliability of quantitative antral follicle counts stratified according to the size of the follicle. Measurement reliability is likely to be relatively poor as it will depend on both the identification and subsequent measurement of each follicle. There is a natural variation in determining the total number of antral follicles, both within and between observers and once a follicle has been identified there will be further variability in its measurement. Any variability is likely to be exaggerated when there are larger numbers of antral follicles as more measures are required overall (Scheffer, Broekmans et al. 2002; Jayaprakasan, Campbell et al. 2008). Assessment of follicle size requires measurement of each follicle in two dimensions and calculation of the mean diameter (Haadsma, Bukman et al. 2007). This can be very labour intensive and the reliability and validity of such measures are likely to be reduced when there are numerous follicles as it is difficult to ensure each follicle is only measured once and none are missed. Three-dimensional ultrasound allows the user to acquire a volume of information which can be examined off-line and facilitates the implementation of various software programmes that enhance measurement accuracy and both intra- and inter-observer reliability (Raine-Fenning, Campbell et al. 2003). The most recent development has seen the introduction of automated data analysis where mathematical algorithms allow the definition and differentiation of hypo

echoic, fluid-filled areas within the acquired volume (Raine-Fenning, Jayaprakasan et al. 2007).

SonoAVC (Sonography based automated volume calculation: GE Medical Systems, Zipf, Austria) is a new software program that identifies and quantifies hypo echoic regions within a three-dimensional dataset and provides automatic estimation of their absolute dimensions, mean diameter, and volume (Raine-Fenning 2008). Each individual volume is given a specific color and the automated measurements of its mean diameter (relaxed sphere diameter), its maximum dimensions (x, y, z diameters), and its volume are displayed in descending order from the largest to the smallest (Raine-Fenning 2008). An unlimited number of volumes can theoretically be quantified, which makes it an ideal tool for follicle tracking. It also identifies small volumes and can theoretically be used to count antral follicles. To date SonoAVC has been used to assess stimulated ovaries only (Raine-Fenning, Jayaprakasan et al. 2007; Raine-Fenning 2008). These studies have shown that SonoAVC provides automatic measurements of follicular diameter and volume that are more reliable and more accurate than comparable estimations made from 2D data (Raine-Fenning, Jayaprakasan et al. 2007; Raine-Fenning 2008). However, its reliability and application in assessing antral follicles has not been tested so far.

## 1.2.2 Physiological variation in the markers of ovarian reserve

The process of folliculogenesis is a continuous process, from the stage of primordial follicles to the preovulatory stage, and therefore at any time, the ovary contains follicles in all stages of development (Gougeon 1998). Tests evaluating ovarian reserve are

classically performed in the early follicular phase and therefore the ranges of normality described also relate to the values in that stage of menstrual cycle. The follicular phase of the menstrual cycle, also the final stages of follicular growth includes the late tertiary antral follicles and preovulatory follicles. During the late luteal phase with the disintegration of corpus luteum and consequent rise in pituitary follicle stimulating hormone (FSH), the 2 – 5 mm follicles, which have entered the preantral stage 70 days earlier, become selectable follicles competing with each other for growth inducing FSH. The selectable antral follicles are gonadotrophin dependent and compete with each other for growth inducing FSH. Age related increases in gonadotrophins have been demonstrated in both basal determinations (Sherman, West et al. 1976; Reyes, Winter et al. 1977; Metcalf and Livesey 1985) and in provocative tests such as clomiphene citrate challenge test (Navot, Rosenwaks et al. 1987; Loumaye, Billion et al. 1990). Therefore, an early serum FSH is commonly determined in the work up of female subfertility. Brown et al investigated the intercycle and day to day early follicular variation in the serum levels of FSH and found a mean variation of up to 25.6% between cycles and a mean variation of up to 15% in the day to day early follicular levels (Brown, Liu et al. 1995). This variation was significantly less than that observed in serum E2 levels (Brown, Liu et al. 1995). This variation could be reflective of the quantity and quality of selectable follicle.

AMH which is expressed in the granulosa cells of growing follicles, in the recent years has been shown in several studies to be a univariate predictor of outcome after IVF. The pool of growing follicles is thought to relate to the number of primordial follicles and therefore considered as an indirect marker of ovarian reserve. As AMH is primarily expressed by small antral follicles, it is thought to relate closely to primordial follicle number (Kevenaar, Meerasahib et al. 2006). Another advantage of using AMH as a marker of ovarian reserve

is that the levels do not fluctuate across and between cycles as much as the other endocrine markers (E2, Inhibin B, FSH, LH) of ovarian reserve (Hehenkamp, Looman et al. 2006; La Marca, Stabile et al. 2006; La Marca, Giulini et al. 2007; Tsepelidis, Devreker et al. 2007).

The antral follicle count (AFC), an ultrasound marker of ovarian reserve can be measured using two-dimensional or three-dimensional ultrasound (Scheffer, Broekmans et al. 2002). A recent meta-analysis done on the accuracy of multivariate models predicting ovarian reserve and pregnancy following ART, has shown that the performance of multivariate models in the prediction of poor ovarian response after IVF is comparable with that of AFC (Verhagen, Hendriks et al. 2008). Jayaprakasan et al looked at the intercycle variability of AFC, ovarian volume, and basal FSH levels and found least variation in AFC (Jayaprakasan, Campbell et al. 2008). Their work also supports the use of 3D ultrasound in improving the reliability of the test (Jayaprakasan, Walker et al. 2007; Jayaprakasan, Campbell et al. 2008). The small antral follicle population which might be a test of functional reserve has not been tested as yet for its physiological variations and its reliability as a test of ovarian reserve.

## *1.3    Benign ovarian cysts*

The most common types of benign ovarian cysts are endometrioma, dermoid, cyst adenoma, and functional ovarian cysts.  The incidence rate for developing a simple cyst is about 8% per year and is more common in pre-menopausal women (Reynolds, Hill et al. 1986). They could be either a functional cyst wherein the dominant follicle does not release the oocyte and the follicle continues to grow forming a cyst, or a luteal cyst which

is formed within the corpus luteum, or a haemorrhagic cyst which is formed by collection of blood in either a functional or a luteal cyst. Mature cystic teratomas or dermoids represent more than 10% of all ovarian neoplasm's and are the most common benign germ cell tumours of the ovary in women of reproductive age.   They arise from ectodermal (hair, skin, sweat glands), mesodermal (bone, muscle, cartilage) and endodermal tissue elements (neural and vascular tissue lines) in variable amounts. Endometriosis affects 3 - 10% of women in their reproductive age and is the leading cause of sub fertility, seen in about 25-35% of women with infertility (Jenkins, Olive et al. 1986; Mahmood and Templeton 1991). Endometriomas are identified in 17-44% of women with endometriosis (Chapron, Vercellini et al. 2002). According to the implantation theory, the endometrial implants cause adherence of the ovary to pelvic peritoneum and progressively invaginate the ovarian cortex (Hughesdon 1957; Brosens, Puttemans et al. 1994). Another theory suggests that endometrioma is formed due to the metaplasia of coelomic epithelium which invaginate into the ovarian cortex (Donnez, Nisolle et al. 1996). These theories suggest that an endometrioma is perhaps a pseudo cyst.

Ovarian cysts can cause pain and discomfort related to pressure on adjacent structures, torsion, rupture, haemorrhage (both within and outside of the cyst), and abnormal uterine bleeding (Chapron, Vercellini et al. 2002). Rarely, they become malignant. Clear cell carcinoma and endometroid adenocarcinoma are the most common type of carcinoma associated with endometriosis (Kobayashi, Kajihara et al.). It is hypothesised that repeated events of haemorrhage in endometriosis can contribute to carcinogenesis and progression via 3 major processes: 1) increasing oxidative stress promotes DNA methylation; 2) activating anti-apoptotic pathways supports tumour promotion; and 3) aberrant expression of stress signalling pathways (Kobayashi, Kajihara et al.). Malignant

transformation in a dermoid cyst of the ovary is a rare complication, occurring in only 1-2% of cases, with squamous cell carcinoma being the most common type (Powell, Stinson et al. 2003; Hurwitz, Fenton et al. 2007). In assisted reproduction treatment, three mechanisms are feared to alter the outcome of treatment, one that ovary containing the cyst may not respond adequately to COH, second that oocytes collected may get contaminated by the cyst fluid, and lastly that inadvertent puncture of cyst during oocyte collection might cause subsequent peritonitis and infection.

*Table 1: Ultrasonic diagnostic features of commonly occurring benign cysts.*

Diagnosis of ovarian cysts is predominantly based on the morphologic features of the cyst on ultrasound as shown in the table below.

| | |
|---|---|
| **Functional cysts** | |
| **Follicular cyst** | Unilocular cyst ≥3cms in diameter with a thin smooth wall and echo free fluid within it. Some may undergo haemorrhagic change producing echogenic fluid (Reynolds, Hill et al. 1986) |
| **Corpus luteum cysts** | Hypo echoic cysts with thick walls and varying size although usually less than 3cms. Haemorrhage is often seen (Reynolds, Hill et al. 1986) |
| **Endometrioma** | Thin walled ovoid or round hypo echoic masses containing fluid with scattered internal echoes or gravity dependent fluid filled levels from layering of haemorrhage. Acoustic enhancement through the lesion (Athey and Diment 1989) |
| **Germ cell tumour**<br><br>**Dermoid / Teratoma** | Echogenic mass of varying density and shadowing. Echogenic particles in a hypo echoic medium (dermoid mesh). Cyst with fat-fluid level where the uppermost oil layer is echo free (Caspi, Appelman et al. 1996). |

Khamsi et al reported a study of 14 women in whom endometrioma was inadvertently entered resulting in retrieved oocytes that were either exposed or not exposed to endometrioma fluid. They found no difference in fertilization or early embryo development between the two groups. The fertilization rate for oocytes exposed to an endometrioma was 60%, versus 56% for controls. The good-quality embryo formation rate for oocytes exposed to an endometrioma was 45%, versus 46% for controls. (Khamsi, Yavas et al. 2001). In contrast, another study found high concentrations of transforming growth factor-B1in the fluid of endometrioma and showed a growth enhancing effect on endometrioma cells (Badawy, Cuenca et al. 1998). It is therefore a common practice to excise the ovarian cysts especially when measuring more than 4cms.

Operative laparoscopy compared with laparotomy has been established as the gold standard surgical approach in the treatment of ovarian cysts in terms of reduced postoperative pain, analgesic requirement, hospitalization, and adhesion formation (Pados, Tsolakidis et al. 2006). Potential problems with surgical treatment of ovarian cysts are the small risk of post surgical ovarian failure of 2.4% especially when dealing with bilateral ovarian cysts (Busacca, Riparini et al. 2006), a moderate 30.4% risk of recurrence (Koga, Takemura et al. 2006) and a reduction in ovarian reserve. The principle of cystectomy is to either remove or destroy the cyst wall or capsule in order to prevent recurrence. The techniques commonly used therefore are stripping of the cyst from underlying normal ovarian tissue or drainage of the cyst with ablation of cyst wall. Following the excision of the cyst or stripping of the cyst wall from the ovarian cortex, it is a normal practice to achieve haemostasis in the cyst bed using some form of coagulation, either monopolar, bipolar, laser, or an ultrasonic device. The current body of evidence suggests that ovarian cysts especially endometrioma can affect the function of the

adjacent normal ovarian tissue (Maneschi, Marasa et al. 1993), and that stripping of ovarian cyst is associated with inadvertent loss of normal ovarian tissue attached to it (Hachisuga and Kawarabayashi 2002; Muzii, Bianchi et al. 2002; Roman, Tarta et al. 2010). Few studies have suggested that damage to ovarian reserve seen with removal of endometriomas might be more pronounced than non-endometriotic cysts (Maneschi, Marasa et al. 1993; Muzii, Bianchi et al. 2002; Exacoustos, Zupi et al. 2004; Chang, Han et al. 2010; Iwase, Hirokawa et al. 2011). There is also suggestion that inexperience of the surgeon can increase the damage caused to the normal ovarian tissue and ovarian reserve thereby (Yu, Huang et al.). When looking at outcomes following assisted reproduction treatment, Esinler et al found that although the ovarian reserve reduces following endometrioma cystectomy, it does not translate into impaired pregnancy outcome. (Esinler, Bozdag et al. 2006). This finding was in agreement with another study reporting a quantitative but not a qualitative damage to ovarian reserve following laparoscopic excision of endometrioma. (Ragni, Somigliana et al. 2005). Somigliana et al observed the response of operated ovary and the non operated contra lateral ovary during ovarian stimulation as part of an IVF cycle, and found a significant reduction in the ovarian reserve in the operated ovary, as measured by the number of follicles measuring >15mm on day of HCG administration (Somigliana, Ragni et al. 2003). Candiani et al also found a reduction in the ultrasound markers of ovarian reserve following ovarian cystectomy for benign cysts when compared to the contra lateral normal ovary, however suggested this effect due to ovulation in the intact gonad and not due to injury to ovarian vascularisation. (Candiani, Barbieri et al. 2005).

## 1.3.1  Systematic review of current literature

Recently a cochrane database systematic review was conducted to determine the effectiveness and safety of surgery, medical treatment, combination therapy or no treatment for improving reproductive outcomes among women with endometriomata, prior to undergoing ART cycles (Benschop, Farquhar et al.). The endometrioma included in analysis were those that were diagnosed by laparoscopy or imaging tests including ultrasound and magnetic resonance imaging (MRI). The primary outcome evaluated was live birth rate and secondary outcomes included ovarian response, clinical pregnancy rate and miscarriage. Four RCT's including 312 participants were further analysed. None of the trials reported on the live birth rates and paucity of evidence on this outcome persists. None of the trials showed a significant effect on reproductive outcomes including clinical pregnancy rate and miscarriage. Laparoscopic aspiration or cystectomy of endometriomata prior to ART did not show evidence of benefit over expectant management with regard to the clinical pregnancy rate (Benschop, Farquhar et al.).

When comparing expectant management to surgical approach, the chance of conception is not the only issue that has to be considered. Whilst costs and hazard of surgical complications support expectant management, oocyte retrieval risks, the possibility of missing occult malignancy and endometriosis progression due to ovarian stimulation would favour surgical treatment. Alternative therapeutical options include medical treatment and ultrasound-guided aspiration. Whereas prolonged GnRH agonist down-regulation may be beneficial, data on ultrasound aspiration are more controversial (Somigliana, Vercellini et al. 2006). The laparoscopic excision of ovarian endometriomas appears to increase the chances of spontaneous conception, but the value of this treatment in women selected for IVF–ICSI cycles is debated. Studies recruiting women

with unilateral disease and comparing the ovarian response in the affected ovary to contralateral normal ovary indicate that excision of endometriomas is associated with a quantitative damage to ovarian reserve (Somigliana, Vercellini et al. 2006).

Various methods have been reported to evaluate ovarian reserve following ovarian cystectomy. These include ovarian response and or pregnancy rate in women undergoing assisted reproduction treatment (ART); two–dimensional ultrasound markers of ovarian reserve such as volume, vascularity, and AFC (2.0-10.0mm); ovarian volume as measured using 3D ultrasound; and endocrine markers of ovarian reserve including AMH and FSH. The ovarian reserve in operated ovary has more commonly been compared to either the contra lateral normal ovary or matched controls, and in very few studies to itself in a longitudinal study design. Studies that have used outcomes of ovarian response to evaluate ovarian reserve have used measures including the duration of ovarian stimulation, the number of oocytes collected, fertilisation rate, and clinical pregnancy rate when examining the effects of ovarian cystectomy in sub-fertile population undergoing assisted reproduction treatment. They have compared the ovarian response of operated ovary either to the contra lateral normal ovary or to the matched case-controls (Loh, Tan et al. 1999; Canis, Pouly et al. 2001; Marconi, Vilela et al. 2002; Somigliana, Ragni et al. 2003; Wong, Gillman et al. 2004; Loo, Lin et al. 2005; Alborzi, Zarei et al. 2006; Esinler, Bozdag et al. 2006; Somigliana, Vercellini et al. 2006; Alborzi, Ravanbakhsh et al. 2007; Nakagawa, Ohgi et al. 2007; Horikawa, Nakagawa et al. 2008; Kahyaoglu, Ertas et al. 2008; Somigliana, Arnoldi et al. 2008; Benaglia, Somigliana et al. 2009; Garcia-Velasco and Somigliana 2009; Tsoumpou, Kyrgiou et al. 2009; Benaglia, Somigliana et al. 2010). Ovarian cystectomy has been shown to impair ovarian reserve following assisted reproduction treatment (ART), but reduced ovarian reserve does not seem to translate

into impaired pregnancy outcome (Demirol, Guven et al. 2006; Esinler, Bozdag et al. 2006; Somigliana, Ragni et al. 2006; Kahyaoglu, Ertas et al. 2008; Garcia-Velasco and Somigliana 2009). Few studies have reported ovarian volume in conjunction with ovarian response and compared to the response in contra lateral ovary (Somigliana, Ragni et al. 2006). Few studies have used ultrasound markers such as volume, antral follicle count, and stromal blood flow in the operated ovary and compared to the contra lateral ovary (Exacoustos, Zupi et al. 2004; Candiani, Barbieri et al. 2005; Benaglia, Somigliana et al. 2010; Coric, Barisic et al. 2010; Donnez, Lousse et al. 2010).   One prospective longitudinal studies comparing postoperative ovary to the preoperative ovary has used endocrine and ultrasound markers of ovarian reserve with post operative follow up (Candiani, Barbieri et al. 2005; Chang, Han et al. 2009; Li, Liu et al. 2009; Tsolakidis, Pados et al. 2009; Chang, Han et al. 2010; Pados, Tsolakidis et al. 2010; Iwase, Hirokawa et al. 2011)).   Both, ultrasound and endocrine markers of ovarian reserve have been used in the literature to quantify the impact of pathology and surgery. The endocrine markers such as Follicle Stimulating Hormone (FSH), Anti-Müllerian Hormone (AMH), Oestradiol, and Inhibin B provide information on ovarian reserve of both ovaries as a combined unit. Ultrasound is the only method so far which allows a direct assessment of each ovary as a separate entity; however the measures derived are presented either as a mean or total value.

In clinical practice, assessing both ovaries as a combined unit is of relevance in managing sub fertile couples, and the only area where examining each ovary as a single unit probably holds its place is when examining the effects of surgery. Majority of the studies looking at effect of surgery on ovarian reserve have done so by either comparing the operated ovary to the non operated contra lateral gonad, or to a control group.

## 1.4.    Rationale for the study and Hypothesis

The evidence suggests that ovarian cystectomy may be detrimental to the ovarian reserve, however there is wide variation in the methods employed to evaluate this effect. The total antral follicle count (2-10mm) and AMH appear to be the two most predictive and reliable markers of ovarian reserve, correlating well with each other and also with ovarian ageing. There is a suggestion that 3D ultrasound provides more reliable total antral follicle counts. Small antral follicle counts (2-6mm) may have a role in prediction of ovarian reserve as it correlates better to ovarian ageing than larger antral follicles. Also, production of AMH is mainly by pre antral and these small antral follicles, thereby providing a possibility of improving upon the predictive ability of total AFC.

My hypothesis was that small antral follicle counts produce better estimates of ovarian reserve and that it would be a reliable test to use when evaluating the effect of ovarian cystectomy on ovarian reserve. My specific objectives to examine this hypothesis were to:

(1)    Determine the intraobserver and interobserver reliability of antral follicle counts made using 3D ultrasound assisted SonoAVC and compare to counts made using 2D ultrasound and manual 3D ultrasound method.

(2)    Examine the optimum means of quantifying antral follicle number and size by comparing 2D and automated 3D ultrasound techniques.

(3)    Validate the antral follicle counts of different follicle size cohorts made using 2D and 3D ultrasound methods

(4)     Determine the predictive value of the automated quantification of the number and size of small antral follicles in women undergoing assisted reproduction treatment.

(5)     Determine the inter-ovarian, intra- and inter-cycle variation in the antral follicle counts of different follicle size cohorts made using semi-automated 3D ultrasound technique in women with normal menstrual cycles.

(6)     Determine the effect of combined oral contraceptive pill on the markers of ovarian reserve in comparison to non pill users.

(7)     Examine the effect of ovarian cystectomy on ovarian reserve using the most reliable method.

**CHAPTER 2.  Materials and Methods**

This chapter includes the materials and methods used to initially evaluate the reliability and validity of method used, followed by methods used to assess factors that could influence ovarian reserve, including, the physiological variation in the markers of ovarian reserve, effect of combined oral contraceptive pills containing low dose estrogen, and effect of ovarian cysts and cystectomy on the markers of ovarian reserve.

## *2.1    Ultrasound Technique*

### 2.1.1  Data acquisition

All the transvaginal ultrasound examinations were performed using a Voluson Expert 730[TM] or Voluson E8 Expert (GE Medical Systems, Zipf, Austria) and a four-dimensional, 5-9 MHz transvaginal transducer. Scans were performed in the standard manner with the patient in a supine position with knees flexed and hips abducted. The assessment comprised of a preliminary conventional 2D ultrasound assessment of the pelvis, to exclude any obvious pathology, followed by visualization of ovaries in their transverse and longitudinal planes and the subsequent acquisition of 3D data using the volume mode. The volume mode, which displays a truncated sector, was then adjusted to precisely define the area of interest; the sweep angle set to $90^0$ and a 3D dataset acquired using the high quality, slow sweep mode, which results in the highest resolution. The resultant multiplanar display of the ovary was examined to confirm its inclusion in entirety. 3D datasets on the blood flow to the ovaries using 3D power Doppler was again acquired using the high quality, and a slow sweep mode with the sweep angle set to $90^0$.  Pulsed wave Doppler was used to measure the ovarian blood flow in the stromal blood vessels.

Similar acquisitions of 3D grey scale dataset and 3D power Doppler was performed on the uterus and cervix, and pulsed wave Doppler of uterine arteries acquired. Datasets from each subject was stored on recordable digital video discs (DVDs) for subsequent off line analysis under a unique identification code.

## 2.1.2   Data display and analysis

4D View ™ (version 7.0, GE Medical Systems) was used for offline data analysis. The 3D dataset were opened and displayed using the multiplanar view (Figure 2.1), which shows three sectional planes (the A, B, and C plane) simultaneously that are mutually related so that movement within one plane produces geometrically equivalent movements in the other two planes. These planes can be used to standardize the view of the ovary, or any 3D dataset, and for this thesis we would ensure that the A plane shows the ovary in its longitudinal section with the iliac vessels inferiolaterally and the B plane the transverse image orthogonal to this. This orientation automatically ensures the image presented in the C plane demonstrates the coronal view of the ovary. This view is described as a multiplanar view and was used to measure the number of antral follicles in each ovary. This display provides the investigator an  advantage of being able to cross-check the number of follicles in any one plane against the two other image planes, which provides the observer with more spatial information than conventional imaging and results in more reliable and valid qualitative and quantitative measurements (Scheffer, Broekmans et al. 2002; Jayaprakasan, Campbell et al. 2008). The quality of the image was adjusted and optimized, in terms of magnification, for all datasets and the 'render mode' entered to generate a 3D rendered view of the ovary. The render box was adjusted to exclude as

much extra-ovarian information as possible and to ensure that the whole ovary was included in the volume of interest (VOI). The threshold settings, which assign transparency associated with fluid to opaque voxels, was maintained for all datasets.

**Figure 2.1 Multiplanar view of the ovary with a render box defining the region of interest.** The figure shows the typical display of an image acquired using 3D ultrasound. Three planes the longitudinal, transverse and the coronal planes of the image acquired are displayed simultaneously on the screen. The render box delineates the area of interest and allows further processing of image.
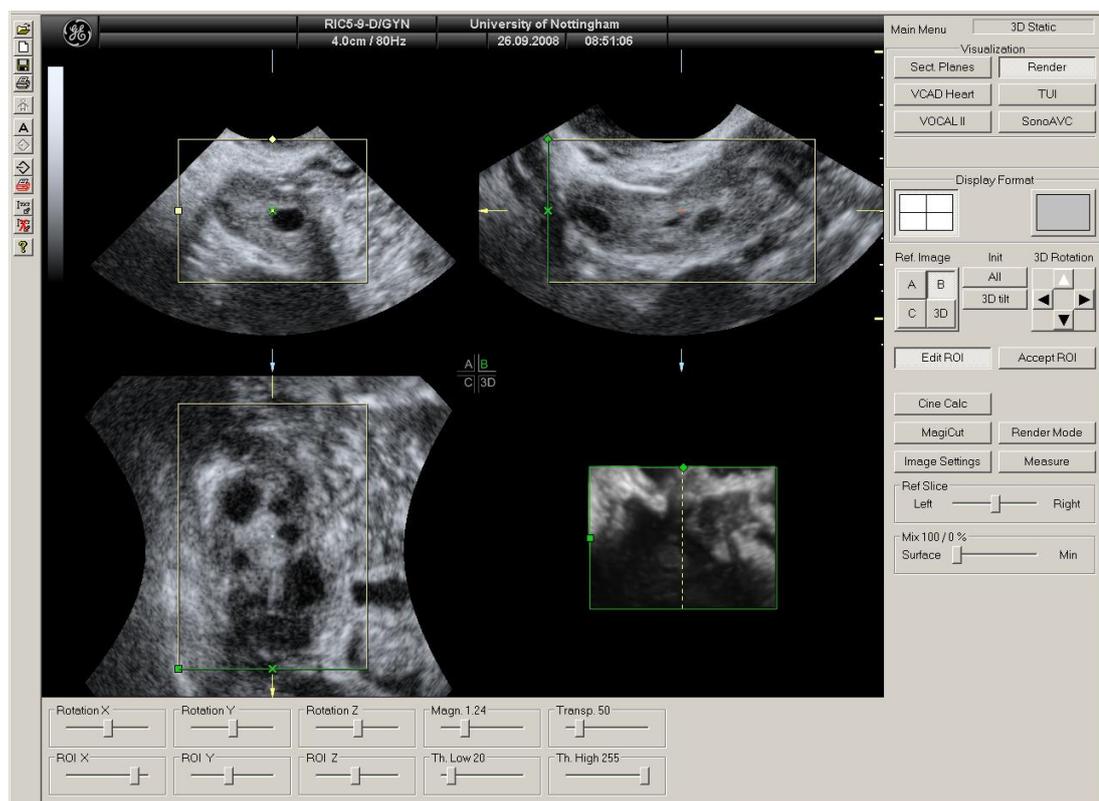


Once the dataset is correctly positioned, SonoAVC was implemented. The individual follicles are then displayed with a specific colour and shown together with their dimensions and relative sizes (Figure 2.2).

**Figure 2.2        SonoAVC - automated measurement of antral follicles**
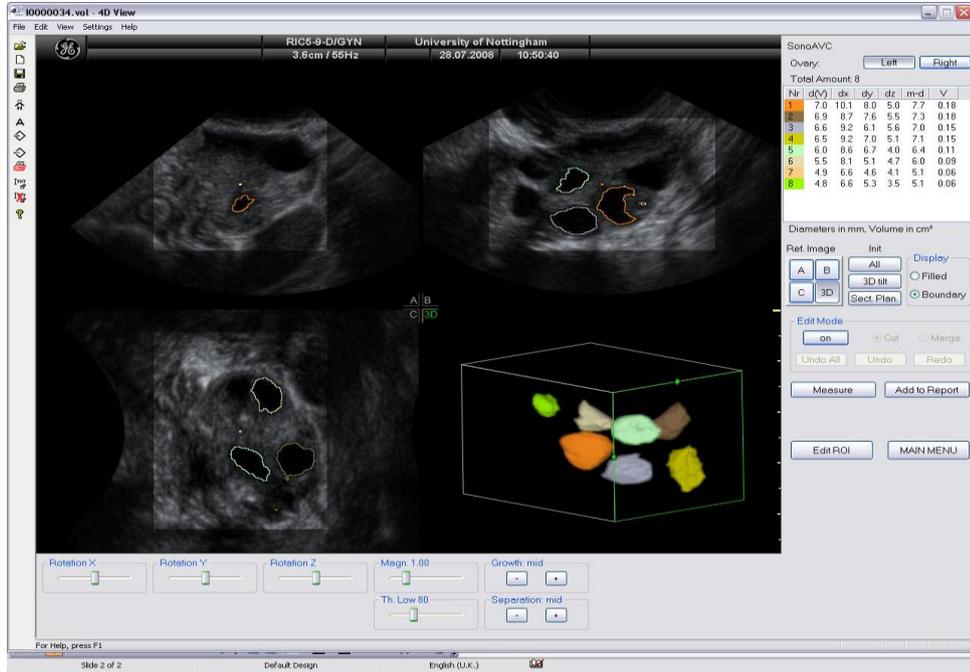


**Figure 2.3        SonoAVC with post processing – semi-automated measurement of**
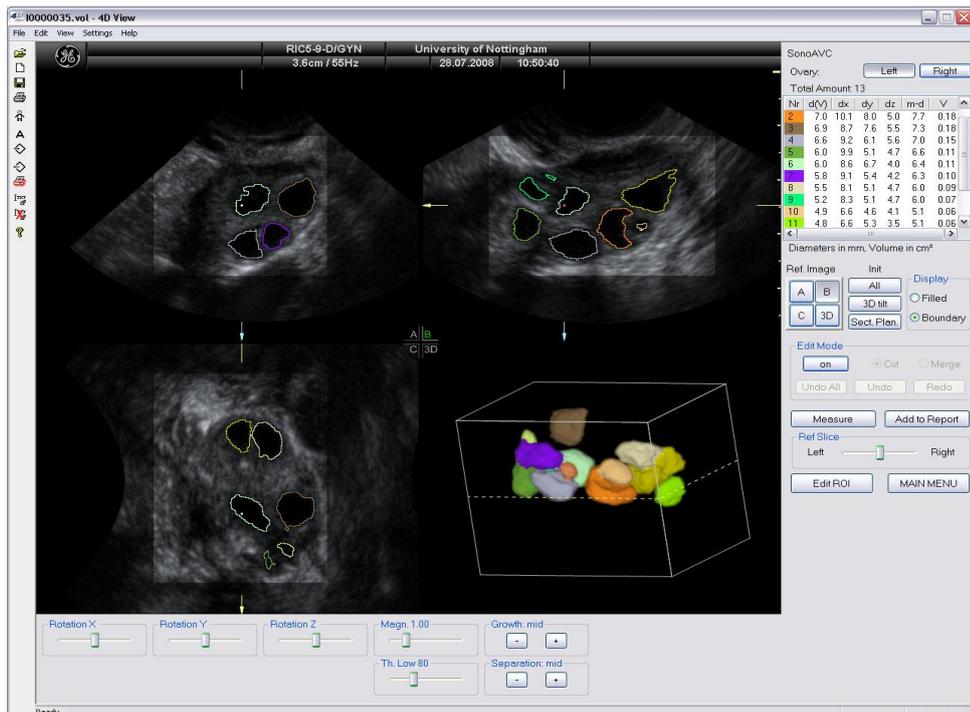
**antral follicles**

Post-processing, involving the manual identification of follicles not included in the automated analysis, was then used to ensure all antral follicles are counted and measured (Figure 2.3). Each additional antral follicle identified in this way is given a new colour and its dimensions displayed together with those follicles that were originally identified. The mean diameter of relaxed sphere of each antral follicle in both ovaries displayed as d (v) was recorded.

**Figure 2.4        Ovarian volume calculated with VOCAL**

Volume of each ovary was analysed using virtual organ computer aided analysis (VOCAL) on 4D view (Figure 2.4). This is done by delineating the ovarian cortex in one standard plane as the volume is rotated $180^0$ through $30^0$ rotational steps.

**Figure 2.5          3D Power Doppler Indices**

To obtain the power Doppler indices (vascularisation index-VI, flow index-FI, and vascularisation flow index-AFI), facility of histogram was applied on the rendered image of the ovary obtained using VOCAL.
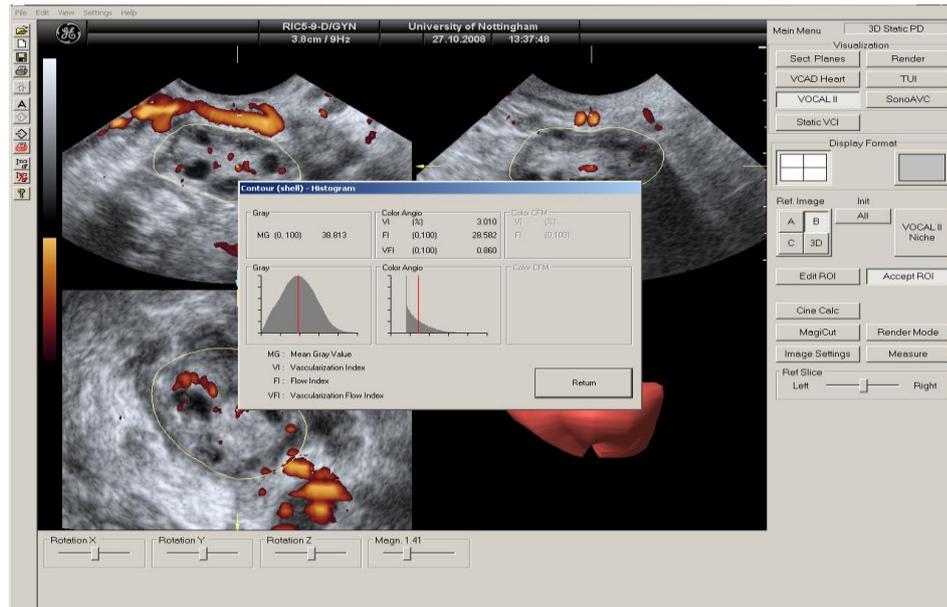


**Figure 2.6          Blood flow in stromal ovarian artery using pulsed wave Doppler indices:** Pulsed wave Doppler indices were recorded from the stored dataset for each ovary and the uterine arteries.
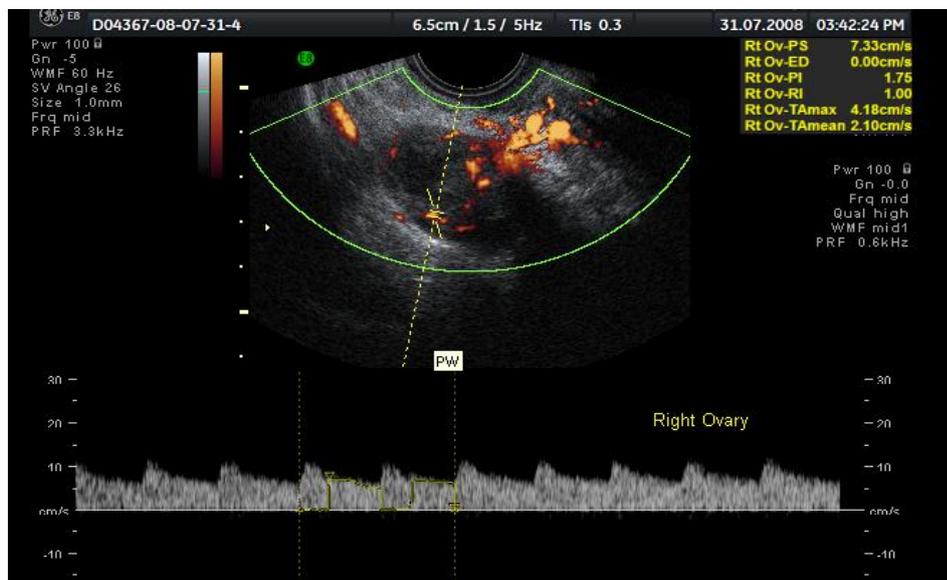
Department of Obstetrics and Gynaecology at Nottingham University Hospitals predominantly uses the Voluson series of ultrasound machines manufactured by *GE.* This is perhaps to help with the purchase power and service contracts. Whilst many other manufacturers including Medison, Toshiba, Siemens and Hitachi have 3D probe incorporated in some of the ultrasound machines, the applications are limited to simply the acquisition of 3D data. "*Voluson"* series has the facility to manipulate and process images off-line using the software '4D view'. Whilst 4D view equivalent is available on Medison manufactured ultrasound machine, automated volume counts using SonoAVC is unique to Voluson series of ultrasound machines manufactured by *GE* and was therefore used for my research project.

## *2.2    Subject profile*

The research studies were conducted in accordance with the ethical principles that have their origin in 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. Ethical approval was granted by the University of Nottingham and Nottingham Research Ethics Committee (REC) (Reference numbers: C/1/2008; D/1/2008; REC 08/H0403/60) and the Nottingham University Hospital's Research and Development Department (Ref: 08GY001). Informed, written consent was obtained prior to the enrolment of all subjects.

### 2.2.1   Reliability of SonoAVC

Fifty-five subjects aged less than 40 years with early follicular phase Follicle Stimulating Hormone (FSH) below 15 IU/L, planning to undergo treatment for their sub fertility, were

prospectively recruited. Ultrasound was performed during the early follicular phase of menstrual cycle between days 2-5. The numbers of follicles measuring between 2 to 10 mm in diameter were counted in each ovary to calculate the total antral follicle count in that ovary. Subjects who had had previous ovarian surgery, had one ovary, and those with ovarian cysts or follicles measuring more than 10 mm were excluded from the study. Three-dimensional datasets were acquired and subsequently analysed. The tAFC (2-10mm antral follicles) was calculated by two observers using three independent methods: 2D real time equivalent (2DRTE), 3D manual multiplanar view (3DMPV), and SonoAVC. For measures made using SonoAVC, the initial automated count (sAVC-AA) was recorded and post-processing (sAVC-PP) then applied to identify follicles that had been missed or incorrectly included. Intraclass correlation and limits of agreement were used to evaluate the methods.

### 2.2.2   SonoAVC in prediction of outcome following assisted reproduction treatment

156 consecutive subjects planning to undergo their first treatment cycle of *in vitro* fertilisation (IVF) were prospectively recruited. Inclusion criteria included age less than 40 years, body mass index (BMI) of less than 30, regular menstrual cycles of 24-32 days duration, planned first cycle of IVF with or without ICSI, and an early follicular phase FSH level of <15IU/L. All subjects underwent a pre-treatment ultrasound assessment in the early follicular phase (cycle day 2-5) of menstrual cycle. Subjects were excluded if they were found to have an ovarian follicle or cyst measuring more than 20 mm in diameter. Recruited subjects were subsequently excluded if their treatment cycle was cancelled or if they had failed fertilisation or embryo cleavage. Subjects wishing to egg share or altruistically donate their oocytes were not included.

A long protocol, involving pituitary suppression with Gonadotrophin Releasing Hormone (GnRH) agonists (500 mcg/day of Buserelin; Suprefact®, Aventis Pharma, Kent, UK or 800 mcg/day of Nafarelin; Synarel®, Pharmacia, Milton Keynes, UK) started in the mid-luteal phase of the menstrual cycle, was used in all subjects. Ovarian stimulation was commenced once down regulation was confirmed with a daily dose of either 225 IU of recombinant Follicle Stimulating Hormone (FSH; Gonal-F; Serono Pharmaceuticals Ltd, Feltham, UK) or 225 IU of human menopausal gonadotrophin (hMG; Menopur; Ferring Pharmaceuticals Ltd, UK). The response to ovarian stimulation was monitored by ultrasound and serum oestradiol levels on an alternate day basis from the fifth day of FSH administration and human Chorionic Gonadotrophin (hCG; 6500 IU of Ovitrelle; Serono Pharmaceuticals Ltd, Feltham, UK when using recombinant FSH or 5000 IU of Pregnyl; Organon Laboratories Ltd when using Menopur) administered when there were at least three follicles measuring 18 mm or more in diameter with transvaginal, ultrasound-guided oocyte retrieval scheduled 36 hours later. One or two embryos were replaced according to the wishes of the couple and the number of embryos available 48 hours later. From the day of embryo transfer, luteal support was provided through the self-administration of progestogen pessaries (Cyclogest; Shire Pharmaceuticals Ltd, Basingstoke, Hants, UK). Clinical pregnancy, as confirmed by a transvaginal ultrasound scan performed 5 weeks following embryo transfer assessing the fetal viability, was taken as the primary outcome measure for analysis. Secondary outcome measures assessed included the number of mature oocytes, fertilised oocytes and cleaved embryos.

### 2.2.3   Physiological variation in ovarian reserve

Healthy volunteers were recruited prospectively through local advertisement. The inclusion criteria included was subjects with regular periods aged between 18 to 45 years, and with no previous history of ovarian surgery.

To assess the intracycle variation in ovarian reserve, assessments were planned for early follicular, mid follicular, peri-ovulatory and mid to late luteal phases of menstrual cycle. The assessments involved transvaginal ultrasound scans for the three-dimensional ultrasound markers and a blood test for endocrine markers of ovarian reserve each visit.

To assess the intercycle variation in ovarian reserve, assessments were planned in the early follicular phase of menstrual cycle at 0, 1, 3, 6 and 12 months. The assessments involved transvaginal ultrasound scans for the three-dimensional ultrasound markers and a blood test for endocrine markers of ovarian reserve each visit.

### 2.2.4   Effect of combined oral contraceptive pills

Healthy volunteers were prospectively recruited with the help of local advertisement. The inclusion criteria included subjects with regular periods aged between 18 and 45 years, with no previous history of surgery on the ovaries, and who were taking combined oral contraceptive pills containing low dose estrogen for more than one year. The control group included subjects with regular periods aged between 18 and 45 years, with no previous history of ovarian surgery, and who had not taken hormonal contraceptive in the last one year.

Assessments involved a transvaginal ultrasound scan for three dimensional ultrasound markers of ovarian reserve and blood test for endocrine markers of ovarian reserve in the early follicular phase of the menstrual cycle.

### 2.2.5  Effect of ovarian cyst and cystectomy

Subjects with regular periods aged between 18 and 45 years planned to have an ovarian cystectomy for benign ovarian cyst were prospectively identified and recruited from the out patients department, gynaecology wards, preoperative clinics, patient waiting lists and operating diary of the gynaecologist at Queen's medical Centre. The assessments involved a transvaginal ultrasound scan and a blood test for the markers of ovarian reserve in the early follicular phase of menstrual cycle pre operatively and 1, 3, and 6 months post operatively.

## *2.3    Statistical analysis*

Statistical analysis was performed using the Statistical Package for the Social Sciences (version 15; SPSS, Chicago, IL, USA). Distribution of data was assessed using Kolmigrov-Smirnov test. For un-paired analysis, student's t test was used for parametric data and Mann-Whitney test for non parametric data. For paired analysis, student's t test was used for parametric data and Wilcoxon signed rank test for non-parametric data. A P value of <0.05 was considered as significant. A general linear model with repeated measures was used to determine differences with time within each group and the interaction between them.

Linear regression analysis was used to assess the value of different variables for the prediction of the outcome following assisted reproduction treatment. Binary logistic regression analysis was used to evaluate the effect of the same variables on the prediction of pregnancy. Receiver operating characteristic (ROC) curve analysis was performed to quantify the ability of any significant predictors to discriminate between pregnant and non-pregnant subjects. Areas under the ROC curves ($AUC_{ROC}$) were

compared using the MEDCALC software package (version 9.5.2.0; MedCalc Software, Mariakerke, Belgium).

Interobserver reliability was assessed by two-way mixed Intraclass Correlation Coefficients (ICCs) with absolute agreement and their 95% confidence intervals. Limits of agreement, as described by Bland and Altman, were used to express inter- and intra observer reliability and to compare the different methods (Bland and Altman 1995; Khan and Chien 2001). Intraclass correlation coefficient (ICC) is a measure of reliability which evaluates the proportion of variance between observations (McGraw 1996). The scale from Altman was used in classification of the reliability values (Altman 1999). ICC values under 0.20 were considered poor, 0.21- 0.40 fair, 0.41-0.60 moderate, 0.61-0.80 good, and 0.81-1.00 very good. Limits of agreement are estimated from the mean and standard deviation of the differences with 95% of the differences lying within two standard deviations either side of mean. These limits, like confidence intervals, give an idea of the spread of variance between the methods**.**

**CHAPTER 3. The intra-observer and inter-observer reliability**

**of automated follicle counts made using three-dimensional**

**ultrasound and SonoAVC**

## *3.1   Abstract*

**Objective**: To assess the reliability of automated measurements of the total antral follicle count (tAFC) made using Sono-Automatic Volume Count (SonoAVC), and to compare these to two-dimensional (2D) and manual three-dimensional (3D) techniques.

**Methods**: 55 subjects aged <40 who had 3D transvaginal ultrasound (Voluson 730 Expert, GE Medical Systems) in early follicular phase of menstrual cycle were prospectively recruited. Three-dimensional datasets were acquired and subsequently analysed. The tAFC (2-10mm antral follicles) was calculated by two observers using three independent methods: 2D real time equivalent (2DRTE), 3D manual multiplanar view (3DMPV), and SonoAVC. For measures made using SonoAVC, the initial automated count (sAVC-AA) was recorded and post-processing (sAVC-PP) then applied to identify follicles that had been missed or incorrectly included. Intraclass correlation and limits of agreement were used to evaluate the methods.

**Results**: The intra- and inter-observer reliability of measurements of tAFC was best with SonoAVC followed by 3D MPV and 2D RTE. The initial count calculated by sAVC-AA missed follicles reflecting in the significantly lower mean tAFC (6.51±4.79) than that made after post-processing (sAVC-PP; 18.42±10.53, p<0.001), 3D-MPV (19.38±10.85, p<0.001) and 2D RTE (19.26±10.55, p<0.001) techniques. The mean tAFC became more comparable with post-processing (sAVC-PP) but still remained significantly lower than counts made with 2D RTE and 3D MPV (P<0.05).

**Conclusion**: SonoAVC is a reliable method for measuring the tAFC. The tAFC made with SonoAVC following post processing although lower are more comparable to 2D and 3D

MPV but at the expense of time. This may have been observed because SonoAVC measures and colour codes each follicle preventing re-counting.

## 3.2    Introduction

Ovarian reserve is estimated by combining certain clinical parameters and a variety of endocrinological or ultrasonographic measurements (Scheffer, Broekmans et al. 1999; Merce, Gomez et al. 2005; Jayaprakasan, Walker et al. 2007; McIlveen, Skull et al. 2007; Soldevila, Carreras et al. 2007). There is debate as to which combination of parameters has the best predictive value and there is no consensus at present. The relative contribution of each individual measure of ovarian reserve is clearer and most authors agree that antral follicle counts and serum anti-Müllerian hormone levels have the most discriminative potential (van Rooij, Broekmans et al. 2005; Visser, de Jong et al. 2006; Nardo, Christodoulou et al. 2007). The total antral follicle count (tAFC), measured using ultrasound, appears to perform as well as AMH and each modality has its own advantages and disadvantages. Serum measurements of AMH are still relatively expensive and there is a definite inter-laboratory variation in the performance of the assay (Visser, de Jong et al. 2006). In contrast, antral follicle counts are easy to perform and cheap in comparison as all units have access to ultrasound facilities. Follicle counts, as a quantitative measure of ovarian reserve, are also subject to 'assay' variation due to intra- and inter- observer differences and require additional time and manpower to perform (Jayaprakasan, Campbell et al. 2008).

Antral follicle counts are typically performed using real-time, two-dimensional ultrasound but may be estimated from three-dimensional ultrasound data. Both methods involve the

identification of follicles measuring between 2-10 mm as the observer manipulates the ultrasound transducer or scrolls through a stored dataset respectively. These techniques take time and are associated with a degree of measurement error as follicles can be missed or counted more than once. Automated measurement of the number of antral follicles has the potential to address both issues (Raine-Fenning, Jayaprakasan et al. 2007; Jayaprakasan, Campbell et al. 2008).

SonoAVC is a new software program that identifies and quantifies hypo echoic regions within a three-dimensional dataset and provides automatic estimation of their absolute dimensions, mean diameter, and volume (Raine-Fenning 2008). Each individual volume is given a specific color and the automated measurements of its mean diameter (relaxed sphere diameter), its maximum dimensions (x, y, z diameters), and its volume are displayed in descending order from the largest to the smallest (Raine-Fenning 2008). An unlimited number of volumes can theoretically be quantified, which makes it an ideal tool for follicle tracking. It also identifies small volumes and can theoretically be used to count antral follicles. To date SonoAVC has been used to assess stimulated ovaries only (Raine-Fenning, Jayaprakasan et al. 2007; Raine-Fenning 2008). These studies have shown that SonoAVC provides automatic measurements of follicular diameter and volume that are more reliable and more accurate than comparable estimations made from 2D data (Raine-Fenning, Jayaprakasan et al. 2007; Raine-Fenning 2008).

This study is the first to assess the inter- and intra-observer reliability of automated antral follicle counts made using SonoAVC and to compare these measures to those made using manual 2D and 3D techniques. This study was designed to test the hypothesis that

automated measurement of the total AFC using SonoAVC would be more reliable and quicker than measures made using conventional 2D and 3D techniques.

## 3.3    Methods

**Experimental Design**

Subjects aged less than 40 years with early follicular phase Follicle Stimulating Hormone (FSH) below 15 IU/L, planning to undergo treatment for their sub fertility, were prospectively recruited. Ultrasound was performed during the early follicular phase of menstrual cycle between days 2-5. The numbers of follicles measuring between 2 to 10 mm in diameter were counted in each ovary to calculate the total antral follicle count in that ovary. Subjects who had had previous ovarian surgery, had one ovary, and those with ovarian cysts or follicles measuring more than 10 mm were excluded from the study.

A sample size of 42 was required if the intraclass correlation coefficient between two observations was assumed at 0.80 with Type I error of 0.05 and Type II error of 0.20.

**Data acquisition**

All transvaginal ultrasound examinations were performed by a single investigator (J.C) using a Voluson Expert 730$^{TM}$ (GE Medical Systems, Zipf, Austria) and 7.5 MHz transvaginal transducer. The scan first involved a conventional 2D ultrasound assessment of the pelvis to exclude any obvious pathology, followed by visualization of ovaries in their transverse and longitudinal planes. This was followed by acquisition of 3D data using the 3D volume mode. The 3D volume mode displayed a truncated sector which was adjusted to define the area of interest; the sweep angle was set to $90^{°}$ so as to include the entire ovary and a

3D dataset was then acquired using the high quality, slow sweep mode. The resultant multiplanar display of the ovary was examined to confirm its inclusion in entirety. Datasets of both ovaries from each subject were stored on recordable digital video discs for subsequent analysis. The ultrasound settings, both grey scale and Doppler, were standardized and identical for all subjects.

**Data measurement**

4D View ™ (version 7.0, GE Medical Systems) was used for all measurements. The 3D dataset was opened and displayed as a single view showing the longitudinal or transverse view available with real-time conventional 2D ultrasound. The quality of the image was adjusted and optimized in terms of magnification for all datasets. Three different methods were then used to measure the number of antral follicles measuring 2-10mm in diameter in each ovary.

*Method 1: 2D real-time equivalent (2D RTE)*

The AFC was measured in each ovary using the longitudinal (A plane) and transverse (B plane) planes in an identical manner to that what would be done in the way real-time antral follicle counts are performed using conventional 2D ultrasound. These two planes are available to the observer with conventional 2D ultrasound but cannot be seen simultaneously. The investigators were able to scroll through the dataset in these two planes simulating a clinical setting of real-time 2D, but were not allowed to rotate the image along the x-axis or to see the two images simultaneously as these functions are not available with real-time scanning (Jayaprakasan, Walker et al. 2007).

### *Method 2: 3D Multiplanar view (3D MPV)*

The multiplanar view provides the observer with the longitudinal (A) and transverse (B) views described above for measurement method 1 but also demonstrates the coronal (C) plane which is not available with conventional 2D ultrasound (Jayaprakasan, Walker et al. 2007). The coronal plane is a reconstructed view of the dataset at 90-degrees to the transducer and provides the user with more spatial information. These three sectional planes are shown simultaneously and are mutually related so that movement within one plane produces geometrically equivalent movements in the other two planes. Method 2 involved measuring the tAFC using all three perpendicular planes. The investigators had the advantage of being able to cross-check the number of follicles in any one plane against the two other image planes (Jayaprakasan, Walker et al. 2007).

### *Method 3: 3D assisted SonoAVC*

The multiplanar view was used to display the dataset as in method 2. Once the three sectional planes were displayed, the render mode was entered to generate a 3D rendered view of the ovary. The render box was adjusted to exclude as much extra-ovarian information as possible and ensure that the whole ovary was included in the region of interest. The threshold settings, which assign transparency associated with fluid to opaque voxels, were maintained for all datasets. Once the dataset had been correctly positioned, SonoAVC was implemented. The individual follicles were then displayed with a specific colour and shown together with their dimensions and relative sizes. The total number of follicles initially identified using this technique was recorded as the 'SonoAVC-automated analysis' (SonoAVC-AA) antral follicle count (Figure 2.2). Preliminary work had shown that this initial automated assessment of the ovary missed many follicles, both within stimulated and unstimulated ovaries, which were readily evident to any observer.

These follicles can be included in the analysis by manually clicking on them. Each additional antral follicle identified in this way is given a new colour and its dimensions are displayed together with those follicles that were originally identified. The total AFC at the end of this process was recorded and referred to as the 'sonoAVC-post processing' (SonoAVC-PP) antral follicle count (Figure 2.3). The time required for the initial, automated assessment and the additional time required for the manual post-processing were recorded separately allowing the total time for measurements to be defined.

