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Endocrine markers of ovarian function: Clinical and biological aspects with focus on Anti Müllerian hormone

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ENDOCRINE MARKERS OF OVARIAN FUNCTION: CLINICAL AND BIOLOGICAL ASPECTS WITH FOCUS ON ANTI MÜLLERIAN HORMONE

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Abstract
Declining birthrates and infertility are now a common problem in the Western World. Due to the tendency to postpone childbearing an increasing age of women who consider motherhood is generally seen. A factor closely related to female fertility is the ovarian reserve, a term used to designate both quantitative as well as qualitative aspects of the remaining gametes in the female gonads. Despite a natural age-dependent decline in terms of the amount of gametes, a substantial variability exists between individuals as to the age at which the actual decrease starts. In this setting biomarkers to assess the individual fertility potential are often requested. Currently the antral follicle count and the Anti Müllerian Hormone (AMH) level are considered to be the most predictive markers. The two markers roughly describe the same issue as they are both related to certain developmental stages of the folliculogenisis. Furthermore, the number of growing follicles is closely related to the activity of the hypothalamic-pituitary axis which controls the secretion of Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH).
The aim of the present thesis was firstly to explore the age-related circadian variation of AMH in normally ovulating women and patients with Polycystic Ovarian syndrome (PCOS) and its relation to gonadotropin secretion; secondly to explore the inter- and intra-cyclic variation of AMH related to the number of antral follicles measured by ultrasound; thirdly to explore AMH as a marker of time to pregnancy in fertile women, and finally, to examine the significance of mid-follicular phase LH levels in patients undergoing IVF.

The results show that, in contrast to women with PCOS, normally ovulating women reveal a significant circadian variation in AMH. The co-variation with androgens and LH, may indicate that LH masters the secretion of AMH. Moreover, in normally ovulating women, AMH shows a significant intra-and inter-cyclic variation which may question the current use of one measurement of the hormone as sufficient to evaluate the ovarian reserve. A significant positive correlation was found between AMH and the number of small antral follicles. Moreover, in a cohort of spontaneously pregnant women, AMH was found to be related to the number of menstrual cycles required to obtain a pregnancy. In women undergoing a long pituitary down-regulation with GnRHa and hormonal stimulation with gonadotropins to obtain multi-follicuar development prior to In Vitro Fertilization, the clinical pregnancy rate as well as the consumption of exogenous gonadotropins was inversely correlated to the mid-follicular LH levels.

In conclusion, the present results demonstrate that normally ovulating women have circadian as well as intra- and inter-cyclic variations in AMH. Moreover, the AMH level seems to be related to time to pregnancy and closely linked to LH as a key player during folliculogenesis.

Key words
Ovarian reserve, Anti Müllerian Hormone, fertility, fecundity, biomarkers, Time To Pregnancy, Luteinizing Hormone, antral follicle count

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"Success is not final, failure is not fatal,
it is the courage to continue that counts."
- Winston Churchill

To Silje, Helle, Linn, Lars, Ola and Ane

CONTENTS

ABBREVIATIONS

AFC	Antral Follicle Count
AFS	Antral Follicle Stage
AIH	Artificial Insemination Husband
AMH	Anti Müllerian Hormone
AMHR	Anti Müllerian Hormone Receptor
ART	Assisted Reproductive Technologies
BMI	Body Mass Index
BMP	Bone Morphogenic Protein
CV	Coefficient of Variation
DFI	DNA Fragmentation Index
FSH	Follicle Stimulating Hormone
GAI	Gestational Age at Inclusion
GDF	Growth and Differentiation Factor
GDNF	Glial cell-derived Neurotrophic Factor
GnRH	Gonadotropin Releasing Hormone
HR	Hazard Ratio
IVF	In Vitro Fertilization
ICSI	Intra Cytoplasmic Sperm Injection
IUI	Intrauterine Insemination
LH	Luteinizing Hormone
OR	Odds Ratio
PMDS	Persistent Müllerian Duct Syndrome
PCOS	Polycystic Ovary Syndrome
SCSA	Sperm Chromatin Structure Assay
SD	Standard Deviation
TFR	Total Fertility Rate
TGF	Transforming Growth Factor
TSH	Thyroid Stimulating Hormone
TTP	Time To Pregnancy
WHO	World Health Organization

PREFACE

This thesis comprises two parts. The first part contains a review of the literature within the field. Subsequently, the aims of the thesis are presented. Moreover, part one contains an overview of the materials and methods used for the studies, presentation of the results as well as a general discussion of the findings of the five studies the thesis is based on. Finally, a conclusion and some future perspectives are drawn. The second part of the thesis comprises the published Papers (I and V), two submitted manuscripts (II and IV) and one manuscript (III) on which the present thesis is based.

LIST OF ORIGINAL PAPERS

This thesis is based on the following papers referred to in the text by their Roman numerals:

I **Leif Bungum,** Anna-Karin Jacobsson, Fredrik Rosen, Charlotte Becker, Claus Yding Andersen, Nuray Güner and Aleksander Giwercman. Circadian variation in concentration of anti-Müllerian hormone in regularly menstruating females: relation to age, gonadotropin and sex steroid levels. *Human Reproduction 2011, Vol.26, No.3, pp. 678–684.*

II **Leif Bungum,** Florencia Franssohn, Mona Bungum, Peter Humaidan, Aleksander Giwercman. The circadian variation in Anti-Müllerian Hormone in patients with polycystic ovary syndrome differs significantly from normally ovulating women. *Submitted.*

III **Leif Bungum,** Julia Tagevi, Mona Bungum, Ligita Jokubkiene, Povilas Sladkevcius, Lil Valentin, Aleksander Giwercman. Menstrual cycle dependent variation in serum levels of Anti-Müllerian Hormone - relation to age, antral follicle count and sex steroids. *Manuscript.*

IV **Bungum L,** Bungum M, Toft G, Axmon A, Bonde JP, Pedersen HS, Ludwicki JK, Zviezdai V, Spano M and Giwercman A. Anti Müllerian Hormone and time to pregnancy in a fertile population. *Submitted.*

V Humaidan P, **Bungum L,** Bungum M and Andersen C.Y. Ovarian response and pregnancy outcome related to mid-follicular LH levels in women undergoing assisted reproduction with GnRh agonist down-regulation and recombinant FSH stimulation. *Human Reproduction 2002, Vol. 17, No.8, pp. 2016-2021.*

POPULAR SCIENTIFIC SUMMARY

During recent decades, significant changes in fertility pattern are seen in the Western World. Declining birth rates and smaller family sizes affect the population size, but also cause unwanted effects for the desired number of children to be achieved by the individual couple. Also involuntary childlessness is a consequence of postponing childbearing to an age where the chance of spontaneous pregnancy is less likely. A major age-related factor to limit a couple's chance of conception is the declining quantity and quality of the eggs in the women's ovary.

The total amount of eggs available for a woman during her reproductive life is deposited in the ovaries as a number of quiescent eggs already in fetal life. On the earliest stages of development the pool of eggs reaches millions in number, but a vast majority of them decay before the female has reached her reproductive age. During women's fertile period of life, a proportion of these non-growing eggs are activated to grow throughout 5-6 months and finally reach a stage where they can ovulate and become fertilized or go through so-called programmed cell death. In this manner, women's supply of eggs is slowly depleted and finally, when all eggs are used, menopause will occur. The term ovarian reserve designates the pool of eggs present in the ovaries at any given time. A huge individual difference is seen in the size of the initial pool and how rapidly it is used.

For a woman or a couple planning to build a family, questions like; when to start to reproduce and how long could pregnancy potentially be postponed without jeopardizing the chance of obtaining a family of a size of choice often are raised. In order to be able to answer these questions professionally, a reliable test to assess ovarian reserve, including the amount of remaining eggs in the ovaries, is requested.

Today, the levels of Anti Müllerian Hormone (AMH) or the number of small follicles measurable by ultrasound are considered to be the best markers of ovarian reserve. Both tests measure certain developing stages in between the quiescent stage and eggs ready to be selected for ovulation, and this number mirrors the number of remaining eggs in the ovaries. Several scientific publications claim AMH to be so stable a test that only one blood test is sufficient for estimating a woman's ovarian reserve. Also different cut-off levels believed to predict the chance of natural as well as assisted conception are suggested.

For a quiescent egg in the ovary to be activated and start growing, a signal from the brain is necessary. The signal originates in the part of the brain called the hypothalamus and is transmitted to the hypophysis via a signal substance named Gonadotropin Releasing Factor (GnRh). This signal controls two hormones secreted from the hypophysis, the Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH), which both are brought to the ovaries by the blood and exert a crucial impact on the recruitment of quiescent follicles to start growing into an ovulating follicle.

In order to add more clinically useful knowledge, the aim of the present thesis was to explore whether there is a variation over time in secretion of AMH, and to study its relation to the two gonadotropins; FSH and LH.