Two investigators (JC and SD) independently counted the total number of antral follicles in each dataset using the 2D RTE, 3D MPV, and the SonoAVC (SonoAVC-AA and SonoAVC-PP) methods. One investigator (SD) measured the datasets twice in no specific order to assess the intra observer variation also to reduce the operator dependent bias. When comparing the methods, average of the counts made by the two observers for each method was considered. The order of measurements and the method used for each dataset was randomly determined and different for each investigator. This was achieved by allocating a unique number to each dataset and ensuring that each dataset had been assessed using each technique. The time taken by each investigator to measure the total AFC within both ovaries per subject was recorded to the nearest second for each of the three methods.

**Statistical analysis**

Statistical analysis was undertaken using the Statistical Package for the Social Sciences (SPSS, version 15.0, Chicago, IL). The total antral follicle count (tAFC) made by adding the number of antral follicles in both ovaries per subject was used as a single variable for analysis.

Interobserver reliability was assessed by two-way mixed Intraclass Correlation Coefficients (ICCs) with absolute agreement and their 95% confidence intervals. Limits of agreement, as described by Bland and Altman, were used to express inter- and intra observer reliability and to compare the different methods (Bland and Altman 1995; Khan and Chien 2001). Intraclass correlation coefficient (ICC) is a measure of reliability which evaluates the proportion of variance between observations (McGraw 1996). The scale from Altman was used in classification of the reliability values (Altman 1999). ICC values under 0.20 were considered poor, 0.21- 0.40 fair, 0.41-0.60 moderate, 0.61-0.80 good, and 0.81-1.00 very good. Intraclass correlation can give a falsely reassuring impression of reliability (Rothwell 2000) and so Bland-Altman plots were also used to estimate the agreement between the two observers (Bland and Altman 1986) and because we were evaluating and comparing a new method to an established one (Bland and Altman 1999). To quantify the inter-observer agreement the differences between the averaged antral follicle counts and SDs were calculated, and limits of agreement were constructed (Bland and Altman 1986). Limits of agreement are estimated from the mean and standard deviation of the differences with 95% of the differences lying within two standard deviations either side of mean. These limits, like confidence intervals, give an idea of the spread of variance between the methods**.** The Bland Altman plots were constructed so that each observer could be identified and any systematic bias, where one observer constantly obtains higher or lower values, identified. No priori definition of the maximum width for limits of agreement was done before analyzing the results since there are no clinical data to define what an acceptable result would be.

The distribution of the data was checked using normal probability plots. The mean and standard deviation ($\pm$ SD) are given for normally distributed data and the median and

range for non-parametric data. Dependent on the normality of the data, a one-way analysis of variance with Wilcoxon Signed Ranks test or a paired student t-test was used to examine for differences in the mean AFC between the observers, within one observer, and according to the measurement method. A P value of <0.05 was considered to be statistically significant.

## 3.4    Results

Of the fifty-five subjects, six were excluded from the study; 3 had follicles measuring ≥ 10mm and another 3 were found to have ovarian cysts. Complete data were available on 49 subjects therefore and 98 datasets were available for analysis.

The data were not normally distributed and are expressed, therefore, as median and inter quartile ranges. The median tAFC (inter-quartile range, 25th and 75th percentile) measured by 2D RTE, 3D MPV, SonoAVC-AA, and SonoAVC-PP were 18 (10.56 and 26.81), 16.5 (10.25 and 26.75), 5 (3.37 and 9.18), and 15.5 (10.25 and 24.75) respectively (Table 3.1). The initial tAFC assessed by SonoAVC-AA missed many follicles which is reflected in a significantly lower median tAFC than for all other methods (P<0.001). The median tAFC with SonoAVC-PP increased to 15.5 but remained significantly lower than counts made with 2D RTE (P=0.006) and 3D MPV (P=0.028).

The Intraclass Correlation Coefficient (ICC) for 2D RTE, 3D MPV, sAVC-AA, and sAVC-PP were 0.979 (95% CI 0.964-0.988), 0.988 (95% CI 0.979-0.993), 0.971 (95% CI 0.949-0.983), and 0.997 (95% CI 0.995-0.998) respectively suggesting good inter-observer reliability for each method (Table 3.1).

The intra-observer and inter-observer limits of agreement for the three methods are expressed in Table 3.1. The limits of agreement suggest measurements made with

SonoAVC (SonoAVC-AA and SonoAVC-PP) are more reliable than those made using the 3D

MPV and 2D RTE techniques. The limits of agreement between the different methods are

expressed in Table 3.2 and shown as Bland-Altman plots in Figure 3.1. These data show a

significant difference in the mean tAFC between sonoAVC-PP, 3D MPV, and 2D RTE. The

best inter-method agreement was between 3D MPV and 2D RTE.

**Table 3.1:** Comparison of different methods of measuring antral follicles using mean AFC ± SD,

median AFC with inter quartile range, inter and intra observer reliability with limits of agreement,

and inter observer reliability with Intraclass correlation coefficient (ICC).

| Method | Median (Inter Quartile Range) | Inter observer upper and lower LOA | Intra observer upper and lower LOA | ICC (95% CI) |
|--------|------------------------------|-----------------------------------|-----------------------------------|--------------|
| 2D RTE | 18 (10.56 and 26.81) | 7.11 and -4.8 | 8 and -6.92 | 0.979 (0.964 -0.988) |
| 3D MPV | 16.5 (10.25 and 26.75) | 5.33 and -3.56 | 5.49 and -3.72 | 0.988 (0.979 -0.993) |
| sAVC-AA | 5 (3.37 and 9.18) | 3.24 and -3.1 | 3.78 and -3.67 | 0.971 (0.949 -0.983) |
| *sAVC-PP* | 15.5 (10.25 and 24.75) | 1.71 and -2.58 | 2.51 and -2.59 | 0.997 (0.995 -0.998) |

\* 2D RTE (2D real time equivalent), 3D MPV (3D multiplanar view), sAVC-AA (automated analysis with

SonoAVC), and sAVC-PP (SonoAVC with post-processing)

**Figure 3.1:** Bland–Altman plot showing the intermethod comparison between two-dimensional real-time equivalent (2D-RTE), three-dimensional multiplanar view (3D-MPV), and automatic volume count with postprocessing (sAVC-PP) using total antral follicle count as the single variable of analysis. The plot shows the overall mean difference between the observations made with each method and 95% coverage interval of the differences. ■ 3D-MPV vs 2D-RTE;  △3D-MPV vs sAVC-PP;    ●2D-RTE vs.sAVC-PP.

The mean of antral follicle count and the differences are expressed in numbers (n).
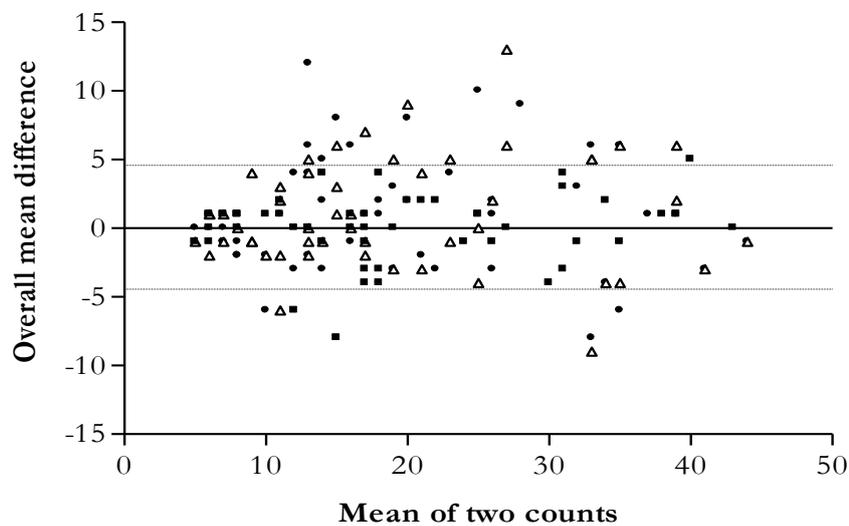


The time taken for measurements was found to be normally distributed. The mean (± SD) time in seconds for measurement of tAFC with 2D RTE, 3D MPV, sAVC-AA, and sAVC-PP was 71 ± 18.48, 97 ± 30.38, 26 ± 2.67, and 112 ± 29.86 respectively. Sono AVC with post processing (SonoAVC-PP) took the longest time followed by the 3D MPV, 2D RTE, and automated mode of Sono AVC (SonoAVC-AA) (Table 3.2).

**Table 3.2:** An inter-method comparison of the different measurement techniques showing the difference in time taken to make total antral follicle count (AFC), the difference in the mean AFC, limits of agreement, and significance (P) of these variables.

| Methods compared | Mean difference in time ± SD in seconds | Significance in time (P value) | Mean difference in tAFC ± SD | Significance in tAFC (P value) | Upper limit of agreement | Lower limit of agreement |
|---|---|---|---|---|---|---|
| sAVC-AA vs sAVC-PP | 86.03 ± 28.78 | P<0.001 | 12 ± 7.27 | P < 0.001 | 26.08 | -2.42 |
| sAVC-PP vs 3D MPV | 14 ± 22.03 | P=0.020 | 1 ± 3.91 | P = 0.006 | 8.66 | -6.66 |
| sAVC-PP vs 2D RTE | 41 ± 23.14 | P<0.001 | 0.92 ± 4.07 | P = 0.028 | 8.90 | -7.06 |
| 3D MPV vs 2D RTE | 26 ± 20.48 | P<0.001 | 0.08 ± 2.30 | P = 0.27 | 4.58 | -4.53 |

## 3.5   Discussion

This is the first study to examine the reliability of automated antral follicle counts made using SonoAVC. The intra-observer and inter-observer reliability of antral follicles made using SonoAVC was better than measurements made using the 3D multiplanar view or the 2D real time equivalent technique but only after post processing had been applied. This was at the expense of time and therefore negates some of the benefit of the software. However, the colour coding provided by sonoAVC ensures follicles are not counted more than once and all are identified. This may explain why the mean and median antral follicle

counts made with SonoAVC following post processing were significantly lower than those made with the 3D multiplanar view and the 2D real time equivalent techniques.

The software also provides an objective evaluation of each follicle as its diameter and volume are calculated. These advantages are specific to the software and have important implications for the research setting and for future clinical studies. The total AFC is calculated by counting the number of follicles measuring less than 10 mm in diameter in both ovaries. This is often done subjectively and even when measurements are made to assess the absolute size of the follicle these are often restricted to one or two follicles within each ovary to gain an impression of the number of follicles smaller than 10 mm. SonoAVC measures the mean diameter and volume of each antral follicle counted, thus providing a more accurate measurement of antral follicles, especially those measuring between 2-6 mm which are thought to relate more closely to age and other endocrine ovarian reserve tests (Haadsma, Bukman et al. 2007).

The main limitation of the new software was the erroneous low total antral follicle counts. The median total antral follicle count based on the initial automated SonoAVC analysis (sAVC-AA), was significantly lower than counts made with the 2D real time equivalent (2D RTE) and 3D MPV techniques. The follicles missed by sAVC-AA were of random sizes. This reflects a current lack of sensitivity of the software to identify all antral follicles as many additional follicles not included in the initial automated assessment were readily evident in the multiplanar view. sAVC-PP was required in all cases, which made the final total antral follicle count more comparable to the other methods. In this study, post processing involved the simple identification of individual follicles that had been missed as well as those that had been included incorrectly. Post processing can also be performed by tracing around follicles to identify them as separate or single entities (Raine-Fenning

2008) but this was not required in this study, presumably due to the small volumes being studied.

Previous work by our own group has shown that 3D ultrasound significantly improves the interobserver reliability of antral follicle counts (Jayaprakasan, Walker et al. 2007; Jayaprakasan, Campbell et al. 2008). Scheffer et al (Scheffer, Broekmans et al. 2002) showed that both 2D and 3D methods of counting antral follicles were comparable and reproducible but raised the possibility of improvement in the use of antral follicle count as a clinical test as a consequence to the use of 3D ultrasound. Although time taken to make off-line measurements using manual 3D techniques is more than 2D, the duration of the actual ultrasound examination and patient exposure is significantly reduced using 3D compared with real-time 2D ultrasound (Jayaprakasan, Campbell et al. 2008). SonoAVC presents a potential solution to this but at present requires too much manual post-processing to make it clinically useful and limits the use of the technology at present as it is not truly automated. The tradeoff between the ability to measure the total AFC reliably and the time required for this must be addressed. The software was designed for the assessment of larger follicles within a stimulated ovary and is currently being recalibrated for the detection of smaller volumes. This will hopefully reduce the need for post-processing and the overall time required. The software is probably more appropriate for use within the research setting at present and the potential advantage of having a reliable assessment of both follicle number and size needs to be addressed in prospective studies.

## 3.6    Conclusions

The intraobserver and interobserver reliability of automated antral follicle counts made with SonoAVC are better than comparable measurements made with manual 2D and 3D ultrasound. The initial automated measurements are spuriously low and significantly underestimate the true follicle count which requires manual post-processing. This allows the identification of missed follicles but at the expense of time and limits the automated nature and potential of the software. The software does provide an objective measurement of each follicle which has important implications for research as it allows investigation into the relative importance of follicles of different sizes.

**CHAPTER 4. Quantitative analysis of antral follicle number**

**and size: a comparison of real-time two-dimensional and**

**automated three-dimensional ultrasound techniques**

## *4.1   Abstract*

**Objective:** To compare two-dimensional (2D) ultrasound to automated three-dimensional (3D) ultrasound for the measurement of antral follicle number and size.

**Methods:** 24 subjects aged <40 underwent transvaginal ultrasound (Voluson E8) in the early follicular phase of menstrual cycle. A 2D ultrasound scan of both ovaries was performed and each antral follicle identified and then measured by taking the mean of two diameters. A 3D ultrasound dataset of both ovaries was then acquired and analysed using "Sonography-based Automated Volume Count" (SonoAVC™). The time taken to measure the size of all antral follicles in both ovaries was recorded in seconds for each technique. The antral follicle counts were then grouped into categories according to size: 2.0–5.0 mm, 2.0–6.0 mm, 2.0–8.0 mm, 2.0–9.0 mm, and 2.0-10mm. Limits of agreement (LOA) and a paired t-test or Wilcoxon signed ranks test was used to analyse the data dependent on its distribution.

**Results:** The AFC in all the categories was significantly lower with SonoAVC™ than with 2D ultrasound (p<0.05). When antral follicles were compared for every mm size, 2D ultrasound measured more follicles measuring 3.0-3.99mm (4.11±3.70 vs 2.63±2.31; P=0.019) and 4.0-4.99mm (4.63±4.86 vs 2.68±2.89; P=0.013) than those measured with SonoAVC™. LOA were widest with follicles measuring 3.0-3.99mm (6.38 and -3.43) and 4.0-4.99mm (7.99 and -4.09). SonoAVC™ took significantly lesser time to measure the number and size of the antral follicles than 2D ultrasound (132.05±56.23 vs 324.47±162.22; p<0.001).

**Conclusion:** Fewer antral follicles are evident overall when SonoAVC™ is used to analyse 3D ultrasound data. The clinical value of these remains to be determined but the automated technique is significantly quicker than measures made using 2D ultrasound which may also overestimate the number and size of follicles.

## *4.2    Introduction*

The antral follicle count is a marker of ovarian reserve and has been shown to be a significant predictor of ovarian reserve (Broekmans, Kwee et al. 2006; Jayaprakasan, Campbell et al. 2008; Verhagen, Hendriks et al. 2008). The highest production of anti-Müllerian hormone (AMH) is seen in the granulosa cells of the pre-antral and small antral follicles measuring 4 mm or less in diameter with expression gradually reducing as follicles become larger (Weenen, Laven et al. 2004). The size of the antral follicle may be more important than the total number therefore and more predictive of ovarian reserve and outcome following assisted reproduction treatment. Very few studies have considered the size of antral follicles other than to define the limits of follicular diameter above and below which follicles should not be included in the final count (Chang, Chiang et al. 1998; Pellicer, Gaitan et al. 1998; Weenen, Laven et al. 2004). Studies that have considered the size of the follicles between and above these extremes suggest that follicles measuring 2-5mm correlate most strongly with assisted reproduction treatment outcomes (Chang, Chiang et al. 1998). Pellicer et al. used 3D ultrasound to assess the population of these 'selectable' follicles in young, low responders and found that the number of antral follicles measuring 2-5 mm in diameter was significantly lesser than the  age-matched normal responders (Pellicer, Ardiles et al. 1998).

The standard technique used to the quantify the size of an antral follicle involves calculation of the mean diameter from two measurements of the follicular diameter (Haadsma, Bukman et al. 2007). No studies have reported on the intra- or inter-observer reliability of quantitative antral follicle counts stratified according to the size of the follicle. Measurement reliability is likely to be relatively poor as it will depend on both the identification and subsequent measurement of each follicle. There is a natural variation in determining the total number of antral follicles, both within and between observers and once a follicle has been identified there will be further variability in its measurement. Any variability is likely to be exaggerated when there are larger numbers of antral follicles as more measures are required overall (Scheffer, Broekmans et al. 2002; Jayaprakasan, Campbell et al. 2008).

SonoAVC identifies and quantifies hypo echoic regions within a three-dimensional dataset and provides automatic estimation of their absolute dimensions, mean diameter, and volume. SonoAVC, when used for follicle tracking during controlled ovarian stimulation, provides automatic measurements of follicular diameter and volume that are more reliable and more accurate than comparable estimations made from 2D data (Raine-Fenning, Jayaprakasan et al. 2007; Raine-Fenning 2008; Raine-Fenning, Jayaprakasan et al. 2008). In chapter 3, I have demonstrated how sonoAVC can be used to automatically determine the total number of antral follicles and that these measurements are more reliable than manual measures of acquired 3D data. This study was specifically designed, therefore, to compare automated analysis of 3D data, made using SonoAVC, and real-time two-dimensional ultrasound for the assessment of the number and size of antral follicles in women undergoing assisted reproduction treatment.

## 4.3    Methodology

Twenty-four subjects aged less than 40 years planning to undergo assisted reproduction treatment for unexplained sub fertility were prospectively recruited. Subjects with a history of previous ovarian surgery including ovarian cystectomy, ovarian drilling and unilateral oophorectomy were excluded from the study. If the entry criteria were met subjects underwent transvaginal ultrasound during the early follicular phase, cycle days 2-5, in the immediate menstrual cycle prior to treatment. Those subjects found to have ovarian cysts or follicles measuring more than 10 mm were excluded at this point.  A total sample size of 32 was required to power the study at 0.85 with a difference in total AFC of 3 between two methods.

A single investigator (J.C) performed all of the ultrasound scans using a Voluson E8 Expert (GE Medical Systems, Zipf, Austria) and a four-dimensional 5-9 MHz transvaginal transducer. The ultrasound assessment involved a conventional real-time 2D ultrasound assessment of the pelvis to exclude any obvious pathology, followed by visualization of ovaries in their transverse and longitudinal planes. Antral follicle number and size were first measured using real-time 2D ultrasound and then, following the acquisition of 3D data, with sonoAVC. The ultrasound settings and technique of data acquisition were standardized and identical for all subjects: gain, -5; speckle reduction imaging (SRI), 2; CrossXBeam CRI (compound resolution imaging), 3; CRI filter, high; enhance, 2; reject, 25; and harmonics, high. Once the dataset had been obtained sonoAVC, which was integrated in the ultrasound machine, was used to provide an automatic assessment of the follicles within each ovary. The precise details of both measurement techniques are detailed below:

### 4.3.1  Measurement method 1: Conventional real-time 2D ultrasound

**Figure 4.1:** The figure below shows an image of ovary acquired using 2D ultrasound in early follicular phase of menstrual cycle. Each antral follicle is measured using 2D imaging in two perpendicular diameters
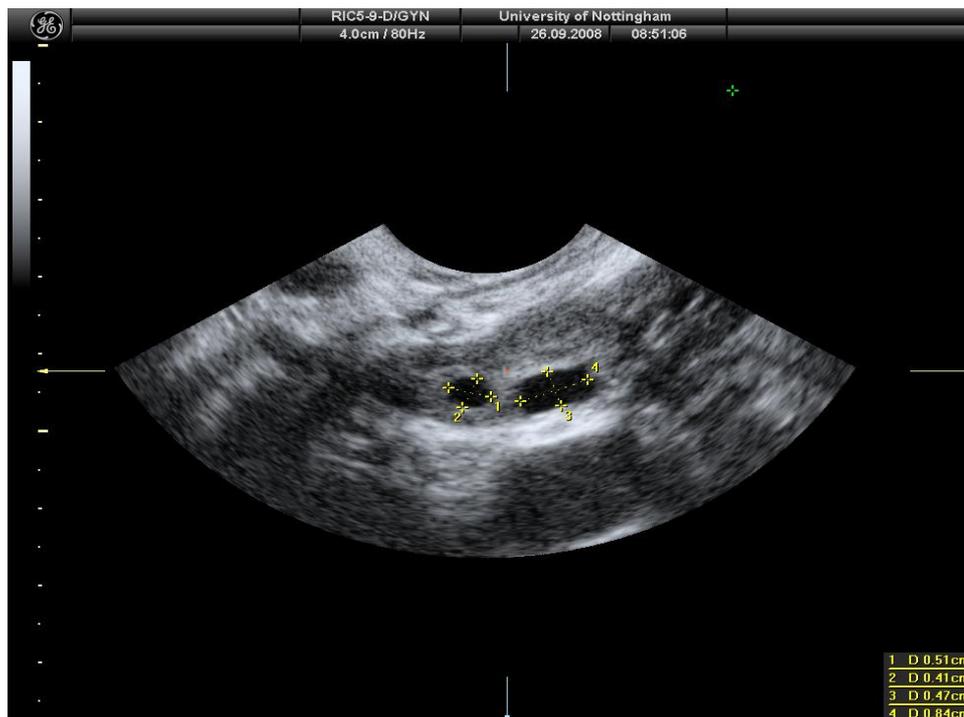


This method involved measurement of antral follicles during the conventional real-time ultrasound assessment of the ovaries and represents the current standard for this. Once the ovary was localized the observer used the transducer to scroll through the ovary in two planes, longitudinal and transverse, and observe the antral follicles. Each antral follicle was then identified and measured in turn until the whole ovary was analysed. Antral follicle size was calculated by taking the mean of two perpendicular diameters, one of which represented the largest dimension, of each follicle (Figure 4.1). The same process was then repeated for the contra lateral ovary.

## 4.3.2  Measurement method 2:  Automated 3D – SonoAVC

SonoAVC was used on the ultrasound machine (version E8, GE Medical Systems) on the dataset which was acquired using 3D and displayed using the multiplanar view, making it closer to real time scanning. The image display was optimized and the render mode entered to generate a three-dimensional volume of interest (VOI). The render box was adjusted to exclude as much extra-ovarian information as possible and ensure that the whole ovary was included in the VOI. The threshold settings, which assign transparency associated with fluid to opaque voxels, were maintained for all datasets at a default setting of 'low'. Once the dataset had been correctly positioned, SonoAVC was implemented. The individual follicles were then displayed with a specific colour and shown together with their dimensions and relative sizes. Post-processing, involving the manual identification of follicles not included in the automated analysis, was then used to ensure all antral follicles were counted and measured. Each additional antral follicle identified in this way was given a new colour and its dimensions displayed together with those follicles that were originally identified. The mean diameter of relaxed sphere of each antral follicle in both ovaries displayed as d(v) was recorded.

The antral follicle population for each subject was grouped into five categories based on the absolute dimension of the follicles as follows: $\leq$ 2mm, 2.1 to 4.0mm, 4.1 to 6.0mm, 6.1 to 8.0mm, and 8.1 to 9.0mm for further analysis. The time taken for the whole process was recorded to the nearest second. The clock was started as soon as the baseline scan had been conducted and the decision made to assess the antral follicle population. The clock was stopped when all antral follicles in both ovaries had been measured and their individual sizes recorded. This was a continuous process for both measurement techniques and included the process of data acquisition for the 3D method.

**Statistical analysis**

Statistical analysis was undertaken using the Statistical Package for the Social Sciences (SPSS, version 15.0, Chicago, IL). The distribution of the data was checked using normal probability plots. The mean and standard deviation (± SD) are given for normally distributed data and the median and range for non-parametric data. Dependent on the normality of the data, a paired student t-test or one-way analysis of variance with Wilcoxon Signed Ranks test was used to examine for differences between the measurement methods in the number of antral follicles overall and within each size category and in the time taken for the measurements.. A P value of <0.05 was considered to be statistically significant.

## 4.4    Results

Three subjects were excluded from the study, as they were found to have follicles measuring 10 mm or more, and complete data were available, therefore, for 21 subjects who had a mean (± SD; range) age of 34.02 (± 4.29; 28.30 to 37.50) years and BMI of 24.22 (± 2.32; 22.20 to 27.00). The cause for their subfertility related to male factors (8; 38.09%), tubal disease (2; 9.52%) or was unexplained (11; 52.38%).

The measurement data and time taken for analysis were both confirmed to be normally distributed. The mean time taken for the automated analysis of the 3D ultrasound dataset with SonoAVC™ (132.05 ± 56.23 seconds) was significantly lesser than that required for the 2D ultrasound assessment (324.47 ± 162.22 seconds), respectively (P<0.001). Post processing was used in all cases and the time recorded for SonoAVC™ includes the additional time needed for this. The mean time taken for SonoAVC™ without post

processing was 41.06 ± 11.12 seconds. The mean time required for the post processing aspect was 90.99 ± 45.11 seconds.  SonoAVC™ recorded a significantly lower number of antral follicles than 2D ultrasound when the total counts were considered and also when the follicles were grouped into the different sub-categories (Table 4.1). The limits of agreement between the two methods were wide as well (Table 4.1). A similar trend was seen when the number of antral follicles were stratified and analysed to the nearest millimeter; a significantly higher number of follicles measuring 3.0 to 3.99 mm (4.11 ± 3.70 vs 2.63 ± 2.31; P=0.019) and 4.0 to 4.99 mm (4.63 ± 4.86 vs 2.68 ± 2.89; P=0.013) were identified with 2D ultrasound than with the automated 3D ultrasound technique (Table 4.2). The limits of agreement between the two methods were widest with follicles measuring 3.0 to 3.99 mm (6.38 and -3.43) and 4.0 to 4.99 mm (7.99 and -4.09) (Table 4.2).

**TABLE 4.1**: A comparison of two-dimensional (2D) ultrasound and automated three-dimensional (SonoAVC™; Sonography-based Automated Volume Count) ultrasound techniques for the assessment of the total antral follicle count according to the absolute size of the antral follicles. The two methods have been compared using limits of agreement and the P value derived using a paired samples *t* test.

| | | Real-time 2D (Mean ± SD) | 3D SonoAVC (Mean ± SD) | Mean difference ± SD (95% CI) | P value | Upper LOA | Lower LOA |
|---|---|---|---|---|---|---|---|
| **Time (seconds)** | | 324.47 ± 162.22 | 132.05 ± 56.23 | 192.42 ± 113.51 (137.71 to 247.13) | <0.001 | 414.91 | -30.06 |
| Antral follicle count (n) according to different group categories | **2.0 to 5.0 mm** | 10.95 ± 8.61 | 8.11 ± 6.22 | 2.84 ± 4.03 (0.90 to 4.79) | 0.007 | 10.74 | -5.06 |
| | **2.0 to 6.0 mm** | 16.63 ± 10.58 | 11.37 ± 8.62 | 5.26 ± 3.19 (3.72 to 6.80) | <0.001 | 11.52 | -1.00 |
| | **2.0 to 8.0 mm** | 18.26 ± 10.75 | 15.53 ± 9.51 | 2.74 ± 2.56 (1.50 to 3.97) | 0.001 | 7.75 | -2.27 |
| | **2.0 to 9.0 mm** | 19.32 ± 10.53 | 16.79 ± 9.79 | 2.53 ± 2.27 (1.43 to 3.62) | 0.001 | 6.98 | -1.92 |
| | **2.0 to 10.0mm** | 19.89 ± 10.33 | 17.16 ± 9.71 | 2.74 ± 2.40 (1.58 to 3.89) | 0.001 | 7.44 | -1.97 |

**TABLE 4.2:** The number of antral follicles stratified by size to the nearest millimeter as measured by two-dimensional (2D) ultrasound and automated three-dimensional (SonoAVC™; Sonography-based Automated Volume Count) ultrasound. The two methods have been compared using limits of agreement and the P value derived using a paired samples *t* test.

| Antral follicle size (n) | Real-time 2D (Mean ± SD) | SonoAVC (Mean ± SD) | Mean difference ± SD (95% CI) | P value | Upper LOA | Lower LOA |
|---|---|---|---|---|---|---|
| **2.0 to 2.99 mm** | 2.21 ± 1.96 | 2.79 ± 1.72 | -0.58 ± 2.19 (-1.64 to 0.48) | 0.265 | 3.72 | -4.88 |
| **3.0 to 3.99 mm (n)** | 4.11 ± 3.70 | 2.63± 2.31 | 1.47 ± 2.50 (0.27 to 2.68) | 0.019 | 6.38 | -3.43 |
| **4.0 to 4.99 mm** | 4.63 ± 4.86 | 2.68 ± 2.89 | 1.95 ± 3.08 (0.46 to 3.43) | 0.013 | 7.99 | -4.09 |
| **5.0 to 5.99 mm** | 3.21 ± 2.30 | 3.26 ± 2.10 | -0.05 ± 1.75 (-0.89 to 0.79) | 0.897 | 3.37 | -3.48 |
| **6.0 to 6.99 mm** | 2.47 ± 1.07 | 2.74 ± 1.05 | -0.26 ± 1.37 (-0.92 to 0.40) | 0.413 | 2.42 | -2.94 |
| **7.0 to 7.99 mm** | 1.63 ± 0.96 | 1.42 ± 1.43 | 0.21 ± 1.08 (-0.31 to 0.73) | 0.408 | 2.34 | -1.91 |
| **8.0 to 8.99 mm** | 1.05 ± 0.91 | 1.26 ± 0.65 | -0.21 ± 1.03 (-0.71 to 0.29) | 0.385 | 1.81 | -2.23 |
| **9.0 to 9.99 mm** | 0.58 ± 0.61 | 0.37 ± 0.60 | 0.21 ± 0.79 (-0.17 to 0.59) | 0.259 | 1.75 | -1.33 |

## 4.5    Discussion

This is the first study to describe the use of SonoAVC™ for the identification and measurement of antral follicle size. Previous work by our own group has shown that the

new automated technique provides a more reliable measure of total follicle number than 2D ultrasound and manual assessment of three-dimensional data both within and between observers (Deb, Jayaprakasan et al. 2009)[5]. The current study extends this work and has shown that SonoAVC™ provides different results to 2D ultrasound when the size of the follicle is considered. Whilst SonoAVC™ is an automated technique, post-processing of the data was required in all cases. The technique is best considered as 'semi-automated' therefore and whilst the additional processing adds to the assessment time it is straightforward and the overall time required for analysis was still significantly quicker than measures made with 2D ultrasound when the size of follicle size was also assessed.

In both studies we found that SonoAVC™ identified and measured significantly less follicles than 2D ultrasound resulting in a consistently lower total antral follicle count than 2D ultrasound regardless of the upper and lower size limits used to define the 'antral follicle population. This may be a true result or reflect fundamental differences in the measurement techniques. An obvious limitation of the current study is the inability to confirm the accuracy of these measurements but histological work suggests that the automated measures are more valid than those derived using 2D ultrasound imaging. Weenen et al. examined the ovaries of 12 regularly cycling subjects aged between 19 and 44 years who had undergone prophylactic oophorectomy as they were genetically predisposed to an increased risk of ovarian cancer or because they had endometriosis (Weenen, Laven et al. 2004). Follicles, at all stages of folliculogenesis, were measured in two perpendicular planes and the mean measure taken as the diameter of follicle. Histologically, an average of 18 follicles measuring 2-6 mm was evident in each ovary. This compared more favorably with the measures made with SonoAVC™ which identified 20 such follicles compared to 2D ultrasound which identified 26. A smaller number of follicles

measuring 1-2mm were evident histologically, approximately six in each ovary. SonoAVC™ identified approximately half this number whilst 2D ultrasound failed to identify any. This almost certainly reflects the resolution limits of ultrasound but may have been further confounded by the subjective nature of 2D ultrasound imaging. The ability of an observer to identify and reliably measure such small structures with 2D ultrasound is likely to be time dependent but a degree of measurement inaccuracy would still be expected even if more time was allowed for assessment. Whilst the subjects in the study by Weeneen et al. may be different from those in the current study with regards to the timing of the assessment in the menstrual cycle and in the population studied, who were having oophorectomy, the data reported represent the best currently available on the human ovary against which ultrasound can be evaluated. There are obvious limitations to the current evidence base and future validation studies may provide a stronger evidence base against which standards could be set.

If we accept that the semi-automated 3D ultrasound measures are more valid than those made with 2D ultrasound imaging we need to consider why this new technique is better. It may relate to the image being displayed in a 3D ultrasound multiplanar view which allows cross-checking of each follicle in three different planes, improving spatial orientation therefore, but is more likely to reflect the fact that each follicle is colour-coded which prevents repeat measures of the same follicle (Raine-Fenning, Jayaprakasan et al. 2007). As regards the objective assessment of follicle size, the 2D ultrasound technique derives the follicular diameter through estimation of the mean of two perpendicular, linear measures whereas SonoAVC™ uses volumetric information to define a 'relaxed sphere (Raine-Fenning, Jayaprakasan et al. 2007). This might explain the differences seen between the two techniques as 2D ultrasound is more likely to disregard

small irregularities in the follicles. Volumetric measurements made with SonoAVC™ have been shown to provide a a more reliable and valid assessment of the estimated diameter of larger follicles than both 2D ultrasound and manual assessment of 3D ultrasound datasets regardless of the number of linear measures taken to define the size of the follicle (Raine-Fenning, Jayaprakasan et al. 2008; Raine-Fenning, Deb et al. 2009).

## 4.6    Conclusion

Our results show that SonoAVC™, despite being a semi-automated method as data post-processing is invariably required, provides quicker measures of antral follicle size than 2D ultrasound. There is least agreement between the two methods with antral follicles measuring 3.0 to 4.99 mm. If the size of the follicles contributing to the overall antral follicle population is more important than the absolute size of the pool, and the current animal and human evidence suggests it is, these findings have important implications for the research setting and clinical environment. Further research is required to evaluate the biological and clinical importance of quantifying antral follicle size and count.

**CHAPTER 5. The predictive value of the automated quantification of the number and size of small antral follicles in women undergoing assisted reproduction treatment.**

## 5.1   Abstract

**Objective:** Sono automatic volume calculation (SonoAVC) automatically identifies and measures the dimensions of hypoechogenic areas within datasets acquired using three-dimensional (3D) ultrasound. The objective of this study was to evaluate the predictive value of automated antral follicle counts according to their relative sizes in women undergoing assisted reproduction treatment (ART).

**Methods**: 156 subjects aged ≤40 years with a baseline FSH ≤15 IU due to undergo their first cycle of ART were prospectively recruited. SonoAVC was used to measure the datasets and record the number of antral follicles measuring ≤9mm in diameter. These follicles were then grouped into subsets according to their relative sizes: ≤2.0 mm, 2.1-4.0 mm, 4.1-6.0 mm, 6.1-8.0 mm, and 8.1-9.0 mm. The primary outcome was viable pregnancy confirmed on ultrasound 5 weeks following embryo transfer.

**Results:** 142 subjects were included for analysis of primary endpoint. Those subjects who conceived had significantly more antral follicles measuring ≤ 2 mm (p=0.041) and 2.1-4.0 mm (p<0.001) than those who had unsuccessful treatment. There were no significant differences between the groups in the number of antral follicles measuring 4.1-6.0 mm (p=0.191), 6.1-8.0 mm (p=0.203), or 8.1-9.0 mm (p=0.601). Multiple logistic regression showed antral follicles measuring 2.1-4.0 mm were an independent predictor of pregnancy (Exp(B)=1.234, 95%CI 1.092 and 1.491; p=0.004; AUC=0.693).

**Conclusion:** SonoAVC provides automated measures of antral follicle number and size. Using this technique, the number of antral follicles measuring 2.1 to 4.0 mm in diameter is an independent, significant predictor of pregnancy following IVF treatment.

## *5.2    Introduction*

Ultrasonographic assessment of the total number of antral follicles measuring 2-9 mm is a reliable determinant of ovarian reserve (Scheffer, Broekmans et al. 2003; Ng, Chan et al. 2005; Jayaprakasan, Campbell et al. 2008). The pool of antral follicles comprises pre-antral and early antral follicles (0.2 – 2.0 mm) that are largely gonadotrophin-independent, small antral follicles (1.0-6.0 mm) selectable due to their responsiveness to gonadotrophins, and larger antral follicles (>6.0 mm) that are gonadotrophin-dependent (Gougeon 1989). At any given timepoint during the menstrual cycle, the ovaries contain follicles at different developmental stages and antral follicle counts must be made during the early follicular phase (Gougeon 1998). The total antral follicle count (tAFC) is made by counting the number of antral follicles measuring 2 to 10 mm in both ovaries and can be estimated using two-dimensional (Scheffer, Broekmans et al. 2002) or three-dimensional ultrasound (Jayaprakasan, Campbell et al. 2008). The majority of studies evaluating the value of the antral follicle counts only consider the overall number and not the absolute size of the follicles, which may be a separate, independent predictor.

Anti-Müllerian hormone (AMH), also known as Müllerian inhibiting substance, is one of the best markers of ovarian reserve (Dehghani-Firouzabadi, Tayebi et al. 2008; Nelson, Yates et al. 2009). AMH is largely expressed by the granulosa cells of pre-antral and small antral follicles measuring 6.0 mm or less (Modi, Bhartiya et al. 2006). Whilst serum levels of AMH correlate with the total number of antral follicles (de Vet, Laven et al. 2002; van Rooij, Broekmans et al. 2005; Fanchin, Mendez Lozano et al. 2007), it is likely that these smaller antral follicles more accurately reflect ovarian reserve than the total population of follicles. The number of small antral follicles is also strongly correlated with other ovarian reserve tests, such as AMH, supporting the concept that these smaller follicles represent

the functional ovarian reserve (Haadsma, Bukman et al. 2007). There is a linear decline in the number of antral follicles with age (Gougeon 1994) and this is more apparent in the smaller antral follicles (<6.0 mm) than the larger ones (>6.0 mm) (Scheffer, Broekmans et al. 2003; Haadsma, Bukman et al. 2007) and their total number is, therefore, more reflective of the primordial follicle pool.

Assessment of the antral follicle population is commonly made using two-dimensional ultrasound, which is simply used to identify and count the number of small follicles within each ovary. The largest follicles should be measured to ensure only follicles measuring 9.0 mm or less in diameter are included in the total count as larger follicles are more likely to be atretic (Khairy, Clough et al. 2008). Assessment of follicle size requires measurement of each follicle in two dimensions and calculation of the mean diameter (Haadsma, Bukman et al. 2007). This can be very labour intensive and the reliability and validity of such measures are likely to be reduced when there are numerous follicles as it is difficult to ensure each follicle is only measured once and none are missed. Three-dimensional ultrasound allows the user to acquire a volume of information which can be examined off-line and facilitates the implementation of various software programmes that enhance measurement accuracy and both intra- and inter-observer reliability (Raine-Fenning, Campbell et al. 2003). The most recent development has seen the introduction of automated data analysis where mathematical algorithms allow the definition and differentiation of hypo echoic, fluid-filled areas within the acquired volume (Raine-Fenning, Jayaprakasan et al. 2007). SonoAVC also provides automatic estimation of the absolute dimensions of each three-dimensional fluid-filled area (Raine-Fenning, Jayaprakasan et al. 2008). Each individual volume is given a specific colour and the automated measurements of its mean diameter (relaxed sphere diameter), its maximum

dimensions (x, y, z diameters), and its volume are displayed in descending order from the largest to the smallest (Raine-Fenning, Jayaprakasan et al. 2008). An unlimited number of volumes can theoretically be quantified and the software lends itself, therefore, to the examination of follicles within the ovary.

The aim of this study was to evaluate the value of antral follicles stratified according to their size, calculated automatically with SonoAVC, in the prediction of viable pregnancy 5 weeks following embryo transfer on ultrasound, ovarian response to controlled ovarian stimulation with gonadotrophins, and fertilisation and cleavage rates, in women undergoing in vitro fertilisation (IVF) or intracytoplasmic sperm injection (ICSI) as part of their assisted reproduction treatment (ART).

## 5.3   Methods

### Study design

Subjects planning to undergo their first treatment cycle of in vitro fertilisation (IVF) were prospectively recruited. Inclusion criteria included age less than 40 years, body mass index (BMI) of less than 30, regular menstrual cycles of 24-32 days duration, planned first cycle of IVF with or without ICSI, and an early follicular phase FSH level of <15IU/L. All subjects underwent a pre-treatment ultrasound assessment in the early follicular phase (cycle day 2-5) of menstrual cycle. Subjects were excluded if they were found to have an ovarian follicle or cyst measuring more than 20 mm in diameter. Recruited subjects were subsequently excluded if their treatment cycle was cancelled or if they had failed fertilisation or embryo cleavage. Subjects wishing to egg share or altruistically donate their oocytes were not included.

Clinical pregnancy, as confirmed by a transvaginal ultrasound scan performed 5 weeks following embryo transfer assessing the fetal viability, was taken as the primary outcome measure for analysis. Secondary outcome measures assessed included the number of mature oocytes, fertilised oocytes and cleaved embryos. To power the study at 0.8 incorporating ten predictor variables in the regression model, a sample size of 130 was required.

**Data acquisition**

A single investigator (JC) performed all of the transvaginal ultrasound examinations using a Voluson Expert 730$^{TM}$ and a four-dimensional, 5-9 MHz transvaginal transducer. A preliminary conventional 2D ultrasound assessment of the pelvis was performed to exclude any obvious pathology, followed by visualization of ovaries in their transverse and longitudinal planes and the subsequent acquisition of 3D data using the volume mode. A 3D dataset was subsequently acquired using the high quality, slow sweep mode resulting in the highest resolution. The resultant multiplanar display of the ovary was examined to confirm its inclusion in entirety. Datasets of both ovaries from each subject were stored on recordable digital video discs (DVDs) for subsequent analysis.

**Data measurement**

4D View $^{TM}$ (version 7.0, GE Medical Systems) was used for offline data analysis. The 3D dataset was opened and displayed using the multiplanar view, which shows three sectional planes (the A, B, and C plane) simultaneously that are mutually related so that movement within one plane produces geometrically equivalent movements in the other two planes. These planes can be used to standardize the view of the ovary, or any 3D dataset, and for this study we ensured the A plane showed the ovary in its longitudinal section with the iliac vessels inferiolaterally and the B plane the transverse image

orthogonal to this. This orientation automatically ensures the image presented in the C plane demonstrates the coronal view of the ovary. The quality of the image was adjusted and optimized, in terms of magnification, for all datasets and the 'render mode' entered to generate a 3D rendered view of the ovary. Once the dataset had been correctly positioned, SonoAVC was implemented. The individual follicles were then displayed with a specific colour and shown together with their dimensions and relative sizes. Post processing involved manually clicking on the follicles that were missed as described in chapter 3. Each additional antral follicle identified in this way was given a new colour and its dimensions displayed together with those follicles that were originally identified. The size of the follicles, as measured automatically and displayed with different colour codes on the screen, was sub-divided according to the mean follicle sizes into those measuring <2 mm, 2.1 to 4.0 mm, 4.1 to 6.0 mm, 6.1 to 8.0 mm, and 8.1 to 9.0 mm.

**Treatment protocol**

A long protocol, involving pituitary suppression with Gonadotrophin Releasing Hormone (GnRH) agonists (500 mcg/day of Buserelin; Suprefact®, Aventis Pharma, Kent, UK or 800 mcg/day of Nafarelin; Synarel®, Pharmacia, Milton Keynes, UK) started in the mid-luteal phase of the menstrual cycle, was used in all subjects. Ovarian stimulation was commenced once down regulation was confirmed with a daily dose of either 225 IU of recombinant Follicle Stimulating Hormone (FSH; Gonal-F; Serono Pharmaceuticals Ltd, Feltham, UK) or 225 IU of human menopausal gonadotrophin (hMG; Menopur; Ferring Pharmaceuticals Ltd, UK). The response to ovarian stimulation was monitored by ultrasound and serum oestradiol levels on an alternate day basis from the fifth day of FSH administration and human Chorionic Gonadotrophin (hCG; 6500 IU of Ovitrelle; Serono Pharmaceuticals Ltd, Feltham, UK when using recombinant FSH or 5000 IU of Pregnyl;

Organon Laboratories Ltd when using Menopur) administered when there were at least three follicles measuring 18 mm or more in diameter with transvaginal, ultrasound-guided oocyte retrieval scheduled 36 hours later. One or two embryos were replaced according to the wishes of the couple and the number of embryos available 48 hours later. From the day of embryo transfer, luteal support was provided through the self-administration of progestogen pessaries (Cyclogest; Shire Pharmaceuticals Ltd, Basingstoke, Hants, UK). A urine pregnancy test was performed 16 days from the day of embryo transfer and subjects with positive tests had the viability, position, and growth of their pregnancy confirmed by a transvaginal ultrasound scan 3 weeks later at 7 weeks of gestation if the date of embryo transfer is considered as the time of implantation.

## Statistical analysis

The Statistical Package for the Social Sciences (version 15.0; SPSS, Chicago, IL) was used for data analysis. The distribution of the data was checked for normality using a normal probability plot. An unpaired t-test or Mann-Whitney U test, applied to normally distributed and skewed data respectively, were used to examine for significant differences in each variable between pregnant and non-pregnant groups. A p-value of <0.05 was considered statistically significant and to have not occurred by chance.

Linear regression analysis was used to assess the value of age and antral follicles, both overall and according to their mean diameter, for the prediction of the number of mature oocytes retrieved, the number of fertilised oocytes, and the number of embryos at cleavage stage. Binary logistic regression analysis was then applied to evaluate the effect of the same variables on the prediction of pregnancy. Receiver operating characteristic (ROC) curve analysis was performed to quantify the ability of any significant predictors to discriminate between pregnant and non-pregnant subjects. Areas under the ROC curves

(AUC$_{ROC}$) were compared using the MEDCALC software package (version 9.5.2.0; MedCalc Software, Mariakerke, Belgium) (Hanley and McNeil 1983).

## 5.4    Results

Of the 156 subjects recruited, a total of 142 were included in the final analysis of the primary endpoint (viable pregnancy on ultrasound scan 5 weeks following embryo transfer). Of the 14 subjects excluded from this analysis, 3 were found to have a well defined ovarian cyst requiring ovarian cystectomy, 2 failed to down regulate adequately requiring cancellation and rescheduling of the treatment cycle, 3 decided to enter the egg-share scheme after recruitment, 3 subjects had failed fertilisation, and 3 had failed cleavage.

A total of 151 subjects were included in the final analysis of secondary endpoint including the number of mature oocytes. Of the 5 subjects excluded from the study, 3 were found to have a well defined ovarian cyst requiring ovarian cystectomy, and 2 failed to down regulate adequately requiring cancellation and rescheduling of the treatment cycle. A total of 148 subjects were included in the final analysis of secondary endpoint including number of fertilised oocytes and cleaved embryos. Of the 8 subjects excluded, 3 were found to have a well defined ovarian cyst requiring ovarian cystectomy,  2 failed to down regulate adequately requiring cancellation and rescheduling of the treatment cycle, and 3 decided to enter the egg-share scheme after recruitment.

The mean (SD; range) age and BMI of the whole study group were 34.30 (4.51; 23.00 - 39.86 years) and 25.67 (3.24; 22.21 - 28.97 Kg/m2) respectively. 76 (51.35%) subjects had standard IVF and the remaining 72 (48.65%) had ICSI to facilitate fertilisation. 52 (35.14%) were given hMG as the ovarian stimulant and 96 (64.86%) rFSH. 73 (51.41%) subjects had

a viable clinical pregnancy confirmed on transvaginal ultrasound scan 5 weeks following embryo transfer, and the remaining 69 (48.59%) had a negative pregnancy outcome, which included a negative pregnancy test in 64 subjects and a non viable pregnancy on ultrasound in 5 subjects. The pregnant and the non-pregnant groups were compared for age, type of treatment (IVF / ICSI), type of gonadotrophin used (rFSH / hMG), size of antral follicles, number of total oocytes, number of mature oocytes, number of fertilised oocytes, number of cleaved embryos, and grade of embryos using a Mann-Whitney U test as they were not normally distributed (Table 5.1). The key findings were that the pregnant group were significantly younger (33.38 ± 4.07; 24.25 - 39.86) than their non-pregnant counterparts (35.29 ± 4.38; 23.00 – 39.56) and had a significantly higher number of antral follicles overall (19.71 ± 10.60; 3 - 48 vs 14.06 ± 8.77; 3 - 43 respectively).

However, sub-group analysis on the basis of absolute follicle size revealed the differences between the groups in the number of antral follicles was restricted to the smaller follicles measuring <2.0 mm and 2.1 to 4.0 mm as there were no differences in the number of follicles measuring 4.1-6.0 mm, 6.1-8.0 mm, or 8.1 to 9.0 mm. There were also no differences between the pregnant and non-pregnant groups in the type of gonadotrophin used for ovarian stimulation, the type of treatment employed for fertilisation or in the grade of embryos (Table 5.1).

**Table 5.1:** This table shows the differences between the group that achieved a clinical pregnancy following ART to the group that did not achieve a clinical pregnancy. Variables examined included the age of participant, type of treatment (IVF / ICSI), type of gonadotrophin used, antral follicles stratified by size, and ovarian response.

| Variable | | Pregnant (n=73) | | Non-Pregnant (n=69) | | P value |
|---|---|---|---|---|---|---|
| **Age (years)** | | 33.38 (4.07; 24.25-39.86) | | 35.29 (4.38; 23.00-39.56) | | 0.006 |
| **Type of treatment** | | IVF 37 (50.68%) | ICSI 36 (49.32%) | IVF 35 (50.72%) | ICSI 34 (49.28%) | 0.996 |
| **Type of gonadotrophin** | | rFSH 46 (63.01%) | hMG 27 (36.99%) | rFSH 48 (69.57%) | hMG 21 (30.43%) | 0.411 |
| **Antral Follicle Count stratified by mean follicle diameter** | **Total** | 19.71 (10.60; 3-48) | | 14.06 (8.77; 3-43) | | 0.001 |
| | **≤ 2.0 mm** | 1.49 (2.07; 0-11) | | 0.87 (1.16; 0-5) | | 0.041 |
| | **2.1-4.0 mm** | 6.97 (5.29; 0-26) | | 3.92 (3.39; 0-14) | | <0.001 |
| | **4.1-6.0 mm** | 6.62 (4.87; 0-25) | | 5.39 (4.56; 0-22) | | 0.069 |
| | **6.1-8.0 mm** | 3.73 (2.66; 0-12) | | 3.13 (2.28; 0-11) | | 0.192 |
| | **8.1-9.0 mm** | 0.90 (1.06; 0-4) | | 0.74 (0.81; 0-3) | | 0.583 |
| **Total oocytes (n)** | | 12.73 (6.42; 2-35) | | 10.86 (5.37; 1-26) | | 0.118 |
| **Mature oocytes (n)** | | 10.73 (5.17; 2-23) | | 9.04 (4.54; 1-20) | | 0.081 |
| **Fertilised oocytes (n)** | | 7.26 (3.70; 1-20) | | 5.81 (4.09; 1-19) | | 0.028 |
| **Cleaved embryos (n)** | | 6.89 (3.54; 1-19) | | 5.53 (3.99; 1-18) | | 0.024 |
| **Mean embryo grade** | | 2.22 (0.61; 1-3) | | 2.41 (0.55; 1-3) | | 0.071 |

Values are presented as mean (SD; range) or as number (percentage). IVF = in vitro fertilisation / ICSI = intracytoplasmic sperm injection, rFSH = recombinant Follicle Stimulating Hormone (Gonal-F; Serono Pharmaceuticals Ltd, Feltham, UK); hMG = human menopausal gonadotrophins (Menopur; Ferring Pharmaceuticals Ltd, UK)

Since age is related to number and size of the antral follicles as well as the outcome of various ovarian reserve tests (Broekmans, Kwee et al. 2006), linear regression analysis of age and the different cohorts of antral follicles, according to their mean diameter, was applied individually for the prediction of the number of mature oocytes retrieved, number of fertilised oocytes, and the number of embryos reaching cleavage stage (Table 5.2). The analysis showed that when regression analysis was applied to each of the variable separately, all the variables except for 8.1 to 9.0 mm follicles were significant. However, when multiple regression analysis including the age and antral follicle number stratified according to their mean diameter was applied, the cohort of antral follicles measuring 2.1 to 4.0 mm in size were the only significant variable in the prediction of these outcome measures of ovarian response (Table 5.3). A significant correlation coefficient (R) was found between the antral follicles measuring 2.1 to 4.0 mm and age (R=-0.189; P=0.024), number of total oocytes retrieved (R=0.453; P<0.001), number of mature oocytes (R=0.431; P<0.001), number of fertilised oocytes (R=0.343; P<0.001), and number of embryos at cleavage stage (R=0.347; P<0.001). However, the correlation was not significant between 2.1 to 4.0 mm follicles and grade of embryos (R=0.144; P=0.087), and total dose of gonadotrophin used for ovarian stimulation (R=-0.084; P=0.303).

**Table 5.2:** Linear regression analysis of each individual predictor variable including age and antral

follicle number stratified according to their mean diameter, for the prediction of the number of

mature oocytes, fertilised oocytes, and cleaved embryos.

| Predictor variable | | Number of mature oocytes (n=151) | | | Number of fertilised oocytes (n=148) | | | Number of cleaved embryos (n=148) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | P value | $R^2$ | ß | P value | $R^2$ | ß | P value | $R^2$ | ß |
| Age | | 0.015 | 0.099 | -0.314 | 0.075 | 0.022 | -0.147 | 0.092 | 0.019 | -0.139 |
| Total antral follicles | | <0.001 | 0.225 | 0.474 | 0.001 | 0.127 | 0.356 | 0.001 | 0.117 | 0.322 |
| Antral follicles by size (mm) | ≤ 2.0 | 0.014 | 0.040 | 0.199 | 0.743 | 0.001 | 0.027 | 0.831 | 0.001 | 0.018 |
| | 2.1–4.0 | <0.001 | 0.249 | 0.499 | <0.001 | 0.139 | 0.359 | <0.001 | 0.120 | 0.346 |
| | 4.1-6.0 | 0.001 | 0.123 | 0.351 | 0.016 | 0.085 | 0.292 | 0.026 | 0.081 | 0.284 |
| | 6.1-8.0 | 0.008 | 0.047 | 0.217 | 0.041 | 0.053 | 0.181 | 0.018 | 0.072 | 0.219 |
| | 8.1-9.0 | 0.992 | 0.000 | 0.001 | 0.947 | 0.000 | -0.006 | 0.942 | 0.000 | 0.006 |

$R^2$: It is a measure of variability in the outcome due to the predictors (Standardised ß): expresses the number

of standard deviation that the outcome changes due to a single standard deviation change in the predictor.

Univariate analysis suggested most of the variables were significant predictors of

pregnancy outcome. However, only the cohort of antral follicles measuring 2.1 to 4.0 mm

in size remained a significant predictor of clinical pregnancy when logistic regression

analysis was applied to all of the variables (Table 5.4). ROC curve analysis of the variables

predictive of pregnancy on univariate analysis also showed a maximum area under curve

(AUC) for antral follicles measuring 2.1 to 4.0 mm; albeit the AUC for age (0.634), fertilised

oocytes (0.633), and cleaved embryos (0.632) did not differ significantly. The AUC for

mature oocytes (0.585) and mean embryo grade (0.577) was significantly lesser than the

AUC for 2.1 to 4 mm follicles (Figure 5.1). The sensitivity, specificity, positive and negative predictive value, positive likelihood ratio, and post-test probability for the prediction of pregnancy at different cut-off levels for the cohort of antral follicles measuring 2.1 to 4.0 mm are shown in Table 5.5. Whilst the optimum cut-off level, as indicated by the highest sum of sensitivity and specificity, was at 3 or more, the post-test probability was highest at a cut-off level of 6 or more.

**Table 5.3:** Multiple linear regression analysis of model including age and antral follicle number stratified according to their mean diameter, for the prediction of the number of mature oocytes, fertilised oocytes, and cleaved embryos.

| Predictor variable | | Number of mature oocytes (n=151); $R^2$=0.296 | | | Number of fertilised oocytes (n=148); $R^2$=0.170 | | | Number of cleaved embryos (n=148); $R^2$=0.159 | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | P value | 95% CI | ß | P value | 95% CI | ß | P value | 95% CI | ß |
| Age | | 0.064 | -0.385 to 0.043 | -0.182 | 0.557 | -0.188 to 0.102 | -0.048 | 0.590 | -0.179 to 0.102 | -0.440 |
| Antral follicles by size (mm) (n) | ≤ 2.0 | 0.462 | -0.279 to 0.611 | 0.055 | 0.165 | -0.587 to 0.101 | 0.088 | 0.154 | -0.578 to 0.092 | 0.075 |
| | >2.0–4.0 | <0.001 | 0.246 to 0.626 | 0.402 | 0.001 | 0.102 to 0.418 | 0.309 | 0.002 | 0.088 to 0.397 | 0.298 |
| | >4.0-6.0 | 0.712 | -0.165 to 0.241 | 0.035 | 0.669 | -0.135 to 0.210 | 0.044 | 0.647 | -0.129 to 0.207 | 0.047 |
| | >6.0-8.0 | 0.427 | -0.208 to 0.490 | 0.066 | 0.088 | -0.08 to 0.602 | 0.128 | 0.068 | -0.15 to 0.569 | 0.063 |
| | >8.0-9.0 | 0.637 | -1.015 to 0.623 | -0.034 | 0.713 | -0.820 to 0.562 | -0.029 | 0.734 | -0.789 to 0.557 | -0.027 |

**Table 5.4:** Logistic regression analysis of age, number of antral follicles stratified according to size, number of mature oocytes, number of fertilised oocytes, number of cleaved embryos, and mean grade of embryos replaced for the prediction of pregnancy following IVF / ICSI treatment.

| Predictor Variable | | Odds Ratio | 95% Confidence Interval | P value |
|---|---|---|---|---|
| Age | | 0.987 | 0.867 – 1.014 | 0.057 |
| Total antral follicles | | 1.072 | 1.014 – 1.094 | 0.047 |
| Antral Follicle Count stratified by mean follicle diameter | ≤ 2.0 mm | 0.935 | 0.644 – 1.082 | 0.173 |
| | 2.1-4.0 mm | 1.234 | 1.092 – 1.491 | 0.004 |
| | 4.1-6.0 mm | 1.055 | 0.951 – 1.172 | 0.311 |
| | 6.1-8.0 mm | 1.020 | 0.853 – 1.219 | 0.831 |
| | 8.1-9.0 mm | 0.758 | 0.497 – 1.154 | 0.196 |
| Mature oocytes | | 1.080 | 0.949 – 1.229 | 0.242 |
| Fertilised oocytes | | 0.815 | 0.446 – 1.487 | 0.505 |
| Cleaved embryos | | 1.128 | 0.623 – 2.045 | 0.691 |
| *Mean grade of embryos* | | 1.753 | 0.868 – 3.543 | 0.118 |

**Table 5.5:** Receiver operator characteristic curve assessment of the best cut-off level for the number of antral follicles measuring 2.1 to 4.0 mm for the prediction of pregnancy following IVF with/without ICSI treatment

| 2.1-4.0 mm AFC Cut-off | Sensitivity (%) | Specificity (%) | +LR | -LR | PPV (%) | NPV (%) |
|---|---|---|---|---|---|---|
| >1 | 87.67 | 23.19 | 1.14 | 0.53 | 54.70 | 64.00 |
| >2 | 82.19 | 36.23 | 1.29 | 0.49 | 57.70 | 65.80 |
| >3[*] | 72.60 | 59.42 | 1.79 | 0.46 | 65.40 | 67.20 |
| >4 | 61.64 | 68.12 | 1.93 | 0.56 | 67.20 | 62.70 |
| >5 | 53.42 | 78.26 | 2.46 | 0.60 | 72.20 | 61.40 |
| >6 | 47.95 | 84.06 | 3.01 | 0.62 | 76.10 | 60.40 |
| >7 | 38.36 | 86.96 | 2.94 | 0.71 | 75.70 | 57.10 |
| >8 | 26.03 | 88.41 | 2.24 | 0.84 | 70.40 | 53.00 |
| >9 | 24.66 | 89.86 | 2.43 | 0.84 | 72.00 | 53.00 |
| >10 | 19.18 | 92.75 | 2.65 | 0.87 | 73.70 | 52.00 |

**\* Optimum cut-off level** is a level at which an acceptable compromise is achieved between sensitivity, specificity, PPV and NPV of the test.

**Positive predictive value (PPV):** It is the proportion of subjects with positive test results who are correctly diagnosed. It is a critical measure of the performance of a diagnostic method, as it reflects the probability that a positive test reflects the underlying condition being tested for.

**Negative predictive value (NPV):** It is defined as the proportion of subjects with a negative test result who are correctly diagnosed.

**Positive and negative likelihood ratio (+LR and −LR):** A likelihood ratio of greater than 1 indicates the test result is associated with the disease. A likelihood ratio less than 1 indicates that the result is associated with absence of the disease.

**Figure 5.1:** Receiver operator characteristic curves of variables predictive of pregnancy
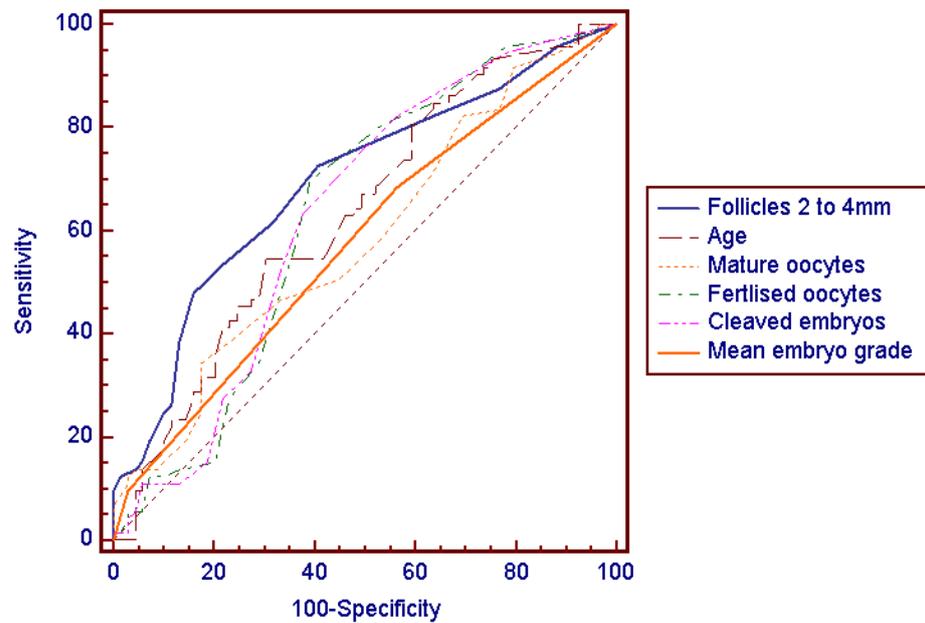


## 5.5    *Discussion*

This is the first study to evaluate different cohorts of antral follicles, based on their absolute size, in the prediction of reproductive outcome in subjects undergoing IVF treatment. Our results show that the small antral follicles measuring between 2.1 to 4.0 mm are a significant predictor of viable pregnancy confirmed on ultrasound 5 weeks following embryo transfer, independent of age, the number of mature oocytes obtained, fertilisation rates, the number of cleaved embryos, and the grade of embryos transferred.

One study  has considered the different sizes of antral follicles and reported that the pregnancy rates were higher in women with higher number of 5-10 mm size antral follicles (Pohl, Hohlagschwandtner et al. 2000). However, they also included follicles measuring up to 20 mm in their 'total antral follicle count' and found that cycle cancellation due to poor ovarian response was higher in women with a dominant number of antral follicles measuring more than 11 mm. Regression analysis, in our study, however demonstrated that the small antral follicles, measuring between 2.1 to 4.0 mm, are the most significant predictor of the number of mature oocytes retrieved following IVF treatment and that this is independent of age and the total number of antral follicles. This is in keeping with work by Pellicer et al. who looked at the number of small, selectable antral follicles in young, low responders with normal basal FSH levels (Pellicer, Ardiles et al. 1998). Those women with an unexpected poor response were found to have a significantly lower number of follicles measuring between 2 to 5 mm than age-matched controls. In our study, linear regression analysis also revealed a significant predictive relationship between follicles measuring 2.1 to 4.0 mm and fertilisation rates and the number of cleaved embryos. This supports the notion that the smaller antral follicles are the follicles that truly reflect the ovarian potential and are most predictive of response to controlled ovarian stimulation during IVF treatment. Several studies suggest, however, that the total number of antral follicles is predictive of reproductive response (van Rooij, Broekmans et al. 2002; Hendriks, Mol et al. 2005; Muttukrishna, McGarrigle et al. 2005). It is possible that the smaller, more responsive follicles comprise the majority of the follicle population included in the total count in these individuals and that this masks the importance of the smaller follicles.

AMH has also been suggested as an important predictor of response to ovarian stimulation. Ficicioglu et al., for example, found that levels of AMH predict the number of oocytes retrieved with a positive predictive value of 96% (Ficicioglu, Kutlu et al. 2006) and other groups have reported a strong correlation between the number of oocytes retrieved and both AMH levels and the total number of antral follicles (de Vet, Laven et al. 2002; van Rooij, Broekmans et al. 2005; Fanchin, Mendez Lozano et al. 2007; Dehghani-Firouzabadi, Tayebi et al. 2008). AMH is expressed by the granulosa cells of the early growing, pre-antral and small antral follicles, which measure less than 6 mm,, but not by non-atretic, larger antral follicles or those that have become atretic, and may reflect or represent the population of smaller antral follicles more than the overall number (Baarends, Hoogerbrugge et al. 1995; Gruijters, Visser et al. 2003; Weenen, Laven et al. 2004; Skalba, Cygal et al. 2008). Based on our results, further studies evaluating ovarian response and reproductive outcome based on the number of small antral follicles and AMH levels are warranted.

The primary outcome measure evaluated in this study was a viable intrauterine pregnancy confirmed on a transvaginal ultrasound scan 5 weeks after embryo transfer. Several factors are thought to influence the chance of pregnancy after IVF treatment and in this study we considered age, the total number of antral follicles and the number of follicles within each size cohort, the type of gonadotrophin used, the technique used for fertilisation (IVF / ICSI), the number of mature oocytes retrieved, the number of fertilised oocytes, the number of cleaved embryos, and the number and grade of embryos transferred. Logistic regression revealed that the only variable predictive of clinical pregnancy was the number of antral follicles measuring between 2.1 to 4.0 mm (Exp(B)=1.221; 95% CI 1.072 and 1.391; p=0.003). Receiver operating characteristic

analysis suggested this cohort of follicles provides the most discriminative power to differentiate those women likely to conceive (AUC: 0.694) from those whose treatment will not be successful; a total count of three providing the optimum sensitivity and specificity with a post-test probability of 53%.

Ovarian ageing is known to influence the ovarian response to stimulation, as measured by the oocyte yield at retrieval, and is characterised by the progressive depletion of the primordial follicular cohort (Broekmans, Knauff et al. 2007). The antral follicle count is positively correlated with the primordial follicular population (Gougeon 1996) and is, therefore, a significant predictor of poor ovarian response. Haadsma et al. evaluated antral follicles of different sizes and found a decline in the number of small antral follicles with increasing age whereas the number of larger follicles remained constant. In our study, we further sub-grouped the small and large antral follicles and found a similar significant correlation between age and the cohort of follicles measuring 2.1 to 4.0 mm (p=0.005) and 4.1 to 6.0 mm (p=0.001). In this study we did not include women above the age of 40 who are expected to have a much lower chance of pregnancy as a result of impaired oocyte quality, as illustrated by lower implantation rates even when the ovarian response is deemed acceptable and when there are an adequate number of embryos available for transfer (van Rooij, Bancsi et al. 2003). It would be interesting to examine the relative proportions of the different cohorts of antral follicle in these women but one would expect a reduction in the smaller follicles.

The results of our study must be interpreted with caution and are not generally applicable as we only included subjects predicted to have a normal response, as assessed by age and FSH levels of <15 IU/L, undergoing their first cycle of IVF. Further work is required in women who have demonstrated a poor or exaggerated response to ovarian stimulation

and the predictive value of the different cohorts of antral follicles compared with other markers of ovarian reserve, especially AMH which has emerged as one of the most powerful predictors of response and reproductive outcome following assisted reproduction treatment (Wunder, Guibourdenche et al. 2008; Nelson, Yates et al. 2009)

## 5.6    *Conclusion*

SonoAVC allows the quantitative assessment of the number and relative sizes of the antral follicle population in women undergoing *in vitro* fertilisation treatment. Small antral follicles, measuring 2.1 to 4.0 mm, appear to be the most significant predictor of the number of mature oocytes retrieved, fertilisation rate and the number of embryos reaching the cleavage stage. This cohort of follicles also appears to predict pregnancy independently of age and the total number of antral follicles. Further work is required in women with poor and exaggerated response to treatment and should be undertake in conjunction with serum AMH measures. This technique may facilitate prospective studies designed to examine the effect of different gonadotrophin preparations and doses and treatment protocols.

# CHAPTER 6. Validation of antral follicle counts of different follicle size cohorts made using two- and three-dimensional ultrasound

## *6.1    Abstract*

**Objectives:** Three-dimensional (3D) ultrasound assisted sonography-based automated volume calculation (SonoAVC) and 2D ultrasound have been described in counting and measuring the size of antral follicles. This study was designed in an in vitro setting to validate the count and size of antral follicles measured using SonoAVC and 2D against the actual size and number of follicles assessed by dissecting the bovine ovaries.

**Methods:** 3D Ultrasound scan (Voluson 730 Expert, GE Medical Systems) was performed on 22 bovine ovaries. Antral follicles were counted and measured on stored 3D dataset using SonoAVC on multiplanar view and 2D real-time equivalent method on longitudinal and transverse planes of the dataset. All visible antral follicles were dissected manually and measured using a microscope. Ovarian volume calculated using volume displacement (VD) method was compared against that measured using Virtual Organ Computer-aided AnaLysis (VOCAL) and 2D method (volume for an ellipsoid). Student's *t* test, limits of agreement (LOA) and Intraclass correlation (ICC) coefficient were used to evaluate the variance and agreement between methods.

**Results:** The mean ovarian volume calculated by volume displacement (VD) was comparable to that using 3D ultrasound (VOCAL) (P=0.338), however significantly larger than 2D (P<0.001). Total AFC (2.0-10.0mm) measured by the manual dissection (MD) method were significantly higher than SonoAVC (P=0.003) and significantly lower than 2D (P<0.001), however LOA between MD and SonoAVC were lower than that between MD and 2D.  AFC of follicles measuring 2.0-4.0mm was significantly higher with MD than SonoAVC (P<0.001) and significantly lower than 2D (P<0.001). LOA were narrower

between MD and SonoAVC than between MD and 2D. Antral follicles measuring >4.0-6.0 mm and >6.0-10.0mm showed no significant difference between either methods (P<0.05), but LOA between MD and SonoAVC were narrower than between MD and 2D.

**Conclusion:** SonoAVC with post-processing significantly underestimates and 2D overestimates the number of antral follicles measuring 4.0mm or less, but both make comparable counts of follicles measuring more than 4.0mm when compared with the antral follicles dissected manually. However, the agreement with SonoAVC with post-processing was more than that with 2D.

## *6.2    Introduction*

Three-dimensional (3D) and two-dimensional (2D) ultrasound have both been used to count the total number of antral follicles and measure their dimensions. Many studies restrict analysis of size to a few follicles focusing on the largest and smallest follicles and then subjectively counting the number between these extremes without measuring their individual sizes. This is necessary as the manual identification and measurement of each and every follicle is unlikely to be reliable or valid and is clearly not practical in the clinical setting. Different authors have suggested different follicle populations should be considered with the upper limit of follicle size varying from 8 to 10 mm (Weenen, Laven et al. 2004; Allemand, Tummon et al. 2006; Lam and Raine-Fenning 2006; Nardo and Gelbaya 2008). Several clinical and laboratory studies have shown that the smaller follicles may be more reflective of functional ovarian reserve and follicle quality suggesting there is a need for a more detailed analysis of antral follicles by size .

Sonography-based automated volume calculation (SonoAVC) is an automated method of volume calculation which can be applied on images acquired using three-dimensional (3D) ultrasound. Few studies that have used SonoAVC to assess follicles in stimulated ovaries, have shown that SonoAVC provides automatic measurements of follicular diameter and volume that are more reliable and more accurate than comparable estimations made from 2D data (Raine-Fenning, Jayaprakasan et al. 2007; Raine-Fenning 2008; Deutch, Joergner et al. 2009). It also identifies small volumes and can theoretically be applied on antral follicles as they contain an antrum which is a fluid filled cavity within the follicle. Our previous work has shown that the initial automated measurements made by SonoAVC are obviously low and significantly underestimate the true follicle count. However, the follicles missed can be included in the final count by manually clicking on them, making the method semi-automated. Each additional antral follicle identified in this way is given a new colour and its dimensions are displayed together with those follicles that were originally identified.  SonoAVC with post-processing is a reliable method for measuring antral follicles and shows better intra- and inter-observer reliability than 3D multiplanar view and two-dimensional (2D) ultrasound (chapter 3). SonoAVC is quicker and provides better estimates of small antral follicle number than conventional real-time 2-D ultrasound. Although time taken by SonoAVC to count the total number of antral follicles is more than 2D ultrasound, our results show that SonoAVC, despite being a semi-automated method as data post-processing is invariably required, provides reliable measures of the size of antral follicles more quickly than real-time 2D ultrasound (chapter 4). This has important implications for both the research setting and clinical environment as it allows a quantitative analysis of antral follicle number and size without disturbing

workflow. It is a more objective and reliable method than real-time 2D ultrasound and takes significantly less time than conventional ultrasound assessments.

The AFC measured using either 2D or 3D ultrasound has emerged as a single independent predictor of ovarian response following *in vitro* fertilization (IVF) treatment.(verhagen 2008) SonoAVC has been able to provide reliable and quick measures of antral follicle sizes, thus enabling more research in to different size groups of antral follicles. Small antral follicles (2.0-6.0mm) could potentially reflect the actual functional or qualitative ovarian reserve as it highly correlates with AMH (Jayaprakasan, Deb et al. 2009; van Disseldorp, Lambalk et al. 2010) and is also a significant predictor of ovarian response and pregnancy outcome following IVF (Jayaprakasan, Campbell et al.; Haadsma, Bukman et al. 2007; Deb, Batcha et al. 2009; Haadsma, Groen et al. 2009). However, it is not clear whether the antral follicles actually seen on ultrasound reflect the true number of antral follicles present in the ovary at that time.  This study was therefore designed in an *in vitro* setting to validate the antral follicle counts and the size of antral follicles measured by SonoAVC and 2D ultrasound against the actual size and number of follicles assessed by dissecting cow ovaries, that resemble human ovaries in terms of numbers and size of antral follicles.

## 6.3    Methods

Ultrasound scan was performed on bovine ovaries in an *in vitro* setting. The ovaries were dissected from the reproductive tract of the cows and any excess tissue manually dissected. A phantom was made out of a latex glove which was filled with water and glycerine. The fluid mixture containing 8 parts of water and 2 parts of glycerine

constituted a solution of desirable density such that the ovary would neither sink nor float in the fluid. The glove was filled to about 60% of its capacity by the fluid mixture and the bovine ovary placed in it just before performing the ultrasound scan.

The ultrasound scans were performed by a single investigator (S.D) using a Voluson E8 Expert (GE Medical Systems, Zipf, Austria) and a three-dimensional 5-9 MHz transvaginal transducer. 3D ultrasound of the ovary was performed which included delineation of the ovary with application of a region of interest and the subsequent acquisition of a series of 2D planes acquired during a high quality slow sweep mode of the ultrasound beam set at a $90^0$ angle. This ensured that data from the whole ovary was acquired and also that the greatest number of 2D planes were acquired giving the highest degree of resolution when the 2D data are reconstructed as a 3D volume. The acquired 3D data were displayed on the ultrasound machine (Voluson E8 Expert: General Electric Medical Systems, Zipf, Austria) in the multiplanar view. To simulate the 2D ultrasound assessment (2D real-time equivalent - 2D RTE), the antral follicles were counted and measured in each ovary using the longitudinal (A plane) and transverse (B plane) planes in an identical manner to that what would be done when performing real time 2D ultrasound (Figure 6.1). The investigators were able to scroll through the dataset in these two planes simulating a clinical setting of real-time 2D, but were not allowed to rotate the image along the x-axis or to see the two images simultaneously as these functions are not available with real-time scanning.

**Figure 6.1:** Dataset acquired of bovine ovary in a glove filled with 8:2 of water and glycerine. Each

antral follicle was measured in two perpendicular planes similar to 2D
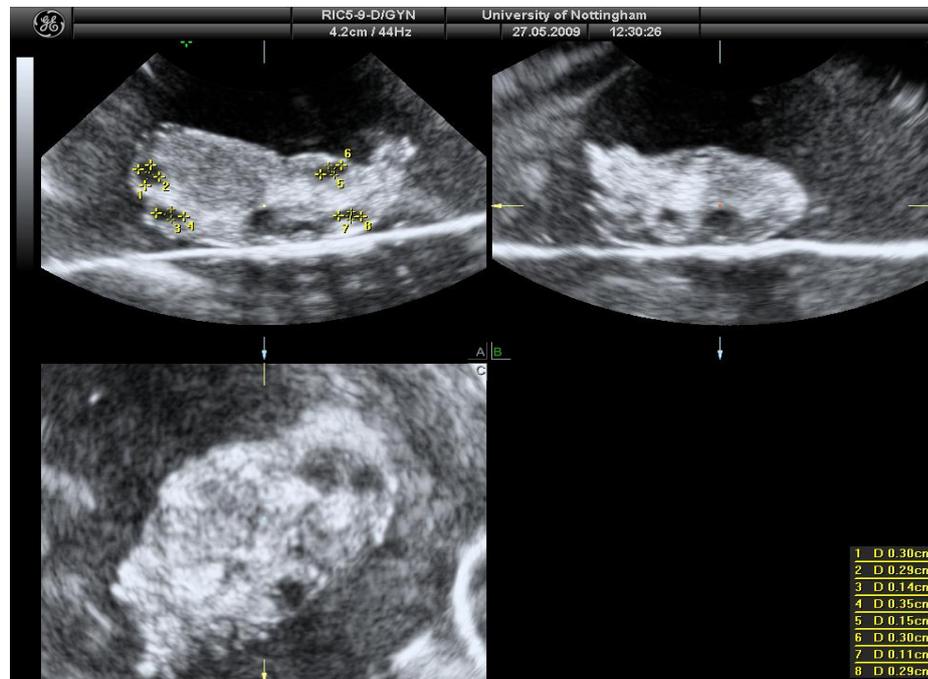


For the application of SonoAVC, the acquired 3D data were displayed on the ultrasound

machine in the multiplanar view (Figure 6.2). The image was optimised and then rendered

to generate a three-dimensional volume of interest (VOI). The render box was adjusted to

exclude as much extra-ovarian information as possible and ensure that the whole ovary

was included in the VOI. The threshold settings, which assign transparency associated

with fluid to opaque voxels, were maintained for all datasets at a default setting of 'low'.

Once the dataset had been correctly positioned, 3D automated software, 'sonography-

based automated volume count' (sonoAVC: GE Medical Systems) was implemented

(Raine-Fenning, Jayaprakasan et al. 2008). Post-processing, involving the manual

identification of follicles, was used to ensure all antral follicles were counted and

measured. The mean 'relaxed sphere diameter', displayed as d (V), of each antral follicle

was recorded and used for data analysis. The antral follicle population for each ovary was

recorded to the nearest millimetre, as this reflects the current resolution of the ultrasound system, starting from 2.0 mm up to a maximum of 10.0 mm. They were analysed and results expressed for follicle size cohorts of 2.0-4.0mm, >4.0-6.0mm, >6.0-10.0mm and 2.0-10.0mm.

**Figure 6.2:** 3D dataset of a bovine ovary in a phantom of glove containing 8:2 of water and glycerine. Antral follicles are measured using SonoAVC
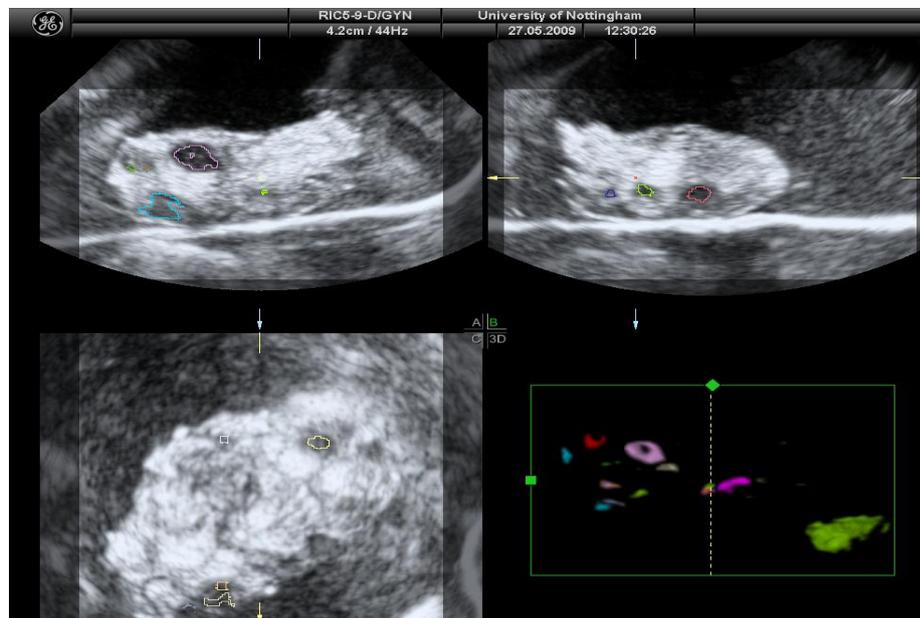


Ovarian volume was calculated before performing the scan using the method of volume displacement. Virtual Organ Computer-aided AnaLysis (VOCAL imaging programme: GE Medical Systems) was used to quantify the ovarian volume. The method has been described in detail previously (Raine-Fenning, Campbell et al. 2002) but briefly involved display of ovary in 3D multiplanar view followed by delineation of ovarian cortex in B plane by manually tracing round the ovary in $30^0$ rotational steps as the dataset was rotated through a total of $180^0$, providing six planes for each volume calculation. To calculate the volume as one would on 2D ultrasound, three diameters were measured

perpendicular to each other on each ovary using the longitudinal (A plane) and transverse (B plane) planes in an identical manner to that what would be done when performing 2D ultrasound. Formula for calculation of volume of an ellipsoid was applied to derive the volume by 2D ultrasound (0.53*length*width*depth)

Soon after the ultrasound scan, the ovary was transported back to the laboratory in normal saline and dissected under a microscope by another investigator (J.A). All visible follicles were dissected out using ultra-fine scissors and forceps (Abir, Franks et al. 1997; Gilchrist, Wicherek et al. 2001) and measured using the microscope eye piece graticule units. Any excess ovarian tissue was gently teased away from the follicles and placed in a medium of normal saline on a heated microscope stage. Three perpendicular diameters were measured for each follicle, the mean of which was then converted to millimetres using the formula, *"mean in graticule units/12.4"*.  The investigator dissecting the ovary was blinded to the ultrasound measurements and the investigator performing the ultrasound was blinded to the findings of the dissected ovary.

A sample size of 17 was required to power the study at 0.8 with a Type I error of 0.05 and Type II error of 0.2. Statistical analysis was undertaken using the Statistical Package for the Social Sciences (SPSS, version 17.0, Chicago, IL). The distribution of the data was checked using normal probability plots. The mean and standard deviation (± SD) are given for normally distributed data and the median and range for non-parametric data. Dependent on the normality of the data, a one-way analysis of variance with Wilcoxon Signed Ranks test or a paired student t-test was used to examine for differences between methods. A P value of <0.05 was considered to be statistically significant. Limits of agreement, as described by Bland and Altman, were used to compare the different methods (Bland and Altman 1995; Khan and Chien 2001). Limits of agreement are

estimated from the mean and standard deviations of the differences with 95% of the differences lying within two standard deviations either side of mean (Bland and Altman 1986; Altman 1999; Bland and Altman 1999). These limits, like confidence intervals, give an idea of the spread of variance between the methods Inter-method correlation was assessed by two-way mixed Intraclass Correlation Coefficients (ICCs) with absolute agreement and their 95% confidence intervals (McGraw 1996).

## 6.4    Results

22 bovine ovaries were assessed and included in the final analysis. Table 6.1 shows the upper and lower limits of agreement between manual dissection (MD) method, SonoAVC with post-processing and 2D, their range of agreements, mean differences between the methods and P value derived from these differences. Our results show that the mean ovarian volume calculated by the method of volume displacement (VD) was comparable to that calculated using 3D ultrasound (VOCAL) (7.68 ± 2.63, 7.86 ± 2.79; P=0.338), however significantly larger than that calculated by 2D (7.68 ± 2.63, 6.08 ± 2.50; P<0.001). The mean total AFC (2.0-10.0mm) measured by the manual dissection (MD) method was significantly higher than that made by SonoAVC (19.27 ± 7.60, 18.18 ± 6.67; P=0.003) and significantly lower than that made by 2D (19.27 ± 7.60, 22.86 ± 8.25; P<0.001), however LOA between MD and SonoAVC were lower than that between MD and 2D (Table 6.1).

Mean AFC of follicles measuring 2.0-4.0mm was significantly higher by MD method than SonoAVC (12.00 ± 5.75, 11.13 ± 5.35; P<0.001) and significantly lower than 2D (12.00 ± 5.75, 15.64 ± 6.36; P<0.001). The LOA, however were narrower between MD and SonoAVC than between MD and 2D (Table 6.1). Mean AFC of antral follicles measuring >4.0-6.0 mm

(MD: 5.14 ± 2.47, SonoAVC: 4.81 ± 2.06, 2D: 5.77 ± 3.38) and >6.0-10.0mm (MD: 1.51 ± 1.50, SonoAVC: 1.68 ± 1.70, 2D: 1.05 ± 1.96) showed no significant difference between either methods (P<0.05). However, more agreement to MD was seen with SonoAVC than 2D, as reflected by the narrow range between their upper and lower limits of agreement (Table 6.1).

 The ICC's suggested an excellent correlation between methods when measuring total AFC of 2.0-10.0mm (0.959, 95% CI: 0.919 to 0.982; P<0.001), AFC of 2.0-4.0mm (0.953, 95% CI: 0.908 to 0.979; P<0.001) and AFC of 2.0-6.0mm (0.956, 95% CI: 0.914 to 0.981; P<0.001); and a moderate correlation when measuring ovarian volume (0.857, 95% CI: 0.735 to 0.933; P=0.01), AFC of >6.0-10.0mm (0.786, 95% CI: 0.621 to 0.896; P=0.01) and AFC of >4.0-6.0mm (0.799, 95% CI: 0.722 to 0.890; P=0.01).

**TABLE 6.1:** Comparison of three different methods (volume displacement, 3D assisted VOCAL and 2D for volume calculation; manual dissection of antral follicles, 3D assisted SonoAVC with post processing and 2D ultrasound for measuring antral follicle counts) using limits of agreement and paired samples *t* test in calculation of ovarian volume and antral follicles stratified by size.

| | Mean ± SD | Mean difference ± (95% CI) | P value | Upper and Lower LOA | Range LOA |
|---|---|---|---|---|---|
| **Ovarian volume Standard: VD** | 7.68 ± 2.63 | | | | |
| VOCAL | 7.86 ± 2.79 | -0.18 ± 0.87 (-0.57 to 0.20) | 0.338 | -1.53 and -1.90 | 3.43 |
| 2D | 6.08 ± 2.50 | 1.60 ± 1.67 (0.87 to 2.34) | <0.001 | 4.88 and -1.67 | 6.55 |
| **AFC 2.0-10.0mm Standard: MD** | 19.27 ± 7.60 | | | | |
| SonoAVC-PP | 18.18 ± 6.67 | 1.09 ± 1.54 (0.41 to 1.77) | 0.003 | 4.11 and -1.93 | 6.04 |
| 2D | 22.86 ± 8.25 | -3.59 ± 2.24 (-4.58 to -2.59) | <0.001 | 0.80 and -7.98 | 8.78 |
| **AFC 2.0-4.0mm MD** | 12.00 ± 5.75 | | | | |
| SonoAVC-PP | 11.14 ± 5.35 | 0.86 ± 0.94 (0.45 to 1.28) | <0.001 | 2.71 and -0.98 | 3.69 |
| 2D | 15.64 ± 6.36 | -3.64 ± 2.13 (-4.58 to -2.69) | <0.001 | 0.53 and -7.81 | 8.34 |
| **AFC >4.0-6.0mm MD** | 5.14 ± 2.47 | | | | |
| SonoAVC-PP | 4.82 ± 2.06 | 0.32 ± 1.17 (-0.20 to 0.84) | 0.216 | 2.61 and -1.98 | 4.59 |
| 2D | 5.77 ± 3.38 | -0.64 ± 1.50 (-1.96 to 0.69) | 0.331 | 3.75 and -5.02 | 8.77 |
| **AFC 2.0-6.0mm MD** | 17.14 ± 7.17 | | | | |
| SonoAVC-PP | 15.95 ± 6.59 | 1.18 ± 1.37 (0.57 to 1.79) | 0.001 | 3.86 and -1.50 | 5.36 |
| 2D | 21.41 ± 8.10 | -4.27 ± 2.25 (-5.27 to -3.27) | <0.001 | 0.14 and -8.68 | 8.82 |
| **AFC >6.0-10.0mm MD** | 1.50 ± 1.54 | | | | |
| SonoAVC-PP | 1.68 ± 1.70 | -0.18 ± 0.80 (-0.53 to 0.17) | 0.296 | 1.38 and -1.74 | 3.12 |
| 2D | 1.05 ± 1.96 | 0.45 ± 1.30 (-0.12 to 1.03) | 0.116 | 3.00 and -2.09 | 5.09 |

## 6.5    *Discussion*

This study has validated the antral follicle counts and ovarian volume measured using 3D ultrasound assisted SonoAVC and 2D real-time equivalent method against the actual number of antral follicles measuring 2.0mm or more that were manually dissected from the bovine ovary. The results show that when validating for antral follicles measuring more than 4.0mm, both SonoAVC and 2D real-time equivalent made counts comparable to the dissection method, but SonoAVC with post processing agreed more than the 2D method. However, this was not true for follicles measuring 4.0mm or less. Whilst SonoAVC made significantly less counts, 2D made significantly more counts than the dissection method, although SonoAVC agreed more with the dissection method than the 2D method.

Although it appears that antral follicle counts made using ultrasound are either underestimated or overestimated, their predictive value has been validated in studies assessing ovarian response following IVF treatment.(Gulekli, Bulbul et al. 1999; Kwee, Elting et al. 2003; Chow, Criniti et al. 2004; Elter, Kavak et al. 2005; Muttukrishna, McGarrigle et al. 2005; Broekmans, Kwee et al. 2006; Haadsma, Bukman et al. 2007; Kwee, Elting et al. 2007; Nardo, Christodoulou et al. 2007; Ireland, Scheetz et al. 2008; Verhagen, Hendriks et al. 2008; Deb, Batcha et al. 2009; Haadsma, Groen et al. 2009; Maheshwari, Gibreel et al. 2009). The AFC has also been strongly correlated to AMH (Jayaprakasan, Campbell et al.; de Vet, Laven et al. 2002; Muttukrishna, McGarrigle et al. 2005; van Rooij, Broekmans et al. 2005; Fanchin, Mendez Lozano et al. 2007), which is another important independent predictor of ovarian reserve and response to controlled ovarian stimulation (van Rooij, Broekmans et al. 2002; Hendriks, Mol et al. 2005; Muttukrishna, McGarrigle et al. 2005; Lekamge, Barry et al. 2007; Nardo, Christodoulou et

al. 2007; Dehghani-Firouzabadi, Tayebi et al. 2008; Riggs, Duran et al. 2008; Verhagen, Hendriks et al. 2008; Wunder, Guibourdenche et al. 2008; Nelson, Yates et al. 2009). Previous studies have shown that small antral follicles measured using SonoAVC with post processing correlate highly to AMH and that they have a significant predictive value in predicting ovarian response to controlled ovarian stimulation (Haadsma, Bukman et al. 2007; Deb, Batcha et al. 2009), poor response (Jayaprakasan, Deb et al. 2009) and pregnancy outcome following IVF (Deb, Batcha et al. 2009; Haadsma, Groen et al. 2009). It is also known that number of small antral follicles progressively decline with age and have been positively correlated to reproductive ageing (Gougeon 1994; Hansen, Morris et al. 2003; Scheffer, Broekmans et al. 2003; Broekmans, Faddy et al. 2004; Haadsma, Bukman et al. 2007; Hansen, Knowlton et al. 2008). This would perhaps make us believe that the current understanding of the AFC cut-offs defining normal from poor or hyper-responders although not accurate are fair assessment tools of ovarian reserve. However, knowing that the current tests for calculating AFC are not accurate, there is a tremendous scope in developing the test accuracies in future, especially the small antral follicles, which might improve upon the current predictive abilities in assessing ovarian response to IVF and also reproductive ageing.

When using SonoAVC, one could argue that since it underestimates AFC, we must exhibit caution with regards to women undergoing first cycle of IVF especially when considering cycle cancellation or refusing IVF treatment. On the other hand, when using 2D to measure antral follicles, one could argue that since it might overestimate AFC, we must consider appropriate dosages when deciding on the amount of gonadotrophin used for ovarian stimulation. SonoAVC was shown to have more agreement with dissection method than 2D, especially with follicles measuring 4.0mm or less.  Since SonoAVC can

reliably measure size of antral follicles, the small antral follicle population must be researched further in studies predicting ovarian response to IVF.


## 6.6    Conclusion

For antral follicles measuring less than 4.0mm, neither 2D nor 3D assisted SonoAVC compare to the actual number in the ovary as measured by manually dissecting them. SonoAVC measures significantly less and 2D measures significantly more follicles. However there was more agreement seen with SonoAVC than with 2D. With antral follicles measuring more than 4.0mm, measures made by both SonoAVC and 2D were comparable to the dissection method, however again the agreement seen was more with SonoAVC than with 2D. It therefore appears that no current method available to measure the count and size of antral follicles is accurate, but SonoAVC with post-processing shows the most agreement with the follicles measured following manual dissection.

**CHAPTER 7. The inter-ovarian variation in three-dimensional ultrasound markers of ovarian reserve in women undergoing baseline investigation for subfertility**

## 7.1    Abstract

**Objective:** To evaluate differences in the three-dimensional (3D) ultrasound markers of ovarian reserve between the ovaries within an individual undergoing investigation for subfertility.

**Methods:** This was a prospective observational study conducted at University-based Assisted Conception Unit. 270 women undergoing baseline early follicular phase ultrasound as an investigation for subfertility were recruited. 3D ultrasound scan in early follicular phase between day 2 to 5 of menstrual cycle was performed. The main outcome assessed was variations in 3D ultrasound markers of ovarian reserve between the two ovaries within same individual.

**Results:** 215 subjects were analysed for ovarian volume and antral follicle count (AFC), and 205 subjects for 3D power Doppler indices. Significant differences were noted (median, range) in the number of antral follicles measuring >6.0mm, and ovarian volume (P<0.05). Significant correlation was noted between the two ovaries in antral follicles measuring 6.0mm or less, ovarian volume, and 3D power Doppler indices (P<0.05). On stratifying the antral follicles according to size using "sonography based automated volume calculation" with post processing, maximum variation was seen in follicles measuring more than 6.0mm as measured using limits of agreement.

**Conclusion:** There are significant differences in the antral follicles measuring >6.0mm and ovarian volume, as measured using 3D ultrasound, that require consideration when comparing the two ovaries within an individual.

## 7.2    Introduction

A true ovarian reserve is the number of primordial follicles present in the ovaries which can currently only be examined histologically. However, ultrasound and endocrine measures of ovarian reserve appear to correlate to this true reserve and have therefore been widely accepted as markers of ovarian reserve

In clinical practice, both ovaries are considered together as a combined unit during ovarian stimulation. The endocrine markers such as Follicle Stimulating Hormone (FSH), Anti-Müllerian Hormone (AMH), Oestradiol, and Inhibin B provide information on ovarian reserve of both ovaries as a combined unit. Ultrasound is the only method so far which allows a direct assessment of each ovary as a separate entity; however the numbers and sizes of follicles are largely presented either as a mean or total value. Ultrasonographic assessment of the total number of antral follicles is an important determinant of ovarian reserve (Scheffer, Broekmans et al. 2003; Ng, Chan et al. 2005; Jayaprakasan, Campbell et al. 2008). The total antral follicle count (tAFC) is typically made by counting the number of follicles measuring 2 to 9 mm (2004; Lam and Raine-Fenning 2006; Nardo and Gelbaya 2008) or 2 to 10 mm (Scheffer, Broekmans et al. 2003; Klinkert, Broekmans et al. 2005) in both ovaries and can be estimated using two-dimensional (Scheffer, Broekmans et al. 2002) or three-dimensional ultrasound (Jayaprakasan, Campbell et al. 2008). The tAFC includes small antral follicles (2.0 - 6.0 mm) that are largely gonadotrophin-independent, but selectable due to their responsiveness to gonadotrophins, and larger antral follicles (>6.0 mm) that are gonadotrophin-dependent (Gougeon 1989).

In the assessment of subfertility, both ovaries are included as a single entity when predicting ovarian reserve or outcomes following assisted reproduction treatment.

However, comparison of one ovary to the other may be warranted in situations such as, when examining the effect of pathology or surgery on the ovary.  Ovarian cystectomy has been shown to impair ovarian reserve (Loh, Tan et al. 1999; Somigliana, Ragni et al. 2003; Candiani, Barbieri et al. 2005; Godinjak, Idrizbegovic et al. 2005; Somigliana, Ragni et al. 2006) and ovarian response (Loo, Lin et al. 2005; Ragni, Somigliana et al. 2005; Demirol, Guven et al. 2006; Esinler, Bozdag et al. 2006; Yazbeck, Madelenat et al. 2006; Kahyaoglu, Ertas et al. 2008; Somigliana, Arnoldi et al. 2008) following assisted reproduction treatment (ART). However, cystectomy and reduced ovarian reserve does not seem to translate into impaired pregnancy outcome (Demirol, Guven et al. 2006; Esinler, Bozdag et al. 2006; Somigliana, Ragni et al. 2006; Kahyaoglu, Ertas et al. 2008; Garcia-Velasco and Somigliana 2009). Data from few studies suggest that cystectomy might even be detrimental to ovarian response when compared to no surgery (Canis, Pouly et al. 2001; Marconi, Vilela et al. 2002; Garcia-Velasco, Mahutte et al. 2004; Demirol, Guven et al. 2006; Alborzi, Ravanbakhsh et al. 2007; Donnez, Lousse et al. 2009; Garcia-Velasco and Somigliana 2009). This might be due to inadvertent removal of normal ovarian tissue along with the cyst or damage caused by the use of diathermy. Some studies have used ultrasound measures including ovarian volume, antral follicle count, and blood flow (Somigliana, Ragni et al. 2003; Candiani, Barbieri et al. 2005; Horikawa, Nakagawa et al. 2008) to compare the operated ovary to the unoperated contra-lateral ovary. Other studies have evaluated the effect of surgery by comparing the ovarian response to controlled ovarian stimulation during ART in the operated ovary to the contra lateral normal ovary (Somigliana, Ragni et al. 2003; Ragni, Somigliana et al. 2005; Esinler, Bozdag et al. 2006; Somigliana, Ragni et al. 2006; Alborzi, Ravanbakhsh et al. 2007; Horikawa, Nakagawa et al. 2008).  Few studies have shown that the tAFC does not differ significantly

between the two ovaries when measured using two-dimensional (2D) ultrasound (Scheffer, Broekmans et al. 1999; Baerwald, Adams et al. 2003; Chow, Criniti et al. 2004).

This study was designed to quantify the 3D ultrasound markers of ovarian reserve within an individual and to evaluate the differences in these parameters between two ovaries. Our hypotheses were that there is minimal variation between the two ovaries such that one could be used as a control for the other, and that an objective assessment of the effect of unilateral ovarian surgery can be made by comparing the two ovaries.

## 7.3    Methodology

### Study design and population

Subjects were prospectively recruited from women attending the general gynaecology and infertility clinics at Queen's Medical Centre in Nottingham. A sample size of 205 was required to power the study at 85%, for an assumed variation of 3 antral follicles between the two ovaries and a standard deviation of 2.5.

270 subjects aged less than or equal to 41 years were prospectively recruited over duration of 12 months.  These were women who had been referred for assisted reproduction treatment and the ultrasound assessment was performed prior to their initial consultation in the assisted conception unit. Subjects were excluded if they had a past history of pelvic surgery or had been objectively shown to have endometriosis. Subjects meeting the inclusion criteria were scheduled to have a baseline ultrasound assessment during the early follicular phase of the menstrual cycle (day 2 to 5). Subjects

were excluded at this stage if they were found to have an ovarian cyst or follicle measuring more than 10 mm or if a persistent corpus luteum was evident, as it may affect the volume or vascularity of the ovary in comparison to the other ovary. 3D ultrasound was used throughout the study as it has been shown to provide more reliable and more valid measures of ovarian volume and antral follicle numbers than conventional 2D ultrasound (Raine-Fenning, Campbell et al. 2003; Raine-Fenning, Campbell et al. 2003; Scheffer, Broekmans et al. 2003; Merce, Gomez et al. 2005; Jayaprakasan, Walker et al. 2007; Jayaprakasan, Campbell et al. 2008; Verhagen, Hendriks et al. 2008). 3D power Doppler ultrasonography was used to examine ovarian blood flow as it provides a global view of the ovary rather than selected information from a single vessel (Kupesic and Kurjak 2002; Pan, Wu et al. 2002; Raine-Fenning, Campbell et al. 2004).

**Data acquisition**

All subjects had a transvaginal ultrasound scan performed by a single investigator (J.C) using a Voluson Expert 730$^{TM}$ (GE Medical Systems, Zipf, Austria) and a 7.5-MHz 3D transvaginal probe to avoid inter-operator variation in acquisition of 3D data. The ultrasound assessment is described in detail in chapter 2. Briefly, it comprised of a preliminary conventional 2D ultrasound assessment of the pelvis to exclude any obvious pathology, followed by visualization of ovaries in their transverse and longitudinal planes and the subsequent acquisition of 3D data using the volume mode. The volume mode, which displays a truncated sector, was adjusted to precisely define the area of interest, the sweep angle set to 90$^{0}$ and a 3D dataset acquired using the high quality, slow sweep mode, which results in the highest resolution. Power Doppler was then applied using predefined settings for every patient (Raine-Fenning, Campbell et al. 2002). The same

process was repeated for the contra-lateral ovary and the resulting 3D datasets were stored on recordable digital videodisc for subsequent off-line analysis.

**Data analysis**

4D View (version 7.0, GE Kretz) software was used for all measurements.

*3D multiplanar view*

The multiplanar view allows simultaneous display of a 3D dataset in three perpendicular planes conventionally referred to as the longitudinal (A), transverse (B), and coronal (C) planes. These three sectional planes are shown simultaneously and are mutually related such that movement within one plane produces geometrically equivalent movements in the other two planes. For the purposes of this study the total antral follicle count (tAFC) was made by counting the number of antral follicles measuring 2 to 9 mm in each ovary.

*Sonography based automated volume calculation (SonoAVC)*

SonoAVC (GE Medical Systems, Zipf, Austria) was used to automatically measure the size of each antral follicle within the grey scale 3D volume. The lower size limit of follicles that SonoAVC can detect is 1 to 2 mm. Our technique has been described in detail before but, in brief involved the following steps. Firstly, the multiplanar view was used to ensure the ovary was centrally placed and the render mode selected to generate a 3D volume of interest (VOI) box. Once the dataset had been correctly positioned, SonoAVC was implemented. The individual follicles identified were then displayed with a specific colour and shown together with their dimensions and relative sizes. Post-processing is required in almost all cases to manually identify those antral follicles that have been missed in the initial automated analysis. Each additional follicle identified in this way is also given a

specific colour and its dimensions presented alongside the other follicles. The total number of antral follicles was recorded together with the mean diameter of each follicle calculated using the relaxed sphere technique (Raine-Fenning 2008; Raine-Fenning, Jayaprakasan et al. 2008). The follicles were categorized into cohorts according to their relative size as follows: < 2.0 mm, ≥2.0 to 4.0 mm, >4.0 to 6.0 mm, >6.0 to 8.0 mm, and >8.0 to 9.0 mm (Gougeon 1998; Weenen, Laven et al. 2004; Allemand, Tummon et al. 2006; Lam and Raine-Fenning 2006; Haadsma, Bukman et al. 2007).

*Volume and blood flow analysis*

The volume of each ovary and its vascularity were quantified with the Virtual Organ Computer-aided AnaLysis (VOCAL imaging programme; GE Medical Systems). VOCAL was used to manually delineate the ovarian cortex in the B plane of multiplanar view as the 3D power Doppler dataset was rotated $180^0$, vertically through $9^0$ steps providing 20 planes for each volumetric measurement (Raine-Fenning, Campbell et al. 2002). The volume of the resultant model was recorded and the histogram facility was then implemented to quantify the power Doppler signal within it. The histogram generates three indices of vascularity: the Vascularisation Index (VI), the Flow Index (FI), and the Vascularisation Flow Index (VFI) (Raine-Fenning, Nordin et al. 2008; Raine-Fenning, Nordin et al. 2008)

## Statistical analysis

Statistical analysis was undertaken using the Statistical Package for the Social Sciences (SPSS, version 15.0, Chicago, IL). One-way analysis of variance was used to examine for differences in the tAFC, ovarian volume, and 3D power Doppler indices between the two ovaries, and expressed as mean and standard deviation (SD) or median and range dependent on the distribution of the data which was checked with a normal probability

plot. Limits of agreement (LOA) and interquartile ranges were used to express the degree of difference between the two ovaries for each ultrasound variables. Correlation coefficient (R) was used to test the correlation between the two ovaries within an individual and regression coefficient ($R^2$) was used to quantify the variation in one ovary due to the other ovary. A P value below 0.05 was considered to be statistically significant.