In studies I and II, the variability of AMH was examined throughout 24 hours (circadian variation), by drawing blood for analysis every second hour from 8:00 a.m. the first morning, until the next. The study was performed both in normal menstruating women as well as in women diagnosed with Polycystic Ovary Syndrome (PCOS), which is a fairly common endocrine disorder in young women, characterized by anovulation, causing infertility and ovaries with numerous of small follicles. This group of women was chosen for the study since AMH is produced in the small follicles. Hence, the levels of AMH in PCOS women are much higher compared to normally menstruating women. The stability of AMH was also tested over three menstrual cycles by blood tests every fifth day. To find out whether the level of AMH was correlated to the number of small follicles, a three-dimensional vaginal ultrasound was performed at the fifth day of the menstrual cycle. In Study III, the variation of AMH and its co-variation to the antral follicle count was explored over 3 menstrual cycles.

In study IV, blood from pregnant women was analyzed and the level of AMH related to the number of months necessary to obtain pregnancy. The aim of the study was to test AMH as marker of fertility in women/couples with normal fertility. The data for this study came from women in Ukraine, Poland and Greenland.

In study V, we wanted to explore if the level of LH in women undergoing In Vitro Fertilization (IVF) could influence the result of the treatment. This was performed by measuring the level of LH in 207 women undergoing hormonal stimulation prior to IVF. According to the level of LH, the women were divided into groups and compared by the number of eggs retrieved, fertilization and pregnancy rate.

The results of this thesis reveal that normal menstruating women show a significant circadian variation in AMH. Also, for this group of women, AMH levels were shown to fluctuate significantly, both between cycle days within one cycle as well as between cycles.

Furthermore, data from the thesis show that in women with PCOS, the level of AMH in average was three-fold higher compared to the normal menstruating women. However, the circadian variation in AMH as seen for the normal menstruating women was not seen for those with PCOS. For both groups, however, a clear correlation between levels of AMH and LH was found, suggesting that LH masters the secretion of AMH. Moreover, a significant positive correlation was found between levels of AMH and the number of small antral follicles. This finding was, however, expected as AMH is produced in such small follicles. Moreover, in a cohort of women obtaining spontaneous pregnancy, levels of AMH were found to be related to the number of months required to obtain pregnancy.

In women undergoing hormonal stimulation prior to IVF, the rate of pregnancy and the consumption of hormones required for the hormonal stimulation were correlated to the LH levels. The number of successful pregnancies, as well as the amount of stimulating hormones needed, decreased by rising levels of LH.

In conclusion, results from the present thesis have demonstrated that AMH is closely linked to LH, both key factors during development and recruitment of follicles and eggs. In women with a normal menstrual cycle, the level of AMH fluctuates significantly, both between cycle days within one cycle as well as between menstrual cycles. These fluctuations reach such an extent, that the current practice of using only one measurement of AMH to evaluate the ovarian reserve in a woman is questioned. Despite this, AMH seems to have the potential to indirectly measure a couple's chance of obtaining pregnancy, as the time to pregnancy is longest in couples where AMH levels are lowest.

BACKGROUND

For humans, as well as for most other species, the ability to reproduce is essential. However, due to numerous factors, the individual fertility capacity varies tremendously. Whilst the term fertility describes the natural capability of producing offspring, fecundity defines the potential for reproduction, influenced by age, the capability of carrying a pregnancy and finally deliver viable offspring. On the other hand, the term infertility is used to describe lack of fecundity, and subfertility any form of reduced fertility with prolonged time of obtaining conception.

Despite a continuous search for a reliable biomarker able to predict female fertility capacity, so far, no other factor than female age has shown to independently predict the ability to reproduce. A reliable biomarker would be useful in counseling women and couples about their potential reproductive performance and answer central questions like i) Can pregnancy, and for how long, be postponed without increasing our risk of infertility; ii) what is the likelihood of spontaneous pregnancy and live birth in the absence of fertility treatment; iii) in case of subfertility, which treatment is the optimal. Adequate answers would provide a guide to correct management of reduced fertility with appropriate timing of infertility investigations and treatment to avoid both over- and under-treatment.

Traditionally, the most frequently used marker of female fertility capacity has been Follicle Stimulating Hormone (FSH). However, as rising levels of FSH first occur close to a woman's peri-menopausal transition, Anti Müllerian Hormone (AMH) has replaced FSH as the clinically best available marker except from female age [1]. However, the argument for the current clinical use of AMH, with different cut-off levels may be questioned. Another hormone playing an important role in folliculogenesis is Luteinizing Hormone (LH), which to a large extent rule the Estradiol-production in theca and granulosa cells. In Assisted Reproductive Technologies (ART), its role has been discussed as both too high or too low levels during folliculogenesis has been connected to adverse effects for the developing oocyte [2].