## 7.4    Results

Of the 270 subjects recruited in the study, 16 were excluded due to the presence of corpus luteum; 18 for an ovarian cyst in either or both ovaries; and 21 excluded because of follicle measuring more than 10mm. After exclusion, a final study group of 215 subjects were available for analysis of ovarian volume and antral follicle count and diameter of follicles. However, a further 10 subjects were excluded from the analysis of ovarian vascularity due to either the presence of artefacts in the acquired 3D power Doppler dataset or incomplete data, giving a final study group of 205 subjects for the examination of ovarian vascularity.

The data were not normally distributed and therefore presented as median with interquartile ranges. The median (IQR) age in years of the study population was 33.23 (28.43 to 38.20). The total antral follicle count between the right and left ovary was significantly different (7, 0 to 22 vs 6, 0 to 26; P=0.015), with LOA ranging between 7.33 and -7.10 (Table 7.1). However, a significant linear correlation was noted between the two ovaries (R: 0.684; P<0.001) with the regression coefficient ($R^2$) of 0.468 suggesting that 46.8 % of variation in the tAFC in one ovary can be predicted by the tAFC in other ovary (Table 7.2).

Table 7.1: Comparison of the three-dimensional ultrasound markers of ovarian reserve between the two ovaries within an individual with significance levels derived from the Wilcoxon signed rank test, and variation expressed as upper and lower limits of agreement (ULOA and LLOA)

| Variable | Right Ovary (Median; Range) | Left Ovary (Median; Range) | P value | ULOA | LLOA |
|---|---|---|---|---|---|
| Total AFC (n) | 7; 0 to 22 | 6; 0 to 26 | 0.015 | 7.33 | -7.10 |
| AFC ≤ 2.0mm (n) | 0; 0 to 5 | 0; 0 to 7 | 0.117 | 2.15 | -1.88 |
| AFC >2.0mm-4.0mm (n) | 2; 0 to 11 | 2; 0 to 13 | 0.852 | 2.53 | -2.64 |
| AFC >4.0mm-6.0mm (n) | 2; 0 to 14 | 2; 0 to 13 | 0.169 | 3.12 | -2.65 |
| AFC >6.0mm-8.0mm (n) | 1; 0 to 7 | 1; 0 to 6 | 0.027 | 3.85 | -3.27 |
| AFC >8.0mm-9.0mm (n) | 0; 0 to 3 | 0; 0 to 2 | 0.028 | 1.78 | -1.49 |
| Ovarian volume (cm$^3$) | 6.85; 2.02 to 15.42 | 6.34; 1.22 to 13.78 | 0.001 | 4.48 | -3.24 |
| Vascularisation Index (VI) (%) | 5.85; 0.01 to 18.98 | 6.16; 0.02 to 22.19 | 0.448 | 9.28 | -9.99 |
| Flow Index (FI) (0-100) | 36.28; 17.34 to 51.53 | 35.39; 14.88 to 59.89 | 0.327 | 14.72 | -13.37 |
| Vascularisation Flow Index (VFI) (0-100) | 2.15; 0.01 to 8.65 | 2.24; 0.01 to 12.34 | 0.417 | 4.22 | -4.64 |

The antral follicles were further stratified according to size in each ovary and grouped as follicles measuring <2mm, ≥2.0mm-4.0mm, >4.0mm-6.0mm, >6.0mm-8.0mm, >8.0mm-9.0mm. There was no significant difference seen in the number of follicles measuring up

to 6.0mm (P>0.05), however the difference was significant with the number of follicles measuring >6.0mm (P<0.05) (Table 7.1). The upper and lower LOA between the antral follicles of different sizes are shown in Table 1, with widest range between upper and lower LOA seen with antral follicles measuring ≥6.1mm-8.0mm.

**Table 7.2:** The table shows the correlation coefficient (R) reflecting the linear correlation between the two ovaries and the regression coefficient ($R^2$) reflecting the ability of one ovary to predict the variation in other ovary. P value indicates the significance of correlation between ovaries.

| Variable | R | P value | $R^2$ |
|---|---|---|---|
| Total AFC in each ovary (n) | 0.684 | <0.001 | 0.468 |
| AFC < 2.0mm (n) | 0.210 | 0.004 | 0.167 |
| AFC ≥ 2.0mm-4.0mm (n) | 0.475 | <0.001 | 0.432 |
| AFC ≥ 4.0mm-6.0mm (n) | 0.465 | <0.001 | 0.263 |
| AFC ≥ 6.0mm-8.0mm (n) | 0.189 | 0.069 | 0.049 |
| AFC ≥ 8.0mm-9.0mm (n) | 0.073 | 0.450 | 0.003 |
| Ovarian volume ($cm^3$) | 0.573 | <0.001 | 0.423 |
| Vascularisation Index (VI) (%) | 0.454 | <0.001 | 0.211 |
| Flow Index (FI) (0-100) | 0.378 | 0.007 | 0.129 |
| Vascularisation Flow Index (VFI) (0-100) | 0.450 | 0.002 | 0.178 |

A significant linear correlation was noted in the antral follicles measuring up to 6.0mm between the two ovaries, however this correlation became not significant with antral

follicles measuring >6.0mm (Table 7.2). The regression coefficients of follicles measuring ≤6.0mm (≥2.0mm-4.0mm: $R^2$=0.432; >4.0mm-6.0mm: $R^2$=0.263) was higher than that of smaller follicles measuring more than 6.0mm (>6.0mm-8.0mm: $R^2$=0.049; >8.0mm-9.0mm: $R^2$=0.003), suggesting that only a small percentage of variation in larger follicles in one ovary can be predicted by the follicle counts in the other ovary (Table 7.2).

The right ovarian volume significantly differed from the left ovarian volume (P=0.001) with the upper and lower LOA between the two ovaries were noted at 4.48 and -3.24 respectively (Table 7.1). A significant linear correlation was seen between the volumes of two ovaries (R: 0.573; P<0.001) with the $R^2$ of 0.423, suggesting that 42.3% of variation in volume in one ovary can be predicted by the other ovary (Table II). There was no significant difference seen in the medians of the 3D power Doppler indices between the two ovaries (Table 7.1). However, a significant linear correlation was noted in these indices between the two ovaries (Table 7.2). Regression analysis showed that 21.1% of variation in VI, 12.9% variation in FI and 12.9% variation in VFI in one ovary can be predicted by the other ovary. The LOA of these indices between the two ovaries are shown in Table 7.1.

## 7.5    Discussion

This is the first study to compare the three-dimensional ultrasound markers of ovarian reserve between the two ovaries within the same individual. Our results suggest that there are significant differences in some ultrasound markers of ovarian reserve such as ovarian volume, total antral follicle count of each ovary, and antral follicles measuring more than 6.0mm.

The results of this study bear significance when evaluating the effect of ovarian pathology or surgery. Studies evaluating the effect of ovarian cystectomy on ovarian reserve and response to ART have either compared the operated ovary to the contra-lateral normal ovary (Loh, Tan et al. 1999; Somigliana, Ragni et al. 2003; Candiani, Barbieri et al. 2005; Ragni, Somigliana et al. 2005; Somigliana, Infantino et al. 2006; Alborzi, Ravanbakhsh et al. 2007; Horikawa, Nakagawa et al. 2008) or to the control group (Canis, Pouly et al. 2001; Marconi, Vilela et al. 2002; Wong, Gillman et al. 2004; Loo, Lin et al. 2005; Esinler, Bozdag et al. 2006; Yazbeck, Madelenat et al. 2006; Nakagawa, Ohgi et al. 2007; Kahyaoglu, Ertas et al. 2008). When comparing the operated ovary, majority have evaluated its response to controlled stimulation during ART by comparing to the response in contra-lateral ovary. Our study shows significant physiological differences in some of the ultrasound markers of ovarian reserve between the two ovaries within an individual. Since these markers have the capacity to predict ovarian response during ART, these findings should be considered when comparing one ovary to another to assess the effects of surgery.  It would appear that antral follicle count data, stratified by size, should be considered when the two ovaries are compared to quantify the effect of surgery.

The variation in the ultrasound markers of ovarian reserve between the ovaries may relate to the effect of ovulation and corpus luteum formation in the preceding cycle or be an early indicator of dominance in the current cycle (Baerwald, Adams et al. 2003). This potential dynamic process can only be tested by evaluating the ovaries over consecutive menstrual cycles and analysing according to ovarian dominance, which was not measured or accounted for in this preliminary study. Several studies have shown that anti-Müllerian hormone (AMH), an important marker of ovarian reserve is largely expressed by antral

follicles measuring less than 6 mm in diameter (Gruijters, Visser et al. 2003; Kevenaar, Meerasahib et al. 2006; Fanchin, Mendez Lozano et al. 2007; Nardo, Christodoulou et al. 2007). The ability to identify and measure these small antral follicles is of emerging importance in assessing ovarian reserve as AMH is also thought to play an important role in the initiation and cyclic recruitment of ovarian follicles (Durlinger, Gruijters et al. 2001; Visser 2006). This cohort of follicles contributes most to the serum AMH level, which is known to demonstrate limited intra- and inter-cycle variation, and represents the recruitable pool and therefore the functional reserve of the ovary (La Marca, Giulini et al. 2007; Tsepelidis, Devreker et al. 2007). The variation between the two ovaries is least with follicles measuring 6.0mm or less. Their measurement allows the two ovaries to be compared ultrasonographically with more confidence.

The inter-ovarian difference in ovarian volume seen in our study could be either due to inherent differences in the two ovaries or due to the number of larger antral follicles. There are no similar studies; however one study that used 3D ultrasound and compared women with polycystic ovaries (PCO) to normal ovaries reported no difference in ovarian volume in spite of significantly higher antral follicle count in PCO group (Jayaprakasan, Jayaprakasan et al. 2009). Although 3D ultrasound was used, our study differs in design and methodology. In our study, there were no significant differences noted in the 3D power Doppler indices between the two ovaries. The ultrasound scans were performed in the early follicular phase when the population of follicles in the ovary is predominantly antral follicles, and therefore perhaps no difference noted in vascularity (Costello, Shrestha et al. 2005). However, this may not be consistent in different phases of the menstrual cycle, as the growing follicle and corpus luteum acquire more blood supply (Zaidi, Collins et al. 1996).

## 7.6    Conclusion

There are significant differences in the ultrasound markers of ovarian reserve between the two ovaries within an individual during the early follicular phase of the menstrual cycle as measured using 3D ultrasound. This variation is least evident in the small antral follicles, measuring 6 mm or less, which correlate with AMH and more accurately reflect the functional reserve of the ovary.

**CHAPTER 8: The intra-cycle variation in the number of antral follicles stratified by size and in the endocrine markers of ovarian reserve in women with normal ovulatory menstrual cycles**

## *8.1    Abstract*

**Objective:** To quantify the intra-cyclical variation in markers of ovarian reserve measured by the antral follicle counts stratified by size using three-dimensional (3D) ultrasound and anti-Müllerian hormone (AMH) in women with normal ovulatory menstrual cycles.

**Methods:** It was a prospective observational study conducted in University-based teaching hospital. 38 healthy volunteers with normal menstrual cycles were recruited. 3D ultrasound scan and blood test in early (F1) and mid follicular (F2) phase, peri-ovulatory (PO) and luteal (LU) phases of one menstrual cycle was performed. The main outcome measure was the variation in markers of ovarian reserve in different phases of one menstrual cycle.

**Results:** 36 volunteers were included in final analysis, of which 34 attended all four visits. Repeated measures analysis showed a significant variation in total antral follicle count (AFC) (P=0.002). However, on stratifying the antral follicles according to size using "sonography based automated volume calculation" with post processing, a non-significant variation (P=0.382) was seen in small AFC (≤6mm) and a significant variation (P<0.001) in large AFC (>6mm).   The ovarian volume showed a significant intra-cyclical variation (P<0.001). A small but significant intra-cyclical variation was noted in AMH levels (P=0.041). A significant variation was seen in levels of serum Follicle stimulating hormone (FSH), Lutenising hormone (LH), and oestradiol (P<0.05).

**Conclusion:** Small antral follicles (≤6.0mm) measured using 3D ultrasound and AMH show little intra-cycle variation and perhaps should be evaluated in prediction of ovarian reserve independent of menstrual cycle.

## *8.2   Introduction*

Ovarian reserve is a widely accepted concept in the field of assisted reproduction and describes the quantitative and qualitative potential of the ovaries to respond to controlled ovarian stimulation. Of the various tests of ovarian reserve currently available, the antral follicle count (AFC) has been shown to be comparable to various multivariate models in the prediction of ovarian response to controlled ovarian stimulation during *in vitro* fertilisation (IVF) treatment (Verhagen, Hendriks et al. 2008). It typically involves counting the number of antral follicles measuring 2 to 10 mm in both ovaries during the early follicular phase of menstrual cycle defined as days 2 to 5 (Chang, Chiang et al. 1998; Pellicer, Ardiles et al. 1998; Pellicer, Gaitan et al. 1998; Scheffer, Broekmans et al. 1999; Scheffer, Broekmans et al. 2003).

More recently, anti-Müllerian hormone (AMH) has been shown to be comparable to AFC in the prediction of ovarian response (Broer, Mol et al.; van Rooij, Broekmans et al. 2005; La Marca, Giulini et al. 2007; Broekmans, Visser et al. 2008; Elgindy, El-Haieg et al. 2008; Kwee, Schats et al. 2008). AMH is expressed by the pre-antral and small antral follicles measuring up to 6 mm (Weenen, Laven et al. 2004) and is thought to have a role in the initial recruitment and subsequent selection of follicular dominance during folliculogenesis (Durlinger, Kramer et al. 1999; McGee 2000; Durlinger, Gruijters et al. 2001). There is a strong positive correlation between the number of small antral follicles and AMH in the early follicular phase (Jayaprakasan, Deb et al. 2009) and this follicle population might be an important independent predictor of pregnancy following IVF treatment (Deb, Batcha et al. 2009).

Most of the evidence on the reliability and validity of the various tests of ovarian reserve have been based on the tests being performed during the early follicular phase of the menstrual cycle. This relatively small window of opportunity is restrictive both to our patients and to the hospitals and clinics performing the tests. An ideal test of ovarian reserve should not only be reliable but also independent of the menstrual cycle to make its application as practical as possible.

Several groups have suggested that there is no significant variation in the levels of AMH throughout the menstrual cycle (Hehenkamp, Looman et al. 2006; La Marca, Stabile et al. 2006; Tsepelidis, Devreker et al. 2007; van Disseldorp, Lambalk et al. 2010) whilst others have shown a presence of significant variation in the levels (Cook, Siow et al. 2000; Eldar-Geva, Ben-Chetrit et al. 2005; Wunder, Bersinger et al. 2008). As a result AMH is becoming the primary test of ovarian reserve as there is no need to standardise the timing of the assessment. The cyclical variation in the number of antral follicles, a longstanding proven test of ovarian reserve, has received less attention but there are suggestions that there is a more profound intra-cycle variation in their number than in AMH levels (van Disseldorp, Lambalk et al. 2010).

Three-dimensional (3D) ultrasound assessment of the AFC has been shown to have high intra- and inter-observer reliability (Scheffer, Broekmans et al. 2002; Jayaprakasan, Walker et al. 2007; Jayaprakasan, Campbell et al. 2008). Semi-automated follicle counts have recently become available and have also been shown to provide highly reliable measures of the total numbers of antral follicles (chapter 3) and their relative sizes (chapter 4). Examining the variance in the markers of ovarian reserve will not only inform the best time to perform these tests, but also help understand the expression of these markers in relation to the menstrual cycle and optimise the use of protocols in assisted

reproduction treatment. SonoAVC, shown in previous chapters as a reliable and valid method of assessing the size and number of antral follicles was used in this study to quantify the number of antral follicles stratified by size.

This study was designed to quantify the intra-cyclical variations in some of the important markers of ovarian reserve including the antral follicle counts of different size cohorts, ovarian volume, AMH, follicle stimulating hormone (FSH), Luteinising hormone (LH) and Oestradiol in normo-ovulatory healthy volunteers. Our hypothesis was that there is minimal intra-cyclical variation in the functional ovarian reserve as measured by the number of small antral follicles and AMH.

## 8.3    Methods

**Study design**

Study participants described as 'healthy volunteers' were recruited prospectively through advertisements on the intranet portal of the University of Nottingham (UoN) website, and posters displayed in staff canteens, notice boards and staff library. Further information about the study was communicated via email and information leaflets to those who expressed interest in participation in the study. Inclusion criteria included age between 18 to 35 years, body mass index (BMI) between 18 and 26 kg/ $m^2$, regular menstrual cycles with a mean length ranging between 26 to 32 days, no history of ovarian surgery, no features suggestive of endocrine disease, and no hormonal contraceptive use within the last 6 months. Serial transvaginal ultrasound scans were performed and blood samples taken during one menstrual cycle. With the first day of menstruation taken as day 1 of the cycle these visits were scheduled for the follicular (days 2-5), peri-ovulatory (days 12-16),

and luteal (days 20-26) phases of the menstrual cycle. Early follicular phase involved study examinations on day 2 and day 5 of the cycle to ensure the absence of corpus luteum and its remnant effect. Peri-ovulatory phase assessment was started on day 12 of the cycle, and the day when a dominant follicle of more than 16 mm was noted, was taken as the time point to include in the study. Luteal phase assessment was performed 7 days following the peri-ovulatory study time point. A sample size of 32 was required to power the study at 0.8 with a type I error of 0.05 and presumed maximum difference of 4 antral follicles across 4 levels of analysis.

## Data acquisition

The ultrasound scans were performed by a single investigator (S.D) using a Voluson E8 Expert (GE Medical Systems, Zipf, Austria) and a three-dimensional, 5-9 MHz, endovaginal transducer. The ultrasound assessment involved a two-dimensional (2D) ultrasound assessment of the pelvis to exclude any pelvic pathology. Our technique of volume ultrasound included delineation of the ovary with application of a region of interest and the subsequent acquisition of a series of 2D planes acquired during a high quality, slow sweep mode of the ultrasound beam through a predefined $90^0$ angle. This ensured that the whole ovary and the greatest number of 2D planes were acquired giving the highest degree of resolution when the 2D data were reconstructed as a 3D volume.

## Data analysis

The acquired 3D data were displayed on the ultrasound machine (Voluson E8 Expert: General Electric Medical Systems, Zipf, Austria) in the multiplanar view. The grey-scale display of image was optimised and then rendered to generate a three-dimensional volume of interest (VOI). The render box was adjusted to exclude as much extra-ovarian

information as possible and ensure that the whole ovary was included in the VOI. The threshold settings, which assign transparency associated with fluid to opaque voxels, were maintained for all datasets at a default setting of 'low'. Once the dataset had been correctly positioned, 3D automated software, 'sonography-based automated volume count' (sonoAVC: GE Medical Systems) was implemented (Raine-Fenning, Jayaprakasan et al. 2008). The use of sonoAVC with post-processing in counting and measuring the size of antral follicles has been previously described in detail (Deb, Jayaprakasan et al. 2009). In brief, the dimensions and relative sizes of individual follicles are displayed with a specific colour. Post-processing, involving the manual identification of follicles, was then used to ensure all antral follicles were counted and measured. The mean 'relaxed sphere diameter', displayed as d(V), of each antral follicle in both ovaries was recorded and used for data analysis as this has been shown to most accurately reflect the true follicle diameter (Raine-Fenning, Jayaprakasan et al. 2009). The antral follicle population for each subject was recorded to the nearest millimetre, as this reflects the current resolution of the ultrasound system, starting from 2.0 mm up to a maximum of 10.0 mm.

VOCAL was used to quantify the volume of each ovary. The method, described in detail previously (Raine-Fenning, Campbell et al. 2002), involved the manual delineation of the ovarian cortex in B plane of the multiplanar view as the dataset was rotated through a series of six consecutive $30^0$ steps, and a therefore a total of $180^0$, for each volume calculation.

**Hormonal Assays**

Blood samples were centrifuged, within 30 minutes of collection, for 20 min at $4^o$C and 4000 rpm spin to separate the serum which was then frozen at -20°C and stored for subsequent analysis of anti-Müllerian hormone (AMH), follicle stimulating hormone (FSH),

luteinising hormone (LH), and Oestradiol (E2) levels. MIS/AMH enzyme-linked immunosorbent assay kit (Diagnostic Systems Laboratories, Webster, Texas, USA) was used to measure serum AMH levels. The lowest detection limit was 0.006 ng/mL and the intra and interassay coefficients of variation below 5% and 8% respectively.

The Micro particle Enzyme Immunoassay (MEIA) method was used to measure the levels of serum FSH, LH, and Oestradiol levels on an AxSYM auto-analyser (AxSYM; Abbott Laboratories, Abbott Park, IL). The lowest detection limit for FSH was 0.37 IU/L, with the intra- and interassay coefficients of variation were below 5% and above 5% respectively. The lowest detection limit for LH was 0.3 IU/L and the intra- and interassay coefficients of variation of 3% and 7% respectively. The lowest detection limit for E2 was 8 pmol/L, with the intra- and interassay coefficients of variation of 2.9% to 11%, and 4.8% to 15.2% respectively.

## Statistical analysis

The Statistical Package for the Social Sciences (version 17.0; SPSS, Chicago, IL) was used for statistical analysis**.** General linear model with repeated measures design was used to perform the analysis of variance. Test of sphericity was performed using Mauchly's test on the data. If the assumption of sphericity was violated, Greenhouse-Geisser correction was applied to the data and P value derived subsequently. If the P value derived following this correction was significant, the significance of the variation was re-confirmed using Hotelling's trace multivariate test and tests of within subject contrasts. The confidence interval was adjusted using Bonferroni correction, which adjusts the observed significance level for the fact that multiple (repeated) contrasts are being tested. The contrast was set to repeated type to ensure comparison of the mean of each level (except the last) to the mean of the subsequent level. A P-value of less than 0.05 was considered statistically

significant. Correlation coefficients were used to analyse the significance of correlation of the markers between the different cycle phases and intraclass correlation coefficients (ICC) were used to evaluate the true intra-individual intra-cycle variation.

## 8.4    Results

38 healthy volunteers were recruited in the study. Two were excluded from the final analysis as a dominant follicle was not confirmed during the ultrasound assessments. Of the 36 included in final analysis, 34 attended all four visits. One subject did not attend the luteal phase visit and the other did not attend the second follicular phase visit. Mean ± SD age and BMI of the participants was 28.12 ± 5.75 years and 22.34 ± 3.08 kg/m$^2$ respectively. The mean ± SD of markers of ovarian reserve in different phases of menstrual cycle are shown in Table 8.1. The total number of antral follicles measuring 2.0 to 10.0 mm varied significantly (P=0.002) across the menstrual cycle. Analysis of levels of contrasts, showed a significant increase in the count from the first follicular (F1) phase to the second follicular phase (F2) (F=27.05, P<0.001) and then a significant drop in the count from the peri-ovulatory phase (PO) to the luteal (LU) phase (F=8.21, P=0.007). There was no significant difference noted, however, in the total number of follicles measuring 2.0 to 10.0 mm between the F1 and LU phases of menstrual cycle (P>0.05).

On stratifying the antral follicles into cohorts of small (2.0 to 4.0mm and >4.0 to 6.0mm) and large (>6.0 to 10.0mm) antral follicles, we found that there was no significant intra-cyclical variation in the small antral follicles measuring up to 6.0mm (P=0.382) but a significant variation in the larger follicles measuring more than 6.0mm (P<0.001). The test for levels of contrast with large antral follicles showed that the count significantly increases from the F2 phase to PO phase (F=36.32, P<0.001) of cycle and then shows a

significant drop from PO to LU phase (F=12.168, P=0.001). The small antral follicles showed no significant variation at different levels of contrast (P>0.05) (Figure 8.1).

**Figure 8.1:** The intra-cycle variation in the mean (error bars of S.E.M) antral follicle counts assessed using repeated measures analysis are shown in figure below. P values are marked at levels of comparison showing significant change (*=P<0.05; **=P<0.01; ***=P<0.001).
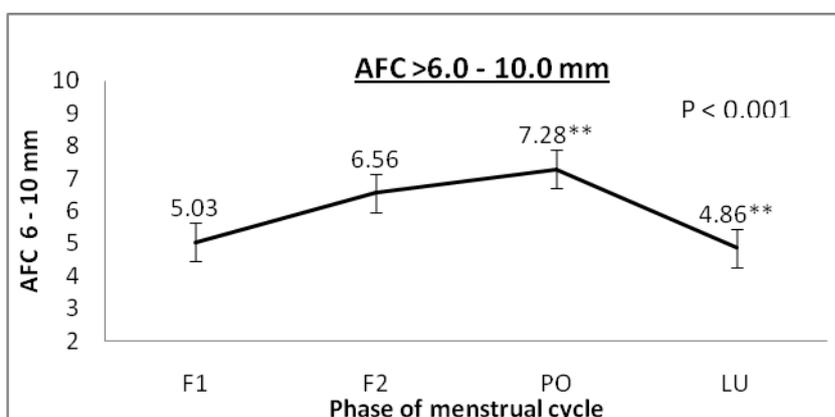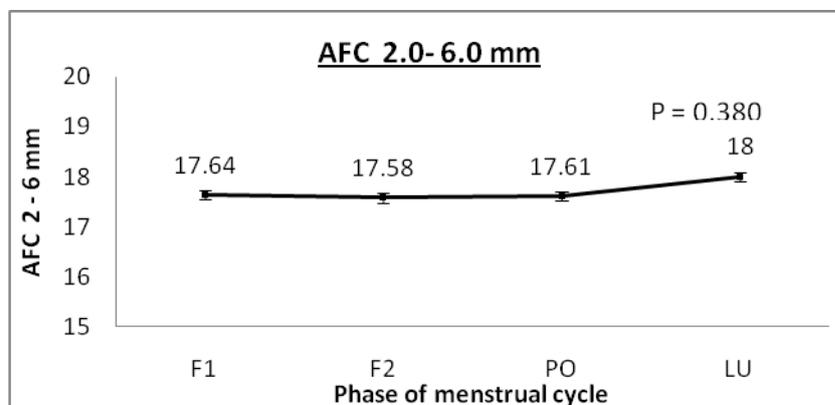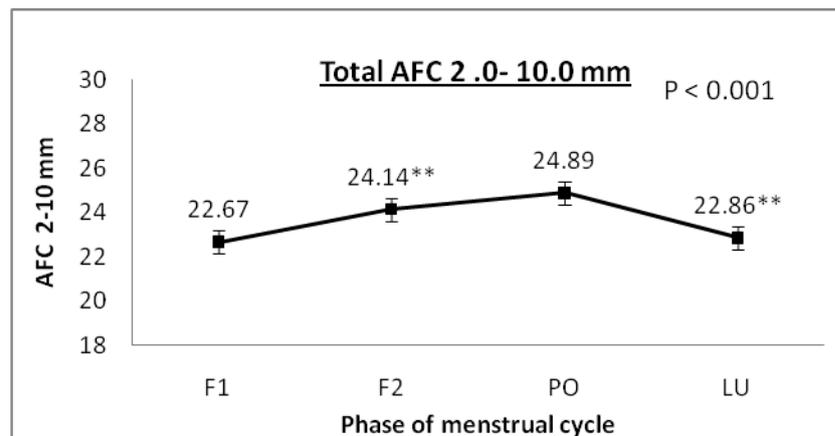
**Table 8.1:** Comparison of the Mean ± SD (95% CI) of the ultrasound and endocrine determinants of ovarian reserve measured over one menstrual cycle in healthy volunteers with normal menstrual cycles. The P value shown is derived using Hotelling's trace multivariate test of repeated measures analysis.

|  | Early follicular N=36 | Mid follicular N=35 | Peri-ovulatory N=36 | Luteal N=35 | P value |
|---|---|---|---|---|---|
| **Total AFC 2-10mm** | 22.67 ± 9.92 | 24.14 ± 10.02 | 24.89 ± 9.88 | 22.86 ± 9.83 | <0.001 |
| **AFC 2-4mm** | 9.39 ± 4.96 | 9.17 ± 4.86 | 9.57 ± 4.98 | 9.81 ± 5.34 | 0.041 |
| **AFC >4-6mm** | 8.25 ± 3.15 | 8.42 ± 3.42 | 8.06 ± 3.36 | 8.19 ± 3.26 | 0.087 |
| **AFC 2-6** | 17.64 ± 7.27 | 17.58 ± 7.40 | 17.61 ± 7.56 | 18.00 ± 7.68 | 0.380 |
| **AFC >6-10mm** | 5.03 ± 3.50 | 6.56 ± 3.46 | 7.28 ± 3.83 | 4.86 ± 3.57 | <0.001 |
| **Volume** | 6.43 ± 2.19 | 6.30 ± 2.01 | 7.28 ± 2.46 | 7.67 ± 2.64 | <0.001 |
| **AMH ng/mL** | 2.61 ± 1.47 | 2.60 ± 1.39 | 2.61 ± 1.42 | 2.92 ± 1.66 | 0.035 |
| **FSH IU/L** | 6.97 ± 2.43 | 6.54 ± 2.84 | 8.33 ± 2.91 | 4.97 ± 1.90 | <0.001 |
| **LH IU/L** | 5.84 ± 2.77 | 6.18 ± 2.74 | 20.18 ± 17.05 | 5.50 ± 3.03 | <0.001 |
| **Oestradiol pmol/L** | 147.72 ± 63.04 | 165.25 ± 64.32 | 893.25 ± 68.38 | 427.78 ± 57.84 | <0.001 |

Two sub-groups of small antral follicles (2.0 to 4.0mm and >4.0 to 6.0mm) were further analysed. Whilst >4-6.0mm follicles showed no significant (p=0.335) intra-cyclical variation, the 2.0-4.0mm follicles showed a small but significant (P=0.042) intra-cyclical variation, and this was noted between the first two follicular phases (F1 and F2) (F=5.091, P=0.033) (Figure 8.2) (Tables 8.3 and 8.4 in Appendix).

The ovarian volume showed a significant intra-cyclical variation (P<0.001). This significance was noted due to the increase in ovarian volume between the F2 and PO

(F=9.44, P=0.004) phases of menstrual cycle, which was mainly contributed by the ovary containing the dominant follicle (Figure 8.2) (Tables 8.3 and 8.4 in appendix).

**Figure 8.2:** The intra-cycle variation in the mean (error bars of S.E.M) of small antral follicle counts assessed using repeated measures analysis are shown in figure below. P values are marked at levels of comparison showing significant change (*=P<0.05; **=P<0.01; ***=P<0.001).
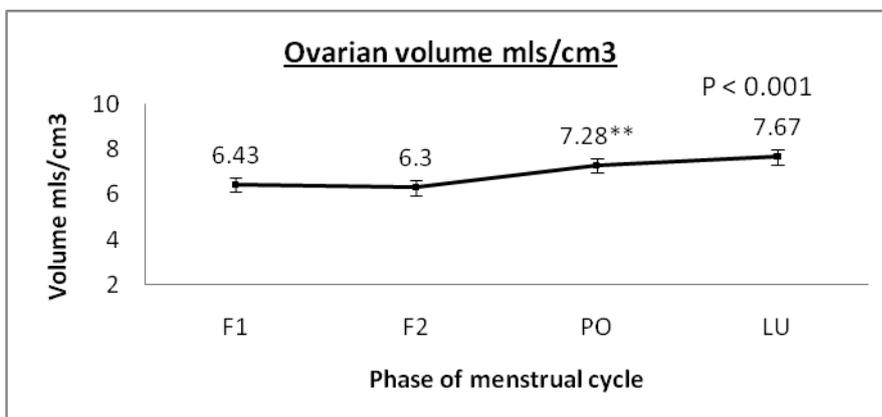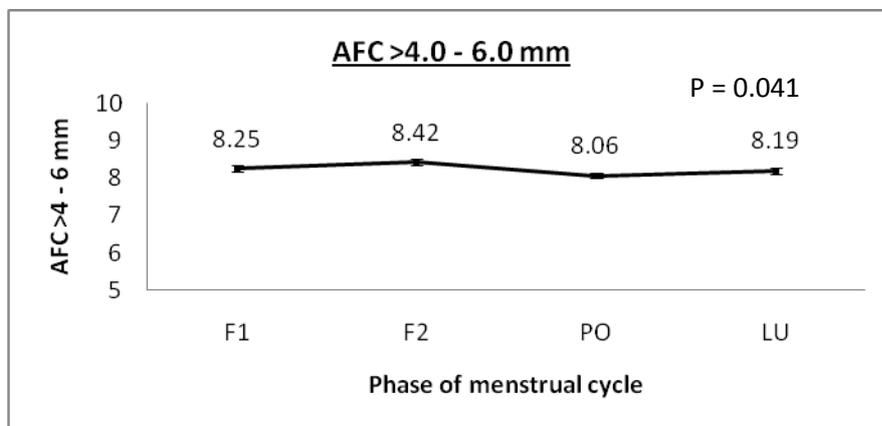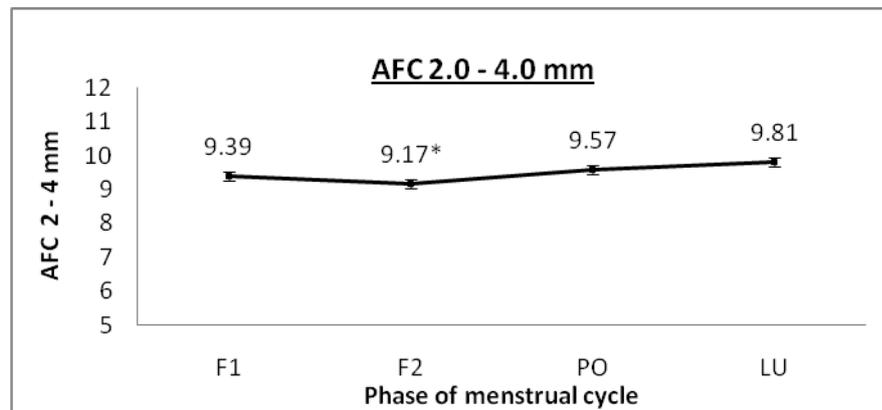
There was a small but significant intra-cyclical variation noted in serum AMH levels (P=0.041). On analysis of levels of within subject contrasts, we found that AMH levels significantly increase between the PO and LU (F=11.89, P=0.039) phases of menstrual cycle, as shown in Figure 8.3.

**Figure 8.3:** The intra-cycle variation in the mean (error bars of S.E.M) AMH levels assessed using repeated measures analysis are shown in figure below. P values are marked at levels of comparison showing significant change (*=P<0.05; **=P<0.01; ***=P<0.001).
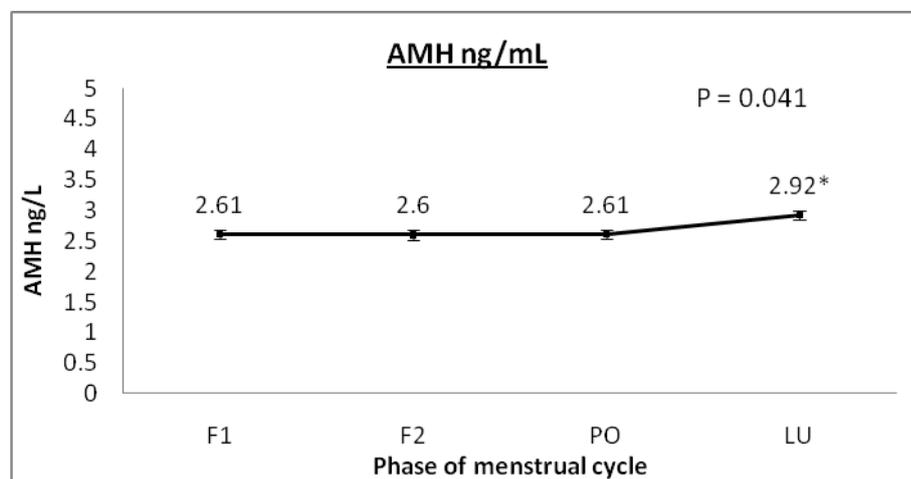


There were expected variations in serum FSH, LH and E2 levels. Serum FSH levels increased between the F2 and PO (F=8.78, P=0.005) phases of the menstrual cycle before falling between the PO and LU (F=58.10, P<0.001) phases when levels were significantly lower (P=0.011) than during the F1 phase of cycle. Serum LH levels showed a similar pattern increasing significantly from the F2 to the PO (F=23.98, P<0.001) phase of the cycle before falling between the PO and LU (F=33.39, P<0.001) phase, when levels were comparable to those seen in the F1 phase of cycle. Oestradiol levels mirrored the gonadotrophins increasing from the F1 to F2 (F=115.48, P=0.001) and F2 to PO (F=385.85, P<0.001) phases before significantly decreasing between the PO and LU (F=129.59,

P<0.001) phases where levels remained significantly higher than those seen during the F1

phase (Figure 8.4) (Tables 8.3 and 8.4 in appendix).

**Figure 8.4:** The intra-cycle variation in the mean (error bars of S.E.M) FSH, LH and Oestradiol

levels assessed using repeated measures analysis are shown in figure below. P values are marked

at levels of comparison showing significant change (*=P<0.05; **=P<0.01; ***=P<0.001).
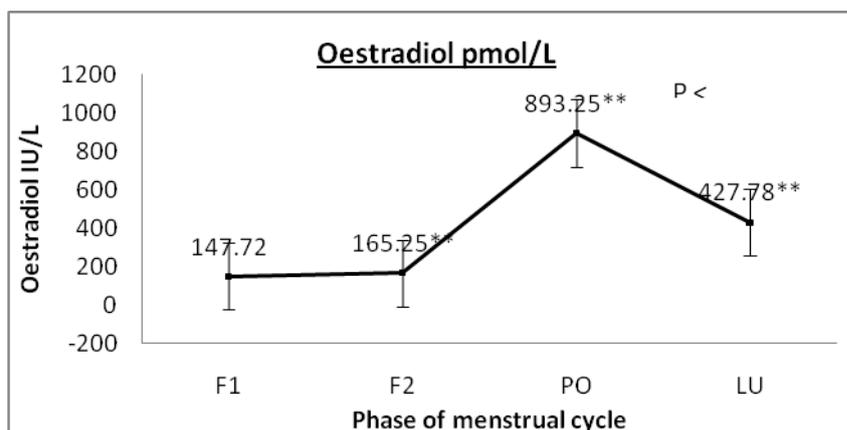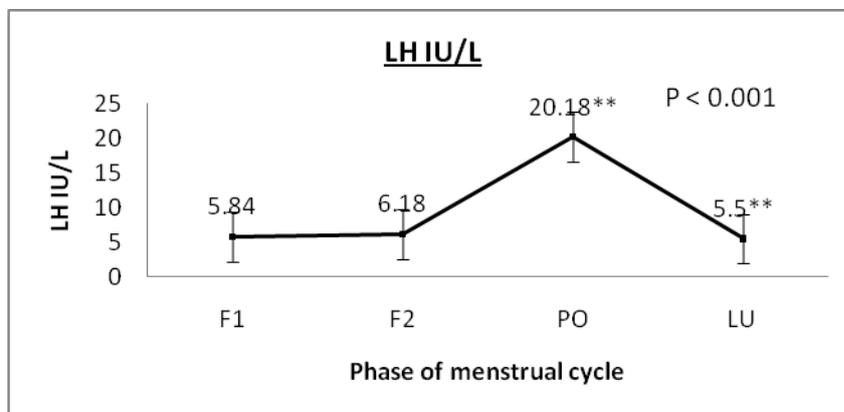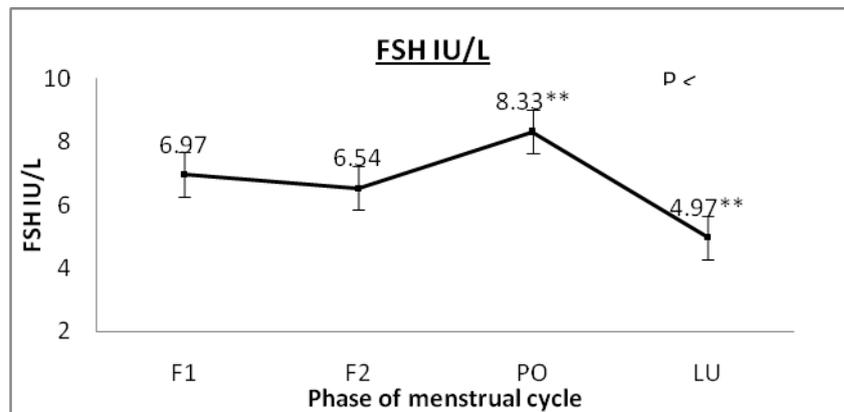
Whilst total AFC (2.0-10.0 mm), small (2.0-6.0 mm) and large (>6.0-10.0 mm) antral follicle counts, ovarian volume and AMH showed significant correlation (R = 0.948, 0.976, 0.575, 0.568 and 0.959 respectively), Oestradiol, FSH and LH showed a non-significant correlation (R = 0.415, 0.408 and 0.373) between different phases of menstrual cycle. AMH, total AFC (2.0-10.0 mm) and small AFC (2.0-6.0 mm) showed excellent ICC of 0.96, 0.94 and 0.94 respectively, suggesting that majority of the intra-cycle variation seen is due to between-subject variation and that the true within-subject variation related to the phase of menstrual cycle was only 4%, 6% and 6% respectively. The ICCs for larger antral follicles (>6.0-10.0 mm), ovarian volume, FSH, LH and Oestradiol suggested high intra-individual intra-cycle variation (Table 8.2).

**Table 8.2:** The table below shows the Intra-class correlation coefficients (ICC) of ultrasound and endocrine markers of ovarian reserve over one menstrual cycle.

|  | ICC | 95% CI | P value |
|---|---|---|---|
| **Total AFC (n)** | 0.939 | 0.901 – 0.965 | <0.001 |
| **AFC 2.0-4.0mm (n)** | 0.931 | 0.890 – 0.961 | <0.001 |
| **AFC >4.0-6.0mm (n)** | 0.898 | 0.839 – 0.941 | <0.001 |
| **AFC 2.0-6.0mm (n)** | 0.938 | 0.900 – 0.965 | <0.001 |
| **AFC >6.0-10.0mm (n)** | 0.518 | 0.353 – 0.681 | <0.001 |
| **Ovarian volume (cm$^3$)** | 0.588 | 0.431 – 0.734 | <0.001 |
| **AMH ng/L** | 0.957 | 0.931 – 0.976 | <0.001 |
| **FSH IU/L** | 0.301 | 0.137 – 0.493 | 0.012 |
| **LH IU/L** | 0.104 | -0.031 – 0.289 | 0.072 |
| **Oestradiol pmol/L** | 0.131 | -0.019 – 0.320 | 0.058 |

## 8.5    *Discussion*

This is the first study to examine the antral follicles stratified into cohorts of small and large antral follicle counts based on their size which was measured using 3D ultrasound assisted semi-automated technique, SonoAVC. The results suggest that the cohort of small antral follicles measuring 2 – 6 mm do not show significant intra-cycle variation and that they show excellent correlation between the different phases of menstrual cycle. The total AFC made by including antral follicles of size 2 – 10 mm significantly varied across the menstrual cycle. This was predominantly due to a variation in the number of larger antral follicles measuring more than 6.0mm and, to a less but still significant extent, in the number of follicles measuring 2.0 to 4.0mm. There was no such variation in the number of follicles measuring 4.0 to 6.0 mm or in the number of small antral follicles measuring 2.0 to 6.0mm as an overall cohort. This latter group of follicles are often referred to as the 'selectable follicles' as they are responsive to gonadotrophins but unlike the larger antral follicles measuring more than 6.0 mm are not dependent on it (Gougeon 1989).

Our results suggest a small but significant intra-cycle variation in serum AMH, mainly attributed by the increase in the levels in luteal phase of menstrual cycle. There was a high correlation noted in the AMH levels through the menstrual cycle. A high level of correlation exist between AMH levels and the small antral follicle population (Jayaprakasan, Deb et al. 2009), which is believed to reflect the expression of AMH by this follicular cohort (Hansen, Morris et al. 2003; Weenen, Laven et al. 2004). The increase in AMH levels seen in the luteal phase of the cycle with no concomitant change in the small antral follicle population seen in this study suggests that there might be a new

recruitment of pre-antral and early antral follicles in the luteal phase that are also known to express AMH (Gougeon 1989; Gougeon 1996; Tsepelidis, Demeestere et al. 2007) but that cannot be identified on ultrasound. The increase in AMH we noted in the luteal phase has been described in one other study (Hehenkemp 2006). They examined 44 healthy volunteers, with an average of 7 visits per participant and described the menstrual cycle starting from the mid luteal phase of previous cycle to the luteal phase of next cycle. The small significant increase in AMH noticed in the luteal phase might actually reflect the levels of subsequent cycle thereby raising the possibility that the number of early and pre-antral follicles expressing AMH may vary between cycles. The authors however believed that the luteal phase increase in AMH levels in their study could be non-significant due to the fewer measurements made and a proportional increase in the number of younger patients assessed at that visit. In our study we only examined four time points in the menstrual cycle and the small but significant increase in luteal phase levels of AMH might have become non-significant with more number of visits in each cycle. Wunder et al showed a peri-ovulatory increase in the levels of AMH in 36 women with normal menstrual cycles (Wunder, Bersinger et al. 2008). They, like in our study, described a period between two menstruations as one menstrual cycle. They examined the AMH levels every other day and found that it increased in the late follicular phase and mid luteal phase of menstrual cycle; and that it decreased in the very early luteal phase possibly due to the negative effect of luteinisation on granulosa cells. In our study, we have measured the size of antral follicles and shown that the small antral follicles that positively correlate with the AMH levels do not vary significantly. However, the luteal phase increase in AMH levels in our study and the peri-ovulatory increase in AMH levels shown by Wunder et al could be attributed to the much smaller or pre-antral follicles that

cannot be seen by ultrasound. It might be possible to explain the new recruitment of these follicles in the mid to late luteal phase of cycle when the levels of gonadotrophins are low, but difficult to explain the increase in AMH levels seen by Wunder et al in the late follicular phase when the high levels of gonadotrophins may have a negative impact on the expression of AMH by granulosa cells. Cook et al examined three time points (early follicular, peri-ovulatory and luteal) in the menstrual cycle of a much smaller population of 20 healthy women (Cook, Siow et al. 2000). They found an increase in AMH levels in the peri-ovulatory phase (LH surge + 1 day). These results are in contrast to what was found in our study and also to that noted by Wunder et al. A small study population with fewer time points in the menstrual cycle may explain these findings.

Several studies have suggested that AMH demonstrates no or minimal variation across the menstrual cycle (Hehenkamp, Looman et al. 2006; La Marca, Stabile et al. 2006; Tsepelidis, Devreker et al. 2007; van Disseldorp, Lambalk et al. 2010).  These findings could be explained by the AMH expression profile. AMH is expressed by the pool of growing antral follicles mainly those measuring less than 6 mm, the number of which is dependent on the follicular pool i.e. the true ovarian reserve and not the cycle phase (Visser, de Jong et al. 2006). This pool of growing follicles is constantly renewed according to the follicular reserve, perhaps therefore making the AMH levels constant throughout the cycle. Follicles measuring more than 10 mm express negligible to small amounts of AMH levels and may be the cause for peri-ovulatory increase in AMH levels seen in some studies.

Only one recent study has examined the intra-cycle variation in AMH along with the antral follicle counts made using two-dimensional ultrasound. The authors concluded that the variation in AMH during the menstrual cycle is significantly less than that seen in the

number of antral follicles and that the AFC is still a cycle-specific test of ovarian reserve. Moreover, they showed higher variation in the small antral follicles measuring 2-5 mm than those measuring 2-10 mm (van Disseldorp, Lambalk et al. 2010). These results contradict to the results from our study, which suggest that the total number of antral follicles measuring 2-10 mm show a significant intra-cycle variation and that the small antral follicles measuring 2-6 mm do not show significant intra-cycle variation. Moreover, the small antral follicles show a high intra-cycle correlation (0.938) comparable to that seen with AMH (0.957). The differences in the result may be due to the difference in methodology used in our study. In our study, the antral follicles were counted and measured using SonoAVC which is a semi-automated 3D assisted method shown to be reliable in counting and measuring antral follicles, superior to 2D ultrasound in measuring the size of follicles. Also, 2D and other manual methods might over-estimate the size of antral follicles (Deb, Jayaprakasan et al. 2009; Deb, Campbell et al. 2010). The findings from our study also compare to the studies showing minimal or no intra-cycle variation in AMH levels, confirming that AMH is mainly expressed by the small antral follicles which in our study shows a non-significant intra-cycle variation.

This is the first study that describes the intra-cycle variation in ovarian volume as measured using 3D ultrasound in normo-ovulatory healthy women. It showed significant intra-cycle variation, especially an increase in the peri-ovulatory and luteal phase of cycle. This could be attributed to the presence of the dominant follicle and the subsequent corpus luteum formation. Inter-cycle variation in ovarian volume has been described in sub-fertile women using two-dimensional (Elter *et al.* 2005) and 3D ultrasound (Jayaprakasan *et al*. 2008). Ovarian volume must be assessed in the early follicular phase therefore before any significant follicle dominance occurs.

FSH and LH, both showed significant intra-cycle variation, mainly due to the increase in the peri-ovulatory phase and a subsequent drop in the luteal phase of menstrual cycle. This variation is well described (Gougeon 1994) and therefore confirms that these tests are also best performed in the early follicular phase. Oestradiol is expressed by the granulosa cells of the growing graffian follicle which shows a peak before the LH peak and ovulation. Our results confirm this feature and also that Oestradiol levels progressively rise till the peri-ovulatory phase and then progressively drop in the luteal phase, although still staying higher than the first follicular phase levels.

## 8.6    Conclusion

The ovarian reserve as measured using small antral follicle count (2.0-6.0mm) and AMH show least intra-cycle variation and an excellent within subject correlation. The small but significant increase in the luteal phase levels of AMH might suggest a new recruitment of early antral and pre-antral follicles and therefore may more adequately predict the actual ovarian response, whilst the follicular phase is more likely to reflect the actual pool of small antral follicles that is likely to respond to ovarian stimulation. Future studies designed to evaluate their ability to predict ovarian response following assisted reproduction treatment should consider performing these tests in different phases of menstrual cycle.

**CHAPTER 9: The inter-cycle variation in the number of antral follicles stratified by size using three-dimensional ultrasound and in the endocrine markers of ovarian reserve in women with normal ovulatory menstrual cycles**

## *9.1    Abstract*

**Objective:** More often, the assisted reproduction treatment (ART) may not begin soon after the test is performed, which raises the question whether the test will be predictive enough of the ovarian response and treatment outcome over time. Whilst there are a number of tests of ovarian reserve, it appears that AMH and AFC are the most predictive tests of ovarian response. The objective of this study was to use 3D ultrasound assisted semi-automated method, SonoAVC to quantify the inter cycle variation in AFC stratified by follicular size and compare it to AMH and other endocrine markers of ovarian reserve over a period of 12 months in normo-ovulatory healthy volunteers.

**Methods:** This was a prospective observational study conducted in a university based assisted conception unit. Study participants described as 'healthy volunteers' were recruited prospectively. Serial transvaginal ultrasound scans were performed and blood samples taken in the early follicular phase of menstrual cycle (days 2-5) at 0, 1, 3, 6, and 12 months. The main outcome measure was the variation in the antral follicles stratified by size over a period of 12 months. Repeated measures analysis was performed on the data.

**Results:** The variation in the mean total number of antral follicles measuring 2.0 to 10.0 mm over 12 months was not significant (P=0.147) with non-significant variation at different levels of contrast (P>0.05). Small antral follicles (2.0 – 6.0 mm) and large antral follicle (>6.0 – 10.0 mm) both showed a non-significant variation in the number of follicles measured over 12 months (P=0.326 and P=0.484 respectively). Both the groups of antral follicles again showed a non-significant variation at different levels of contrast (P>0.05).

Antral follicle count measuring 2.0-10.0 mm and 2.0-6.0 mm showed excellent correlation in their counts across 5 cycles in 12 months examined (0.876, P<0.001 and 0.916, P<0.001 respectively). Antral follicles measuring >6.0-10mm showed a significant but poorer correlation than the smaller antral follicles (0.379, P=0.027).

**Conclusion:** The ovarian reserve as measured using small antral follicle count (2.0-6.0mm) and AMH show least inter-cycle variation and an excellent within subject correlation. The inter-cycle stability of total antral follicle count (2.0-10.0 mm) when performed in the early follicular phase of menstrual cycle appears to be comparable to AMH and small AFC.

## *9.2    Introduction*

Ovarian reserve reflects the potential of the ovaries to produce oocytes leading to embryo formation and pregnancy. In physiological terms, it is perhaps also a test to predict ovarian ageing which has implications on both fertility and menopause (Broekmans, Soules et al. 2009). A test of ovarian reserve is primarily aimed at predicting the ovarian response to ART and also to inform treatment protocols to prevent unfavourable outcomes such as cycle cancellations and ovarian hyper stimulation. More often, the assisted reproduction treatment (ART) may not begin soon after the test is performed, which raises the question whether the test will be predictive enough of the ovarian response and treatment outcome over time. Ageing affects ovarian reserve and therefore the tests of ovarian reserve is unlikely to be independent of time. However, an ideal test of ovarian reserve should be reliable and predictive of response for at least a few months, such that the woman waiting to undergo treatment does not require frequent tests before starting treatment. Furthermore, these tests should have minimal variation over

time such that they could be reliably used to evaluate the effect of pelvic surgery including ovarian cystectomy on ovarian reserve. Generally, a test is thought to be valid for up to 6 months before starting treatment. The only subjective data to support such a practice would be the population studies on pregnancy and ageing (Poma 1981; Milner, Barry-Kinsella et al. 1992; Faddy and Gosden 1996; Gougeon 1998; te Velde and Pearson 2002; Broekmans, Soules et al. 2009). It would also be important for such a test to be reliable showing minimal inter cycle variation.

Whilst there are a number of tests of ovarian reserve, it appears that AMH and AFC are the most predictive tests of ovarian response. Moreover, a good test should show high reproducibility and be valid. A moderate degree of inter-observer reliability of both AFCs and ovarian volume measurements has been reported with both 2D and 3D ultrasound (Scheffer, Broekmans et al. 2002; Merce, Gomez et al. 2005). 3D ultrasound is shown to improve reproducibility of measurements of ovarian volume, blood flow indices (Raine-Fenning, Campbell et al. 2003; Raine-Fenning, Campbell et al. 2003; Raine-Fenning, Campbell et al. 2004), and recently that of total antral follicle count (Jayaprakasan, Campbell et al. 2008). 3D assisted semi-automated antral follicle counts show further improvement in intra and inter observer reproducibility (chapter 3). Also, this technique might provide more reliable and valid measurement of size of antral follicles, thereby enabling the objective evaluation of small antral follicles (chapter 4, 5 and 6).

A significant inter-cycle variability has been reported for basal FSH (Scott, Hofmann et al. 1990) and ovarian volume (Elter, Sismanoglu et al. 2005). Similarly a high degree of variation in the number of antral follicles between cycles was demonstrated both in fertile (Scheffer, Broekmans et al. 1999) and sub fertile  populations (Elter, Sismanoglu et al. 2005). A recent study on infertile women has shown more inter-cycle variability with AFC

than AMH over four consecutive cycles (van Disseldorp, Lambalk et al. 2010). However, all these studies have used 2D ultrasound which is less reliable than 3D ultrasound. AFC measured using 3D ultrasound over two consecutive IVF cycles is shown to demonstrate lesser inter-cycle variability than ovarian volume and basal FSH levels (Jayaprakasan, Campbell et al. 2008). Levels of serum AMH show minimal variation both intra cycle (Hehenkamp, Looman et al. 2006; La Marca, Stabile et al. 2006; La Marca, Giulini et al. 2007; Tsepelidis, Devreker et al. 2007; van Disseldorp, Lambalk et al. 2010) and inter cycle (McIlveen, Skull et al. 2007; van Disseldorp, Lambalk et al. 2010) and there is also some suggestion that the variation is lower than that seen with AFC (van Disseldorp, Lambalk et al. 2010).

The objective of this study was to use 3D ultrasound assisted semi-automated method, SonoAVC to quantify the inter cycle variation in AFC stratified by follicular size using SonoAVC and compare it to AMH and other endocrine markers of ovarian reserve over a period of 12 months in normo-ovulatory healthy volunteers.

## 9.3    Methods

**Study design**

Study participants described as 'healthy volunteers' were recruited prospectively through advertisements on the intranet portal of the University of Nottingham (UoN) website, and posters displayed in staff canteens, notice boards and staff library. Further information about the study was communicated via email and information leaflets to those who expressed interest in participation in the study. The main inclusion criteria were: age between 18 to 35 years, body mass index (BMI) between 18 and 26 kg/ m$^2$, regular

menstrual cycles with a mean length ranging between 26 to 32 days, no history of ovarian surgery, no history suggestive of endocrine disease and no use of hormonal contraceptives in the last 6 months. Serial transvaginal ultrasound scans were performed and blood samples taken in the early follicular phase of menstrual cycle (days 2-5) at 0, 1, 3, 6, and 12 months. A sample size of 28 was required to power the study at 0.8 with a type I error of 0.05 and presumed maximum difference of 0.9 in the mean values across 5 levels of analysis.

**Data acquisition and analysis**

The ultrasound scans were performed by a single investigator (S.D) using a Voluson E8 Expert (GE Medical Systems, Zipf, Austria) and a three-dimensional 5-9 MHz transvaginal transducer. The ultrasound assessment involved a two-dimensional (2D) ultrasound assessment of the pelvis to exclude any pelvic pathology followed by acquisition of 3D data, similar to that in previous chapters. The acquired 3D data were displayed on the ultrasound machine (Voluson E8 Expert) in the multiplanar view. SonoAVC, as described in previous chapters was applied to the data and size of each antral follicle measured thereby was recorded as d(v). Virtual Organ Computer-aided AnaLysis (VOCAL imaging programme: GE Medical Systems) was used to quantify the ovarian volume in each individual ovary. The method has been described in detail previously (Raine-Fenning, Campbell et al. 2002) but briefly involved display of ovary in multiplanar view followed by delineation of ovarian cortex in B plane by manually tracing round the ovary in $30^0$ rotational steps as the dataset was rotated through a total of $180^0$, providing six planes for each volume calculation.

**Hormonal Assays**

The method used was similar to that described in chapter 8.

**Statistical analysis**

The Statistical Package for the Social Sciences (version 17.0; SPSS, Chicago, IL) was used for statistical analysis. Descriptive statistics are displayed as either mean ± SD or median with range. General linear model with repeated measures design was used to perform the analysis of variance (described in detail in chapter 8). A P-value of less than 0.05 was considered statistically significant. Correlation coefficients were used to analyse the correlation of the markers over 5 cycles in 12 months and their significance expressed as P value. Intraclass correlation coefficients (ICC) were used to assess the inter-cycle variation and also between the markers of ovarian reserve.

## 9.4   Results

38 healthy volunteers were recruited in the study. Of these, 2 subjects decided to commence contraceptive pills and therefore could not be followed up after their visit at 3 months. A further 6 subjects could not be followed up after their visit at 6 months as 5 subjects became pregnant and one decided to have an intrauterine contraceptive device. 30 subjects attended all 5 visits at 0, 1, 3, 6, and 12 months. Mean ± SD age and BMI of the participants was 28.12 ± 5.75 years and 22.34 ± 3.08 kg/m$^2$ respectively. The mean ± SD of markers of ovarian reserve over 12 months are shown in Table 9.1. The mean total number of antral follicles measuring 2.0 to 10.0 mm over 12 months did not differ (P=0.147) showing a non-significant variation at different levels of contrast (P>0.05) (Figure 9.1).

**Table 9.1:** Comparison of the Mean ± SD (95% CI) of the ultrasound determinants of ovarian reserve measured in five menstrual cycles at 0, 1, 3, 6, and 12 months in healthy volunteers with normal menstrual cycles. The size of antral follicles was measured using 3D assisted SonoAVC and grouped into cohorts of small (2.0-6.0 mm), large (>6.0-10.0 mm) and total (2.0-10.0 mm) antral follicle counts. The P value was derived using Hotelling's trace multivariate test of repeated measures analysis.

| | 0 month N=38 | 1 month N=38 | 3 months N=38 | 6 months N=36 | 12 months N=30 | P value |
|---|---|---|---|---|---|---|
| **Ovarian volume** | 6.29 ± 1.76 (5.69-6.89) | 6.09 ± 1.63 (5.55-6.65) | 6.21 ± 1.81 (5.59-6.82) | 6.05 ± 1.52 (5.54-6.57) | 6.03 ± 1.78 (5.43-6.64) | 0.434 |
| **Antral follicle counts of both ovaries** | | | | | | |
| **2.0 – 6.0 mm (n)** | 14.08 ± 5.92 (12.05-16.12) | 13.51 ± 5.91 (11.49-15.54) | 14.08 ± 5.92 (12.05-16.12) | 13.88 ± 5.88 (11.86-15.91) | 13.51 ± 5.91 (11.49-15.54) | 0.326 |
| **>6.0 – 10.0 mm (n)** | 5.97 ± 1.46 (5.47-6.47) | 5.60 ± 1.83 (4.97-6.23) | 5.97 ± 1.47 (5.47-6.47) | 5.89 ± 2.03 (5.19-6.58) | 5.60 ± 1.83 (4.97-6.23) | 0.484 |
| **2.0 – 10.0 mm (n)** | 20.05 ± 6.78 (17.76-22.35) | 19.08 ± 6.89 (16.75-21.42) | 20.05 ± 6.78 (17.76-22.35) | 19.22 ± 7.83 (16.57-21.87) | 19.08 ± 6.89 (16.75-21.42) | 0.147 |
| **Antral follicle counts of right ovary** | | | | | | |
| **2.0 – 6.0 mm (n)** | 7.00 ± 3.05 (5.95-8.05) | 6.86 ± 2.83 (5.88-7.83) | 7.00 ± 3.05 (5.95-8.05) | 6.89 ± 2.86 (5.90-7.87) | 6.86 ± 2.83 (5.88-7.83) | 0.870 |
| **>6.0 – 10.0 mm (n)** | 3.08 ± 1.27 (2.65-3.52) | 2.74 ± 1.62 (2.19-3.29) | 3.08 ± 1.27 (2.65-3.52) | 3.14 ± 1.88 (2.49-3.79) | 2.74 ± 1.62 (2.19-3.29) | 0.316 |
| **2.0 – 10.0 mm (n)** | 10.08 ± 3.90 (8.76-11.40) | 9.61 ± 3.74 (8.34-10.88) | 10.08 ± 3.90 (8.76-11.40) | 9.75 ± 4.38 (8.27-11.23) | 9.61 ± 3.74 (8.34-10.88) | 0.870 |
| **Antral follicle counts of left ovary** | | | | | | |
| **2.0 – 6.0 mm (n)** | 7.08 ± 3.16 (6.00-8.17) | 6.66 ± 3.35 (5.50-7.81) | 7.09 ± 3.16 (6.00-8.17) | 6.66 ± 2.82 (5.69-7.62) | 6.66 ± 3.35 (5.51-7.81) | 0.191 |
| **>6.0 – 10.0 mm (n)** | 2.89 ± 1.08 (2.52-3.26) | 2.86 ± 1.29 (2.42-3.30) | 2.88 ± 1.07 (2.52-3.26) | 2.74 ± 1.34 (2.28-3.20) | 2.86 ± 1.29 (2.41-3.30) | 0.885 |
| **2.0 – 10.0 mm (n)** | 9.97 ± 3.34 (8.84-11.10) | 9.47 ± 3.67 (8.23-10.71) | 9.97 ± 3.34 (8.84-11.10) | 9.14 ± 3.70 (7.88-10.39) | 9.47 ± 3.67 (8.23-10.71) | 0.112 |

**Figure 9.1:** The inter-cycle variation in the mean (error bars of S.E.M) antral follicle counts of both and individual ovaries assessed using repeated measures analysis are shown in figure below. P values are marked at levels of comparison showing significant change (*=P<0.05; **=P<0.01; ***=P<0.001).
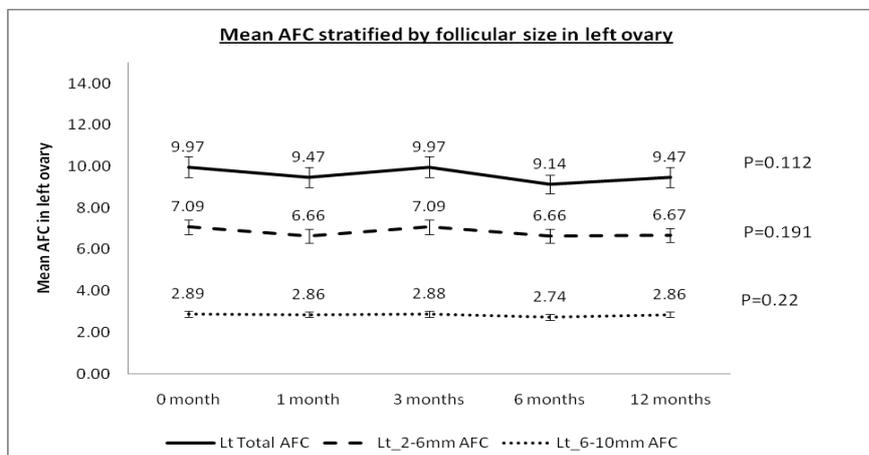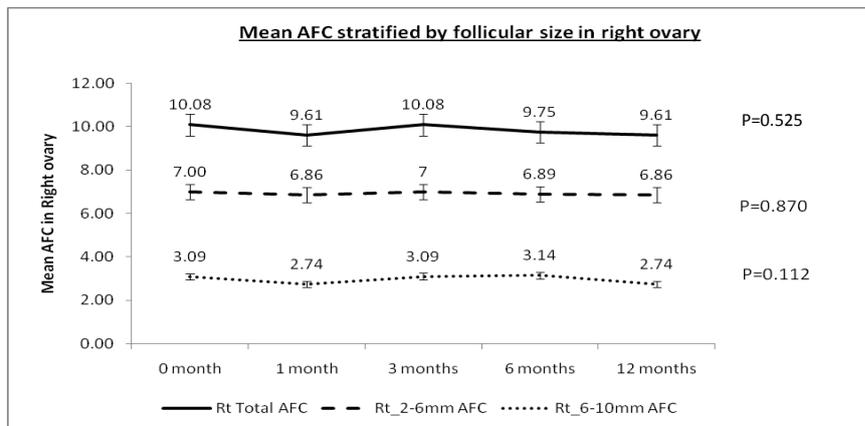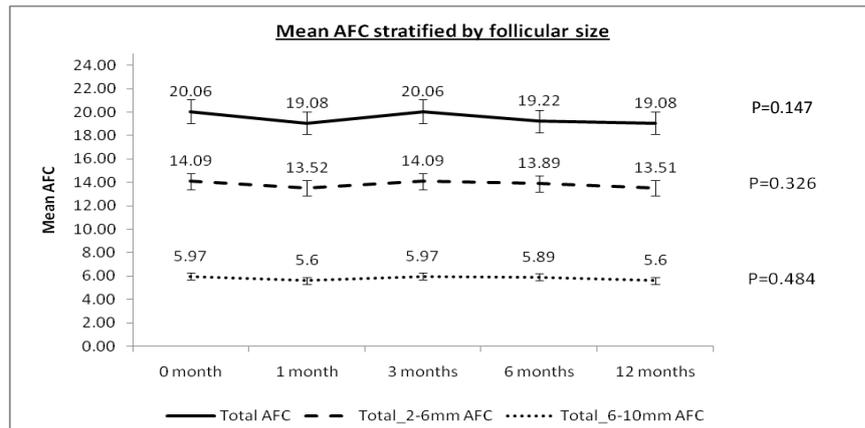
Following the stratification of antral follicles based on their size, two main groups of small antral follicles (2.0 – 6.0 mm) and large antral follicle (>6.0 – 10.0 mm) were further analysed. Both, small and large antral follicle numbers did not differ over 12 months (P=0.326 and P=0.484 respectively). Both the groups of antral follicles again did not change significantly at different levels of contrast (P>0.05) (Figure 9.1). A similar non-significant difference was noted when the antral follicle counts of each right and left ovaries were analysed (P>0.05) (Figure9.1). Antral follicle count measuring 2.0-10.0 mm and 2.0-6.0 mm showed excellent correlation in their counts across 5 cycles in 12 months examined (0.876, P<0.001 and 0.916, P<0.001 respectively). Antral follicles measuring >6.0-10mm showed a significant but lower correlation than the smaller antral follicles (0.379, P=0.027). The ICCs for AFC measuring 2.0-10.0 mm, 2.0-6.0mm, and >6.0-10 mm across 5 cycles over 12 months were 0.89 (95% CI, 0.83 – 0.94), 0.95 (95% CI, 0.92 – 0.97) and 0.51 (95% CI, 0.35 – 0.67) respectively. 89%, 95% and 51% of variation in AFC respectively therefore could be attributed to between-subject variation; and that 11%, 5% and 49% respectively could be due to intra-subject variation.

The inter-cycle change in mean AMH levels over a period of 12 months was not significant (P=0.063) with a non-significant change in means at different levels of contrast (P>0.05) (Table 9.2 and Figure 9.2). The correlation of AMH levels between cycles was excellent, with a correlation coefficient of 0.918 (P<0.001). The ICC for AMH across five cycles over 12 months was 0.91 (95% CI, 0.85 – 0.95). 91% of variation therefore could be attributed to between-subject variation and that only 9% is the intra-individual variation between cycles.

Mean serum FSH, LH and progesterone levels differed significantly across five cycles over a period of 12 months (P=0.027, P=0.033 and P=0.023 respectively). However, Oestradiol levels did not show a significant inter-cycle change (P=0.517) (Table 9.2).

**Table 9.2:** Comparison of the Mean ± SD (95% CI) of the endocrine determinants of ovarian reserve measured in five menstrual cycles at 0, 1, 3, 6, and 12 months in healthy volunteers with normal menstrual cycles. The P value was derived using Hotelling's trace multivariate test of repeated measures analysis.

| | 0 month N=38 | 1 month N=38 | 3 months N=38 | 6 months N=36 | 12 months N=30 | P value |
|---|---|---|---|---|---|---|
| **AMH ng/mL** | 2.76 ± 1.45 (2.20–3.33) | 2.66 ± 1.84 (1.95-3.38) | 2.88 ± 1.78 (2.19-3.57) | 3.10 ± 1.68 (2.45-3.75) | 2.99 ± 1.65 (2.36-3.64) | 0.053 |
| **FSH IU/L** | 6.64 ± 2.75 (5.57-7.70) | 7.81 ± 2.48 (6.84-8.77) | 6.97 ± 1.76 (6.29-7.66) | 7.06 ± 3.45 (5.73-8.40) | 7.84 ± 2.91 (6.71-8.97) | 0.027 |
| **LH IU/L** | 5.86 ± 2.45 (4.91-6.81) | 4.87 ± 1.89 (4.13-5.60) | 5.71 ± 1.76 (5.02-6.39) | 5.58 ± 2.02 (4.79-6.36) | 5.69 ± 2.27 (4.81-6.58) | 0.033 |
| **Oestradiol pmol/L** | 149.04 ± 51.79 (131.09-160.30) | 147.05 ± 42.35 (136.83-175.48) | 157.39 ± 51.39 (144.10-183.84) | 159.07 ± 54.21 (142.23-188.23) | 148.36 ± 42.17 (138.89-172.23) | 0.517 |
| **Progesterone pmol/L** | 1.34 ± 0.67 (1.12-1.57) | 1.25 ± 0.86 (0.96-1.54) | 1.03 ± 0.38 (0.91-1.16) | 1.53 ± 1.27 (1.10-1.96) | 1.32 ± 0.59 (1.13-1.52) | 0.023 |

**Figure 9.2:** The variation in the mean (error bars of S.E.M) levels of endocrine markers of ovarian reserve using repeated measures analysis are shown in figure below. P values are marked at levels of comparison showing significant change (*=P<0.05; **=P<0.01; ***=P<0.001).
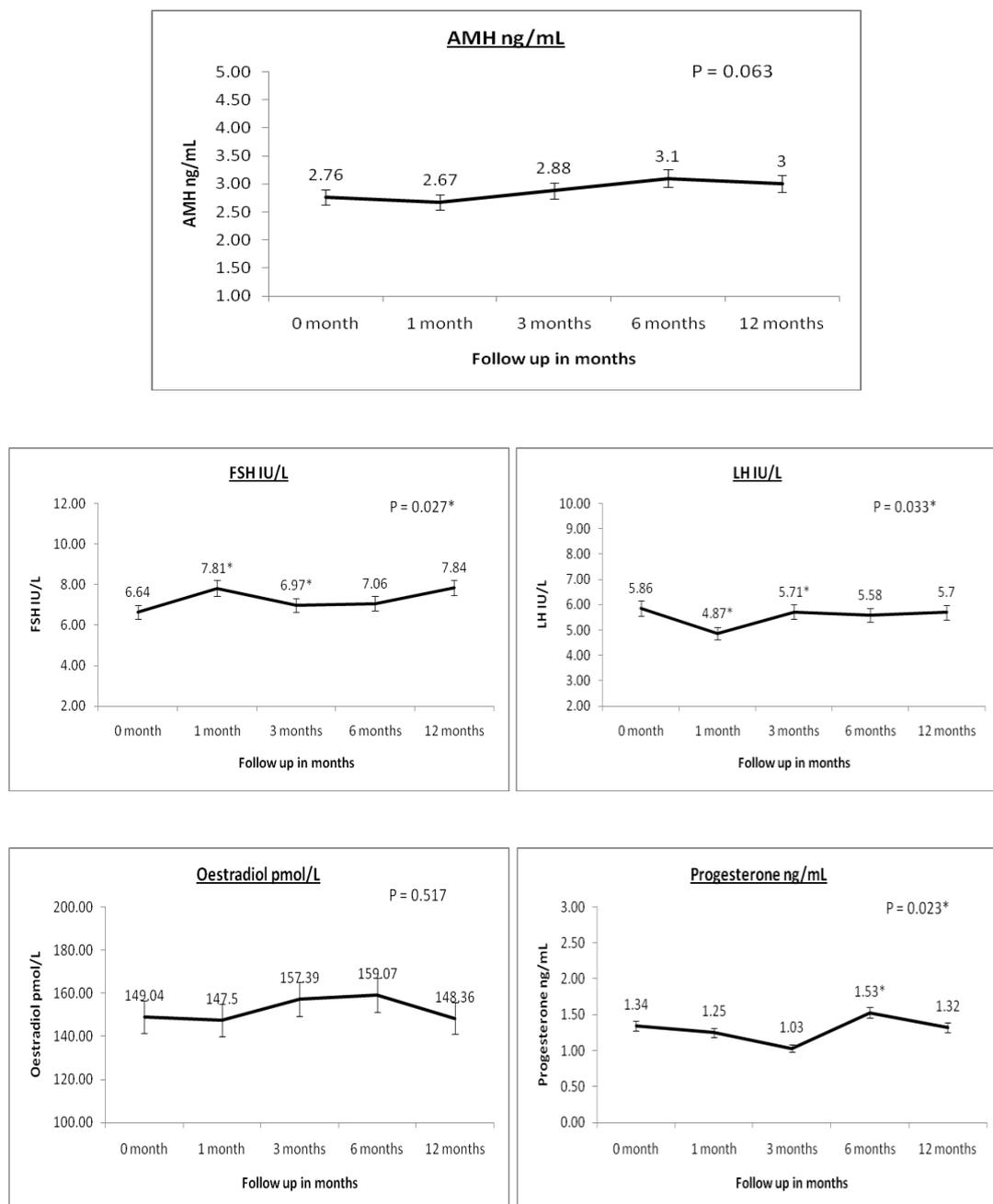


A significant inter-cycle correlation in FSH levels was seen, with a correlation coefficient of 0.599 (P=0.042). A poor correlation between cycles was seen in LH, Oestradiol and

progesterone levels, with correlation coefficients of 0.433 (P=0.108), 0.446 (P=0.135) and 0.090 (P=0.618) respectively. The ICCs for FSH, LH, Oestradiol and progesterone were 0.67 (95% CI, 0.52 – 0.80), 0.50 (95% CI, 0.32 – 0.68), 0.27 (95% CI, 0.11 – 0.48) and 0.19 (95% CI, 0.06 – 0.36) respectively. In other words, variation of 67% in FSH, 50% in LH, 27% in Oestradiol and 19% in progesterone levels is due to between-subject variation; and that a much higher proportion of variation (33%, 50%, 73% and 81% respectively) due to intra-individual fluctuation of levels between cycles.

## 9.5  *Discussion*

This is the first study to quantify markers of ovarian reserve at five time points across a 12 month period. The results indicate that the small AFC measuring 2.0-6.0 mm showed the least variation between five cycles over a period of 12 months followed by serum AMH and total AFC measuring 2.0-10.0 mm. A high inter-cycle correlation was observed in these markers with significant intra class correlation coefficients suggesting that there is only a small intra-individual variation in these markers between cycles.

A high level of correlation exists between AMH levels and the small antral follicle population (Jayaprakasan, Deb et al. 2009), which is believed to reflect the expression of AMH by this follicular cohort (Hansen, Morris et al. 2003; Weenen, Laven et al. 2004).The larger antral follicles express negligible amounts of AMH and are fewer in number in the early follicular phase (mean ± SD: 5.08 ± 1.78) than small AFC measuring 2.0-6.0 mm (mean ± SD: 13.82 ± 5.91). The chapter on intra-cycle variation in antral follicle counts shows that the number of larger antral follicles significantly increase from the late follicular to the peri-ovulatory phase of menstrual cycle and then the numbers drop in the

luteal phase to levels comparable to those in the early follicular phase, perhaps indicating that this trend persists between cycles showing little inter-cycle change in the early follicular phase of the menstrual cycle. The small antral follicles are often referred to as the 'selectable follicles' as they are responsive to gonadotrophins but unlike the larger antral follicles measuring more than 4.0 mm are not dependent on it (Gougeon 1989). The levels of gonadotrophins are low in the early follicular phase and therefore perhaps the numbers of larger antral follicles measuring more than 6.0 mm are fewer in this phase of menstrual cycle. The total AFC measuring 2-10 mm in the early follicular phase mainly comprises of antral follicles less than 6.0mm thereby demonstrating a non-significant inter-cycle change in this study. The results from this study in conjunction with those from Chapter 8 suggest that to ensure a reliable measure of ovarian reserve, the tests including the total AFC (2.0-10.0 mm) and the AFC of larger antral follicles (> 6.0 mm) are best measured in the early follicular phase, whereas the small AFC (2.0-6.0 mm) could be measured in any phase of menstrual cycle.

Studies that have looked at inter-cycle variation in the total AFC (2.0-10.0 mm) over more than two cycles, showed a non-significant change in the mean counts between cycles, but a greater intra-individual variation between cycles when compared to our study (Fanchin, Taieb et al. 2005; van Disseldorp, Lambalk et al. 2010). Both groups studied a similar group of sub-fertile women who underwent assisted reproduction treatment (ART) including intrauterine insemination (IUI) followed by IVF. Fanchin et al examined variation over three consecutive cycles and vanDisseldorp et al examined four cycles. They reported ICCs of 0.73 and 0.71 respectively for tAFC (2.0-10.0 mm), which suggested significantly higher within-subject cycle variation than those observed in our study (ICC 0.89). Furthermore, the ICC for small AFC (2.0-6.0 mm) at 0.95 in our study was even better, but

unfortunately small antral follicles were not examined in the above two studies. The within-subject variation in AMH levels in our study were comparable to those reported in these two studies.  The difference in results, in relation to AFC could be attributed to the use of 3D ultrasound and SonoAVC; and also that 5 cycles were examined in our study over a period of twelve months. SonoAVC is shown to make more reliable and valid counts as shown in previous chapters (Deb, Batcha et al. 2009; Deb, Jayaprakasan et al. 2009; Deb, Campbell et al. 2010). Inter-cycle variability of tAFC (2.0-10.0 mm) alone measured using 2D ultrasound  has also been studied previously and shown to be moderate in most studies (Scheffer, Broekmans et al. 1999; Hansen, Morris et al. 2003; Elter, Sismanoglu et al. 2005). A degree of variation in antral follicle counts and levels of AMH might prove desirable when selecting an optimum time for commencing ART thereby improving ovarian response and outcome. Future trials designed to look at this concept might provide further understanding of physiological variations in the markers of ovarian reserve.

Only one study that used 3D ultrasound to examine the inter-cycle variation between two cycles in sub-fertile population, measured tAFC (2.0-10.0) using the manual method of counting antral follicles in a multiplanar view (Jayaprakasan, Campbell et al. 2008). They reported that the inter-cycle variability in tAFC may mainly be caused by observer variability and that the true biological variation may be minimal. In our study, the use of SonoAVC by the same experienced observer on the same machine is likely to reduce the inter- and intra-observer variation and thereby enable true reflection of within-subject inter-cycle variation.

The current data also suggest a non-significant inter-cycle change in the mean AMH levels; and a high correlation between cycles over 12 months duration. The ICC of 0.91 seen in

our study was comparable to ICC of 0.89 reported in previous studies (Fanchin, Taieb et al. 2005; van Disseldorp, Lambalk et al. 2010). Other studies looking at variation between two cycles, have reported minimal inter-cycle variation in AMH levels (McIlveen, Skull et al. 2007; Streuli, Fraisse et al. 2008). In our study, the inter-cycle stability of AMH (ICC - 0.91) was comparable to that seen with tAFC (2.0-10.0 mm) (ICC - 0.89) and small AFC (2.0-6.0 mm) (ICC - 0.95).

A significant inter-cycle variation was seen in the mean FSH, LH and progesterone levels; however variation in mean oestradiol levels over 12 months was not significant. The correlation of LH, progesterone and Oestradiol between cycles was poor; however a moderate correlation was seen with FSH between cycles. Since there is no consensus on the basal Oestradiol levels that could predict ovarian response and pregnancy outcome following ART, Oestradiol is not routinely performed as a test for ovarian reserve (Broekmans, Kwee et al. 2006). Although, in our study the mean Oestradiol levels did not a show a significant variation between cycles, the correlation was poor and the ICC low at 0.27, suggesting that the majority of variation seen between cycles is due to intra-individual fluctuation in levels between cycles.  Elter et al investigated inter-cycle variability of FSH, total AFC and ovarian volume in 50 infertile women undergoing ART. They found the least inter-cycle variation in FSH levels followed by volume and tAFC (Elter, Sismanoglu et al. 2005). In contrast, the results from our study show a significantly lower inter-cycle variation in tAFC than FSH (ICC: 0.89 vs 0.67). The difference in these findings could be due to the use of 3D ultrasound assisted SonoAVC and the larger number of cycles examined in our study.

## *9.6    Conclusion*

The ovarian reserve as measured using small antral follicle count (2.0-6.0mm) and AMH show little inter-cycle variation and an excellent within subject correlation. The inter-cycle stability of total antral follicle count (2.0-10.0 mm) when performed in the early follicular phase of menstrual cycle appears to be comparable to AMH and small AFC. These determinants of ovarian reserve demonstrate inter-cycle stability for up to 12 months and hence future studies designed to evaluate their ability to predict ovarian response following assisted reproduction treatment should consider performing these tests of ovarian reserve once in a year, irrespective of the number of ART cycles required.

**CHAPTER 10: Quantifying the effect of the combined oral contraceptive pill on the functional ovarian reserve as measured by serum anti-Müllerian hormone and the small antral follicle count made using three-dimensional ultrasound**

## 10.1  Abstract

**Objective:** Oral contraceptive pills suppress the hypothalomo-pituitary-axis. This can affect the ultrasound and endocrine markers used to examine ovarian reserve. The objective of this study was to quantify the ultrasound and endocrine markers of functional ovarian reserve in women using combined oral contraceptive pill (COCP) for a prolonged duration of more than a year.

**Methods:** This study is a prospective case-control study on healthy volunteers. 34 subjects using COCP with hormone-free-interval (HFI) for more than one year were compared to 36 normo-ovulatory, age-matched controls that had not used hormonal contraception for more than one year. Volunteers taking COCP had a 3D ultrasound scan and blood sample within the first 4 days of active pill ingestion and those in the control group had the scan and blood test in the early follicular phase (days 2-5) of menstrual cycle. The main outcome measure was the differences in antral follicle counts stratified according to size, AMH, FSH, LH and oestradiol levels.

**Results:** There were no significant differences in the number of small antral follicles measuring 2-6mm. The COCP group had significantly lesser antral follicles measuring ≥ 6mm (P<0.001), had significantly smaller ovaries (P<0.001) which also had lower vascular indices than the control group (P<0.05). Whilst serum FSH, LH and oestradiol levels were significantly lower in COCP group (P<0.05), there was no significant difference in serum AMH levels between the two groups.

**Conclusion:** Prolonged use of COCP suppressed pituitary gonadotrophins and antral follicle development beyond 6mm but had no effect on levels of serum AMH and small antral follicles.

## 10.2  Introduction

The combined oral contraceptive pill (COCP) is an effective and popular method of reversible contraception (2001; Skouby 2004). The contraceptive effect is brought about by suppression of the hypothalamo-pituitary-ovarian axis, which inhibits follicular growth, maturation of the dominant follicle and ovulation, and as a result of their affect on the endometrium and cervical mucus (Mishell, Kletzky et al. 1977; group 2001). The hormone-free interval (HFI) during which a withdrawal bleed normally occurs in a standard oral contraceptive regime, results in some recovery of the pituitary-ovarian axis (Montloin 1990; van der Spuy, Sohnius et al. 1990), which leads to an increase in the levels of gonadotrophins and, eventually, in the resumption of follicle growth and sex steroid production (van Heusden and Fauser 1999; Schlaff, Lynch et al. 2004). Some ovarian activity, as measured by follicular growth beyond 10 mm, is present without ovulation during the pill use (Crosignani, Testa et al. 1996) but a HFI of up to 7 days does not affect the contraceptive efficacy of the COCP (Fraser and Jansen 1983; Killick, Bancroft et al. 1990). It is less clear whether the pituitary-ovarian suppression induced by the COCP or its recovery during the HFI has any impact on functional ovarian reserve, which is reflected by the number of small antral follicles and serum anti-Müllerian hormone (AMH) levels (La Marca, Sighinolfi et al.; Broekmans, Visser et al. 2008; Deb, Batcha et al. 2009; Jayaprakasan, Deb et al. 2009).

The pool of primordial follicles is finite and folliculogenesis a continuous process that depletes this reserve. During the late luteal phase, with the regression of the corpus luteum and consequent rise in  follicle stimulating hormone (FSH), the 2–5 mm antral follicles are recruited into a pool of selectable follicles from which the follicle destined to ovulate in the subsequent cycle will be selected  (Gougeon and Lefevre 1983). It is not known whether suppression of pituitary-ovarian axis by the COCP influences the recruitment and growth of these selectable antral follicles.

This study was therefore designed to quantify the effect of pituitary-ovarian axis suppression induced by use of the COCP on functional ovarian reserve as estimated by the number of small antral follicles and serum AMH levels. Our hypothesis was that ovarian reserve will be affected with the use of COCP.

## 10.3   Methods

**Study design**

The study design was that of a prospective observational nature comparing the experimental group to age matched controls. Study participants described as 'healthy volunteers' were recruited through advertisements on the intranet portal of the University of Nottingham (UoN) website, and posters displayed in staff canteens, notice boards and staff library. Further information about the study was communicated via email and information leaflets to those who expressed interest in participation in the study.

The main inclusion criteria were: age between 18 to 35 years, non-smoker, absence of menstrual irregularities, and no past history of ovarian surgery. Those volunteers who had

been using COCP containing 30 micrograms of ethinyloestradiol and 150 micrograms of levonorgestrel (Microgynon 30, Schering AG, Germany) for more than a year were included in the experimental group (COCP group). They had been using the COCP on a regular basis and were having monthly withdrawal bleeds during the hormone-free-interval. Those volunteers who had not used COCP or any other form of hormonal contraceptive for more than a year were included in the control group. The main exclusion criterion was presence of ovarian pathology such as ovarian cysts identified on a baseline ultrasound scan undertaken after recruitment. This scan was conducted during the first 4 days of active pill ingestion in the experimental group and during the early follicular phase of the menstrual cycle (between days 2-5) in the control group and if the patient had no pelvic pathology, venepuncture was undertaken and serum stored for subsequent analysis.

A sample size of 30 was required in each arm to power the study at 0.85, for an assumed variation of 4 antral follicles in the total AFC between groups and a standard deviation of 2.5.

**Data acquisition and analysis**

A single investigator (S.D) performed all of the ultrasound scans using a Voluson E8 Expert (GE Medical Systems, Zipf, Austria) and a three-dimensional 5-9 MHz transvaginal transducer. The ultrasound assessment involved a two-dimensional (2D) ultrasound assessment of the pelvis to exclude any pelvic pathology, followed by acquisition of 3D volume data. The acquired 3D data were displayed on the ultrasound machine (Voluson E8 Expert) in the multiplanar view and processed further using SonoAVC and VOCAL to

obtain antral follicle sizes and ovarian volume respectively. The technique and method used was similar to that used for studies in previous chapters.

**Hormonal Assays**

Blood samples were collected from each subject and centrifuged within 30 min of venepuncture for 20 min at 4$^{\circ}$C and 4000 rpm spin to separate the serum. The processing, storage and analysis of the samples was similar to that used for studies in previous chapters.

**Statistical analysis**

The Statistical Package for the Social Sciences (version 16.0; SPSS, Chicago, IL) was used for statistical analysis**.** The distribution of the data was checked for normality using a normal probability plot. The data are expressed as mean (± SD) or median (± range) and differences between groups were examined using an independent student's t test or Mann Whitney U test dependent on the distribution of the data. The relationship of each follicular cohort, stratified according to the mean diameter of the follicles, with the endocrine markers including AMH, FSH, LH , and Oestradiol was evaluated using Pearson's or Spearman's correlation coefficient (r). A P-value of less than 0.05 was considered statistically significant.

## *10.4  Results*

34 subjects that were taking combined oral contraceptive pill containing 30 micrograms of ethinyloestradiol and 150 micrograms of levonorgestrel (Microgynon 30, Schering AG, Germany) for more than one year were evaluated in the experimental group. 36 subjects who had not used any form of hormonal contraception for more than one year were

evaluated in the control group. There were no differences between the groups in age or body mass index. The mean ± SD (range) age of the COCP group was 25.35 ± 4.23 (20 – 34.6) years and that of control group was 27.16 ± 4.63 (20.30 – 35) years (P=0.137).  The mean ± SD (range) BMI of the COCP group was 23.32 ± 3.41 (16.60 – 30.41) and that of control group was 23.76 ± 2.70 (18.31 – 29.69) (P=0.599).

The experimental group had used the COCP for an average of 3.16 years (range: 2.4 to 6 years). In this group, a significant correlation was seen between AMH and the total number of antral follicles measuring 2 to 10 mm (r=0.741, P<0.001). However, on evaluating the relationship between AMH and antral follicles stratified into cohorts of 1 mm, a significant correlation was only seen with the smaller antral follicles that measured less than 6 mm (r=0.724, P<0.001). Larger antral follicles, measuring 6 to 10 mm, did not correlate with serum AMH levels (r=0.284, P=0.121). There was no significant correlation seen between the total antral follicle counts (AFC) or follicles of different sizes and any of the other endocrine markers including FSH, LH, and Oestradiol in the experimental group using the COCP (Table 10.1).

**Table 10.1**: The correlation between various antral follicle cohorts stratified by size, AMH, FSH, LH, and Oestradiol in women using the combined oral contraceptive pill (30 mcg of ethinyl Oestradiol and 150 mcg of levonorgesterel) for more than a year. The relationships are presented as the Pearson's correlation coefficient (r) and its level of significance (P value)

| Antral follicle size (mm) | AMH r (p value) | FSH r (P value) | LH r (P value) | E2 r (P value) |
|---|---|---|---|---|
| 2.0 to 2.99 | 0.281 (0.126) | -0.154 (0.408) | -0.074 (0.692) | 0.133 (0.475) |
| 3.0 to 3.99 | 0.492 (0.010) | 0.108 (0.563) | -0.184 (0.322) | 0.231 (0.211) |
| 4.0 to 4.99 | 0.627 (<0.001) | -0.023 (0.903) | 0.183 (0.324) | -0.015 (0.937) |
| 5.0 to 5.99 | 0.700 (<0.001) | -0.032 (0.866) | 0.150 (0.419) | 0.246 (0.182) |
| 6.0 to 6.99 | 0.302 (0.082) | -0.066 (0.723) | -0.72 (0.701) | 0.303 (0.098) |
| 7.0 to 7.99 | 0.284 (0.121) | -0.007 (0.968) | 0.121 (0.518) | -0.007 (0.969) |
| 8.0 to 8.99 | 0.320 (0.079) | -0.368 (0.042) | -0.330 (0.70) | -0.103 (0.581) |
| 9.0 to 9.99 | -0.048 (0.800) | -0.035 (0.852) | 0.002 (0.989) | 0.129 (0.488) |
| **Antral follicle counts** | | | | |
| 2.0 to 3.99 (2-4) | 0.396 (0.028) | -0.159 (0.393) | -0.165 (0.375) | 0.230 (0.214) |
| 2.0 to 4.99 (2-5) | 0.625 (<0.001) | -0.121 (0.518) | -0.007 (0.9710 | 0.147 (0.431) |
| 2.0 to 5.99 (2-6) | 0.724 (<0.001) | -0.098 (0.60) | 0.056 (0.767) | 0.203 (0.274) |
| 2.0 to 8.99 (2-9) | 0.750 (<0.001) | -0.115 (0.538) | 0.021 (0.910) | 0.236 (0.201) |
| 2.0 to 9.99 (2-10) | 0.741 (<0.001) | -0.116 (0.535) | 0.021 (0.910) | 0.241 (0.192) |
| 6.0 to 9.99 (6-10) | 0.284 (0.121) | 0.027 (0.782) | 0.102 (0.504) | 0.098 (0.571) |

**Figure 10.1:** Comparison of the antral follicles stratified by size between the experimental group (n

= 34), who had been using the COCP for more than one year, and the control group (n = 36).
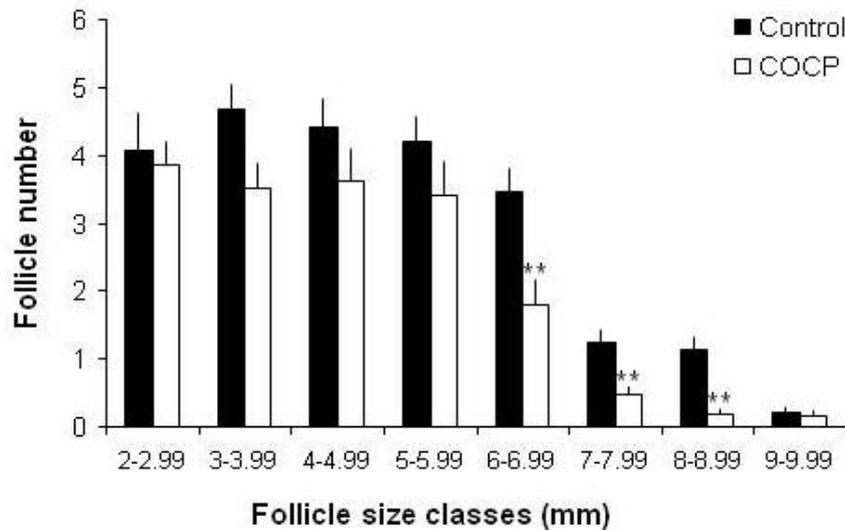


An 'independent samples t test' confirmed there was no significant difference between

the experimental group and the control group in the number of small antral follicles

measuring less than 6 mm (P=0.127). The COCP group, that had used hormonal

contraceptive for more than a year, had significantly lesser antral follicles measuring 6 to

10 mm than the control group (2.65 ± 2.93 versus 6.04 ± 3.25; P<0.001). This was

reflected in the total AFC, which was significantly lower as a result of these differences in

the COCP group (17.06 ± 8.55 vs 23.42 ± 8.00; P=0.007). The COCP users had significantly

smaller ovaries (4.38 ± 2.37 vs 7.44 ± 2.66 $cm^3$; P<0.001) which were also less vascular

than the control group (VI: P=0.008; FI: P=0.047; and VFI: P=0.002) (Table 10.2 and Figure

10.1). Comparison of the endocrine markers, including AMH, FSH, LH, and E2, between

the two groups is shown in Table 10.3. Whilst there was no significant difference seen in

the levels of serum AMH between the two groups, serum FSH, LH, and E2 were all significantly lower in the COCP group than the control group (P < 0.05).

**Table 10.2**: Comparison of the ultrasound markers of ovarian reserve between the experimental group (n = 34), who had been using the COCP for more than one year, and the control group (n = 36).

| Variable | Experimental group: COCP user (n=34) | | Control group (n=36) | | P value |
|---|---|---|---|---|---|
| | Mean ± SD | Range | Mean ± SD | Range | |
| **Antral follicle size (n)** | | | | | |
| **2.0 – 2.99 mm** | 3.87 ± 1.88 | 1 – 8 | 4.08 ± 3.35 | 0 – 13 | 0.767 |
| **3.0 – 3.99 mm** | 3.52 ± 2.23 | 0 – 9 | 4.67 ± 2.35 | 0 – 9 | 0.070 |
| **4.0 – 4.99 mm** | 3.61 ± 2.81 | 0 – 11 | 4.42 ± 2.45 | 1 – 9 | 0.272 |
| **5.0 – 5.99 mm** | 3.42 ± 2.80 | 0 – 9 | 4.21 ± 2.21 | 0 – 10 | 0.262 |
| **6.0 – 6.99 mm** | 1.81 ± 2.09 | 0 – 10 | 3.46 ± 2.04 | 0 – 7 | 0.005 |
| **7.0 – 7.99 mm** | 0.48 ± 0.63 | 0 – 2 | 1.25 ± 1.11 | 0 – 3 | 0.002 |
| **8.0 – 8.99 mm** | 0.19 ± 0.40 | 0 – 1 | 1.13 ± 1.15 | 0 – 3 | 0.001 |
| **9.0 – 9.99 mm** | 0.16 ± 0.45 | 0 – 2 | 0.21 ± 0.41 | 0 – 1 | 0.694 |
| **Antral follicle counts (n)** | | | | | |
| **2 to 4 mm** | 7.39 ± 3.33 | 2 – 17 | 8.75 ± 4.84 | 2 – 19 | 0.222 |
| **2 to 5 mm** | 11 ± 4.93 | 4 – 23 | 13.17 ± 6.08 | 3 – 24 | 0.156 |
| **2 to 6 mm** | 14.42 ± 6.97 | 5 – 32 | 17.38 ± 7.08 | 7 – 33 | 0.127 |
| **2 to 9 mm** | 16.90 ± 8.47 | 5 – 36 | 23.21 ± 7.85 | 10 – 43 | 0.007 |
| **2 to 10 mm** | 17.06 ± 8.55 | 5 – 36 | 23.42 ± 8.00 | 10 – 43 | 0.007 |
| **6 to 10 mm** | 2.65 ± 2.93 | 0 – 14 | 6.04 ± 3.25 | 1 - 13 | <0.001 |
| **Ovarian volume (cm$^3$)** | 4.38 ± 2.37 | 1.56 – 11.02 | 7.44 ± 2.66 | 3.15 – 14.21 | <0.001 |
| **Vascularisation Index (VI: %)** | 3.46 ± 2.72 | 0.09 – 13.4 | 5.58 ± 2.96 | 0.09 – 13.04 | 0.008 |
| **Flow Index (FI: 0_100)** | 29.70 ± 5.22 | 18.12 – 42.87 | 32.68 ± 5.59 | 16.95 – 43.17 | 0.047 |
| **Vascularisation Flow Index (VFI: 0_100)** | 1.19 ± 1.11 | 0.03 – 5.36 | 2.39 ± 1.67 | 0.03 – 6.32 | 0.002 |

**Table 10.3**: Comparison of the endocrine markers of ovarian reserve between the experimental group (n = 34), who had been using the COCP for more than one year, and the control group (n = 36). $*$ Estimated values of AMH in pmol/L using the conversion factor 1 ng/mL = 7.14 pmol/L.

| Variable | Experimental group: COCP user (n=34) | | Control group (n=36) | | P value |
|---|---|---|---|---|---|
| | Mean ± SD | Range | Mean ± SD | Range | |
| FSH (IU/L) | 4.73 ± 3.86 | 0.44 – 15.39 | 6.59 ± 0.93 | 4.99 – 8.33 | 0.015 |
| LH (IU/L) | 3.46 ± 3.17 | 0.38 – 10.39 | 5.76 ± 2.52 | 1.43 – 11.29 | 0.005 |
| Oestradiol (pmol/L) | 95.58 ± 56.77 | 2 – 217 | 149.58 ± 8.27 | 84 – 361 | 0.001 |
| AMH (ng/mL) | 2.75 ± 1.59 | 0.23 – 6.34 | 3.06 ± 1.27 | 1.46 – 5.88 | 0.440 |
| AMH (pmol/L)[*] | 19.63 ± 11.36 | 1.65 – 45.30 | 21.83 ± 9.05 | 10.45 – 42 | 0.440 |

## 10.5  Discussion

This is the first study to quantify the effect of the combined oral contraceptive pill with hormone free interval on the small antral follicle counts and AMH using 3D ultrasound assisted semi-automated method, SonoAVC. Our results show that despite lower serum levels of gonadotrophins in the COCP users, the population of small antral follicles measuring 2 to 6 mm and serum AMH levels are comparable to those seen in non-pill users. These results are consistent with the fact that the pool of selectable antral follicles (2-5 mm) are gonadotrophin responsive but not dependant and that the antral follicles measuring more than 6 mm are gonadotrophin dependant (Gougeon and Lefevre 1983).

In this study, serum AMH concentrations were the only endocrine marker that was comparable between the COCP users and the control group. Further, a significant positive correlation was noted between AMH and small antral follicles measuring up to 6mm in both the experimental and the control group, but there was no relationship with larger antral follicles, FSH, LH and E2 levels. These results are consistent with the observations of Weenen et al who have shown that AMH is mainly expressed by the pre antral and small antral follicle population up to a diameter of around 4mm (Weenen, Laven et al. 2004). Since the majority (70%) of the total antral follicle counts include these small antral follicles, a significant positive correlation was also observed between AMH and total AFC in the current study and these results are comparable to the correlation seen between AMH and antral follicles in normal (van den Berg, van Dulmen-den Broeder et al.; La Marca, Stabile et al. 2006) and sub-fertile populations (Weenen, Laven et al. 2004; Broekmans, Visser et al. 2008; Haadsma, Groen et al. 2009; Jayaprakasan, Deb et al. 2009). As with the current study, Jayaprakasan et al also have shown a strong significant correlation between AMH and follicles measuring less than 6mm (Jayaprakasan, Campbell et al. 2008; Jayaprakasan, Deb et al. 2009). Further, results from chapter 5 have shown that small antral follicles are a significant independent predictor of ovarian response to controlled stimulation, and clinical pregnancy rates following IVF treatment and these findings are in agreement with those reported by other investigators (Fanchin, Schonauer et al. 2003; Eldar-Geva, Ben-Chetrit et al. 2005; Fanchin, Taieb et al. 2005; Penarrubia, Fabregues et al. 2005; Fleming, Deshpande et al. 2006). Overall, these findings support the interpretation that the number of small antral follicles and circulating AMH concentrations more accurately reflect the functional ovarian reserve. Although some studies suggest, that the total number of antral follicles is predictive of reproductive

response (van Rooij, Broekmans et al. 2002; Hendriks, Mol et al. 2005; Muttukrishna, McGarrigle et al. 2005), this can be explained by the fact that the smaller, more responsive antral follicles comprise the majority of the follicle population included in the total antral follicle count in these individuals.

Although the HFI has an impact on reversal of the inhibition of pituitary ovarian axis, it is interesting to note that the number of antral follicles measuring more than 6mm were significantly lower in COCP users when compared to the control group, suggesting that the reduction in gonadotrophins in COCP users affects the growth of gonadotrophin dependent antral follicles but not the gonadotrophin responsive preantral and small antral follicles. It is well established from both in vitro (Spears 1994; Chun, Eisenhauer et al. 1996; Newton, Picton et al. 1999; Wright, Hovatta et al. 1999) and in vivo (Hsueh, Eisenhauer et al. 1996; McGee, Perlas et al. 1997; Hsueh, McGee et al. 2000; Campbell, Telfer et al. 2004) studies that FSH is a major survival factor for preantral and early antral follicles, the stage during which a majority of follicle undergo atresia under physiological conditions (Gougeon 1996). However, due to the range of approaches and doses used in these animal studies, it is difficult to determine the minimum amount of FSH that is required to influence the survival of these preantral and small antral follicles. From the present study, it is clear that a relatively modest suppression of pituitary gonadotrophins in our study did not significantly affect the number of selectable antral follicles but was enough to significantly reduce the number of large antral follicles greater than 6mm in diameter.

Surprisingly, there are relatively fewer publications in the literature examining the effect of the COCP on ovarian function in women. Van den berg et al. studied 25 women who had been taking low dose COCP for an average of 1.8years and followed them for two

subsequent natural menstrual cycles after stopping the pill.  They found that FSH and

inhibin B levels significantly decreased; AMH, total AFC (antral follicles measuring 2 to

10mm), and ovarian volume significantly increased; and that levels of LH and E2 did not

significantly change after discontinuation of hormonal contraception (van den Berg, van

Dulmen-den Broeder et al.). In our study the total AFC was significantly higher in the

control group which is comparable to the above study, but this was mainly due to the

large antral follicles. The results of endocrine profile in the above study contrast with

those of our study. We found that AMH does not significantly differ between the two

groups, but the FSH, LH, and E2 levels reduce significantly in pill users. These differences

may perhaps be due to the low dose COCP used in this study or the timing of blood

samples collected. This interpretation is supported by the results of Somunkiran et al, who

studied 15 normal women who were given COCP containing 35 mcg of ethinyl oestradiol

and 2mg cyproterone acetate for 6 months and the endocrine and ultrasound markers of

ovarian reserve compared pre and post treatment (Somunkiran, Yavuz et al. 2007). They

found that AMH levels did not change significantly with the use of COCP, but FSH, LH, and

Oestradiol levels significantly reduced with the use of COCP. Further, a reduction in

ovarian volume and follicle number following COCP use was found and we have extended

this observation to show that only the larger antral follicle population measuring 6 mm or

more was significantly reduced by the use of COCP. We also found that the vascularity of

the ovaries was significantly lesser in COCP users than the control group. This could be

attributed to smaller ovaries and lesser number of larger antral follicles than the control

group.