INTRODUCTION

Trends in fertility

During the past century, a global increase in economic and social development coinciding with a substantial decline in human fertility has been observed [3]. In the industrialized countries, a significant contribution to involuntary childlessness is the current postponing of parenthood to an age where especially female fertility capacity is declining. This trend will inevitably affect the population size as the Total Fertility Rate (TFR) falls below the population replacement level of 2,1 births per couple [4], but also cause unwanted effects for the desired number of children to be achieved by the individual couple [5].

The age where women start trying to conceive, will significantly affect her probability to actually give birth or her ability to create a family of a size due to her wishes and those of her partner. In the course of the last 40 years, where efficient contraceptive medication and devices has been available, especially higher educated women have postponed pregnancy. Whilst in the European Union, a rise in the mean age of motherhood started in the late 1970s, in Canada from 1970 to 1999, the average age of a woman delivering her first child increased from 24.6 to 29.1 years [6].

A natural age-dependent decline in fertility exists in both genders, however the drop in fecundity starts earlier in women and show a substantial variability in which age it actually starts. The advancing maternal age is associated with an increasing rates of aneuploidy in oocytes coinciding with decreasing numbers of primordial follicles and quality of oocytes [7]. This coincides with a significant increase in the number of couples seeking medical help for involuntary childlessness. Actually, infertility affects approximately 15% of all couples trying to conceive [8]. However, remarkably many women are ignorant of the potential consequences of delayed childbearing. Even in well-educated couples, unawareness to the negative age effects combined with unrealistic expectations to ART exists [9].

Assisted Reproductive Technologies (ART)

The term ART defines all technologies that involve handling of gametes outside the body, either sperm alone as in Intrauterine Insemination (IUI), or both eggs and sperm as in *In Vitro* Fertilization (IVF) and Intracytoplasmic Sperm Injection (ICSI) [10].

While insemination of prepared sperm had been performed in animals, the fully use of in-vitro handling of both gametes was, for the first time, reported successful in 1978 by birth of the first IVF baby [11]. Assisted reproductive technologies are applied worldwide and it is estimated that more than five million babies have been born as a result of ART [12].

The most common indications for treatment with the least invasive form of ART, i.e. IUI, where prepared semen is inseminated in the women's uterus, are ovulatory dysfunction, unexplained subfertility and milder forms of male subfertility. *In Vitro* Fertilization is primarily used in female subfertility or as a second line treatment in unexplained infertility where less invasive treatment had proved unsuccessful [10]. Since 1992 ICSI has been used to treat male infertility [13] but also to an increasing degree for other indications [14, 15].

The principle behind hormonal stimulation by the use of exogenous injected gonadotropins is to rescue and grow multiple pre-ovulary follicles. In order to avoid premature ovulation caused by raising estrogen levels triggering LH surges, a GnRh agonist applied by injections or nasal spray is used. This treatment is called "long protocol", due to the pre-treatment period necessary to control pituitary release of FSH and LH. The daily application of the agonist will cause a steadily declining LH level as shown in Figure 1.

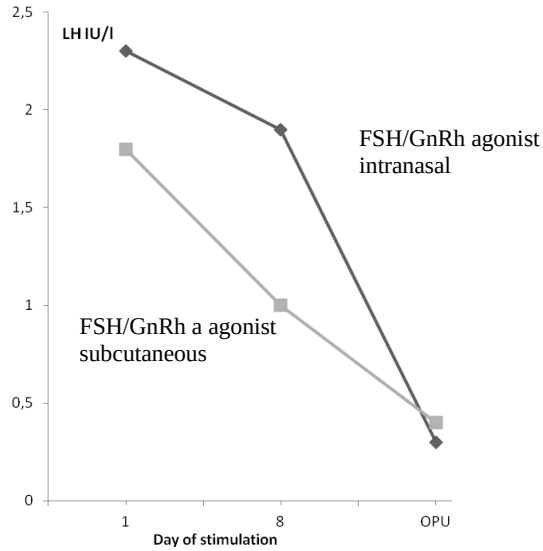

Figure 1. The effect of different administration forms of GnRh agonist on LH levels during controlled ovarian stimlation

Prevention of premature luteinization may also be achieved by the use of a GnRh antagonist. In this treatment, the gonadotropin stimulation starts in connection to a menstrual period and after some days supplied by the antagonist. This treatment is of shorter duration and hence called the "short protocol". Actually, hormonal stimulation and prevention of premature luteinization in connection to ART, is the most widespread indication for blocking pituitary gonadotropin secretion.