The results of the current study are relevant to the use of COCP in assisted reproduction

treatment, including both IVF and ICSI. Paradoxically, oral contraceptive pills have been

used for down-regulation in both poor responders (Fisch, Royburt et al. 1996; al-Mizyen, Sabatini et al. 2000; Keltz, Gera et al. 2007) and in women at risk of ovarian hyper stimulation syndrome (OHSS) (Damario, Barmat et al. 1997) with beneficial effects having been reported in both the groups. In poor responders, the use of COCP prior to using GnRH analogue in short protocol in IVF has been shown to blunt the LH flare and it is thought that this might reduce the early rise in follicular androgens (Keltz, Gera et al. 2007), and prevent the rescue of corpus luteum resulting in better follicular recruitment and ovarian response (Gelety, Pearlstone et al. 1995; Biljan, Mahutte et al. 1998). Conversely, the pre treatment use of COCP in hyper-responders in order to improve the outcome and incidence of OHSS following IVF has been attributed to a reduction in LH levels leading to a fall in follicular androgens and less FSH-induced granulosa cell aromatase activity (Franks and Mason 1991; Damario, Barmat et al. 1997). Our results, which show that the functional ovarian reserve as measured by small antral follicles and AMH does not change with the COCP use; do not clearly support either of these interpretations although the fall in large antral follicles resulting from COCP use might be beneficial in patients at risk of OHSS. The benefits, therefore, of using the COCP as part of an assisted reproduction treatment cycles are unclear although it is possible that this may lead to a homogenous response to controlled ovarian stimulation by influencing the recruitment and growth of follicles and the yield and quality of the oocytes harvested. This possibility, however, requires evaluation in future trials.

## 10.6 Conclusion

This study has shown that prolonged use of combined oral contraceptive pill with hormone free interval suppressed pituitary gonadotrophins and decreased the volume and blood flow within the ovary.  It also suppressed the antral follicle development beyond 6 mm but had no effect on the smaller antral follicles measuring less than 6 mm or on serum AMH levels both of which are thought to be most reflective of functional ovarian reserve.

# CHAPTER 11: Effect of ovarian cystectomy on ovarian reserve as quantified by AMH and antral follicle counts measured by SonoAVC

## 11.1  *Abstract*

**Objective:** This study was designed to quantify the effect of ovarian cyst per se and that of laparoscopic ovarian cystectomy on the markers of ovarian reserve as measured using AMH, and 3D ultrasound assisted measures of small antral follicle count and total antral follicle count. The hypothesis was that ovarian cystectomy has a significant effect on the ovarian reserve.

**Methods:** It was a prospective observational study conducted in a university based assisted conception unit. Women diagnosed with an ovarian cyst of presumed benign nature on ultrasound scan were recruited. Ovarian reserve was evaluated in women with unilateral benign ovarian cyst pre operatively and then following laparoscopic cystectomy at one, three, and 6 months. AMH, FSH, and 3D assisted software - SonoAVC that made semi-automated antral follicle counts were used to quantify ovarian reserve. Repeated measures analysis was performed to examine the effect.

**Results:** AMH and FSH levels showed a significant decline post-operatively (P<0.05). A similar significant decline in antral follicle counts measuring 2.0-10.0mm and 2.0-6.0mm was observed in the ovary that underwent cystectomy (P<0.05). These total and small AFC's, however showed a non-significant decline in the contra-lateral normal ovary (P>0.05). A non-significant decline was observed in the number of larger antral follicles measuring >6.0 mm in both, the diseased and contra-lateral normal ovaries. Analysis of within-subject levels of follow up showed that total AFC and small AFC in the operated ovary continued to decline up until 3 months (P<0.05) post-operatively before stabilising at 6 months. Levels of serum AMH significantly dropped and that of FSH increased one

month post operatively and stayed at those levels 3 and 6 months later. On analysis based on cyst types, both endometrioma and dermoid cysts showed a significant drop in ovarian reserve which persisted up to 6 months following cystectomy (P<0.05), whereas a simple cyst showed a non-significant change (P>0.05). Effect with endometrioma was more pronounced than that with dermoid cyst (P<0.05)

**Conclusion:** Laparoscopic ovarian cystectomy is associated with a significant loss of ovarian reserve measured using AMH and small antral follicle counts, the effect lasting at least up to 6 months. Endometrioma itself significantly affects the ovarian reserve of the diseased ovary and that removal of endometrioma might cause more damage to ovarian reserve than the non-endometriotic cysts.

## 11.2  Introduction

Benign ovarian cysts commonly occur in premenopausal women and in majority are either endometrioma, mature cystic teratomas called dermoid cyst, benign cystadenomas or functional ovarian cysts. Endometriosis affects 3 - 10% of women in their reproductive age and is the leading cause of sub fertility, seen in about 25-35% of women with infertility (Jenkins, Olive et al. 1986; Mahmood and Templeton 1991). Endometriomas are identified in 17-44% of women with endometriosis (Chapron, Vercellini et al. 2002).  According to the implantation theory, the endometrial implants cause adherence of the ovary to pelvic peritoneum and progressively invaginate the ovarian cortex (Hughesdon 1957; Brosens, Puttemans et al. 1994). Another theory suggests that endometrioma is formed due to the metaplasia of coelomic epithelium which invaginate into the ovarian cortex (Donnez, Nisolle et al. 1996). These theories suggest that an endometrioma is perhaps a pseudo

cyst. Mature cystic teratomas or dermoids represent more than 10% of all ovarian neoplasm's and are the most common benign germ cell tumours of the ovary in women of reproductive age.

Laparoscopic cystectomy is usually performed for symptomatic ovarian cysts as can cause further pain and discomfort related to pressure on adjacent structures, torsion, rupture, haemorrhage (both within and outside of the cyst), and abnormal uterine bleeding (Chapron, Vercellini et al. 2002). In sub fertile women, they are often removed to improve chances of conception. Operative laparoscopy compared with laparotomy has been established as the gold standard surgical approach in the treatment of ovarian cysts in terms of reduced postoperative pain, analgesic requirement, hospitalization, and adhesion formation (Pados, Tsolakidis et al. 2006). Potential problems with surgical treatment of ovarian cysts are the small risk of post surgical ovarian failure of 2.4% especially when dealing with bilateral ovarian cysts (Busacca, Riparini et al. 2006), a moderate 30.4% risk of recurrence (Koga, Takemura et al. 2006) and a reduction in ovarian reserve. The principle of cystectomy is to either remove or destroy the cyst wall or capsule in order to prevent recurrence. The techniques commonly used therefore are stripping of the cyst from underlying normal ovarian tissue or drainage of the cyst with ablation of cyst wall. Following the excision of the cyst or stripping of the cyst wall from the ovarian cortex, it is a normal practice to achieve haemostasis in the cyst bed using some form of coagulation, either monopolar, bipolar, laser, or an ultrasonic device. The current body of evidence suggests that ovarian cysts, especially endometrioma, can affect the function of the adjacent normal ovarian tissue (Maneschi, Marasa et al. 1993), that stripping of ovarian cyst is associated with inadvertent loss of normal ovarian tissue

attached to it (Hachisuga and Kawarabayashi 2002; Muzii, Bianchi et al. 2002; Roman, Tarta et al. 2010), and finally coagulation or ablation achieved using electrical energy, laser, or ultrasonics is associated with loss of ovarian reserve (Li, Liu et al. 2009; Tsolakidis, Pados et al. 2009; Coric, Barisic et al. 2010). There are some recent studies showing less ovarian damage with the use of $CO_2$ laser and with the use of three-stage procedures when treating endometriomas (Donnez, Lousse et al. 2010; Pados, Tsolakidis et al. 2010; Tsolakidis, Pados et al. 2010). Few studies have suggested that damage to ovarian reserve see with removal of endometriomas might be more pronounced than non-endometriotic cysts (Maneschi, Marasa et al. 1993; Muzii, Bianchi et al. 2002; Exacoustos, Zupi et al. 2004; Chang, Han et al. 2010; Iwase, Hirokawa et al. 2011). There is also suggestion that inexperience of the surgeon can increase the damage caused to the normal ovarian tissue and ovarian reserve thereby (Yu, Huang et al.).

Various methods have been reported to evaluate ovarian reserve following ovarian cystectomy. These include ovarian response and / or pregnancy rate in women undergoing assisted reproduction treatment (ART); two–dimensional ultrasound markers of ovarian reserve such as volume, vascularity, and AFC (2.0-10.0mm); ovarian volume as measured using 3D ultrasound; and endocrine markers of ovarian reserve including AMH and FSH. The ovarian reserve in the operated ovary has more commonly been compared to either the contra lateral normal ovary or matched controls, and in very few studies to itself in a longitudinal study design. Studies that have used outcomes of ovarian response to evaluate ovarian reserve have used measures including the duration of ovarian stimulation, the number of oocytes collected, fertilisation rate, and clinical pregnancy rate when examining the effects of ovarian cystectomy in sub-fertile population undergoing

assisted reproduction treatment. They have compared the ovarian response of operated ovary either to the contra lateral normal ovary or to the matched case-controls (Loh, Tan et al. 1999; Canis, Pouly et al. 2001; Marconi, Vilela et al. 2002; Somigliana, Ragni et al. 2003; Wong, Gillman et al. 2004; Loo, Lin et al. 2005; Alborzi, Zarei et al. 2006; Esinler, Bozdag et al. 2006; Somigliana, Vercellini et al. 2006; Alborzi, Ravanbakhsh et al. 2007; Nakagawa, Ohgi et al. 2007; Horikawa, Nakagawa et al. 2008; Kahyaoglu, Ertas et al. 2008; Somigliana, Arnoldi et al. 2008; Benaglia, Somigliana et al. 2009; Garcia-Velasco and Somigliana 2009; Tsoumpou, Kyrgiou et al. 2009; Benaglia, Somigliana et al. 2010). Ovarian cystectomy has been shown to impair ovarian reserve following assisted reproduction treatment (ART), but reduced ovarian reserve does not seem to translate into impaired pregnancy outcome (Demirol, Guven et al. 2006; Esinler, Bozdag et al. 2006; Somigliana, Ragni et al. 2006; Kahyaoglu, Ertas et al. 2008; Garcia-Velasco and Somigliana 2009). A few studies have reported ovarian volume in conjunction with ovarian response and compared to the response in contra lateral ovary (Somigliana, Ragni et al. 2006). Other studies have used ultrasound markers such as volume, antral follicle count, and stromal blood flow in the operated ovary and compared to the contra lateral ovary (Exacoustos, Zupi et al. 2004; Candiani, Barbieri et al. 2005; Benaglia, Somigliana et al. 2010; Coric, Barisic et al. 2010; Donnez, Lousse et al. 2010).  Very few prospective longitudinal studies comparing postoperative ovary to the preoperative ovary have used varying combinations of endocrine and ultrasound markers of ovarian reserve with post operative follow up of varying number and duration (Candiani, Barbieri et al. 2005; Chang, Han et al. 2009; Li, Liu et al. 2009; Tsolakidis, Pados et al. 2009; Chang, Han et al. 2010; Pados, Tsolakidis et al. 2010; Iwase, Hirokawa et al. 2011).

To further compound the assessment of ovarian reserve following ovarian cystectomy, there are inherent physiological variations in some of the markers of ovarian reserve as shown in previous chapters. When using ultrasound markers of ovarian reserve, it is important to note that there is an inter-ovarian variation in the markers of ovarian reserve including the AFC, ovarian volume, vascularity as measured using 3D power Doppler indices and pulsed wave Doppler, such that one ovary may not be reliably compared to the other contra lateral ovary (chapter 7). The small antral follicles measuring less than 6mm show least variation between the two ovaries and hence if the two ovaries had to be compared, they would be the most reliable marker of reserve to use (chapter 7). Small antral follicles show the least inter-cycle variation and could be used reliably in longitudinal studies (chapter 9). There is a significant inter-ovarian, intra-cyclical and inter-cyclical variation seen with ovarian volume and therefore might not be a reliable test to assess the effect of ovarian cystectomy (chapters 7, 8 and 9). Although, ovarian volume assessed using 3D ultrasound has been shown to have good intra- and inter-observer reliability (Scheffer, Broekmans et al. 2002; Raine-Fenning, Campbell et al. 2003; Raine-Fenning, Campbell et al. 2003; Merce, Gomez et al. 2005; Merce, Bau et al. 2006), it shows significant inter-cycle  (Elter, Sismanoglu et al. 2005) and intra- cycle variation. A significant inter-cycle variability has been reported for basal FSH (Scott, Hofmann et al. 1990) and an expected intra cycle variation as shown in previous chapter (chapter). Several studies have confirmed intra- and inter-cycle stability of AMH (La Marca, Malmusi et al. 2004; Fanchin, Taieb et al. 2005; Hehenkamp, Looman et al. 2006; La Marca, Stabile et al. 2006; Tsepelidis, Devreker et al. 2007; Elgindy, El-Haieg et al. 2008; van Disseldorp, Lambalk et al. 2010) Moreover, amongst all the endocrine markers of ovarian reserve, AMH is a very good predictor of ovarian response and pregnancy (La

Marca, Sighinolfi et al.; Broekmans, Kwee et al. 2006; Dehghani-Firouzabadi, Tayebi et al. 2008; Verhagen, Hendriks et al. 2008; Maheshwari, Gibreel et al. 2009; Jayaprakasan, Campbell et al. 2010).

It therefore appears that amongst all the ultrasound and endocrine markers of ovarian reserve, the small antral follicle (≤6.0mm) population and AMH emerge as the markers of choice followed by total antral follicle count (2.0-10.0mm). Bearing in mind the within and inter subject variations, it would appear that a longitudinal design of study wherein the operated ovary is compared to itself is a desirable method of assessing ovarian reserve following cystectomy.

This study was designed to quantify the effect of ovarian cyst per se and that of laparoscopic ovarian cystectomy on the markers of ovarian reserve as measured using AMH, and 3D ultrasound assisted measures of small antral follicle count and total antral follicle count. The hypothesis was that ovarian cystectomy has a significant effect on the ovarian reserve.

## 11.3   Methods

**Study design**

This was a quantitative prospective observation study designed to evaluate ovarian reserve in women with unilateral benign ovarian cyst pre operatively and then evaluate the effect of laparoscopic cystectomy on ovarian reserve post operatively at one, three, and 6 months. AMH, FSH, and 3D assisted software - SonoAVC that made semi-automated antral follicle counts were used to quantify ovarian reserve. A sample size of 30 was

required to power the study at 0.8 with a type I error of 0.05 and presumed maximum difference of 0.8 ng/mL in the mean values of AMH across 5 levels of analysis.

Study participants were recruited prospectively through advertisements and posters displayed in Gynaecology out-patients department, patient toilets, staff canteens and notice boards. The main inclusion criteria were: age between 18 to 35 years, body mass index (BMI) between 18 and 28 kg/ m$^2$, regular menstrual cycles with a mean length ranging between 26 to 35 days, presence of unilateral benign looking ovarian cyst on ultrasound scan, no history of ovarian surgery, no history suggestive of endocrine disease and no use of hormonal contraceptives in the last 6 months. On identification of suitable participant who met the above inclusion criteria, study information was explained by the investigators and information leaflet given to those who expressed interest in participation in the study. Subsequently, a consent form was signed and plan made for their future visits. A transvaginal ultrasound scan was performed and blood samples taken during each patient visit. First visit was arranged preoperatively in the early follicular phase of the menstrual cycle and involved performing an ultrasound scan and blood test. Subsequent visits were arranged at one month, 3 months, and 6 months following the laparoscopic ovarian cystectomy when a TVS was performed and blood test taken.

### *Operative technique:*

Laparoscopic pneumoperitoneum was created with CO2, followed by insertion of a sub umbilical 10 mm port and two to three 5 mm lateral ports under direct laparoscopic observation. Monopolor scissors were used to open the ovarian cortex and identify the cyst wall. The cyst was subsequently enucleated from the ovarian cortex by blunt and sharp dissection. Every effort was made to excise the cyst in its entirety without spillage

of contents. Haemostasis was achieved using bipolar forceps. The cyst was removed in an endoscopic bag and sent for histopathological analysis.

*Outcome measures:*

Primary outcome measures were changes in the serum AMH and small antral follicle population following the ovarian cystectomy. This was done by comparing the post operative levels at 1, 3, and 6 months to the pre operative levels. Secondary outcome measures were total AFC and serum FSH levels. These primary and secondary measures of ovarian reserve were also applied to the different types of cyst on sub group analysis.

## Data acquisition

The ultrasound scans were performed by a single investigator (S.D) using a Voluson E8 Expert (GE Medical Systems, Zipf, Austria) and a three-dimensional 5-9 MHz transvaginal transducer. The ultrasound assessment involved a two-dimensional (2D) ultrasound assessment of the pelvis to confirm the presence of ovarian cyst. Size of the cyst was measured by taking the average of three perpendicular diameters measured in longitudinal and transverse planes. A wide angle of $180^0$ was set to examine the ovary with the cyst in its entirety when required. The technique of volume ultrasound as explained in the chapter of methods included delineation of the ovary with application of a region of interest and the subsequent acquisition of a series of 2D planes acquired during a high quality slow sweep mode of the ultrasound beam set at a $120^0$ angle for the ovary with cyst and $90^0$ angle for the normal ovary and the operated ovary. This ensured that data from the whole ovary was acquired and also that the greatest number of 2D planes were acquired giving the highest degree of resolution when the 2D data are reconstructed as a 3D volume.

**Data analysis**

Multiplanar view was used to display the acquired 3D data (Voluson E8 Expert: General Electric Medical Systems, Zipf, Austria). The grey-scale display of image was optimised and then rendered to generate a three-dimensional volume of interest (VOI). The render box was adjusted to exclude as much extra-ovarian information as possible and ensure that the whole ovary was included in the VOI. The threshold settings, which assign transparency associated with fluid to opaque voxels, were maintained for all datasets at a default setting of 'low'. Once the dataset had been correctly positioned, 3D automated software, 'sonography-based automated volume count' (sonoAVC: GE Medical Systems) was implemented (Raine-Fenning, Jayaprakasan et al. 2008). The use of sonoAVC with post-processing in counting and measuring the size of antral follicles has been described in detail in chapters 2 and 3. The mean 'relaxed sphere diameter', displayed as d (V), of each antral follicle in both ovaries was recorded and used for data analysis. The antral follicle population for each subject was recorded to the nearest millimetre, as this reflects the current resolution of the ultrasound system, starting from 2.0 mm up to a maximum of 10.0 mm.

**Hormonal Assays**

Within 30 minutes of collection of blood samples, they were centrifuged for 20 min at $4^{o}$C and 4000 rpm spin to separate the serum. The serum sample was frozen at -20°C and stored for subsequent analysis of anti-Müllerian hormone (AMH) and follicle stimulating hormone (FSH) MIS/AMH enzyme-linked immunosorbent assay kit (Diagnostic Systems Laboratories, Webster, Texas, USA) was used to measure the serum AMH levels. The lowest detection limit was 0.006 ng/mL and the intra and interassay coefficients of variation below 5% and 8% respectively.

Micro particle Enzyme Immunoassay (MEIA) method on an AxSYM auto-analyser (AxSYM; Abbott Laboratories, Abbott Park, IL) was used to measure the levels of serum FSH. The lowest detection limit for FSH was 0.37 IU/L, with the intra- and interassay coefficients of variation were below 5% and above 5% respectively.

**Statistical analysis**

The Statistical Package for the Social Sciences (version 17.0; SPSS, Chicago, IL) was used for statistical analysis. Descriptive statistics are displayed as mean ± SD with 95% CI or as median with range. The difference in pre operative and post operative means are also expressed in percentages. General linear model with repeated measures design was used to perform the analysis of variance. A P-value of less than 0.05 was considered statistically significant. In order to examine the effect of cyst on ovarian reserve paired samples *t* test was used to compare the difference in small antral follicles between the diseased and contra lateral normal ovary.

## *11.4   Results*

### 11.4.1 Overall analysis of all cyst types

The mean ± SD (95% CI) of the pre operative and post operative levels of serum AMH, FSH, AFC's of the diseased ovary, contra-lateral normal ovary  and those of both ovaries are shown in Table 11.1. AMH and FSH levels showed a significant decline post-operatively (P<0.05). A similar significant decline in antral follicle counts measuring 2.0-10.0mm and 2.0-6.0mm was observed in the ovary that underwent cystectomy (P<0.05). These total and small AFC's, however showed a non-significant decline in the contra-

lateral normal ovary (P>0.05). A non-significant decline was observed in the number of larger antral follicles measuring >6.0 mm in both, the diseased and contra-lateral normal ovaries. The decline in mean AFC including antral follicles measuring 2.0-10.0mm and 2.0-6.0mm from both ovaries reflected the significant decline observed in the diseased ovary (P<0.05), the larger AFC (>6.0-10.0mm) however showed no significant change (P>0.05) (Table 11.1).

Analysis of within-subject levels of follow up showed that total AFC and small AFC in the operated ovary continued to decline up until 3 months (P<0.05) post-operatively before stabilising at 6 months. Total AFC and small AFC of both ovaries significantly declined at 1 month (P<0.05) before stabilising at 3 months (P>0.05. The contra lateral normal ovary does not appear to compensate as shown by the statistically in significant change in the AFC from pre operative to post operative levels in that ovary (Figure 11.1 and 11.2).

**Table 11.1:** Mean ± SD (95% CI) of cohorts of antral follicle counts stratified by size, AMH and FSH before and up to 6 months following ovarian cystectomy for benign disease. The table also compares the cohorts of total, small and large antral follicle counts in both, the normal and diseased ovary following ovarian cystectomy.

| | Pre operative N=38 | 1 month N=38 | 3 months N=37 | 6 months N=34 | P value |
|---|---|---|---|---|---|
| AMH ng/mL | 2.71 ± 2.15 (1.90 – 3.51) | 1.69 ± 1.96 (1.45 – 2.93) | 2.09 ± 1.88 (1.63 – 3.04) | 2.29 ± 2.03 (1.73 – 3.25) | 0.001 |
| FSH IU/L | 6.38 ± 2.13 (5.58 – 7.17) | 7.72 ± 1.96 (6.99 – 8.45) | 7.26 ± 2.61 (6.28 – 8.23) | 7.17 ± 2.76 (6.14 – 8.20) | 0.024 |
| **Antral Follicle Counts of ovary with cyst** | | | | | |
| 2.0 – 6.0 mm (n) | 10.33 ± 7.55 (7.66 – 13.01) | 7.00 ± 6.99 (4.52 – 9.48) | 7.73 ± 7.5 (5.06 – 10.39) | 7.30 ± 7.05 (4.80 – 9.80) | <0.001 |
| >6.0 – 10.0 mm (n) | 1.68 ± 1.05 (1.29 – 2.06) | 1.23 ± 1.02 (0.85 – 1.60) | 1.32 ± 0.75 (1.05 – 1.60) | 1.55 ± 1.12 (1.14 – 1.96) | 0.167 |
| 2.0 – 10.0 mm (n) | 12.03 ± 7.90 (9.23 – 14.83) | 8.21 ± 7.37 (5.60 – 10.83) | 9.03 ± 7.69 (6.31 – 11.76) | 8.76 ± 7.83 (5.98 – 11.54) | <0.001 |
| **Antral Follicle Counts of contra lateral normal ovary** | | | | | |
| 2.0 – 6.0 mm (n) | 11.56 ± 6.66 (9.24 – 13.88) | 10.59 ± 5.05 (8.83 – 12.35) | 10.56 ± 4.95 (8.83 – 12.29) | 9.65 ± 6.48 (7.39 - 11.91) | 0.062 |
| >6.0 – 10.0 mm (n) | 1.90 ± 1.56 (1.33 – 2.47) | 1.58 ± 1.26 (1.12 – 2.04) | 1.77 ± 1.52 (1.22 – 2.33) | 1.87 ± 1.02 (1.49 – 2.25) | 0.464 |
| 2.0 – 10.0 mm (n) | 13.53 ± 7.34 (10.97 – 16.09) | 12.15 ± 5.42 (10.26 – 14.04) | 12.29 ± 5.57 (10.35 – 14.24) | 11.35 ± 7.10 (8.88 – 13.83) | 0.058 |
| **Total Antral Follicle Counts of both ovaries** | | | | | |
| 2.0 – 6.0 mm (n) | 22.89 ± 13.46 (17.45 – 26.84) | 17.14 ± 11.46 (13.85 – 21.85) | 17.68 ± 10.99 (14.66 – 22.34) | 16.95 ± 12.35 (12.43 – 21.04) | <0.001 |
| >6.0 – 10.0 mm (n) | 3.58 ± 2.16 (2.89 – 4.40) | 2.81 ± 1.58 (2.18 – 3.29) | 3.09 ± 1.66 (2.45 – 3.61) | 3.42 ± 1.93 (2.44 – 3.79) | 0.071 |
| 2.0 – 10.0 mm (n) | 26.54 ± 15.04 (20.70 – 30.89) | 19.93 ± 12.08 (16.51 – 24.66) | 20.73 ± 12.72 (17.53 – 25.53) | 20.20 ± 14.05 (15.15 – 24.55) | <0.001 |

**Figure 11.1:** The variation in the mean (error bars of S.E.M) AFCs in the diseased and contra-lateral normal ovary as measured pre-operatively and 1, 3, and 6 months post-operatively. P value was derived using repeated measures analysis and are marked at levels of comparison showing significant change (*=P<0.05; **=P<0.01; ***=P<0.001).
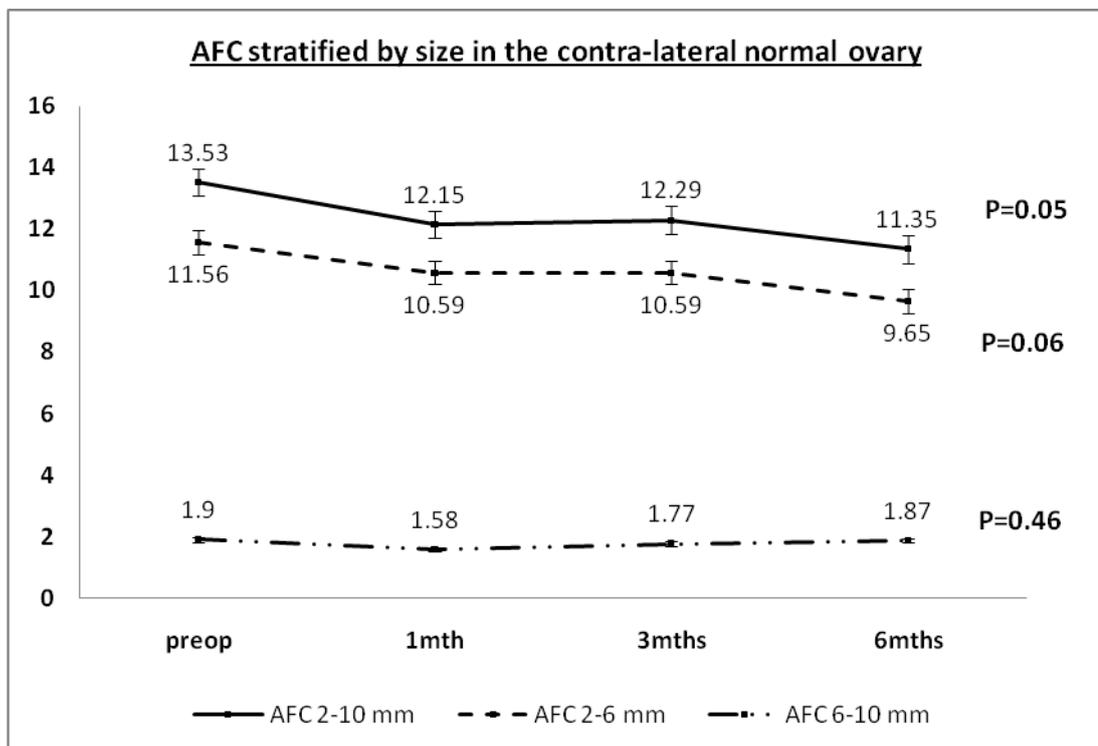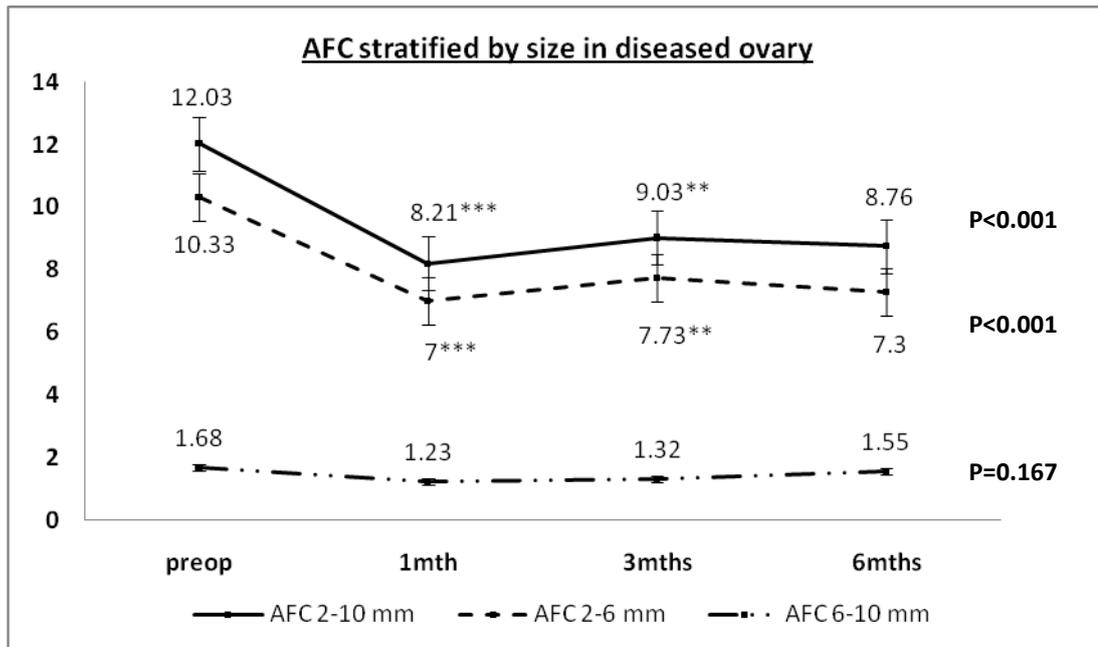
**Figure 11.2:** The variation in the mean (error bars of S.E.M) AFCs of both ovaries as measured pre-operatively and 1, 3, and 6 months post-operatively. P value was derived using repeated measures analysis and are marked at levels of comparison showing significant change (*=P<0.05; **=P<0.01; ***=P<0.001).



Levels of serum AMH significantly dropped and that of FSH increased one month post operatively and stayed at those levels 3 and 6 months later. This suggests an immediate effect of cystectomy on ovarian reserve which persisted up to 6 months post operatively (Figure 11.3).

**Figure 11.3:** The variation in the mean (error bars of S.E.M) levels of serum AMH and FSH as measured pre-operatively and 1, 3, and 6 months post-operatively. P value was derived using repeated measures analysis and are marked at levels of comparison showing significant change (*=P<0.05; **=P<0.01; ***=P<0.001).
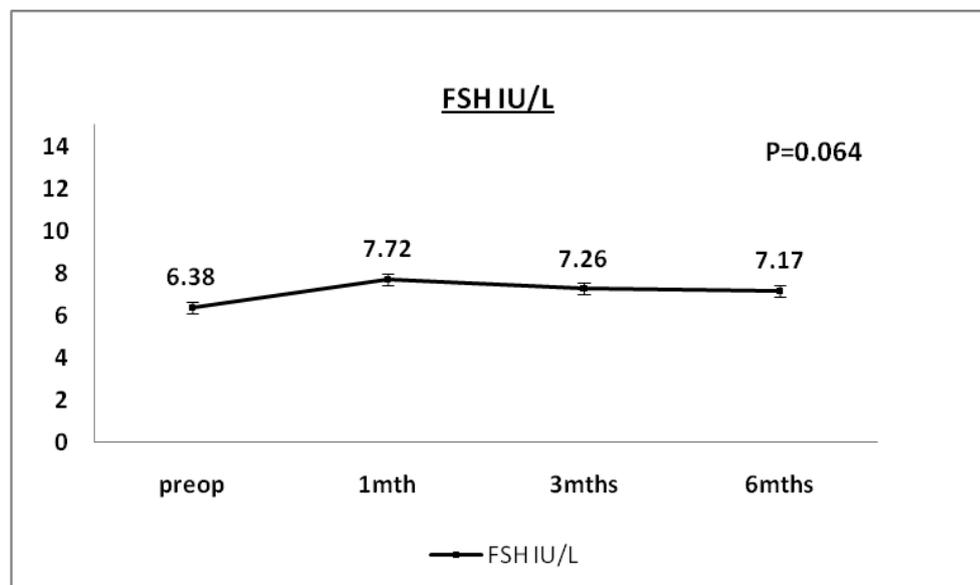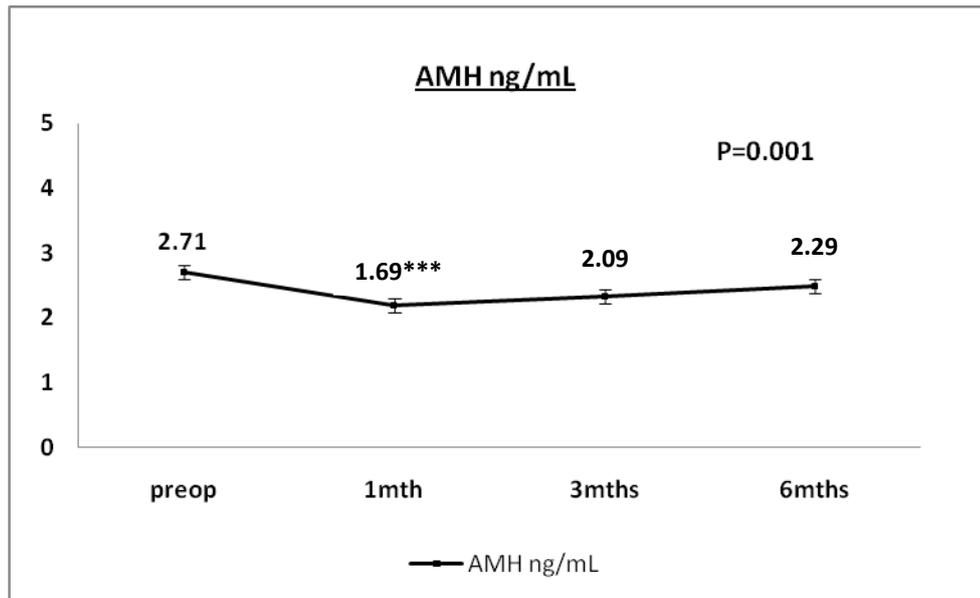




The variation in the levels of AMH, FSH and antral follicle counts was further quantified with percent change in post operative levels when compared to pre operative levels.

There was a significant drop in levels of AMH post operatively at 1 month, 3 months and 6 months by 38%, 23% and 16% respectively. There was an increase in FSH by 21% at 1 month, 14% at 3 months and 12% at 6 months noted but not statistically significant. The number of small antral follicles in the operated ovary showed a significant drop by 42% at 1 month, 37% at 3 months and 35 % at 6 months. Total number of antral follicles in the operated ovary reduced significantly by 40% at 1 month, 35% at 3 months and 32% at 6 months. Since the contra lateral normal ovary was not affected by the cystectomy, the reduction in total AFC (antral follicles of both ovaries) showed a lesser but significant drop by 25% at 1 month, 22% at 3 months and 24% at 6 months.

Pre operative AMH levels showed higher correlation with total AFC including antral follicles in both ovaries (2.0-6.0mm: 0.884, 2.0-10.0mm: 0.841) than with the AFC in the ovary with the cyst alone (2.0-6.0mm: 0.742, 2.0-10.0mm: 0.742). One month postop, correlation between AMH and tAFC (2.0-6.0mm: 0.862, 2.0-10.0mm: 0.848) were better than with operated ovary (2.0-6.0mm: 0.697, 2.0-10.0mm: 0.777). A similar trend was noted 3 months post op (with tAFC 2.0-6.0mm and 2.0-10.0mm: 0.873 and 0.857; with AFC of operated ovary: 0.721 and 0.645) and 6 months postop (with tAFC 2.0-6.0mm and 2.0-10.0mm: 0.870 and 0.825; with AFC of operated ovary: 0.761 and 0.620).

## 11.4.2 Sub group analysis by type of cysts

Three types of benign cysts were identified histologically, 18 dermoid cysts, 15 endometriomas, and 5 functional cysts. The average size of dermoid cysts was 72.28 mm, that of endometrioma was 62.34 mm and that of simple cyst was 52.45 mm.

### 11.4.2a          Dermoid cyst

The mean ± SD (95% CI) of the pre operative and post operative levels of serum AMH, FSH, AFC's of the diseased ovary, contra-lateral normal ovary  and those of both ovaries are shown in Table 11.2. AMH levels showed a significant decline post-operatively (P<0.05), however the change in FSH levels remained non-significant overall (P>0.05).  A significant decline in antral follicle counts measuring 2.0-10.0mm and 2.0-6.0mm was observed in the ovary that underwent cystectomy (P<0.05). These total and small AFC's, however showed a non-significant decline in the contra-lateral normal ovary (P>0.05). A non-significant decline was observed in the number of larger antral follicles measuring >6.0 mm in both, the diseased and contra-lateral normal ovaries. The decline in mean AFC including antral follicles measuring 2.0-10.0mm and 2.0-6.0mm from both ovaries reflected the significant decline observed in the diseased ovary (P<0.05), the larger AFC (>6.0-10.0mm) however showed no significant change (P>0.05) (Table 11.2).

Analysis of within-subject levels of follow up showed that total AFC and small AFC in the operated ovary continued to decline up until 3 months (P<0.05) post-operatively before stabilising at 6 months. Total AFC and small AFC of both ovaries significantly declined at 1 month (P<0.05) before stabilising at 3 months (P>0.05). The contra lateral normal ovary does not appear to compensate as shown by the statistically in significant change in the AFC from pre- to post operative levels in that ovary (Figure 11.4).

Levels of serum AMH significantly dropped on the visit at one month post operatively (P<0.05), stabilising at 3 months visit but showing a significant recovery at 6 months visit post-operatively (P<0.05).  FSH levels significantly increased on the visit at one month post operatively (P<0.05) and then stabilising thereafter (Figure 11.5).

**Table 11.2:** Mean ± SD (95% CI) of cohorts of antral follicle counts stratified by size, AMH and FSH

before and up to 6 months following ovarian cystectomy for **dermoid cyst**.

| | Pre operative N=18 | 1 month N=18 | 3 months N=17 | 6 months N=16 | P value |
|---|---|---|---|---|---|
| AMH ng/mL | 2.84 ± 2.46 (1.57 – 4.11) | 2.27 ± 2.46 (1.10 – 3.64) | 2.15 ± 2.25 (1.39 – 3.71) | 2.22 ± 2.44 (1.63 – 4.14) | 0.002 |
| FSH IU/L | 6.40 ± 2.11 (5.32 – 7.49) | 8.18 ± 1.47 (7.43 – 8.94) | 7.99 ± 1.83 (7.06 – 8.94) | 7.53 ± 2.59 (6.19 – 8.86) | 0.021 |
| **Antral Follicle Counts of ovary with cyst** | | | | | |
| 2.0 – 6.0 mm (n) | 11.50 ± 9.36 (6.84 – 16.16) | 8.22 ± 8.71 (3.89 – 12.56) | 9.22 ± 9.29 (4.60 – 13.84) | 8.56 ± 8.69 (4.24 – 12.88) | <0.001 |
| >6.0 – 10.0 mm (n) | 2.00 ± 0.79 (1.59 – 2.41) | 1.06 ± 0.66 (0.72 – 1.39) | 1.53 ± 0.79 (1.12 – 1.94) | 1.65 ± 1.11 (1.07 – 2.22) | 0.077 |
| 2.0 – 10.0 mm (n) | 13.39 ± 9.60 (8.61 – 18.17) | 9.22 ± 9.01 (4.74 – 13.70) | 10.67 ± 9.48 (5.95 – 15.38) | 10.11 ± 9.39 (5.44 – 14.79) | <0.001 |
| **Antral Follicle Counts of contra lateral normal ovary** | | | | | |
| 2.0 – 6.0 mm (n) | 12.89 ± 8.15 (8.97 – 16.82) | 11.79 ± 6.05 (8.87 – 14.71) | 10.42 ± 5.73 (7.66 – 13.18) | 9.84 ± 7.57 (6.19 – 13.49) | 0.065 |
| >6.0 – 10.0 mm (n) | 2.12 ± 1.62 (1.28 – 2.95) | 1.53 ± 1.18 (0.92 – 2.14) | 1.71 ± 1.57 (0.89 – 2.51) | 1.65 ± 1.17 (1.05 – 2.25) | 0.693 |
| 2.0 – 10.0 mm (n) | 15.05 ± 8.92 (10.76 – 19.35) | 13.26 ± 6.36 (10.19 – 16.33) | 12.11 ± 6.19 (9.12 – 15.09) | 11.32 ± 8.24 (7.34 – 15.29) | 0.099 |
| **Total Antral Follicle Counts of both ovaries** | | | | | |
| 2.0 – 6.0 mm (n) | 24.79 ± 16.62 (16.78 – 32.79) | 20.42 ± 14.04 (13.65 – 27.19) | 19.95 ± 13.48 (13.45 – 26.44) | 17.95 ± 14.93 (10.75 – 25.14) | 0.002 |
| >6.0 – 10.0 mm (n) | 4.00 ± 1.79 (3.13 – 4.86) | 2.42 ± 1.02 (1.93 – 2.91) | 3.11 ± 1.49 (2.39 – 3.82) | 2.95 ± 2.04 (1.96 – 3.93) | 0.078 |
| 2.0 – 10.0 mm (n) | 28.79 ± 17.85 (20.19 – 37.39) | 22.84 ± 14.08 (16.06 – 29.63) | 23.05 ± 13.67 (16.47 – 29.64) | 20.89 ± 15.97 (13.19 – 28.59) | 0.001 |

### 11.4.2b         Endometrioma

The mean ± SD (95% CI) of the pre operative and post operative levels of serum AMH, FSH, AFC's of the diseased ovary, contra-lateral normal ovary  and those of both ovaries are shown in Table 11.3. AMH levels showed a significant decline post-operatively (P<0.05), however the change in FSH levels remained non-significant overall (P>0.05).  A significant decline in antral follicle counts measuring 2.0-10.0mm and 2.0-6.0mm was observed in the ovary that underwent cystectomy (P<0.05). Unlike the dermoid cysts, the small AFC measuring 2.0-6.0mm in the contra-lateral normal ovary showed a significant overall variation (P<0.05), however the total AFC (2.0-10.0mm) remained non-significant (P>0.05). A non-significant decline was observed in the number of larger antral follicles measuring >6.0 mm in both, the diseased and contra-lateral normal ovaries. The decline in mean AFC including antral follicles measuring 2.0-10.0mm and 2.0-6.0mm from both ovaries reflected the significant decline observed in the diseased ovary (P<0.05), the larger AFC (>6.0-10.0mm) however showed no significant change (P>0.05) (Table 11.3).

Analysis of within-subject levels of follow up showed that total AFC and small AFC in the operated ovary continued to decline up until 3 months (P<0.05) post-operatively before stabilising at 6 months. Total AFC and small AFC of both ovaries significantly declined up until 3 months (P<0.05) before stabilising at 6 months (P>0.05). The overall significant variation seen in the contra lateral normal ovary was perhaps due to a compensatory increase in the small and total AFC at 3 months post-operatively (P<0.05) (Figure 11.4).

AMH levels significantly declined up until 3 months post operatively (P<0.05), whilst FSH levels stabilised from 0ne month post-operatively (Figure 11.5).

**Table 11.3:** Mean ± SD (95% CI) of cohorts of antral follicle counts stratified by size, AMH and FSH

before and up to 6 months following ovarian cystectomy for **endometrioma**.

| | Pre operative N=15 | 1 month N=15 | 3 months N=15 | 6 months N=14 | P value |
|---|---|---|---|---|---|
| **AMH ng/mL** | 2.72 ± 1.96 (1.09 – 4.36) | 1.58 ± 1.42 (0.39 – 2.76) | 1.78 ± 1.56 ( 0.48 – 3.08) | 1.71 ± 1.47 (0.48 – 2.94) | 0.015 |
| **FSH IU/L** | 7.58 ± 2.16 (5.78 – 9.39) | 9.53 ± 1.99 (7.87 – 11.19) | 9.29 ± 1.09 (8.38 – 10.21) | 9.19 ± 1.47 (7.96 – 10.42) | 0.134 |
| **Antral Follicle Counts of ovary with cyst** | | | | | |
| **2.0 – 6.0 mm (n)** | 9.50 ± 3.07 (6.93 – 12.09) | 4.50 ± 1.93 (2.89 – 6.11) | 5.38 ± 2.07 (3.65 – 7.10) | 5.38 ± 2.45 (3.33 – 7. | 0.002 |
| **>6.0 – 10.0 mm (n)** | 1.75 ± 1.58 (0.43 – 3.07) | 1.72 ± 0.89 (1.01 – 2.49) | 1.38 ± 0.52 (0.94 – 1.81) | 1.38 ± 0.74 (0.75 – 1.99) | 0.769 |
| **2.0 – 10.0 mm (n)** | 11.25 ± 3.11 (8.65 – 13.85) | 6.25 ± 2.38 (4.26 – 8.24) | 6.75 ± 2.12 (4.98 – 8.52) | 7.13 ± 2.53 (5.01 – 9.24) | <0.001 |
| **Antral Follicle Counts of contra lateral normal ovary** | | | | | |
| **2.0 – 6.0 mm (n)** | 10.50 ± 5.04 (6.28 – 14.72) | 9.00 ± 3.59 (6.01 – 11.99) | 12.25 ± 4.23 (8.71 – 15.79) | 10.88 ± 5.06 (6.65 – 15.10) | 0.007 |
| **>6.0 – 10.0 mm (n)** | 2.38 ± 1.77 (0.89 – 3.85) | 2.13 ± 1.73 (0.68 – 3.57) | 2.13 ± 1.55 (0.83 – 3.42) | 2.13 ± 0.64 (1.59 – 2.66) | 0.068 |
| **2.0 – 10.0 mm (n)** | 12.88 ± 5.46 (8.31 – 17.44) | 11.13 ± 4.76 (7.14 – 15.11) | 14.37 ± 5.24 (9.99 – 18.75) | 13.00 ± 5.24 (8.62 – 17.38) | 0.029 |
| **Total Antral Follicle Counts of both ovaries** | | | | | |
| **2.0 – 6.0 mm (n)** | 20.00 ± 7.69 (13.57 – 26.43) | 13.50 ± 5.37 (9.01 – 17.99) | 17.63 ± 6.25 (12.39 – 22.85) | 16.87 ± 6.17 (11.71 – 22.03) | 0.034 |
| **>6.0 – 10.0 mm (n)** | 4.12 ± 3.14 (1.50 – 6.75) | 3.87 ± 2.17 (2.06 – 5.69) | 3.50 ± 1.85 (1.95 – 5.05) | 3.50 ± 1.19 (2.50 – 4.49) | 0.639 |
| **2.0 – 10.0 mm (n)** | 24.13 ± 8.37 (17.12 – 31.13 | 17.38 ± 6.95 (11.57 – 23.18) | 21.13 ± 7.24 (15.07 – 27.17) | 20.38 ± 6.84 (14.65 – 26.09) | 0.005 |

**Figure 11.4:** The variation in the mean (error bars of S.E.M) levels of serum AMH and FSH in different types of ovarian cysts as measured pre-operatively and 1, 3, and 6 months post-operatively. P value was derived using repeated measures analysis and are marked at levels of comparison showing significant change (*=P<0.05; **=P<0.01; ***=P<0.001).

**Figure 11.5:** The variation in the mean (error bars of S.E.M) of small AFCs in the diseased and contra-lateral normal ovary with different type of cyst as measured pre-operatively and 1, 3, and 6 months post-operatively. P value was derived using repeated measures analysis and are marked at levels of comparison showing significant change (*=P<0.05; **=P<0.01; ***=P<0.001).



**2.0-6.0 mm AFC in the diseased ovary following cystectomy and the cyst type**

Endometrioma ─── Dermoid ─ ● ─ Simple cyst



**2.0-6.0 mm AFC in the contra-lateral normal ovary and the cyst type**

Endometrioma (P=0.029) ─ ● ─ Dermoid (P=0.152) ─── Simple cyst (P=0.324)
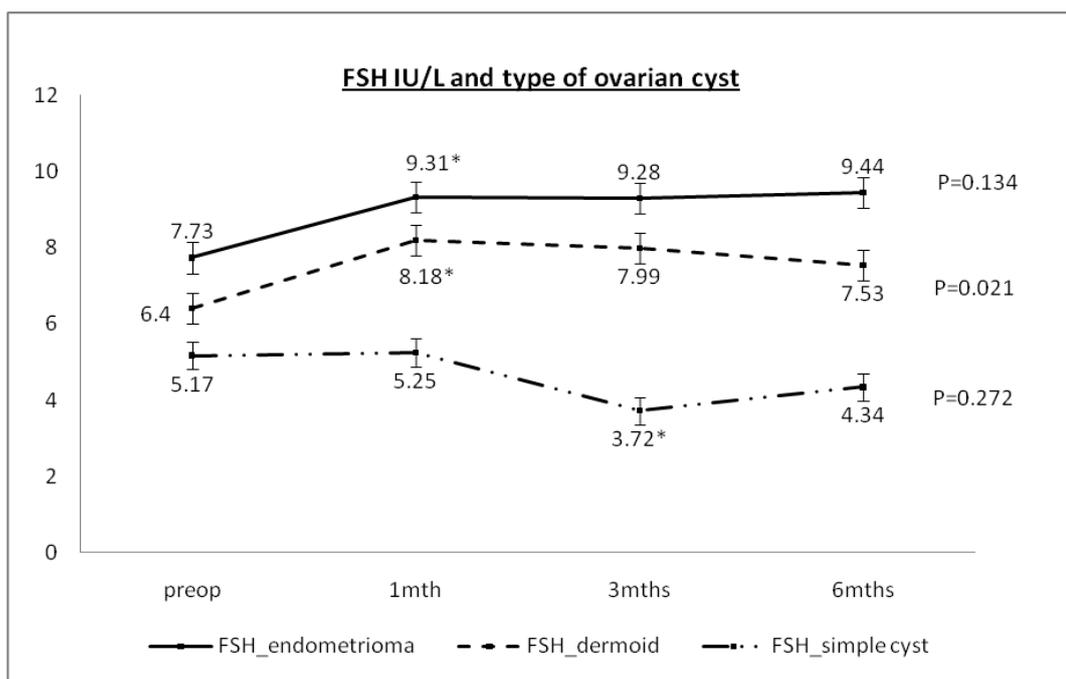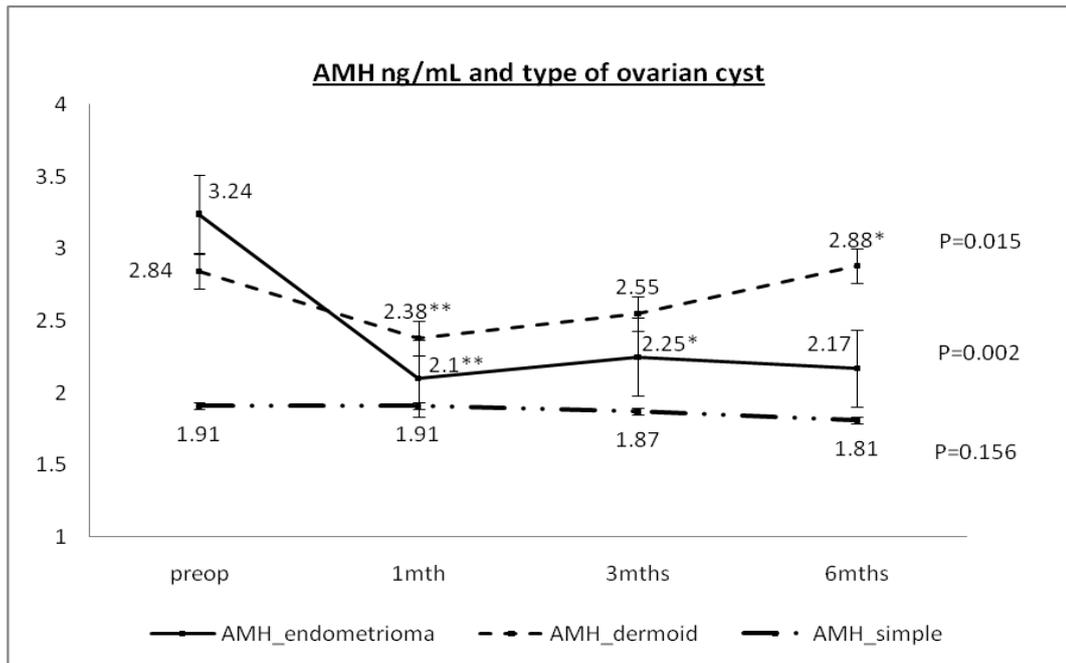
**11.4.2c          Simple cyst**

Repeated measures analysis of variance showed that none of the markers of ovarian reserve in women operated for simple ovarian cyst showed any significant change post operatively (P>0.05) (Table 11.4 and Figures 11.4 and 11.5).