Mature eggs are retrieved from the ovaries by trans-vaginal ovarian aspiration. By the use of an ultrasound probe inserted into the vagina creating an image of the ovaries and other nearby pelvic organs, the follicles can be emptied by means of a thin needle using suction to aspirate the follicular fluid containing the oocytes.

Fertilization is performed either by conventional IVF or by ICSI. In IVF around 100 000 spermatozoa are co-incubated with the oocytes for 1 ½ hour or longer and in ICSI one single sperm is injected directly into the oocyte. If fertilization is obtained and an appropriate cleavage and embryo development follows, one or more of the embryos are transferred to the woman's uterus. Embryos may be transferred at the cleavage stage (2-3 days after oocyte retrieval) or at the blastocyst stage (day 5 after oocyte retrieval). For the embryo transfer, one or more embryos and a droplet of the culture fluid will be loaded into a soft catheter and disposed into the uterine cavity.

The human ovary

Anatomy

The primary function of the human ovary is to produce and release competent oocytes for fertilization and embryonic development, and to secrete steroid hormones. The human ovaries, in size approximately 4 x 2 x 0.8 cm, are paired organs located at the lateral wall of the pelvis. Laterally, they attach to the pelvic wall by the suspensory ligament, and medially to the uterus by the ovarian ligament. As an additional support, the ovary is wrapped in a peritoneal fold called the mesovarium, a part of the broad ligament (Figure 2).

The cortex forms an outer sheath of the ovary holding the gametes in different stages of development, and medulla is the central part consisting of stromal cells which is connective tissue supporting the 3-dimensional structure of the organ.

The female gametes, i.e. the oocytes, are located in a rich vascularized bed beneath the firm tunica albuginea on the cortically medullary border surrounded by stromal cells, blood vessels and nerves. The different developmental stages found are the primordial follicles, primary and secondary oocytes, antral follicles, preovulatory follicles and the corpus luteum [16].