**Table 11.4:** Mean ± SD (95% CI) of cohorts of antral follicle counts stratified by size, AMH and FSH before and up to 6 months following ovarian cystectomy for simple cyst. The table also compares the cohorts of total, small and large antral follicle counts in both, the normal and diseased ovary following ovarian cystectomy.

| | Pre operative N=5 | 1 month N=5 | 3 months N=5 | 6 months N=4 | P value |
|---|---|---|---|---|---|
| **AMH ng/mL** | 1.91 ± 0.98 (0.66 – 3.16) | 1.91 ± 1.21 (1.08 – 2.74) | 1.87 ± 1.11 (0.82 – 2.92) | 1.81 ± 0.88 (0.80 – 2.82) | 0.156 |
| **FSH IU/L** | 5.17 ± 2.22 (4.10 – 6.23) | 5.25 ± 3.32 (4.31 – 6.18) | 3.72 ± 2.38 (2.14 – 5.30) | 4.34 ± 2.12 (3.09 – 5.58) | 0.272 |
| **Antral Follicle Counts of ovary with cyst** | | | | | |
| **2.0 – 6.0 mm (n)** | 8.28 ± 4.22 (0.15 – 2.13) | 6.71 ± 4.14 (0.41 – 2.70) | 6.57 ± 5.12 (0.22 – 1.50) | 6.43 ± 4.88 (0.03 – 2.83) | 0.245 |
| **2.0 – 10.0 mm (n)** | 9.43 ± 7.65 (3.14 – 15.72) | 7.86 ± 5.55 (1.69 – 14.03) | 7.43 ± 5.87 (1.78 – 13.08) | 7.86 ± 4.55 (1.37 – 14.34) | 0.095 |
| **Antral Follicle Counts of contra lateral normal ovary** | | | | | |
| **2.0 – 6.0 mm (n)** | 9.14 ± 5.15 (8.15 – 10.13) | 9.14 ± 6.05 (7.11 – 11.17) | 9.00 ± 4.73 (6.28 – 11.72) | 8.86 ± 7.57 (5.77 – 11.94) | 0.324 |
| **2.0 – 10.0 mm (n)** | 10.14 ± 7.92 (8.89 – 11.39) | 10.29 ± 6.36 (8.38 – 12.19) | 10.43 ± 6.19 (7.06 – 13.80) | 10.86 ± 7.24 (7.07 – 14.65) | 0.123 |
| **Total Antral Follicle Counts of both ovaries** | | | | | |
| **2.0 – 6.0 mm (n)** | 17.43 ± 8.97 (11.25 – 23.61) | 15.86 ± 8.08 (9.58 – 22.14) | 15.57 ± 9.19 (8.57 – 22.57) | 15.29 ± 8.18 (7.41 – 23.16) | 0.256 |
| **2.0 – 10.0 mm (n)** | 19.57 ± 8.88 (12.59 – 26.55) | 18.14 ± 7.26 (10.81 – 25.47) | 17.86 ± 9.18 (9.69 -26.03) | 18.71 ± 9.67 (9.09 – 28.34) | 0.145 |

## *11.5   Discussion*

This is the first study that has evaluated the effects of ovarian cyst and cystectomy on ovarian reserve using AMH and a 3D ultrasound assisted novel technique of measuring and counting antral follicles (sonoAVC), thereby enabling the use of small antral follicles in the measurement of ovarian reserve in a reliable and reproducible manner. The results suggest that laparoscopic ovarian cystectomy for a benign cyst is associated with a significant loss of ovarian reserve lasting up to at least 6 months post operatively. Expectedly, the damage caused is more in the ovary that is operated upon than the other normal ovary. The results also suggest that the damage caused by the stripping of endometrioma might be more pronounced than the damage caused by stripping of dermoid cyst.

### 11.5.1 Effect of ovarian cyst per se on the ovarian reserve

This is the first study that has quantified the effect of cyst on the ovary using 3D ultrasound. We found that there was no significant difference in the small and total antral follicle counts in the ovary with the cyst when compared to the contra lateral normal ovary. The limitation of such a comparison is that there are inherent physiological differences between the two ovaries within the same individual as shown in chapter 7. However, since the small antral follicles measuring up to 6.0 mm show minimal inter-ovarian variation, and that it has very good correlation with AMH (Jayaprakasan, Deb et al. 2009), we would have to conclude that the cyst per se does not affect the ovarian reserve as measured by small antral follicle count made by using 3D assisted semi-automated technique (sonoAVC). On sub group analysis of the type of cysts, we found

that there was a significant reduction (P=0.042) in the number of small antral follicles in the ovary with endometrioma, but not in the ovary with non-endometriomas when compared to their contra lateral normal ovary. These findings are supported by the histopathology study done by Maneschi et al where they showed that the presence of cyst itself may affect the adjacent normal ovarian tissue (Maneschi, Marasa et al. 1993). They examined three types of benign cysts, 13 mature teratomas, 32 endometriomas and 9 cyst adenomas. The group showed that the ovarian cortex which is stretched and thinned by the growth of benign tumour is not altered morphologically in the presence of teratomas and benign cyst adenomas, however with endometriomas, there was presence of microscopic implants in the stroma and that the follicular number and activity was significantly reduced. Recently, Roman et al reported that the size of the endometrioma is directly related to the proportional increase in the damage to normal ovarian parenchyma at cystectomy (Roman, Tarta et al. 2010). Based on these findings, we could hypothesise that increase in the size of endometrioma could directly have an effect on the adjacent normal ovarian tissue and ovarian reserve thereby. However, to be able to make such assumptions, future trials are warranted where small antral follicle counts could be used to examine the effect of cyst when comparing to contra lateral normal ovary; and where oophorectomy is required, no opportunity is lost in examining the adjacent ovarian parenchyma histologically.

We still retain the comment made in a recent review article that there is insufficient data to suggest whether endometrioma related damage to ovarian reserve precedes or follows surgery (Garcia-Velasco and Somigliana 2009). We believe that perhaps the damage to ovarian reserve is two-fold with endometriotic cyst, one caused by the presence of cyst per se as shown in this study and supported by some histological data, and the other

related to cystectomy. Unless histology available, perhaps the best tool to assess ovarian reserve in these women would be to use the small antral follicle count and compare it to the contra lateral normal ovary.

## 11.5.2        Ovarian cystectomy and damage assessed histologically

Few studies have quantified histologically the amount of normal ovarian tissue removed at the time of cystectomy. It would be reasonable to presume that this would have an impact on the quantitative ovarian reserve; however whether it has an impact on the qualitative aspect of a woman's life in terms of pregnancy and premature menopause is uncertain.

Muzii et al examined 42 benign cysts removed laparoscopically by the stripping technique, of which 26 were endometriomas, 6 dermoids, 7 serous and 3 mucinous cystadenomas. They showed that in 36% of the cysts, normal ovarian tissue is excised together with the cyst wall. On sub group analysis, this was more pronounced with endometriomas than with non-endometriomas (54% vs. 6%) (Muzii, Bianchi et al. 2002). These results are comparable to our study. There was a 52% reduction noted in the small antral follicle count in the ovary that was operated upon for an endometrioma. These findings may further emphasise the reliability of using small antral follicle count as a marker of ovarian reserve when evaluating ovarian cysts. Muzii et al later evaluated the laparoscopic technique of stripping endometriotic ovarian cyst and the associated inadvertent removal of normal ovarian tissue. 48 women were randomised to two consecutive independent randomised trials. One trial evaluated the technique of circular excision and subsequent stripping versus immediate stripping at the initial adhesion site. The other trial evaluated the technique of stripping versus coagulating and cutting at the ovarian hilus. They

concluded that ovarian tissue is inadvertently excised along with the endometrioma in most cases irrespective of the technique used, although the excised tissue is at normal functional developmental stage (primary and secondary follicles) only at the hilus and the rest of the tissue contained primordial follicles alone. (Muzii, Bellati et al. 2005). Hachisuga et al histologically examined 72 women that underwent laparoscopic cystectomy for ovarian endometrioma. They divided them into two groups, one where the cyst was easily stripped and the other where stripping of the cyst was difficult. They found that easy stripping of the cyst was associated with more loss of follicles and ovarian stroma. (Hachisuga and Kawarabayashi 2002). Roman et al in their recent study demonstrated that adjacent ovarian parenchyma was present in 97% of cysts removed and that the thickness of ovarian tissue removed increases proportionally with cyst diameter (Roman, Tarta et al.; Roman, Tarta et al. 2010). There is also a possibility that with endometriotic cysts more amount of normal ovarian tissue is inadvertently removed due to the difficulty in stripping the cyst leading to bleeding and extensive use of bipolar diathermy as compared to non-endometriotic cysts (Exacoustos, Zupi et al. 2004).

The results from our study and those reported in the studies above confirm that there is a significant loss of normal ovarian parenchyma when stripping the ovarian cysts, more so in the hilar area and that the small antral follicle count correlates with the loss of ovarian tissue quantified histologically when removing endometriomas.

### 11.5.3        Ovarian cystectomy and clinical data on ovarian reserve

The technique of cystectomy used in our study has been described in the past and is a method commonly used. Laparoscopic cystectomy has been shown to have many advantages over conventional laparotomy and suturing of the ovary following removal of

cyst, including lesser recurrence, reduced morbidity, shorter hospital stay, fewer adhesions, and faster recovery (Yuen, Yu et al. 1997; Saleh and Tulandi 1999; Alborzi, Zarei et al. 2006). In comparison with traditional surgery by laparotomy and suturing of ovarian cortex, laparoscopic cystectomy is currently considered the treatment of choice for benign ovarian cysts (Chapron, Fauconnier et al. 2002; Chapron, Vercellini et al. 2002; Alborzi, Zarei et al. 2006). There are largely two ways of dealing with an ovarian cyst of endometriotic origin. One method is to strip the cyst from normal ovarian parenchyma and the other method is to drain the cyst and coagulate the endometrial lining of the cyst. The main outcome measure following the treatment of cyst is a reduction in the symptoms of pain and reduction in the incidence of recurrence (Saleh and Tulandi 1999). According to the recently published Cochrane review (Hart, Hickey et al. 2008), only three randomized controlled trials have been conducted comparing cystectomy to drainage with ablation by electrosurgery (Beretta, Franchi et al. 1998; Alborzi, Momtahan et al. 2004; Alborzi, Ravanbakhsh et al. 2007). Beretta et al randomised 64 patients to either have a cystectomy or drainage with ablation of endometrial lining, and evaluated the outcome measures of pain and pregnancy rate. They found a statistically significant favourable outcome in women who underwent cystectomy (Beretta, Franchi et al. 1998). Another RCT randomised 100 sub fertile patients to the above two techniques and found lower recurrence rate, lesser pain and better cumulative pregnancy rates with cystectomy than drainage and coagulation (Alborzi, Momtahan et al. 2004). One trial comparing the two techniques showed that there was no difference noted in the ovarian response to controlled ovarian stimulation (Alborzi, Ravanbakhsh et al. 2007). These findings suggest that it would be appropriate to consider the technique of cystectomy when outcomes of pain relief and recurrence were considered. Few cohort studies have shown favourable

outcome following ablation of endometrial capsule but they used laser to coagulate the capsule (Bellina, Fick et al. 1984; Bellina, Voros et al. 1984; Donnez 1987; Sutton and Jones 2002; Azem, Hasson et al. 2009)

One prospective study of 191 women comparing bipolar coagulation, coagulation with ultrasonic harmonic scalpel and suturing of the ovarian cortex (Li, Liu et al. 2009) showed that electrocoagulation causes damage to healthy ovarian parenchyma leading to reduced ovarian reserve as measured using FSH, AFC (3-10mm) and blood flow. One study looking at the effect of bilateral cystectomy with and without electrocoagulation for endometrioma showed that bilateral stripping of the endometrioma decreased the ovarian reserve as measured by FSH and AFC and that it was even more with electro coagulation (Zhang, Zhou et al. 2009). These studies suggest a detrimental effect of electro coagulation on ovarian reserve as measured using FSH, AFC and blood flow indices, however these markers especially FSH and blood flow indices show a significant within and inter-subject variation. Moreover, they are less reliable tests of ovarian reserve (Broekmans, Kwee et al. 2006).

The introduction of $CO_2$ laser in the laparoscopic management of endometriomas has been well established as an alternative, safe, and effective modality (Bellina, Fick et al. 1984; Bellina, Voros et al. 1984; Donnez 1987; Sutton and Jones 2002; Azem, Hasson et al. 2009). It is claimed to provide precise tissue dissection and ablation, controlled penetration, and tissue thermal damage. These advantages justify the reason why lasers may be potentially more tissue sparing than other sources of energy. Another study confirmed that destruction of the filmy superficial internal lining of endometriomas was a safe and effective approach for the treatment of endometriotic cysts without sacrificing the adjacent healthy ovarian cortex (Brosens, Van Ballaer et al. 1996). Donnez et al

published a large series of 814 women with endometriomas treated with a three step procedure (drainage followed by GnRh analogues followed by laser ablation) and showed a postoperative cumulative PR of 51% and the recurrence rate of 8% for a follow-up of 2–11 years (Donnez, Nisolle et al. 1996). Few studies have questioned laparoscopic stripping as an ideal surgical approach for endometriomas because it is associated with excessive removal of ovarian tissue and loss of ovarian follicles with subsequent reduction of ovarian reserve (Candiani, Barbieri et al. 2005; Busacca, Riparini et al. 2006; Busacca and Vignali 2009). A recent study by Donnez et al showed that stripping a large part of the cyst wall and coagulating the remaining 10-20% of cyst around the hilus with Co2 laser was not deleterious to the ovary. Normal ovarian tissue adjacent to cyst was found in only 2% of cases with a recurrence of small endometrioma in 2% (Donnez, Lousse et al. 2010). However, they used ovarian volume and AFC (2-10mm) as markers of ovarian reserve and compared it to that of the contra lateral normal ovary. Both these markers show significant inter ovarian variation as shown in chapter 7. Two recent studies have compared the three step procedure to cystectomy, showing a lesser damage to ovarian reserve as measured with AMH and AFC (2-9mm) in the 3 step procedure (Pados, Tsolakidis et al. 2010; Tsolakidis, Pados et al. 2010). The results from these two trials suggest that CO2 laser may cause lesser damage to ovarian reserve than bipolar electrocoagulation and also that 3 stage procedure might have a favourable impact on ovarian reserve. However, it means the patient will require two operative laparoscopies and an interim 3 months of GnRh analogues, the risks of which would have to be balanced against the advantages of the procedure. Also, as shown in the previous chapters, the use of small antral follicles in assessment of ovarian reserve may be ideal in combination with AMH in future trials.

### 11.5.4        Ovarian cystectomy and subfertility

In sub fertile women undergoing ART, it is unclear whether removal of cyst improves the fertilisation and pregnancy rates; however it is shown that the ovarian response is reduced as evident by the increase in gonadotophins used, reduction in the number of follicles stimulated and the number of oocytes collected. Some believe that the presence of an endometrioma may affect the quality of oocytes collected, but a RCT has shown no difference in fertilisation, pregnancy and implantation rates between surgery versus conservative management of cysts (Demirol, Guven et al. 2006). Several studies have associated ovarian cystectomy with impaired ovarian reserve (Loh, Tan et al. 1999; Somigliana, Ragni et al. 2003; Candiani, Barbieri et al. 2005; Godinjak, Idrizbegovic et al. 2005; Somigliana, Ragni et al. 2006) and ovarian response following assisted reproduction treatment (ART) (Loo, Lin et al. 2005; Ragni, Somigliana et al. 2005; Demirol, Guven et al. 2006; Esinler, Bozdag et al. 2006; Yazbeck, Madelenat et al. 2006; Kahyaoglu, Ertas et al. 2008; Somigliana, Arnoldi et al. 2008).   However, recent meta-analysis (Tsoumpou, Kyrgiou et al. 2009) of studies comparing surgical treatment with no treatment of endometrioma before IVF showed no significant difference in ovarian response and pregnancy rates.

The results from our study confirm the damage caused to ovarian reserve following cystectomy when using the technique of stripping the cyst from normal ovarian parenchyma. However, they cannot be directly compared to majority of the studies in the literature as comparisons are made with either the contra lateral ovary or a matched control. Only a few studies have used a longitudinal design where the ovarian reserve is compared pre and post operatively (Li, Liu et al. 2009; Chang, Han et al. 2010; Pados,

Tsolakidis et al. 2010; Tsolakidis, Pados et al. 2010; Iwase, Hirokawa et al. 2011). Chang et al evaluated ovarian reserve with AMH and ovarian volume measured using 3D ultrasound in 20 women who had undergone cystectomy (13 endometrioma, 6 dermoid and 1 cyst adenoma). They assessed women up to 3 months showing that the reserve reduced immediately post op (1 week) but restored to 65% at 3 months (Chang, Han et al. 2010). Our results are comparable to the immediate post operative drop but in our series we found that the effect persisted up to at least 6 months and that with endometrioma there was no significant recovery in ovarian reserve noted. This might be due to the use of AFC especially the small AFC and also that our sample size was larger than this study. Iwase et al assessed ovarian reserve in 51 patients (29 endometrioma and 21 non-endometrioma) using AMH and FSH. They examined women pre and one month post operatively. They found that AMH levels significantly reduced in all cysts but was more with endometrioma. Although these results are comparable to our study, we believe that our study is superior with regards to a longer follow up of 6 months and that small antral follicle counts were used in the assessment of ovarian reserve.

## 11.6   Conclusions

In conclusion, laparoscopic ovarian cystectomy is associated with a significant loss of ovarian reserve measured using AMH, and small antral follicle counts, the effect lasting at least up to 6 months. Endometrioma itself significantly affects the ovarian reserve of the diseased ovary and that removal of endometrioma might cause more damage to ovarian reserve than the non-endometriotic cysts.  Counting of antral follicles enables assessment of ovarian reserve in individual ovary and therefore provides more accurate assessment of

the damage caused to the operated ovary which otherwise could only be quantified using histological data.  Small antral follicles measuring 2.0-6.0 mm provide more reliable, accurate and comparable assessment of ovarian reserve. The longitudinal prospective nature of the study enables assessment of ovaries pre and post operatively which we believe is a more reliable method of assessing ovarian reserve following cystectomy.

**CHAPTER 12: General Discussion**

In this thesis, I have examined the ultrasound and endocrine markers of ovarian reserve in order to establish an optimum method of evaluating the effect of ovarian cystectomy on ovarian reserve. In this process, I studied a semi-automated 3D ultrasound assisted method, SonoAVC to measure the size and number of antral follicles and found that it made more reliable measures than other methods of measuring antral follicles, thereby defining a reliable and valid method of measuring small antral follicles. I then studied the physiological variation in these small antral follicles and also in other markers of ovarian reserve.  Since the small antral follicle population showed the least variation, I used it as a marker of ovarian reserve along with AMH in the evaluation of ovarian cysts and cystectomy. I also examined the effect of COCP on the markers of ovarian reserve as the study population that I planned to study might wish to use them for contraception during the study period.

## 12.1   Summary of Research Findings

I have shown that the intra-observer and inter-observer reliability of antral follicles made using SonoAVC was better than measurements made using the 3D multiplanar view or the 2D real time equivalent technique but only after post processing had been applied. Furthermore, measurement of the size of antral follicles was quicker and reliable with sonoAVC than with 2D ultrasound. *In vitro* validation study using bovine ovaries confirmed more agreement with sonoAVC than with 2D ultrasound. I further showed that the small antral follicle count as measured using sonoAVC was an independent significant predictor of pregnancy following in vitro fertilization. Having confirmed the reliability and validity of small antral follicle counts, I showed that there was minimal inter-ovarian, intra-cycle and

inter cycle variation in these follicles. I also went on to show that combined oral contraceptive pills did not affect the small antral follicle count and serum AMH levels. Finally, but not the least, I have shown that the benign ovarian cyst, especially endometriotic cyst and laparoscopic ovarian cystectomy of endometriotic and dermoid cyst using the stripping technique cause damage to the ovarian reserve as measured using AMH and small antral follicle count.

I began my research by examining the reliability of automated antral follicle counts made using 3D ultrasound. Sonography based automated volume counts is new software that can only be applied on data acquired using 3D ultrasound. Here, I found that the AFC calculated by the software in its complete automated format erroneously missed antral follicles of random sizes. This could be corrected by application of a feature called 'post-processing', which allowed inclusion of the missed follicles by manually clicking on them. This made the technique semi-automated and increased the time taken to measure antral follicle count. However, the colour coding provided by sonoAVC ensured that follicles were not counted more than once and that all were identified. In chapter 3, I have therefore reported reliability on four methods of counting antral follicles: automated sonoAVC (sAVC-AA), semi-automated sAVC (sAVC-PP), 3D multiplanar view which has mainly been the method of counting antral follicles using 3D ultrasound, and 2D ultrasound. The intra-observer and inter-observer reliability of antral follicles made using SonoAVC with post processing i.e. semi automated method was better than measurements made using the 3D multiplanar view or the 2D real time equivalent technique.

The main limitation of this software was the erroneously low total antral follicle counts made in its automated format which was overcome by post processing but at the expense of time and making the technique semi-automated. The main advantage of the software is its ability to measure diameter and volume of each follicle objectively and provide a unique colour to each follicle measured. This allowed accurate measurement of antral follicles, especially the small antral follicles. The potential advantage of having a reliable assessment of both follicle number and size as suggested in this study informed the work done in the next few chapters.

Having established that antral follicle count made using sonoAVC with post processing was more reliable than 2D and 3D multiplanar view methods, in chapter 4, I have compared its ability to measure the size of antral follicles to that made using 2D ultrasound. No such study has been reported in the literature. The relaxed sphere diameter, as used in this study to define the follicle diameter, was closely correlated to the follicular volume (Raine-Fenning 2008). I found that sonoAVC identified and measured significantly less number of total antral follicles than 2D ultrasound. This may relate to the image being displayed in a 3D multiplanar view which allows crosschecking of each follicle in three different planes and improves spatial orientation but is more likely to reflect the fact that each follicle is colour-coded preventing repeated measures of the same follicle. When stratified according to size, the main differences between the two techniques were between the follicle sizes less than 2 mm and more than 6 mm. Another difference was the time taken by 2D ultrasound to measure each antral follicle, which was significantly more than that taken by sonoAVC with post processing. The results from this study suggested that sonoAVC, despite being a semi-automated method as data post-processing

is invariably required, provides reliable and objective measures of the size of antral follicles more quickly than 2D ultrasound. This has important implications for both the research setting and clinical environment as it allows a quantitative analysis of antral follicle number and size without disturbing workflow.

Having shown that sonoAVC was reliable in measuring and counting the antral follicles and that it was quicker in measuring the size of follicles than 2D ultrasound, it was important to assess its ability as a test of ovarian reserve. Chapter 5 is about the study that I conducted to evaluate different cohorts of antral follicles, based on their absolute sizes measured using sonoAVC, in the prediction of reproductive outcome in subjects undergoing IVF treatment. The results showed that the small antral follicles, especially those measuring between 2.1 to 4.0 mm are a significant predictor of pregnancy independent of age, the number of mature oocytes obtained, fertilisation rates, the number of cleaved embryos, and the grade of embryos transferred. The primary outcome measure evaluated in this study was a viable intrauterine pregnancy confirmed on a transvaginal ultrasound scan 5 weeks after embryo transfer. Taking into account age, total number of antral follicles and the number of follicles within each size cohort, type of gonadotrophin used, technique used for fertilisation (IVF / ICSI), number of mature oocytes retrieved, number of fertilised oocytes, number of cleaved embryos, and the number and grade of embryos transferred, Logistic regression revealed that the only variable predictive of clinical pregnancy was the number of antral follicles measuring between 2.0 to 4.0 mm (Exp(B)=1.221; 95% CI 1.072 and 1.391; p=0.003). Receiver operating characteristic analysis suggested this cohort of follicles provided the most discriminative power to differentiate those women likely to conceive (AUC: 0.694) from

those whose treatment will not be successful; a total count of three providing the optimum sensitivity and specificity with a post-test probability of 53%. There was also a significant correlation found between age and the cohort of follicles measuring 2.1 to 4.0 mm (p=0.005) and 4.1 to 6.0 mm (p=0.001). These results further support the results from a study by Haadasma et al that the small antral follicles decline in number with increasing age and larger antral follicles remain constant. Linear regression analysis showed significant predictive relationship between small antral follicles and fertilisation rates and the number of cleaved embryos. This supports the notion that the smaller antral follicles are the follicles that truly reflect the ovarian potential and are most predictive of response to controlled ovarian stimulation during IVF treatment. Several studies suggest, however, that the total number of antral follicles is predictive of reproductive response (van Rooij, Broekmans et al. 2002; Hendriks, Mol et al. 2005; Muttukrishna, McGarrigle et al. 2005). It is possible that the smaller, more responsive follicles comprise the majority of the follicle population included in the total count in these individuals and that this masks the importance of the smaller follicles.

One limitation of this study was that only women predicted to have a normal response, as assessed by age and FSH levels of <15 IU/L, undergoing their first cycle of IVF were included in this study. Women above the age of 40 or with FSH ≥ 15IU/L that are likely to have an unfavourable outcome following IVF were not included in our study. Further work is required in women with poor and exaggerated response to treatment and would be ideal to undertake in conjunction with serum AMH measures.

Having established that small antral follicle counts measured using sonoAVC was reliable and predictive of ovarian response and pregnancy, it was important to establish that it was a true reflection of the existing follicles in the ovary. Study in chapter 6 was therefore designed to validate the counts made by 3D ultrasound assisted SonoAVC and 2D against the antral follicles manually dissected from the bovine ovary. The results showed that when validating for antral follicles measuring more than 4.0mm, both SonoAVC and 2D real-time equivalent made counts comparable to the dissection method, but SonoAVC with post processing agreed more than the 2D method. However, this was not true for follicles measuring 4.0mm or less. Whilst SonoAVC made significantly fewer counts, 2D made significantly greater counts than the dissection method, although SonoAVC agreed more with the dissection method than the 2D method. It therefore appears that no current method available to measure the count and size of antral follicles is accurate, but SonoAVC with post-processing shows the most agreement with the follicles measured following manual dissection.

Several studies have reported good predictive value of antral follicle counts in assessing response following IVF treatment, their strong correlation to AMH and finally ovarian ageing. This would perhaps make one believe that the current understanding of the AFC cut-offs defining normal from poor or hyper-responders although not accurate are fair assessment tools of ovarian reserve. However, knowing that the current tests for calculating AFC are not accurate, there is a tremendous scope in developing the test accuracies in future, especially the small antral follicles, which might improve upon the current predictive abilities in assessing ovarian response to IVF and also reproductive ageing. When using SonoAVC, one could argue that since it underestimates AFC, we must exhibit caution with regards to women undergoing first cycle of IVF especially when

considering cycle cancellation or refusing IVF treatment. On the other hand, when using 2D to measure antral follicles, one could argue that since it might overestimate AFC, we must consider appropriate dosages when deciding on the amount of gonadotrophin used for ovarian stimulation. SonoAVC was shown to have more agreement with dissection method than 2D, especially with follicles measuring 4.0mm or less.  Since SonoAVC can reliably measure size of antral follicles, the small antral follicle population must be researched further in studies predicting ovarian response to IVF.

When evaluating the effects of ovarian cystectomy, several studies have used the contra lateral ovary as a control. The study in chapter 7 was therefore designed to compare the three-dimensional ultrasound markers of ovarian reserve between the two ovaries within the same individual. Our results suggest that there are significant differences in the ultrasound markers of ovarian reserve between the two ovaries within an individual during the early follicular phase of the menstrual cycle as measured using 3D ultrasound. Ovarian volume, total antral follicle count of each ovary and antral follicles measuring more than 6 mm showed significant differences; however this variation was least evident in the small antral follicles, measuring 6 mm or less. These results would have to be considered when evaluating the effect of ovarian pathology or surgery. Moreover, since these ultrasound markers have the capacity to predict ovarian response during ART, these findings should be considered when comparing one ovary to another to assess the effects of surgery.  The results suggested that antral follicle count data, stratified by size, be considered when the two ovaries are compared to quantify the effect of surgery. The variation in the ultrasound markers of ovarian reserve between the ovaries may relate to the effect of ovulation and corpus luteum formation in the preceding cycle or be an early

indicator of dominance in the current cycle (Baerwald, Adams et al. 2003). The inter-ovarian difference in ovarian volume seen could be either due to inherent differences in the two ovaries or due to the number of larger antral follicles. There were no significant differences noted in the 3D power Doppler indices between the two ovaries. The ultrasound scans were performed in the early follicular phase when the population of follicles in the ovary is predominantly antral follicles, and therefore perhaps no difference noted in vascularity (Costello, Shrestha et al. 2005). However, this may not be consistent in different phases of the menstrual cycle, as the growing follicle and corpus luteum acquire more blood supply (Zaidi, Collins et al. 1996).

The study in chapter 8 was designed to examine the variation in the 3D ultrasound markers of ovarian reserve and the endocrine markers including serum AMH, FSH, LH and Oestradiol in one menstrual cycle. The results suggest that there is a small but significant intra-cyclical variation in serum AMH, which is mainly due to the increase in levels in the luteal phase of the menstrual cycle. The total AFC significantly varied intra-cyclically, however this was mainly contributed to by the large antral follicles measuring more than 6.0mm, and to a smaller but significant extent by the 2.0 to 4.0mm follicles. Overall, the small antral follicles measuring 2.0 to 6.0mm did not show a significant intra-cyclical variation. At any given time point during the menstrual cycle, the ovaries contain follicles at different developmental stages (Gougeon 1998). This explains why a significant intra-cyclical variation in the number of large antral follicles was noted in our study, especially the increase in the number from follicular to peri-ovulatory phase of cycle and then a decrease in number in the luteal phase.

The increase in AMH levels in the luteal phase of the cycle with no change in the small antral follicle count confirming that there exists a continuous recruitment of pre-antral and very small antral follicles that are also known to produce AMH (Gougeon 1989; Gougeon 1996; Tsepelidis, Demeestere et al. 2007) but that cannot be identified on ultrasound. Alternatively, this may be due to the small sample size, albeit we chose a targeted study population that were young normal healthy women with normal ovulatory cycles. Several studies have reported intra-cycle stability of AMH and that it might even show lower variation than total AFC. Significant variation seen in ovarian volume was mainly due to an increase in the peri-ovulatory and luteal phase of cycle and could be attributed to the presence of the dominant follicle and the subsequent corpus luteum formation. FSH and LH, both showed significant intra-cycle variation, mainly due to the increase in the peri-ovulatory phase and a subsequent drop in the luteal phase of menstrual cycle. This variation is well described (Gougeon 1994) and therefore confirms that these tests are best performed in the early follicular phase. Oestradiol levels expectedly showed significant variation.

The results from this study suggested that small antral follicles and AMH showed least intra-cycle variation and an excellent within subject correlation. The main advantage of this study was the objective and reliable assessment of the size of antral follicles using 3D ultrasound and examining the variation in both small and large antral follicles.

Study in chapter 9 was designed to evaluate the inter-cycle variation in the 3D ultrasound and endocrine determinants of ovarian reserve, such that the best test of ovarian reserve could be used when examining the effect of ovarian cystectomy. The small AFC measuring 2.0-6.0 mm showed the least variation between five cycles over a period of 12 months

followed by serum AMH and total AFC measuring 2.0-10.0 mm. A high inter-cycle correlation was observed in these markers with significant intra class correlation coefficient suggesting a small non-significant intra-individual variation between cycles. The results from this study in conjunction with those from Chapter (intra-cycle variation) suggest that to ensure a reliable measure of ovarian reserve, the tests including the total AFC (2.0-10.0 mm) and the AFC of larger antral follicles (> 6.0 mm) are best measured in the early follicular phase, whereas the small AFC (2.0-6.0 mm) could be measured in any phase of menstrual cycle. In our study, the inter-cycle stability of AMH (ICC - 0.91) was comparable to that seen with tAFC (2.0-10.0 mm) (ICC - 0.89) and small AFC (2.0-6.0 mm) (ICC - 0.95). A significant inter-cycle change in the mean FSH, LH and progesterone levels was noted; however a non-significant change in oestradiol levels over 12 months. The inter-cycle stability of total antral follicle count (2.0-10.0 mm) when performed in the early follicular phase of menstrual cycle appears to be comparable to AMH and small AFC. These determinants of ovarian reserve demonstrate inter-cycle stability for up to 12 months and hence future studies designed to evaluate their ability to predict ovarian response following assisted reproduction treatment should consider performing these tests of ovarian reserve once in a year irrespective of the number of ART cycles required.

Study in chapter 10 was designed to further evaluate the effect of combined oral contraceptive pills on ovarian reserve. The results suggested that despite lower serum levels of gonadotrophins in the COCP users, the population of small antral follicles measuring 2 to 6 mm and serum AMH levels were comparable to those seen in non-pill users; however growth of antral follicles beyond 6 mm was significantly reduced in pill users. These results are consistent with the fact that the pool of selectable antral follicles

(2-5 mm) are gonadotrophin responsive but not dependant and that the antral follicles measuring more than 6 mm are gonadotrophin dependant (Gougeon and Lefevre 1983). Overall, these findings support the interpretation that the number of small antral follicles and circulating AMH concentrations more accurately reflect the functional ovarian reserve. Although some studies suggest, that the total number of antral follicles is predictive of reproductive response (van Rooij, Broekmans et al. 2002; Hendriks, Mol et al. 2005; Muttukrishna, McGarrigle et al. 2005) this can be explained by the fact that the smaller, more responsive antral follicles comprise the majority of the follicle population included in the total antral follicle count in these individuals.  FSH is considered to be a major survival factor for gonadotrophin responsive follicles, the stage during which a majority of follicles undergo atresia under physiological conditions (Gougeon and Busso 2000). However, due to the range of approaches and doses used in these animal studies, it is difficult to determine the minimum amount of FSH that is required to influence the survival of these gonadotrophin responsive antral follicles. From the present study, it is clear that a relatively modest suppression of pituitary gonadotrophins in our study did not significantly affect the number of selectable antral follicles. The reduction in ovarian volume may be due to the combined effect of a lower number of large antral follicles and reduced vascularity as shown by 3D power Doppler indices.  The results of the current study are relevant to the use of COCP in assisted reproduction treatment, including both IVF and ICSI. The benefits of using the COCP as part of an assisted reproduction treatment cycles are unclear although it is possible that this may lead to a homogenous response to controlled ovarian stimulation by influencing the recruitment and growth of follicles and the yield and quality of the oocytes harvested. This possibility, however, requires evaluation in future trials.

Having confirmed the functional role of small antral follicles in assessment of ovarian reserve, and its reliability and validity in evaluating ovarian reserve, they were then used in examining the effects of ovarian cyst and cystectomy along with serum AMH in the study in chapter 11. The results suggest that laparoscopic ovarian cystectomy for a benign cyst is associated with a significant loss of ovarian reserve lasting up to at least 6 months post operatively. The damage caused is more in the ovary that is operated upon than the other normal ovary. The results also suggest that the damage caused by the stripping of endometrioma is more pronounced than the damage caused by stripping of dermoid cyst. The positive aspects of this study were that it used 3D ultrasound assisted sonoAVC to measure and count antral follicles and AMH to assess ovarian reserve in a prospective longitudinal observational design. Use of small antral follicles enabled comparison of diseased ovary to the contra lateral ovary thus quantifying the effect of cyst on the affected ovary. Only endometriotic cysts per se affected the ovarian reserve of the ovary containing the cyst. These findings were comparable to some of the histological studies reported emphasising the value of these small follicles in assessing the damage caused by cyst itself. Few studies have quantified histologically the amount of normal ovarian tissue removed at the time of cystectomy. It would be reasonable to presume that this would have an impact on the quantitative ovarian reserve; however whether it has an impact on the qualitative aspect of a woman's life in terms of pregnancy and premature menopause is uncertain. The results from our study and those reported in the past confirm that there is a significant loss of normal ovarian parenchyma when stripping the ovarian cysts, more so in the hilar area and that the small antral follicle count correlates with the loss of ovarian tissue quantified histologically when removing endometriomas. In sub fertile

women undergoing ART, it is unclear whether removal of cysts improves the fertilisation and pregnancy rates; however it is shown that the ovarian response is reduced as evident by the increase in gonadotophins used, reduction in the number of follicles stimulated and the number of oocytes collected. Some believe that the presence of an endometrioma may affect the quality of oocytes collected, but recent meta-analysis of studies has shown no difference in fertilisation, pregnancy and implantation rates between surgery versus conservative management of cysts (Tsoumpou, Kyrgiou et al. 2009). The results of our study confirm the damage caused to ovarian reserve following cystectomy when using the technique of stripping the cyst from normal ovarian parenchyma. However, they cannot be directly compared to majority of the studies in the literature as comparisons are made with either the contra lateral ovary or a matched control. Only a few studies have used a longitudinal design where the ovarian reserve is compared pre and post operatively; however our study differs in the use of small antral follicle count and 3D ultrasound. The main limitation of this study has been a small sample size and a post operative follow up of up to 6 months only, albeit a better design and a longer follow up than what has been reported in the literature so far.

## 12.2   New findings in relation to ovarian reserve assessment and its application in assessment of ovarian cysts

Several new findings relating to ovarian reserve assessment and prediction of ovarian response, particularly when ovarian parameters are defined by 3D ultrasound, have been made in this thesis.

I have evaluated the role of small antral follicles in assessment of ovarian reserve and shown that these follicles are significant predictors of ovarian response and pregnancy following in vitro fertilisation. I have confirmed one of the presumptions that AMH is expressed by pre antral and small antral follicles by demonstrating an excellent correlation between the two (Deb, Batcha et al. 2009).

This thesis evaluates the role of 3D assisted technique of measuring and counting antral follicles called the 'sonography based automated volume calculation'. I have contributed towards its application in the assessment of antral follicles by demonstrating the limitations of completely automated version of sonoAVC whereby it erroneously missed antral follicles of random sizes in the analysis. To overcome these limitations, I have applied the feature of post processing and then evaluated its application in clinical practice. I have shown that sonoAVC with post processing had an excellent intra- and inter-observer reliability in measuring antral follicles (Deb, Jayaprakasan et al. 2009).

Furthermore, I have shown that antral follicles measured by sonoAVC take much lesser time than those measured by 2D ultrasound (Deb, Campbell et al. 2010). There was good agreement between the two methods when measuring antral follicles between 2 and 6 mm, however lesser agreement when measuring more than 6 mm and less than 2mm size follicles (Deb, Campbell et al. 2010). With the help of *in vitro* study on bovine ovaries, I have shown that sonoAVC perhaps underestimates and 2D overestimates the antral follicles measuring less than 4mm, albeit sonoAVC shows more agreement with the manually dissected follicles from the ovary. The two methods compared well with the actual number of follicles when considering follicles measuring more than 4 mm. These findings will further help in its evolution as a test of ovarian reserve.

I have contributed to the current understanding of inter ovarian, intra cycle and inter cycle variations in the small antral follicles when compared to larger antral follicles, total antral follicle count, AMH, and FSH (Deb, Kannamannadiar et al.). Small antral follicles along with AMH show the least physiological variation. This finding makes these two tests reliable in the assessment of ovarian reserve. I have also contributed to the understanding of partial suppression of pituitary ovarian axis following prolonged use of COCP with HFI and its effect on antral follicles, AMH and gonadotrophins (Submitted to journal of *Ultrasound Obstet Gynecol)*. The work from this thesis has shown that COCP does not affect the small antral follicles and AMH.

Finally, I have demonstrated a new objective, reliable and valid method of assessing ovarian reserve following ovarian cystectomy and also a potential method of assessing effect of ovarian cyst per se on ovarian reserve.

## 12.3   Future suggested direction of work

This study has shown the potential benefits of using a semi-automated 3D method (SonoAVC with post processing) for the identification and measurement of antral follicles. It is a more objective and reliable method than real-time 2D ultrasound and takes significantly less time than conventional ultrasound assessments. Further research is required to evaluate the biological and clinical importance of quantifying antral follicle size. Knowing that the current tests for calculating AFC are not accurate, there is a tremendous scope in developing the test accuracies in future, especially the small antral follicles, which might improve upon the current predictive abilities in assessing ovarian response to IVF and also reproductive ageing.

The small antral follicle count appears to predict pregnancy independently of age and the total number of antral follicles. Further work is required in women with poor and exaggerated response to treatment and should be undertake in conjunction with serum AMH measures. This technique may facilitate prospective studies designed to examine the effect of different gonadotrophin preparations and doses and treatment protocols.

The functional ovarian reserve as measured using small antral follicle count (2.0-6.0mm) and AMH show least intra-cycle variation and an excellent within subject correlation. Future studies designed to evaluate their ability to predict ovarian response following assisted reproduction treatment should consider performing these tests in various time-points in the menstrual cycle.

The benefits of using the COCP as part of an assisted reproduction treatment cycles are unclear although it is possible that this may lead to a homogenous response to controlled ovarian stimulation by influencing the recruitment and growth of follicles and the yield and quality of the oocytes harvested. This possibility, however, requires evaluation in future trials.

Future trials evaluating the effect of ovarian cystectomy should be designed to look at standardised techniques of cystectomy on specific types of ovarian cyst using small antral follicle count and AMH in a longitudinal fashion over a longer period of follow up. Whilst examining the quantitative loss of ovarian reserve, qualitative measures such as pregnancy should be considered in the outcome measures.

**CHAPTER 13:   Bibliography**

(2001). "Social determinants of human reproduction." <u>Hum Reprod</u> **16**(7): 1518-26.

(2004). "Revised 2003 consensus on diagnostic criteria and long-term health risks related
to polycystic ovary syndrome (PCOS)." <u>Hum Reprod</u> **19**(1): 41-7.

Abir, R., S. Franks, et al. (1997). "Mechanical isolation and in vitro growth of preantral and
small antral human follicles." <u>Fertil Steril</u> **68**(4): 682-8.

Adler, R. S., J. M. Rubin, et al. (1995). "Ultrasonic estimation of tissue perfusion: a
stochastic approach." <u>Ultrasound Med Biol</u> **21**(4): 493-500.

al-Mizyen, E., L. Sabatini, et al. (2000). "Does pretreatment with progestogen or oral
contraceptive pills in low responders followed by the GnRHa flare protocol
improve the outcome of IVF-ET?" <u>J Assist Reprod Genet</u> **17**(3): 140-6.

Alborzi, S., M. Momtahan, et al. (2004). "A prospective, randomized study comparing
laparoscopic ovarian cystectomy versus fenestration and coagulation in patients
with endometriomas." <u>Fertil Steril</u> **82**(6): 1633-7.

Alborzi, S., R. Ravanbakhsh, et al. (2007). "A comparison of follicular response of ovaries
to ovulation induction after laparoscopic ovarian cystectomy or fenestration and
coagulation versus normal ovaries in patients with endometrioma." <u>Fertil Steril</u>
**88**(2): 507-9.

Alborzi, S., A. Zarei, et al. (2006). "Management of ovarian endometrioma." <u>Clin Obstet
Gynecol</u> **49**(3): 480-91.

Allemand, M. C., I. S. Tummon, et al. (2006). "Diagnosis of polycystic ovaries by three-
dimensional transvaginal ultrasound." <u>Fertil Steril</u> **85**(1): 214-9.

Altman, D. G. (1999). "Practical statistics for medical research." <u>London: Chapman &
Hall/CRC</u>.

Ashrafi, M., T. Madani, et al. (2005). "Follicle stimulating hormone as a predictor of ovarian response in women undergoing controlled ovarian hyperstimulation for IVF." Int J Gynaecol Obstet **91**(1): 53-7.

Athey, P. A. and D. D. Diment (1989). "The spectrum of sonographic findings in endometriomas." J Ultrasound Med **8**(9): 487-91.

Azem, F., J. Hasson, et al. (2009). "Retrieval of immature oocytes after chemotherapy for Hodgkin's disease and prolonged ovarian down-regulation with gonadotropin-releasing hormone agonist." Fertil Steril **92**(2): 828 e1-2.

Baarends, W. M., J. W. Hoogerbrugge, et al. (1995). "Anti-mullerian hormone and anti-mullerian hormone type II receptor messenger ribonucleic acid expression during postnatal testis development and in the adult testis of the rat." Endocrinology **136**(12): 5614-22.

Badawy, S. Z., V. Cuenca, et al. (1998). "Effects of chocolate cyst fluid on endometrioma cell growth in culture." Fertil Steril **70**(5): 827-30.

Baerwald, A. R., G. P. Adams, et al. (2003). "A new model for ovarian follicular development during the human menstrual cycle." Fertil Steril **80**(1): 116-22.

Bellina, J. H., A. C. Fick, et al. (1984). "Application of the CO2 laser to infertility surgery." Surg Clin North Am **64**(5): 899-904.

Bellina, J. H., J. I. Voros, et al. (1984). "Surgical management of endometriosis with the carbon dioxide laser." Microsurgery **5**(4): 197-201.

Benaglia, L., E. Somigliana, et al. (2009). "Endometriotic ovarian cysts negatively affect the rate of spontaneous ovulation." Hum Reprod **24**(9): 2183-6.

Benaglia, L., E. Somigliana, et al. (2010). "Rate of severe ovarian damage following surgery for endometriomas." Hum Reprod **25**(3): 678-82.

Benschop, L., C. Farquhar, et al. "Interventions for women with endometrioma prior to assisted reproductive technology." Cochrane Database Syst Rev(11): CD008571.

Beretta, P., M. Franchi, et al. (1998). "Randomized clinical trial of two laparoscopic treatments of endometriomas: cystectomy versus drainage and coagulation." Fertil Steril **70**(6): 1176-80.

Biljan, M. M., N. G. Mahutte, et al. (1998). "Effects of pretreatment with an oral contraceptive on the time required to achieve pituitary suppression with gonadotropin-releasing hormone analogues and on subsequent implantation and pregnancy rates." Fertil Steril **70**(6): 1063-9.

Bland, J. M. and D. G. Altman (1986). "Statistical methods for assessing agreement between two methods of clinical measurement." Lancet **1**(8476): 307-10.

Bland, J. M. and D. G. Altman (1995). "Comparing methods of measurement: why plotting difference against standard method is misleading." Lancet **346**(8982): 1085-7.

Bland, J. M. and D. G. Altman (1999). "Measuring agreement in method comparison studies." Stat Methods Med Res **8**(2): 135-60.

Bonilla-Musoles, F., F. Raga, et al. (1995). "Three-dimensional ultrasound evaluation of ovarian masses." Gynecol Oncol **59**(1): 129-35.

Bordes, A., A. M. Bory, et al. (2002). "Reproducibility of transvaginal three-dimensional endometrial volume measurements with virtual organ computer-aided analysis (VOCAL) during ovarian stimulation." Ultrasound Obstet Gynecol **19**(1): 76-80.

Bowen, S., J. Norian, et al. (2007). "Simple tools for assessment of ovarian reserve (OR): individual ovarian dimensions are reliable predictors of OR." Fertil Steril **88**(2): 390-5.

Broekmans, F. J., M. J. Faddy, et al. (2004). "Antral follicle counts are related to age at natural fertility loss and age at menopause." Menopause **11**(6 Pt 1): 607-14.

Broekmans, F. J., E. A. Knauff, et al. (2007). "Female reproductive ageing: current knowledge and future trends." Trends Endocrinol Metab **18**(2): 58-65.

Broekmans, F. J., J. Kwee, et al. (2006). "A systematic review of tests predicting ovarian reserve and IVF outcome." Hum Reprod Update **12**(6): 685-718.

Broekmans, F. J., M. R. Soules, et al. (2009). "Ovarian aging: mechanisms and clinical consequences." Endocr Rev **30**(5): 465-93.

Broekmans, F. J., J. A. Visser, et al. (2008). "Anti-Mullerian hormone and ovarian dysfunction." Trends Endocrinol Metab **19**(9): 340-7.

Broer, S. L., B. Mol, et al. "The role of anti-Mullerian hormone assessment in assisted reproductive technology outcome." Curr Opin Obstet Gynecol **22**(3): 193-201.

Brosens, I. A., P. J. Puttemans, et al. (1994). "The endoscopic localization of endometrial implants in the ovarian chocolate cyst." Fertil Steril **61**(6): 1034-8.

Brosens, I. A., P. Van Ballaer, et al. (1996). "Reconstruction of the ovary containing large endometriomas by an extraovarian endosurgical technique." Fertil Steril **66**(4): 517-21.

Brown, J. R., H. C. Liu, et al. (1995). "Variability of day 3 follicle-stimulating hormone levels in eumenorrheic women." J Reprod Med **40**(9): 620-4.

Busacca, M., J. Riparini, et al. (2006). "Postsurgical ovarian failure after laparoscopic excision of bilateral endometriomas." Am J Obstet Gynecol **195**(2): 421-5.

Busacca, M. and M. Vignali (2009). "Endometrioma excision and ovarian reserve: a dangerous relation." J Minim Invasive Gynecol **16**(2): 142-8.

Campbell, B. K., E. E. Telfer, et al. (2000). "Ovarian autografts in sheep as a model for studying folliculogenesis." <u>Mol Cell Endocrinol</u> **163**(1-2): 131-9.

Campbell, B. K., E. E. Telfer, et al. (2004). "Evidence of a role for follicle-stimulating hormone in controlling the rate of preantral follicle development in sheep." <u>Endocrinology</u> **145**(4): 1870-9.

Candiani, M., M. Barbieri, et al. (2005). "Ovarian recovery after laparoscopic enucleation of ovarian cysts: insights from echographic short-term postsurgical follow-up." <u>J Minim Invasive Gynecol</u> **12**(5): 409-14.

Canis, M., J. L. Pouly, et al. (2001). "Ovarian response during IVF-embryo transfer cycles after laparoscopic ovarian cystectomy for endometriotic cysts of >3 cm in diameter." <u>Hum Reprod</u> **16**(12): 2583-6.

Caspi, B., Z. Appelman, et al. (1996). "Pathognomonic echo patterns of benign cystic teratomas of the ovary: classification, incidence and accuracy rate of sonographic diagnosis." <u>Ultrasound Obstet Gynecol</u> **7**(4): 275-9.

Chang, H. J., S. H. Han, et al. (2009). "Impact of laparoscopic cystectomy on ovarian reserve: serial changes of serum anti-Mullerian hormone levels." <u>Fertil Steril</u>.

Chang, H. J., S. H. Han, et al. (2010). "Impact of laparoscopic cystectomy on ovarian reserve: serial changes of serum anti-Mullerian hormone levels." <u>Fertil Steril</u>.

Chang, M. Y., C. H. Chiang, et al. (1998). "Use of the antral follicle count to predict the outcome of assisted reproductive technologies." <u>Fertil Steril</u> **69**(3): 505-10.

Chapron, C., A. Fauconnier, et al. (2002). "Laparoscopic surgery is not inherently dangerous for patients presenting with benign gynaecologic pathology. Results of a meta-analysis." <u>Hum Reprod</u> **17**(5): 1334-42.

Chapron, C., P. Vercellini, et al. (2002). "Management of ovarian endometriomas." <u>Hum Reprod Update</u> **8**(6): 591-7.

Chow, G. E., A. R. Criniti, et al. (2004). "Antral follicle count and serum follicle-stimulating hormone levels to assess functional ovarian age." <u>Obstet Gynecol</u> **104**(4): 801-4.

Chun, S. Y., K. M. Eisenhauer, et al. (1996). "Hormonal regulation of apoptosis in early antral follicles: follicle-stimulating hormone as a major survival factor." <u>Endocrinology</u> **137**(4): 1447-56.

Cook, C. L., Y. Siow, et al. (2000). "Serum mullerian-inhibiting substance levels during normal menstrual cycles." <u>Fertil Steril</u> **73**(4): 859-61.

Coric, M., D. Barisic, et al. (2010). "Electrocoagulation versus suture after laparoscopic stripping of ovarian endometriomas assessed by antral follicle count: preliminary results of randomized clinical trial." <u>Arch Gynecol Obstet</u>.

Costello, M. F., S. M. Shrestha, et al. (2005). "Power Doppler ultrasound assessment of ovarian perifollicular blood flow in women with polycystic ovaries and normal ovaries during in vitro fertilization treatment." <u>Fertil Steril</u> **83**(4): 945-54.

Crosignani, P. G., G. Testa, et al. (1996). "Ovarian activity during regular oral contraceptive use." <u>Contraception</u> **54**(5): 271-3.

Damario, M. A., L. Barmat, et al. (1997). "Dual suppression with oral contraceptives and gonadotrophin releasing-hormone agonists improves in-vitro fertilization outcome in high responder patients." <u>Hum Reprod</u> **12**(11): 2359-65.

de Boer, E. J., I. den Tonkelaar, et al. (2003). "Increased risk of early menopausal transition and natural menopause after poor response at first IVF treatment." <u>Hum Reprod</u> **18**(7): 1544-52.

de Carvalho, B. R., A. C. Rosa e Silva, et al. (2008). "Ovarian reserve evaluation: state of the art." <u>J Assist Reprod Genet</u> **25**(7): 311-22.

de Vet, A., J. S. Laven, et al. (2002). "Antimullerian hormone serum levels: a putative marker for ovarian aging." <u>Fertil Steril</u> **77**(2): 357-62.

Deb, S., M. Batcha, et al. (2009). "The predictive value of the automated quantification of the number and size of small antral follicles in women undergoing ART." <u>Hum Reprod</u> **24**(9): 2124-32.