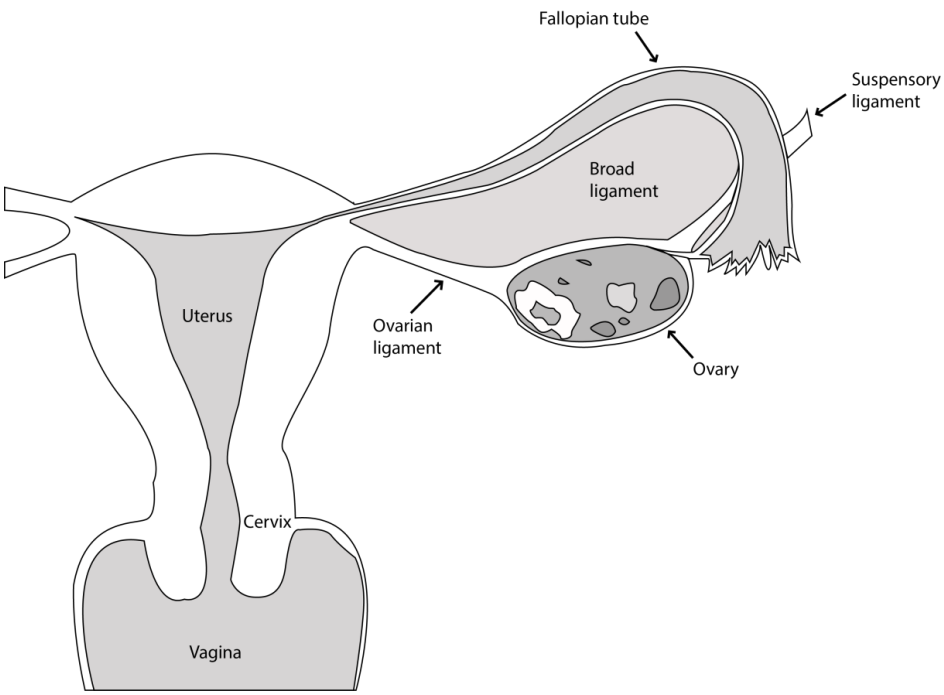

Figure 2. The female reproductive anatomy

Primordial follicle assembly and development

The mammalian oocyte originates from primordial germ cells in the yolk sac epithelium, which early in fetal life migrate into the genital ridge of the naive gonads (ovaries). Hereafter, the epiblast-cells initiate high mitotic activity and becomes oogonia [17], which cluster in nests connected to each other by bridges. The bridges disappear and each oocyte becomes an independent unit called a primordial follicle with the potential for progress into primary, secondary and antral follicles. The peak count around gestational week 20 reach 6-7 million germ cells to be present in the gonads [18] followed by a rapid decline in quantity due to atresia, reducing the count to approximately 3-500 000 in each ovary of the newborn female [19]. At the start of her reproductive period, marked by the menarche, around 500 000 oocytes in total remain [20]. Each oocyte, arrested in the prophase of the first meiotic division, is surrounded by one layer of granulosa cells. The hypothalamic-pituitary axis is functioning in the female fetus from the second trimester of the pregnancy, but the placental secretion of steroids exerts a strong negative feedback to the output of gonadotropins [21].

After birth, the gonadotropin secretion is reactivated with a temporary elevated secretion of FSH, activating the ovarian sex steroid secretion. However, the duration is short and within four months the production of Estradiol falls to a very low level [22]. At menarche, the hypothalamic-pituitary-ovarian axis is activated and becomes fully functioning (Figure 3). Stimulation by FSH produces multi-follicular ovaries, but a coordinated LH surge is initially often lacking causing irregular bleeding patterns due to anovulation and absence of luteal phase transition [23, 24].

Folliculogenesis is a long process, requiring several months for a primordial follicle to develop to the ovulatory stage [25] (Figure 4). The phase of oocyte growth and differentiation up the antral follicle stage is predominantly regarded to be gonadotropin-independent. In the second gonadotropin-dependent stage, the follicle extends to 25-30 mm before it ovulates. In the luteal to follicular phase transition just prior to menstruation, a rapid increase in FSH secretion occurs. Once exceeding the “FSH threshold”, several follicles initiate growth, gain size and secrete Estradiol. The increased production of Estradiol and Inhibin then exerts a negative feedback to the hypothalamus and the pituitary, causing the secretion of FSH to diminish. This is the concept of the “FSH threshold” proposed by Brown [26]. Usually, only the best follicle is able to grow, gain dominance and has the competence to ovulate, triggered by a surge of LH released from the pituitary.

The transition of primordial into primary follicle includes enlargement of the oocyte and proliferation of the flat granulosa cells into a more cuboidal structure. Within the granulosa cells the genome becomes activated and the endoplasmic reticulum develops to meet the requirement for protein production. The granulosa cells are enveloped by a basal lamina separating them from the surrounding stromal/thecal elements, and intercellular connections for exchange of paracrine signaling are established. Shortly after initiation of follicular growth the zona pellucida around the oocyte starts to develop. The granulosa cells divide and expand to multiple layers and each follicle becomes vascularized by arterioles building a network just outside the basal lamina [27] implying a directly blood bourn exposure of growth factors and hormones. Theca cells develop from stromal cells, and as the follicle further advance, an external and internal theca layer is created to form the stage of a secondary oocyte.

The next stage, the antral follicle, is created when small cytoplasmic droplets of fluid unify to form a cavity within the follicle called the antrum. Whilst the cells beneath the zona pellucida becomes corona radiata, the periantral granulosa cells border the antrum and the granulosa cells closest to the oocyte constitute specialized cells called the cumulus oophorus. Reaching the antral follicles stage, the meiosis resumes and as the preovulary LH-surge signals the completion of the first meiotic division, the first polar body extrudes and the oocyte becomes arrested in the metaphase II stage, ready for fertilization.

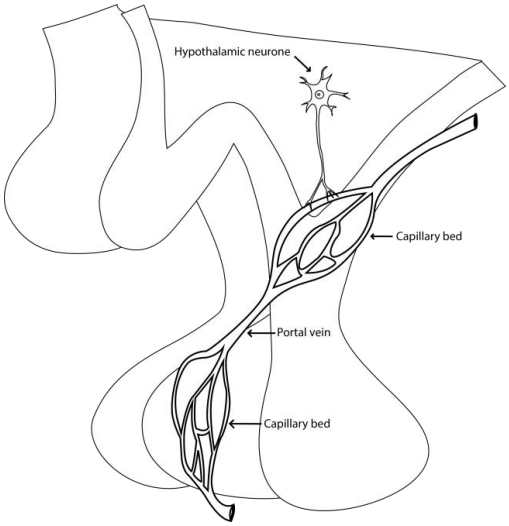

Figure 3. Secretion of gonadotropin is controlled by the hypothalamus. The gonadotropin-releasing hormone (GnRh) from approx. 1000 hypothalamic neurons is discharged in the portal circulation where the hypofysal artery ramifies into a capillary bed and brought to the anterior pituitary by the portal vein

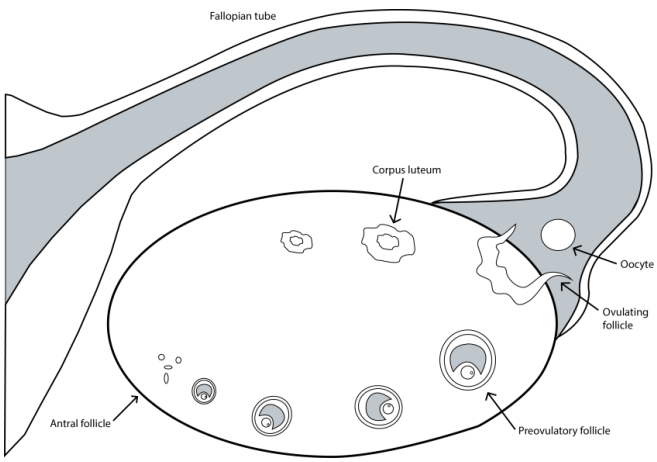

Figure 4. Follicle dynamics in the human ovary

Regulation of follicular growth

The complex growth from a primordial to an ovulating follicle and formation of the corpus luteum requires a strict conduction of stimuli of both extra- and intra-ovarian origin, encompassing the action of both hormones and growth factors. In a balance between factors activating and retarding follicle development, the presence of a sufficient amount of available gametes for selection and fertilization is weighed against a too early depletion of the gamete store.

The hypothalamic-pituitary-ovarian axis constitutes a central unit controlling the dual action of gonadotropins secreted by the same pituitary gonadotropic cells [28]. Both LH and FSH consist of a structurally shared alpha and a different beta subunit encoded by three separate genes [29]. The

production and secretion of gonadotropins. The pattern of GnRH pulses changes during the ovulatory menstrual cycle, with pulse frequency and amplitude gradually increasing during the follicular phase [30, 31]. An initial increase of FSH during the follicular phase abates concomitant to a progressively rise in LH surges which peak at the mid-cycle LH surge initiating ovulation. Increasing FSH levels stimulates follicular recruitment and maturation, and the subsequent enlarged Estradiol secretion stimulates an accelerated GnRH pulse frequency triggering the mid-cyclic LH surge. The synthesis of sex steroids take place in theca and granulosa cells within the developing follicle, illustrated by the 'two-cell two gonadotropin model" (Figure 5). In the theca cells holding LH receptors, cholesterol is converted to androgens via pregnenolol which are subsequently transported to the granulosa cells where FSH via its receptor stimulates aromatase activity converting androgens to Estradiol [32] in an increasing manner as the follicle increase in size. The sex steroids produced are accumulated in the follicular fluid or via blood vessels transported via the general circulation to end organs outside the ovary.

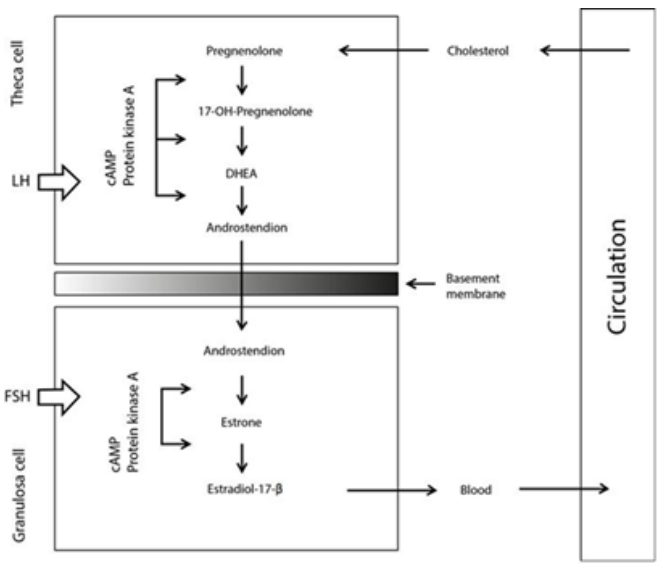

Figure 5. The "Two-cell two gonadotropin concept" for sex steroid production.