Deb, S., B. K. Campbell, et al. (2010). "Quantitative analysis of antral follicle number and size: a comparison of two-dimensional and automated three-dimensional ultrasound techniques." <u>Ultrasound Obstet Gynecol</u> **35**(3): 354-60.

Deb, S., K. Jayaprakasan, et al. (2009). "Intraobserver and interobserver reliability of automated antral follicle counts made using three-dimensional ultrasound and SonoAVC." <u>Ultrasound Obstet Gynecol</u> **33**(4): 477-83.

Deb, S., J. Kannamannadiar, et al. "The interovarian variation in three-dimensional ultrasound markers of ovarian reserve in women undergoing baseline investigation for subfertility." <u>Fertil Steril</u> **95**(2): 667-72.

Dehghani-Firouzabadi, R., N. Tayebi, et al. (2008). "Serum level of anti-mullerian hormone in early follicular phase as a predictor of ovarian reserve and pregnancy outcome in assisted reproductive technology cycles." <u>Arch Iran Med</u> **11**(4): 371-6.

Demirol, A., S. Guven, et al. (2006). "Effect of endometrioma cystectomy on IVF outcome: a prospective randomized study." <u>Reprod Biomed Online</u> **12**(5): 639-43.

Deutch, T. D., I. Joergner, et al. (2009). "Automated assessment of ovarian follicles using a novel three-dimensional ultrasound software." <u>Fertil Steril</u> **92**(5): 1562-8.

Donnez, J. (1987). "CO2 laser laparoscopy in infertile women with endometriosis and women with adnexal adhesions." Fertil Steril **48**(3): 390-4.

Donnez, J., J. C. Lousse, et al. (2009). "Laparoscopic management of endometriomas using a combined technique of excisional (cystectomy) and ablative surgery." Fertil Steril.

Donnez, J., J. C. Lousse, et al. (2010). "Laparoscopic management of endometriomas using a combined technique of excisional (cystectomy) and ablative surgery." Fertil Steril **94**(1): 28-32.

Donnez, J., M. Nisolle, et al. (1996). "Large ovarian endometriomas." Hum Reprod **11**(3): 641-6.

Durlinger, A. L., M. J. Gruijters, et al. (2001). "Anti-Mullerian hormone attenuates the effects of FSH on follicle development in the mouse ovary." Endocrinology **142**(11): 4891-9.

Durlinger, A. L., P. Kramer, et al. (1999). "Control of primordial follicle recruitment by anti-Mullerian hormone in the mouse ovary." Endocrinology **140**(12): 5789-96.

Eldar-Geva, T., A. Ben-Chetrit, et al. (2005). "Dynamic assays of inhibin B, anti-Mullerian hormone and estradiol following FSH stimulation and ovarian ultrasonography as predictors of IVF outcome." Hum Reprod **20**(11): 3178-83.

Elgindy, E. A., D. O. El-Haieg, et al. (2008). "Anti-Mullerian hormone: correlation of early follicular, ovulatory and midluteal levels with ovarian response and cycle outcome in intracytoplasmic sperm injection patients." Fertil Steril **89**(6): 1670-6.

Elter, K., Z. N. Kavak, et al. (2005). "Antral follicle assessment after down-regulation may be a useful tool for predicting pregnancy loss in in vitro fertilization pregnancies." Gynecol Endocrinol **21**(1): 33-7.

Elter, K., A. Sismanoglu, et al. (2005). "Intercycle variabilities of basal antral follicle count and ovarian volume in subfertile women and their relationship to reproductive aging: a prospective study." Gynecol Endocrinol **20**(3): 137-43.

Engmann, L., P. Sladkevicius, et al. (1999). "Value of ovarian stromal blood flow velocity measurement after pituitary suppression in the prediction of ovarian responsiveness and outcome of in vitro fertilization treatment." Fertil Steril **71**(1): 22-9.

Esinler, I., G. Bozdag, et al. (2006). "Outcome of in vitro fertilization/intracytoplasmic sperm injection after laparoscopic cystectomy for endometriomas." Fertil Steril **85**(6): 1730-5.

Evers, J. L., P. Slaats, et al. (1998). "Elevated levels of basal estradiol-17beta predict poor response in patients with normal basal levels of follicle-stimulating hormone undergoing in vitro fertilization." Fertil Steril **69**(6): 1010-4.

Exacoustos, C., E. Zupi, et al. (2004). "Laparoscopic removal of endometriomas: sonographic evaluation of residual functioning ovarian tissue." Am J Obstet Gynecol **191**(1): 68-72.

Faddy, M. J. and R. G. Gosden (1996). "A model conforming the decline in follicle numbers to the age of menopause in women." Hum Reprod **11**(7): 1484-6.

Fanchin, R., D. H. Mendez Lozano, et al. (2007). "Anti-Mullerian hormone concentrations in the follicular fluid of the preovulatory follicle are predictive of the implantation potential of the ensuing embryo obtained by in vitro fertilization." J Clin Endocrinol Metab **92**(5): 1796-802.

Fanchin, R., L. M. Schonauer, et al. (2003). "Serum anti-Mullerian hormone is more strongly related to ovarian follicular status than serum inhibin B, estradiol, FSH and LH on day 3." Hum Reprod **18**(2): 323-7.

Fanchin, R., J. Taieb, et al. (2005). "High reproducibility of serum anti-Mullerian hormone measurements suggests a multi-staged follicular secretion and strengthens its role in the assessment of ovarian follicular status." Hum Reprod **20**(4): 923-7.

Ficicioglu, C., T. Kutlu, et al. (2006). "Early follicular antimullerian hormone as an indicator of ovarian reserve." Fertil Steril **85**(3): 592-6.

Findlay, J. K. (1993). "An update on the roles of inhibin, activin, and follistatin as local regulators of folliculogenesis." Biol Reprod **48**(1): 15-23.

Fisch, B., M. Royburt, et al. (1996). "Augmentation of low ovarian response to superovulation before in vitro fertilization following priming with contraceptive pills." Isr J Med Sci **32**(12): 1172-6.

Fleming, R., N. Deshpande, et al. (2006). "Dynamics of FSH-induced follicular growth in subfertile women: relationship with age, insulin resistance, oocyte yield and anti-Mullerian hormone." Hum Reprod **21**(6): 1436-41.

Fortune, J. E., R. A. Cushman, et al. (2000). "The primordial to primary follicle transition." Mol Cell Endocrinol **163**(1-2): 53-60.

Fortune, J. E., S. Kito, et al. (1998). "Activation of bovine and baboon primordial follicles in vitro." Theriogenology **49**(2): 441-9.

Fowler, P. A., U. Fahy, et al. (1995). "Gonadotrophin surge-attenuating factor bioactivity is present in follicular fluid from naturally cycling women." Hum Reprod **10**(1): 68-74.

Franks, S. and H. D. Mason (1991). "Polycystic ovary syndrome: interaction of follicle stimulating hormone and polypeptide growth factors in oestradiol production by human granulosa cells." J Steroid Biochem Mol Biol **40**(1-3): 405-9.

Fraser, I. S. and R. P. Jansen (1983). "Why do inadvertent pregnancies occur in oral contraceptive users? Effectiveness of oral contraceptive regimens and interfering factors." Contraception **27**(6): 531-51.

Frattarelli, J. L., P. A. Bergh, et al. (2000). "Evaluation of basal estradiol levels in assisted reproductive technology cycles." Fertil Steril **74**(3): 518-24.

Garcia-Velasco, J. A., N. G. Mahutte, et al. (2004). "Removal of endometriomas before in vitro fertilization does not improve fertility outcomes: a matched, case-control study." Fertil Steril **81**(5): 1194-7.

Garcia-Velasco, J. A. and E. Somigliana (2009). "Management of endometriomas in women requiring IVF: to touch or not to touch." Hum Reprod **24**(3): 496-501.

Gaulden, M. E. (1992). "Maternal age effect: the enigma of Down syndrome and other trisomic conditions." Mutat Res **296**(1-2): 69-88.

Gelety, T. J., A. C. Pearlstone, et al. (1995). "Short-term endocrine response to gonadotropin-releasing hormone agonist initiated in the early follicular, midluteal, or late luteal phase in normally cycling women." Fertil Steril **64**(6): 1074-80.

Gilchrist, R. B., M. Wicherek, et al. (2001). "Changes in follicle-stimulating hormone and follicle populations during the ovarian cycle of the common marmoset." Biol Reprod **64**(1): 127-35.

Godinjak, Z., E. Idrizbegovic, et al. (2005). "[Pregnancy after laparoscopic treatment of ovarian endometriotic cysts]." Med Arh **59**(6): 351-3.

Gosling, R. G. and D. H. King (1974). "Arterial assessment by Doppler-shift ultrasound." Proc R Soc Med **67**(6 Pt 1): 447-9.

Gougeon, A. (1984). "Influence of cyclic variations in gonadotrophin and steroid hormones on follicular growth in the human ovary." Clinical Pathology of the Endocrine ovary. Lancaster: MTP press: 63-72.

Gougeon, A. (1989). "Some aspects of the dynamics of ovarian follicular growth in the human." Acta Eur Fertil **20**(4): 185-92.

Gougeon, A. (1994). "[Intragonadal regulation of human follicular genesis: facts and hypotheses]." Ann Endocrinol (Paris) **55**(2): 63-73.

Gougeon, A. (1996). "Regulation of ovarian follicular development in primates: facts and hypotheses." Endocr Rev **17**(2): 121-55.

Gougeon, A. (1998). "Ovarian follicular growth in humans: ovarian ageing and population of growing follicles." Maturitas **30**(2): 137-42.

Gougeon, A. and D. Busso (2000). "Morphologic and functional determinants of primordial and primary follicles in the monkey ovary." Mol Cell Endocrinol **163**(1-2): 33-42.

Gougeon, A., R. Ecochard, et al. (1994). "Age-related changes of the population of human ovarian follicles: increase in the disappearance rate of non-growing and early-growing follicles in aging women." Biol Reprod **50**(3): 653-63.

Gougeon, A. and B. Lefevre (1983). "Evolution of the diameters of the largest healthy and atretic follicles during the human menstrual cycle." J Reprod Fertil **69**(2): 497-502.

Groome, N. P., P. J. Illingworth, et al. (1996). "Measurement of dimeric inhibin B throughout the human menstrual cycle." J Clin Endocrinol Metab **81**(4): 1401-5.

group, T. E. C. w. (2001). "Ovarian and endometrial function during hormonal contraception." Hum Reprod **16**(7): 1527-35.

Gruijters, M. J., J. A. Visser, et al. (2003). "Anti-Mullerian hormone and its role in ovarian function." Mol Cell Endocrinol **211**(1-2): 85-90.

Gulekli, B., Y. Bulbul, et al. (1999). "Accuracy of ovarian reserve tests." Hum Reprod **14**(11): 2822-6.

Haadsma, M. L., A. Bukman, et al. (2007). "The number of small antral follicles (2-6 mm) determines the outcome of endocrine ovarian reserve tests in a subfertile population." Hum Reprod **22**(7): 1925-31.

Haadsma, M. L., H. Groen, et al. (2009). "The predictive value of ovarian reserve tests for miscarriage in a population of subfertile ovulatory women." Hum Reprod **24**(3): 546-52.

Hachisuga, T. and T. Kawarabayashi (2002). "Histopathological analysis of laparoscopically treated ovarian endometriotic cysts with special reference to loss of follicles." Hum Reprod **17**(2): 432-5.

Hanley, J. A. and B. J. McNeil (1983). "A method of comparing the areas under receiver operating characteristic curves derived from the same cases." Radiology **148**(3): 839-43.

Hansen, K. R., N. S. Knowlton, et al. (2008). "A new model of reproductive aging: the decline in ovarian non-growing follicle number from birth to menopause." Hum Reprod **23**(3): 699-708.

Hansen, K. R., J. L. Morris, et al. (2003). "Reproductive aging and variability in the ovarian antral follicle count: application in the clinical setting." Fertil Steril **80**(3): 577-83.

Hart, R. J., M. Hickey, et al. (2008). "Excisional surgery versus ablative surgery for ovarian endometriomata." Cochrane Database Syst Rev(2): CD004992.

Hayes, F. J., J. E. Hall, et al. (1998). "Clinical review 96: Differential control of gonadotropin secretion in the human: endocrine role of inhibin." J Clin Endocrinol Metab **83**(6): 1835-41.

Hehenkamp, W. J., C. W. Looman, et al. (2006). "Anti-Mullerian hormone levels in the spontaneous menstrual cycle do not show substantial fluctuation." J Clin Endocrinol Metab **91**(10): 4057-63.

Hendriks, D. J., J. Kwee, et al. (2007). "Ultrasonography as a tool for the prediction of outcome in IVF patients: a comparative meta-analysis of ovarian volume and antral follicle count." Fertil Steril.

Hendriks, D. J., B. W. Mol, et al. (2005). "Antral follicle count in the prediction of poor ovarian response and pregnancy after in vitro fertilization: a meta-analysis and comparison with basal follicle-stimulating hormone level." Fertil Steril **83**(2): 291-301.

Horikawa, T., K. Nakagawa, et al. (2008). "The frequency of ovulation from the affected ovary decreases following laparoscopic cystectomy in infertile women with unilateral endometrioma during a natural cycle." J Assist Reprod Genet **25**(6): 239-44.

Hsueh, A. J., K. Eisenhauer, et al. (1996). "Gonadal cell apoptosis." Recent Prog Horm Res **51**: 433-55; discussion 455-6.

Hsueh, A. J., E. A. McGee, et al. (2000). "Hormonal regulation of early follicle development in the rat ovary." Mol Cell Endocrinol **163**(1-2): 95-100.

Hughesdon, P. E. (1957). "The structure of endometrial cysts of the ovary." J Obstet Gynaecol Br Emp **64**(4): 481-7.

Hurwitz, J. L., A. Fenton, et al. (2007). "Squamous cell carcinoma arising in a dermoid cyst of the ovary: a case series." BJOG **114**(10): 1283-7.

Ireland, J. L., D. Scheetz, et al. (2008). "Antral Follicle Count Reliably Predicts Number of Morphologically Healthy Oocytes and Follicles in Ovaries of Young Adult Cattle." Biol Reprod.

Iwase, A., W. Hirokawa, et al. (2011). "Serum anti-Mullerian hormone level is a useful marker for evaluating the impact of laparoscopic cystectomy on ovarian reserve." Fertil Steril.

Jaffe, R. (1992). Color Doppler imaging: a new interpretation of the Doppler effect. New York, McGraw-Hill.

Jayaprakasan, K., B. Campbell, et al. (2008). "Establishing the intercycle variability of three-dimensional ultrasonographic predictors of ovarian reserve." Fertil Steril **90**(6): 2126-32.

Jayaprakasan, K., B. Campbell, et al. "A prospective, comparative analysis of anti-Mullerian hormone, inhibin-B, and three-dimensional ultrasound determinants of ovarian reserve in the prediction of poor response to controlled ovarian stimulation." Fertil Steril **93**(3): 855-64.

Jayaprakasan, K., B. Campbell, et al. (2010). "A prospective, comparative analysis of anti-Mullerian hormone, inhibin-B, and three-dimensional ultrasound determinants of ovarian reserve in the prediction of poor response to controlled ovarian stimulation." Fertil Steril **93**(3): 855-64.

Jayaprakasan, K., B. K. Campbell, et al. (2008). "Three-dimensional ultrasound improves the interobserver reliability of antral follicle counts and facilitates increased clinical work flow." Ultrasound Obstet Gynecol **31**(4): 439-44.

Jayaprakasan, K., B. K. Campbell, et al. (2008). "Effect of pituitary desensitization on the early growing follicular cohort estimated using anti-Mullerian hormone." Hum Reprod **23**(11): 2577-83.

Jayaprakasan, K., S. Deb, et al. (2009). "The cohort of antral follicles measuring 2-6 mm reflects the quantitative status of ovarian reserve as assessed by serum levels of anti-Mullerian hormone and response to controlled ovarian stimulation." Fertil Steril.

Jayaprakasan, K., N. Hilwah, et al. (2007). "Does 3D ultrasound offer any advantage in the pretreatment assessment of ovarian reserve and prediction of outcome after assisted reproduction treatment?" Hum Reprod **22**(7): 1932-41.

Jayaprakasan, K., R. Jayaprakasan, et al. (2009). "Can quantitative three-dimensional power Doppler angiography be used to predict ovarian hyperstimulation syndrome?" Ultrasound Obstet Gynecol **33**(5): 583-91.

Jayaprakasan, K., K. F. Walker, et al. (2007). "The interobserver reliability of off-line antral follicle counts made from stored three-dimensional ultrasound data: a comparative study of different measurement techniques." Ultrasound Obstet Gynecol **29**(3): 335-41.

Jenkins, S., D. L. Olive, et al. (1986). "Endometriosis: pathogenetic implications of the anatomic distribution." Obstet Gynecol **67**(3): 335-8.

Jonassen, J. A., K. Bose, et al. (1982). "Enhancement and desensitization of hormone-responsive adenylate cyclase in granulosa cells of preantral and antral ovarian follicles: effects of estradiol and follicle-stimulating hormone." Endocrinology **111**(1): 74-9.

Kahyaoglu, S., E. Ertas, et al. (2008). "Does laparoscopic cystectomy and cauterization of endometriomas greater than 3 cm diminish ovarian response to controlled ovarian hyperstimulation during IVF-ET? A case-control study." J Obstet Gynaecol Res **34**(6): 1010-3.

Keltz, M. D., P. S. Gera, et al. (2007). "Comparison of FSH flare with and without pretreatment with oral contraceptive pills in poor responders undergoing in vitro fertilization." Fertil Steril **88**(2): 350-3.

Kevenaar, M. E., M. F. Meerasahib, et al. (2006). "Serum anti-mullerian hormone levels reflect the size of the primordial follicle pool in mice." Endocrinology **147**(7): 3228-34.

Khairy, M., A. Clough, et al. (2008). "Antral follicle count at down-regulation and prediction of poor ovarian response." Reprod Biomed Online **17**(4): 508-14.

Khamsi, F., Y. Yavas, et al. (2001). "Exposure of human oocytes to endometrioma fluid does not alter fertilization or early embryo development." J Assist Reprod Genet **18**(2): 106-9.

Khan, K. S. and P. F. Chien (2001). "Evaluation of a clinical test. I: assessment of reliability." BJOG **108**(6): 562-7.

Killick, S. R., K. Bancroft, et al. (1990). "Extending the duration of the pill-free interval during combined oral contraception." Adv Contracept **6**(1): 33-40.

Kim, S. H., S. Y. Ku, et al. (2002). "Clinical significance of transvaginal color Doppler ultrasonography of the ovarian artery as a predictor of ovarian response in controlled ovarian hyperstimulation for in vitro fertilization and embryo transfer." J Assist Reprod Genet **19**(3): 103-12.

Kingsley, D. M. (1994). "The TGF-beta superfamily: new members, new receptors, and new genetic tests of function in different organisms." Genes Dev **8**(2): 133-46.

Klein, N. A., D. E. Battaglia, et al. (1996). "Reproductive aging: accelerated ovarian follicular development associated with a monotropic follicle-stimulating hormone rise in normal older women." J Clin Endocrinol Metab **81**(3): 1038-45.

Klein, N. A., P. J. Illingworth, et al. (1996). "Decreased inhibin B secretion is associated with the monotropic FSH rise in older, ovulatory women: a study of serum and follicular fluid levels of dimeric inhibin A and B in spontaneous menstrual cycles." J Clin Endocrinol Metab **81**(7): 2742-5.

Klinkert, E. R., F. J. Broekmans, et al. (2005). "The antral follicle count is a better marker than basal follicle-stimulating hormone for the selection of older patients with acceptable pregnancy prospects after in vitro fertilization." Fertil Steril **83**(3): 811-4.

Klinkert, E. R., F. J. Broekmans, et al. (2004). "A poor response in the first in vitro fertilization cycle is not necessarily related to a poor prognosis in subsequent cycles." Fertil Steril **81**(5): 1247-53.

Kobayashi, H., H. Kajihara, et al. "Risk of carcinoma in women with ovarian endometrioma." Front Biosci (Elite Ed) **3**: 529-39.

Koga, K., Y. Takemura, et al. (2006). "Recurrence of ovarian endometrioma after laparoscopic excision." Hum Reprod **21**(8): 2171-4.

Kupesic, S. and A. Kurjak (2002). "Predictors of IVF outcome by three-dimensional ultrasound." Hum Reprod **17**(4): 950-5.

Kupesic, S., A. Kurjak, et al. (2003). "Three-dimensional ultrasonographic ovarian measurements and in vitro fertilization outcome are related to age." Fertil Steril **79**(1): 190-7.

Kwee, J., M. E. Elting, et al. (2007). "Ovarian volume and antral follicle count for the prediction of low and hyper responders with in vitro fertilization." Reprod Biol Endocrinol **5**: 9.

Kwee, J., M. W. Elting, et al. (2003). "Comparison of endocrine tests with respect to their predictive value on the outcome of ovarian hyperstimulation in IVF treatment: results of a prospective randomized study." Hum Reprod **18**(7): 1422-7.

Kwee, J., R. Schats, et al. (2008). "Evaluation of anti-Mullerian hormone as a test for the prediction of ovarian reserve." Fertil Steril **90**(3): 737-43.

Kyei-Mensah, A., N. Maconochie, et al. (1996). "Transvaginal three-dimensional ultrasound: reproducibility of ovarian and endometrial volume measurements." Fertil Steril **66**(5): 718-22.

La Marca, A., S. Giulini, et al. (2007). "Anti-Mullerian hormone measurement on any day of the menstrual cycle strongly predicts ovarian response in assisted reproductive technology." Hum Reprod **22**(3): 766-71.

La Marca, A., S. Malmusi, et al. (2004). "Anti-Mullerian hormone plasma levels in spontaneous menstrual cycle and during treatment with FSH to induce ovulation." Hum Reprod **19**(12): 2738-41.

La Marca, A., G. Sighinolfi, et al. "Anti-Mullerian hormone (AMH) as a predictive marker in assisted reproductive technology (ART)." Hum Reprod Update **16**(2): 113-30.

La Marca, A., G. Stabile, et al. (2006). "Serum anti-Mullerian hormone throughout the human menstrual cycle." Hum Reprod **21**(12): 3103-7.

Lam, P. M. and N. Raine-Fenning (2006). "The role of three-dimensional ultrasonography in polycystic ovary syndrome." Hum Reprod **21**(9): 2209-15.

Lawson, R., T. El-Toukhy, et al. (2003). "Poor response to ovulation induction is a stronger predictor of early menopause than elevated basal FSH: a life table analysis." Hum Reprod **18**(3): 527-33.

Lee, S. J., E. A. Lenton, et al. (1988). "The effect of age on the cyclical patterns of plasma LH, FSH, oestradiol and progesterone in women with regular menstrual cycles." Hum Reprod **3**(7): 851-5.

Lekamge, D. N., M. Barry, et al. (2007). "Anti-Mullerian hormone as a predictor of IVF outcome." Reprod Biomed Online **14**(5): 602-10.

Li, C. Z., B. Liu, et al. (2009). "The impact of electrocoagulation on ovarian reserve after laparoscopic excision of ovarian cysts: a prospective clinical study of 191 patients." Fertil Steril **92**(4): 1428-35.

Licciardi, F. L., H. C. Liu, et al. (1995). "Day 3 estradiol serum concentrations as prognosticators of ovarian stimulation response and pregnancy outcome in patients undergoing in vitro fertilization." Fertil Steril **64**(5): 991-4.

Liu, X., K. Andoh, et al. (1998). "Effects of growth hormone, activin, and follistatin on the development of preantral follicle from immature female mice." Endocrinology **139**(5): 2342-7.

Loh, F. H., A. T. Tan, et al. (1999). "Ovarian response after laparoscopic ovarian cystectomy for endometriotic cysts in 132 monitored cycles." Fertil Steril **72**(2): 316-21.

Loo, T. C., M. Y. Lin, et al. (2005). "Endometrioma undergoing laparoscopic ovarian cystectomy: its influence on the outcome of in vitro fertilization and embryo transfer (IVF-ET)." J Assist Reprod Genet **22**(9-10): 329-33.

Loumaye, E., J. M. Billion, et al. (1990). "Prediction of individual response to controlled ovarian hyperstimulation by means of a clomiphene citrate challenge test." Fertil Steril **53**(2): 295-301.

Luna, M., L. Grunfeld, et al. (2007). "Moderately elevated levels of basal follicle-stimulating hormone in young patients predict low ovarian response, but should not be used to disqualify patients from attempting in vitro fertilization." Fertil Steril **87**(4): 782-7.

Maheshwari, A., A. Gibreel, et al. (2009). "Dynamic tests of ovarian reserve: a systematic review of diagnostic accuracy." Reprod Biomed Online **18**(5): 717-34.

Mahmood, T. A. and A. Templeton (1991). "Prevalence and genesis of endometriosis." Hum Reprod **6**(4): 544-9.

Maneschi, F., L. Marasa, et al. (1993). "Ovarian cortex surrounding benign neoplasms: a histologic study." Am J Obstet Gynecol **169**(2 Pt 1): 388-93.

Marconi, G., M. Vilela, et al. (2002). "Laparoscopic ovarian cystectomy of endometriomas does not affect the ovarian response to gonadotropin stimulation." Fertil Steril **78**(4): 876-8.

Markstrom, E., E. Svensson, et al. (2002). "Survival factors regulating ovarian apoptosis -- dependence on follicle differentiation." Reproduction **123**(1): 23-30.

Martinoli, C., L. E. Derchi, et al. (1998). "Power Doppler sonography: general principles, clinical applications, and future prospects." Eur Radiol **8**(7): 1224-35.

McGee, E. A. (2000). "The regulation of apoptosis in preantral ovarian follicles." <u>Biol Signals Recept</u> **9**(2): 81-6.

McGee, E. A. and A. J. Hsueh (2000). "Initial and cyclic recruitment of ovarian follicles." <u>Endocr Rev</u> **21**(2): 200-14.

McGee, E. A., E. Perlas, et al. (1997). "Follicle-stimulating hormone enhances the development of preantral follicles in juvenile rats." <u>Biol Reprod</u> **57**(5): 990-8.

McGraw, K. O., Wong, S.P. (1996). "Forming inferences about some Intraclass correlation coefficients." <u>Psychological Methods</u> **1:1**(1): 30-46.

McIlveen, M., J. D. Skull, et al. (2007). "Evaluation of the utility of multiple endocrine and ultrasound measures of ovarian reserve in the prediction of cycle cancellation in a high-risk IVF population." <u>Hum Reprod</u> **22**(3): 778-85.

McNatty, K. P., D. A. Heath, et al. (1999). "Control of early ovarian follicular development." <u>J Reprod Fertil Suppl</u> **54**: 3-16.

McNatty, K. P., A. Makris, et al. (1979). "The production of progesterone, androgens and oestrogens by human granulosa cells in vitro and in vivo." <u>J Steroid Biochem</u> **11**(1C): 775-9.

McNatty, K. P., A. Makris, et al. (1979). "Metabolism of androstenedione by human ovarian tissues in vitro with particular reference to reductase and aromatase activity." <u>Steroids</u> **34**(4): 429-43.

McNatty, K. P., K. Reader, et al. (2007). "Control of ovarian follicular development to the gonadotrophin-dependent phase: a 2006 perspective." <u>Soc Reprod Fertil Suppl</u> **64**: 55-68.

Merce, L. T., S. Bau, et al. (2006). "Assessment of the ovarian volume, number and volume of follicles and ovarian vascularity by three-dimensional ultrasonography and

power Doppler angiography on the HCG day to predict the outcome in IVF/ICSI cycles." Hum Reprod **21**(5): 1218-26.

Merce, L. T., B. Gomez, et al. (2005). "Intraobserver and interobserver reproducibility of ovarian volume, antral follicle count, and vascularity indices obtained with transvaginal 3-dimensional ultrasonography, power Doppler angiography, and the virtual organ computer-aided analysis imaging program." J Ultrasound Med **24**(9): 1279-87.

Merz, E. (1999). "Three-dimensional transvaginal ultrasound in gynecological diagnosis." Ultrasound Obstet Gynecol **14**(2): 81-6.

Metcalf, M. G. and J. H. Livesey (1985). "Gonadotrophin excretion in fertile women: effect of age and the onset of the menopausal transition." J Endocrinol **105**(3): 357-62.

Milner, M., C. Barry-Kinsella, et al. (1992). "The impact of maternal age on pregnancy and its outcome." Int J Gynaecol Obstet **38**(4): 281-6.

Mishell, D. R., Jr., O. A. Kletzky, et al. (1977). "The effect of contraceptive steroids on hypothalamic-pituitary function." Am J Obstet Gynecol **128**(1): 60-74.

Modi, D., D. Bhartiya, et al. (2006). "Developmental expression and cellular distribution of Mullerian inhibiting substance in the primate ovary." Reproduction **132**(3): 443-53.

Montloin, A. (1990). "[Oral contraception]." Rev Infirm **40**(18): 46-52.

Muttukrishna, S., H. McGarrigle, et al. (2005). "Antral follicle count, anti-mullerian hormone and inhibin B: predictors of ovarian response in assisted reproductive technology?" BJOG **112**(10): 1384-90.

Muzii, L., F. Bellati, et al. (2005). "Laparoscopic stripping of endometriomas: a randomized trial on different surgical techniques. Part II: pathological results." Hum Reprod **20**(7): 1987-92.

Muzii, L., A. Bianchi, et al. (2002). "Laparoscopic excision of ovarian cysts: is the stripping technique a tissue-sparing procedure?" Fertil Steril **77**(3): 609-14.

Nakagawa, K., S. Ohgi, et al. (2007). "Impact of laparoscopic cystectomy on fecundity of infertility patients with ovarian endometrioma." J Obstet Gynaecol Res **33**(5): 671-6.

Nardo, L. G., D. Christodoulou, et al. (2007). "Anti-Mullerian hormone levels and antral follicle count in women enrolled in in vitro fertilization cycles: relationship to lifestyle factors, chronological age and reproductive history." Gynecol Endocrinol **23**(8): 486-93.

Nardo, L. G., D. Christodoulou, et al. (2007). "Anti-Mullerian hormone levels and antral follicle count in women enrolled in in vitro fertilization cycles: Relationship to lifestyle factors, chronological age and reproductive history." Gynecol Endocrinol: 1-8.

Nardo, L. G. and T. A. Gelbaya (2008). "Evidence-based approach for the use of ultrasound in the management of polycystic ovary syndrome." Minerva Ginecol **60**(1): 83-9.

Navot, D., Z. Rosenwaks, et al. (1987). "Prognostic assessment of female fecundity." Lancet **2**(8560): 645-7.

Nelson, S. M., R. W. Yates, et al. (2009). "Anti-Mullerian hormone-based approach to controlled ovarian stimulation for assisted conception." Hum Reprod.

Nelson, T. R. and D. H. Pretorius (1998). "Three-dimensional ultrasound imaging." Ultrasound Med Biol **24**(9): 1243-70.

Newton, H., H. Picton, et al. (1999). "In vitro growth of oocyte-granulosa cell complexes isolated from cryopreserved ovine tissue." J Reprod Fertil **115**(1): 141-50.

Ng, E. H., C. C. Chan, et al. (2005). "Antral follicle count and FSH concentration after clomiphene citrate challenge test in the prediction of ovarian response during IVF treatment." Hum Reprod **20**(6): 1647-54.

Ng, E. H., O. S. Tang, et al. (2006). "Ovarian stromal vascularity is not predictive of ovarian response and pregnancy." Reprod Biomed Online **12**(1): 43-9.

Pados, G., D. Tsolakidis, et al. (2010). "Sonographic changes after laparoscopic cystectomy compared with three-stage management in patients with ovarian endometriomas: a prospective randomized study." Hum Reprod **25**(3): 672-7.

Pados, G., D. Tsolakidis, et al. (2006). "Laparoscopic management of the adnexal mass." Ann N Y Acad Sci **1092**: 211-28.

Paeng, D. G., P. J. Cao, et al. (2001). "Doppler power variation from porcine blood under steady and pulsatile flow." Ultrasound Med Biol **27**(9): 1245-54.

Pan, H. A., M. H. Wu, et al. (2002). "Quantification of Doppler signal in polycystic ovary syndrome using three-dimensional power Doppler ultrasonography: a possible new marker for diagnosis." Hum Reprod **17**(1): 201-6.

Pellicer, A., G. Ardiles, et al. (1998). "Evaluation of the ovarian reserve in young low responders with normal basal levels of follicle-stimulating hormone using three-dimensional ultrasonography." Fertil Steril **70**(4): 671-5.

Pellicer, A., P. Gaitan, et al. (1998). "Ovarian follicular dynamics: from basic science to clinical practice." J Reprod Immunol **39**(1-2): 29-61.

Penarrubia, J., F. Fabregues, et al. (2005). "Basal and stimulation day 5 anti-Mullerian hormone serum concentrations as predictors of ovarian response and pregnancy

in assisted reproductive technology cycles stimulated with gonadotropin-releasing hormone agonist--gonadotropin treatment." Hum Reprod **20**(4): 915-22.

Pohl, M., M. Hohlagschwandtner, et al. (2000). "Number and size of antral follicles as predictive factors in vitro fertilization and embryo transfer." J Assist Reprod Genet **17**(6): 315-8.

Poma, P. A. (1981). "Effect of maternal age on pregnancy outcome." J Natl Med Assoc **73**(11): 1031-8.

Popovic-Todorovic, B., A. Loft, et al. (2003). "A prospective study of predictive factors of ovarian response in 'standard' IVF/ICSI patients treated with recombinant FSH. A suggestion for a recombinant FSH dosage normogram." Hum Reprod **18**(4): 781-7.

Pourcelot, L., P. Arbeille, et al. (1985). "Doppler spectrum analysis and vascular imaging in the diagnosis of extracranial carotid artery disease." Int Angiol **4**(1): 59-68.

Powell, J. L., J. A. Stinson, et al. (2003). "Squamous cell carcinoma arising in a dermoid cyst of the ovary." Gynecol Oncol **89**(3): 526-8.

Ragni, G., E. Somigliana, et al. (2005). "Damage to ovarian reserve associated with laparoscopic excision of endometriomas: a quantitative rather than a qualitative injury." Am J Obstet Gynecol **193**(6): 1908-14.

Raine-Fenning, N., B. Campbell, et al. (2002). "The reproducibility of endometrial volume acquisition and measurement with the VOCAL-imaging program." Ultrasound Obstet Gynecol **19**(1): 69-75.

Raine-Fenning, N., S. Deb, et al. (2009). "Timing of oocyte maturation and egg collection during controlled ovarian stimulation: a randomized controlled trial evaluating manual and automated measurements of follicle diameter." Fertil Steril.

Raine-Fenning, N., K. Jayaprakasan, et al. (2008). "Automated measurements of follicle diameter: a chance to standardize?" <u>Fertil Steril</u>.

Raine-Fenning, N., K. Jayaprakasan, et al. (2009). "Automated measurements of follicle diameter: a chance to standardize?" <u>Fertil Steril</u> **91**(4 Suppl): 1469-72.

Raine-Fenning, N., K. Jayaprakasan, et al. (2007). "Automated follicle tracking facilitates standardization and may improve work flow." <u>Ultrasound Obstet Gynecol</u> **30**(7): 1015-8.

Raine-Fenning, N., K. Jayaprakasan, et al. (2008). "SonoAVC: a novel method of automatic volume calculation." <u>Ultrasound Obstet Gynecol</u> **31**(6): 691-6.

Raine-Fenning, N., K. Jayaprakasan, J. Clewes, I. Joergner, S. D. Bonaki, S. Chamberlain, L. Devlin, H. Priddle, and I. Johnson. (2008). "SonoAVC: a novel method of automatic volume calculation." <u>Ultrasound Obstet Gynecol</u> **31**: 691-6.

Raine-Fenning, N., K. Jayaprakasan, S. Chamberlain, L. Devlin, H. Priddle, and I. Johnson (2008). "Automated measurements of follicle diameter: a chance to standardize?" <u>Fertil Steril</u>.

Raine-Fenning, N. J., B. K. Campbell, et al. (2003). "The interobserver reliability of ovarian volume measurement is improved with three-dimensional ultrasound, but dependent upon technique." <u>Ultrasound Med Biol</u> **29**(12): 1685-90.

Raine-Fenning, N. J., B. K. Campbell, et al. (2003). "The reliability of virtual organ computer-aided analysis (VOCAL) for the semiquantification of ovarian, endometrial and subendometrial perfusion." <u>Ultrasound Obstet Gynecol</u> **22**(6): 633-9.

Raine-Fenning, N. J., B. K. Campbell, et al. (2004). "The interobserver reliability of three-dimensional power Doppler data acquisition within the female pelvis." Ultrasound Obstet Gynecol **23**(5): 501-8.

Raine-Fenning, N. J., B. K. Campbell, et al. (2004). "Quantifying the changes in endometrial vascularity throughout the normal menstrual cycle with three-dimensional power Doppler angiography." Hum Reprod **19**(2): 330-8.

Raine-Fenning, N. J., J. S. Clewes, et al. (2003). "The interobserver reliability and validity of volume calculation from three-dimensional ultrasound datasets in the in vitro setting." Ultrasound Obstet Gynecol **21**(3): 283-91.

Raine-Fenning, N. J., N. M. Nordin, et al. (2008). "Determining the relationship between three-dimensional power Doppler data and true blood flow characteristics: an in-vitro flow phantom experiment." Ultrasound Obstet Gynecol **32**(4): 540-50.

Raine-Fenning, N. J., N. M. Nordin, et al. (2008). "Evaluation of the effect of machine settings on quantitative three-dimensional power Doppler angiography: an in-vitro flow phantom experiment." Ultrasound Obstet Gynecol **32**(4): 551-9.

Reame, N. E., T. L. Wyman, et al. (1998). "Net increase in stimulatory input resulting from a decrease in inhibin B and an increase in activin A may contribute in part to the rise in follicular phase follicle-stimulating hormone of aging cycling women." J Clin Endocrinol Metab **83**(9): 3302-7.

Reyes, F. I., J. S. Winter, et al. (1977). "Pituitary-ovarian relationships preceding the menopause. I. A cross-sectional study of serum follice-stimulating hormone, luteinizing hormone, prolactin, estradiol, and progesterone levels." Am J Obstet Gynecol **129**(5): 557-64.

Reynolds, T., M. C. Hill, et al. (1986). "Sonography of hemorrhagic ovarian cysts." <u>J Clin Ultrasound</u> **14**(6): 449-53.

Riggs, R. M., E. H. Duran, et al. (2008). "Assessment of ovarian reserve with anti-Mullerian hormone: a comparison of the predictive value of anti-Mullerian hormone, follicle-stimulating hormone, inhibin B, and age." <u>Am J Obstet Gynecol</u> **199**(2): 202 e1-8.

Roman, H., O. Tarta, et al. "Direct proportional relationship between endometrioma size and ovarian parenchyma inadvertently removed during cystectomy, and its implication on the management of enlarged endometriomas." <u>Hum Reprod</u> **25**(6): 1428-32.

Roman, H., O. Tarta, et al. (2010). "Direct proportional relationship between endometrioma size and ovarian parenchyma inadvertently removed during cystectomy, and its implication on the management of enlarged endometriomas." <u>Hum Reprod</u> **25**(6): 1428-32.

Rothwell, P. M. (2000). "Analysis of agreement between measurements of continuous variables: general principles and lessons from studies of imaging of carotid stenosis." <u>J Neurol</u> **247**(11): 825-34.

Roy, S. K. and B. J. Treacy (1993). "Isolation and long-term culture of human preantral follicles." <u>Fertil Steril</u> **59**(4): 783-90.

Rubin, J. M., R. S. Adler, et al. (1995). "Fractional moving blood volume: estimation with power Doppler US." <u>Radiology</u> **197**(1): 183-90.

Rubin, J. M., R. O. Bude, et al. (1994). "Power Doppler US: a potentially useful alternative to mean frequency- based color Doppler US." <u>Radiology</u> **190**(3): 853-6.

Saleh, A. and T. Tulandi (1999). "Reoperation after laparoscopic treatment of ovarian endometriomas by excision and by fenestration." Fertil Steril **72**(2): 322-4.

Sasano, H., M. Okamoto, et al. (1989). "Immunolocalization of aromatase, 17 alpha-hydroxylase and side-chain-cleavage cytochromes P-450 in the human ovary." J Reprod Fertil **85**(1): 163-9.

Scaramuzzi, R. J., N. R. Adams, et al. (1993). "A model for follicle selection and the determination of ovulation rate in the ewe." Reprod Fertil Dev **5**(5): 459-78.

Scheffer, G. J., F. J. Broekmans, et al. (2002). "Quantitative transvaginal two- and three-dimensional sonography of the ovaries: reproducibility of antral follicle counts." Ultrasound Obstet Gynecol **20**(3): 270-5.

Scheffer, G. J., F. J. Broekmans, et al. (1999). "Antral follicle counts by transvaginal ultrasonography are related to age in women with proven natural fertility." Fertil Steril **72**(5): 845-51.

Scheffer, G. J., F. J. Broekmans, et al. (2003). "The number of antral follicles in normal women with proven fertility is the best reflection of reproductive age." Hum Reprod **18**(4): 700-6.

Schlaff, W. D., A. M. Lynch, et al. (2004). "Manipulation of the pill-free interval in oral contraceptive pill users: the effect on follicular suppression." Am J Obstet Gynecol **190**(4): 943-51.

Scott, R. T., Jr., G. E. Hofmann, et al. (1990). "Intercycle variability of day 3 follicle-stimulating hormone levels and its effect on stimulation quality in in vitro fertilization." Fertil Steril **54**(2): 297-302.

Scott, R. T., J. P. Toner, et al. (1989). "Follicle-stimulating hormone levels on cycle day 3 are predictive of in vitro fertilization outcome." Fertil Steril **51**(4): 651-4.

Seifer, D. B., G. Lambert-Messerlian, et al. (1997). "Day 3 serum inhibin-B is predictive of assisted reproductive technologies outcome." Fertil Steril **67**(1): 110-4.

Sherman, B. M., J. H. West, et al. (1976). "The menopausal transition: analysis of LH, FSH, estradiol, and progesterone concentrations during menstrual cycles of older women." J Clin Endocrinol Metab **42**(4): 629-36.

Skalba, P., A. Cygal, et al. (2008). "[The influence of anti-Mullerian hormone on folliculogenesis]." Ginekol Pol **79**(2): 137-40.

Skouby, S. O. (2004). "Contraceptive use and behavior in the 21st century: a comprehensive study across five European countries." Eur J Contracept Reprod Health Care **9**(2): 57-68.

Smotrich, D. B., E. A. Widra, et al. (1995). "Prognostic value of day 3 estradiol on in vitro fertilization outcome." Fertil Steril **64**(6): 1136-40.

Soldevila, P. N., O. Carreras, et al. (2007). "Sonographic assessment of ovarian reserve. Its correlation with outcome of in vitro fertilization cycles." Gynecol Endocrinol **23**(4): 206-12.

Somigliana, E., M. Arnoldi, et al. (2008). "IVF-ICSI outcome in women operated on for bilateral endometriomas." Hum Reprod **23**(7): 1526-30.

Somigliana, E., M. Infantino, et al. (2006). "The presence of ovarian endometriomas is associated with a reduced responsiveness to gonadotropins." Fertil Steril **86**(1): 192-6.

Somigliana, E., G. Ragni, et al. (2003). "Does laparoscopic excision of endometriotic ovarian cysts significantly affect ovarian reserve? Insights from IVF cycles." Hum Reprod **18**(11): 2450-3.

Somigliana, E., G. Ragni, et al. (2006). "Does laparoscopic removal of nonendometriotic benign ovarian cysts affect ovarian reserve?" Acta Obstet Gynecol Scand **85**(1): 74-7.

Somigliana, E., P. Vercellini, et al. (2006). "Should endometriomas be treated before IVF-ICSI cycles?" Hum Reprod Update **12**(1): 57-64.

Somunkiran, A., T. Yavuz, et al. (2007). "Anti-Mullerian hormone levels during hormonal contraception in women with polycystic ovary syndrome." Eur J Obstet Gynecol Reprod Biol **134**(2): 196-201.

Spears, N. (1994). "In-vitro growth of oocytes. In-vitro growth of ovarian oocytes." Hum Reprod **9**(6): 969-70.

Streuli, I., T. Fraisse, et al. (2008). "Serum antimullerian hormone levels remain stable throughout the menstrual cycle and after oral or vaginal administration of synthetic sex steroids." Fertil Steril **90**(2): 395-400.

Sutton, C. J. and K. D. Jones (2002). "Laser laparoscopy for endometriosis and endometriotic cysts." Surg Endosc **16**(11): 1513-7.

Syrop, C. H., J. D. Dawson, et al. (1999). "Ovarian volume may predict assisted reproductive outcomes better than follicle stimulating hormone concentration on day 3." Hum Reprod **14**(7): 1752-6.

Tamura, T., J. Kitawaki, et al. (1992). "Immunohistochemical localization of 17 alpha-hydroxylase/C17-20 lyase and aromatase cytochrome P-450 in the human ovary during the menstrual cycle." J Endocrinol **135**(3): 589-95.

Tarin, J. J. (1995). "Aetiology of age-associated aneuploidy: a mechanism based on the 'free radical theory of ageing'." Hum Reprod **10**(6): 1563-5.

te Velde, E. R. and P. L. Pearson (2002). "The variability of female reproductive ageing."

   Hum Reprod Update **8**(2): 141-54.

Tinkanen, H., M. Blauer, et al. (2001). "Correlation between serum inhibin B and other

   indicators of the ovarian function." Eur J Obstet Gynecol Reprod Biol **94**(1): 109-

   13.

Tsepelidis, S., I. Demeestere, et al. (2007). "[Anti-mullerian hormone and its role in the

   regulation of ovarian function. Review of the literature]." Rev Med Brux **28**(3):

   165-71.

Tsepelidis, S., F. Devreker, et al. (2007). "Stable serum levels of anti-Mullerian hormone

   during the menstrual cycle: a prospective study in normo-ovulatory women."

   Hum Reprod **22**(7): 1837-40.

Tsolakidis, D., G. Pados, et al. (2009). "The impact on ovarian reserve after laparoscopic

   ovarian cystectomy versus three-stage management in patients with

   endometriomas: a prospective randomized study." Fertil Steril.

Tsolakidis, D., G. Pados, et al. (2010). "The impact on ovarian reserve after laparoscopic

   ovarian cystectomy versus three-stage management in patients with

   endometriomas: a prospective randomized study." Fertil Steril **94**(1): 71-7.

Tsoumpou, I., M. Kyrgiou, et al. (2009). "The effect of surgical treatment for

   endometrioma on in vitro fertilization outcomes: a systematic review and meta-

   analysis." Fertil Steril **92**(1): 75-87.

van den Berg, M. H., E. van Dulmen-den Broeder, et al. "Comparison of ovarian function

   markers in users of hormonal contraceptives during the hormone-free interval

   and subsequent natural early follicular phases." Hum Reprod.

van den Hurk, R. and J. Zhao (2005). "Formation of mammalian oocytes and their growth, differentiation and maturation within ovarian follicles." Theriogenology **63**(6): 1717-51.

van der Spuy, Z. M., U. Sohnius, et al. (1990). "Gonadotropin and estradiol secretion during the week of placebo therapy in oral contraceptive pill users." Contraception **42**(6): 597-609.

van der Steeg, J. W., P. Steures, et al. (2007). "Predictive value and clinical impact of Basal follicle-stimulating hormone in subfertile, ovulatory women." J Clin Endocrinol Metab **92**(6): 2163-8.

van Disseldorp, J., C. B. Lambalk, et al. (2010). "Comparison of inter- and intra-cycle variability of anti-Mullerian hormone and antral follicle counts." Hum Reprod **25**(1): 221-7.

van Heusden, A. M. and B. C. Fauser (1999). "Activity of the pituitary-ovarian axis in the pill-free interval during use of low-dose combined oral contraceptives." Contraception **59**(4): 237-43.

van Noord-Zaadstra, B. M., C. W. Looman, et al. (1991). "Delaying childbearing: effect of age on fecundity and outcome of pregnancy." BMJ **302**(6789): 1361-5.

van Rooij, I. A., L. F. Bancsi, et al. (2003). "Women older than 40 years of age and those with elevated follicle-stimulating hormone levels differ in poor response rate and embryo quality in in vitro fertilization." Fertil Steril **79**(3): 482-8.

van Rooij, I. A., F. J. Broekmans, et al. (2005). "Serum antimullerian hormone levels best reflect the reproductive decline with age in normal women with proven fertility: a longitudinal study." Fertil Steril **83**(4): 979-87.

van Rooij, I. A., F. J. Broekmans, et al. (2002). "Serum anti-Mullerian hormone levels: a novel measure of ovarian reserve." Hum Reprod **17**(12): 3065-71.

van Zonneveld, P., G. J. Scheffer, et al. (2003). "Do cycle disturbances explain the age-related decline of female fertility? Cycle characteristics of women aged over 40 years compared with a reference population of young women." Hum Reprod **18**(3): 495-501.

Verhagen, T. E., D. J. Hendriks, et al. (2008). "The accuracy of multivariate models predicting ovarian reserve and pregnancy after in vitro fertilization: a meta-analysis." Hum Reprod Update **14**(2): 95-100.

Visser, J. (2006). "[Role of anti-Mullerian hormone in follicle recruitment and maturation]." J Gynecol Obstet Biol Reprod (Paris) **35**(5 Pt 2): 2S30-2S34.

Visser, J. A., F. H. de Jong, et al. (2006). "Anti-Mullerian hormone: a new marker for ovarian function." Reproduction **131**(1): 1-9.

Visser, J. A. and A. P. Themmen (2005). "Anti-Mullerian hormone and folliculogenesis." Mol Cell Endocrinol **234**(1-2): 81-6.

Vitale, A. M., O. M. Gonzalez, et al. (2002). "Inhibin a increases apoptosis in early ovarian antral follicles of diethylstilbestrol-treated rats." Biol Reprod **67**(6): 1989-95.

Watt, A. H., A. T. Legedza, et al. (2000). "The prognostic value of age and follicle-stimulating hormone levels in women over forty years of age undergoing in vitro fertilization." J Assist Reprod Genet **17**(5): 264-8.

Webb, R. and B. K. Campbell (2007). "Development of the dominant follicle: mechanisms of selection and maintenance of oocyte quality." Soc Reprod Fertil Suppl **64**: 141-63.

Webb, R., B. K. Campbell, et al. (1999). "Molecular mechanisms regulating follicular recruitment and selection." J Reprod Fertil Suppl **54**: 33-48.

Weenen, C., J. S. Laven, et al. (2004). "Anti-Mullerian hormone expression pattern in the human ovary: potential implications for initial and cyclic follicle recruitment." Mol Hum Reprod **10**(2): 77-83.

Whittingham, T. A. (2007). "Medical diagnostic applications and sources." Prog Biophys Mol Biol **93**(1-3): 84-110.

Wong, B. C., N. C. Gillman, et al. (2004). "Results of in vitro fertilization in patients with endometriomas: is surgical removal beneficial?" Am J Obstet Gynecol **191**(2): 597-606; discussion 606-7.

Wright, C. S., O. Hovatta, et al. (1999). "Effects of follicle-stimulating hormone and serum substitution on the in-vitro growth of human ovarian follicles." Hum Reprod **14**(6): 1555-62.

Wunder, D. M., N. A. Bersinger, et al. (2008). "Statistically significant changes of antimullerian hormone and inhibin levels during the physiologic menstrual cycle in reproductive age women." Fertil Steril **89**(4): 927-33.

Wunder, D. M., J. Guibourdenche, et al. (2008). "Anti-Mullerian hormone and inhibin B as predictors of pregnancy after treatment by in vitro fertilization/intracytoplasmic sperm injection." Fertil Steril.

Yaman, C., M. Sommergruber, et al. (1999). "Reproducibility of transvaginal three-dimensional endometrial volume measurements during ovarian stimulation." Hum Reprod **14**(10): 2604-8.

Yazbeck, C., P. Madelenat, et al. (2006). "[Ovarian endometriomas: Effect of laparoscopic

cystectomy on ovarian response in IVF-ET cycles]." Gynecol Obstet Fertil **34**(9):

808-12.

Yu, H. T., H. Y. Huang, et al. "Laparoscopic ovarian cystectomy of endometriomas:

surgeons' experience may affect ovarian reserve and live-born rate in infertile

patients with in vitro fertilization-intracytoplasmic sperm injection." Eur J Obstet

Gynecol Reprod Biol **152**(2): 172-5.

Yuen, P. M., K. M. Yu, et al. (1997). "A randomized prospective study of laparoscopy and

laparotomy in the management of benign ovarian masses." Am J Obstet Gynecol

**177**(1): 109-14.

Zaidi, J., J. Barber, et al. (1996). "Relationship of ovarian stromal blood flow at the baseline

ultrasound scan to subsequent follicular response in an in vitro fertilization

program." Obstet Gynecol **88**(5): 779-84.

Zaidi, J., W. Collins, et al. (1996). "Blood flow changes in the intraovarian arteries during

the periovulatory period: relationship to the time of day." Ultrasound Obstet

Gynecol **7**(2): 135-40.

Zhang, J., Y. F. Zhou, et al. (2009). "[Influence of various hemostatic methods on ovarian

reserve function in women with ovarian endometriotic cyst treated by

laparoscopic cystectomy]." Zhonghua Fu Chan Ke Za Zhi **44**(8): 583-7.