As FSH rises above the threshold for follicle recruitment, an appropriate number of follicles start to grow. The magnitude and duration of the FSH level above the threshold value, decides how many follicles that will be recruited, a mechanism called the" FSH/threshold window concept" [33]. Only the follicle with the highest mitotic activity and best functioning Estradiol synthesis will be able to survive the feed-back induced lowering of FSH secretion, and the selected (dominant) follicle proceed to ovulation as all the other follicles not meeting the threshold-requirements will go into atresia.

Although the most obvious role for FSH is the final maturation from follicle selection to ovulation, an important role of FSH even at the initial step, the primordial follicle transition, seems likely. This is supported by the observation that perimenopausal women with rising levels of FSH have an accelerated loss of primordial follicles [34, 35]. Also, women diagnosed with hypothalamic hypogonadism with low FSH levels and undetectable AMH levels might ovulate after prolonged FSH-stimulation [36].

Ovulation

Follicular ovulation may occur at most 400 times in a woman's reproductive life, provided spontaneous ovulatory cycles and no use of hormonal contraception. The process includes enzymatic destruction of the cells surrounding the oocytes, the cumulus oophorus. Three different collaborators are essential for a normal ovulation; the oocyte itself, LH receptors in sufficient numbers in mature follicles and the pre-ovulatory LH surge from the pituitary. The process has elements of inflammation afforded by prostaglandins stimulating proteolytic enzymes able of disrupting the follicle wall [37].

The corpus luteum is a unique temporary endocrine structure, formed on the remains from of the ovulating follicle with a time-limited function and existence. During its formation and life span, a number of cells (granulosa, theca) remodel, grow, differentiate and vanish [38]. The corpus luteum plays a vital role in regulation of the menstrual cycle and in the maintenance of early pregnancy through an active LH driven production Progesterone synthesized from Cholesterol involving the mitochondrial P450 coenzyme. In humans, Progesterone is an end-product, which cannot be further processed [39]. At the end of its existence and disappearance from the ovary, a new cycle with growth of new follicles is allowed to start.

Transforming growth factor superfamily (TGF-β)

In the ovary, a complex bidirectional signal-system between the oocyte, granulosa - theca and stromal cells [40] ensure the synchronization of endocrine signals and a variety of growth factors. A majority of these growth factors and signal molecules belong to the transforming growth factor superfamily (TGF-β), however, numerous other factors are also crucial in this regulation. For the initial activation of primordial follicles, kit ligand, leukemia inhibiting factor and fibroblast growth factor have shown to be active. Even Progesterone and Estrogens may be involved in the slowing of activation, as these hormones from maternal or placental origin are high in late stage of the female intrauterine life.

The TGF-β family is divided into three subgroups; ligands, signaling receptors and non-signaling binding proteins [28]. The ligands, defined as molecules that after binding to a receptor create a post-receptor signal, share in common that they are homo- or hetero-dimers covalently linked by disulphide bonds. They include TGF-β factors 1-3, Bone Morphogenic Protein (BMP), Growth and Differentiation Factor (GDF) subfamily, the activin/inhibin system, Glial Cell-Derived Neurotrophic Factor (GDNF), AMH together with a number of less defined members [41]. For transformation of extracellular signals from TGF-β factors to the nucleus, in order to activate downstream gene transcription, so-called SMAD molecules are used to form transcriptional factors for gene expression regulation. Molecules at the cell surface can form non-signaling co-receptors for TGF-β members like inhibins and TGF-β. The complexes formed may be seen as both co-activators and co-repressors as they are able to both depress and enhance the effect of the ligand.

BMP factors 4 and 7, produced in stromal and theca cells, can in cooperation with growth and differentiation factor 9 (GDF-9) [42] initiate activation of primordial follicles [43, 44]. For the opposite effect, slowing down the primordial-to-primary follicle transition, AMH has been identified as an important substance [45].

In the process of progression from primary to early antral follicles, the most important modifications include oocyte enlargement, formation of zona pellucida, creation of multilayer granulosa cell surface, formation of a basal lamnia and the condensation between basal lamnia and stromal cells. The major players in this step include GDP 9 and BMP 15 originated from the oocyte and activin, AMH, BMP 4 and BMP 7 from the theca cells.

The step from pre-antral to follicle selection, includes proliferation of the granulosa and theca cells, vascularization and oocyte enlargement. Activin and BMP 6 from the granulosa cells and GDF 9 from the oocyte promote while AMH from the granulosa cells counteracts the progression of this step. Curiously, also FSH promote this step [46] although progression is not that FSH dependent like follicles beyond this stage. Different exposure to TGF-β members within a cohort of growing follicles may prime the individual follicle and form the base for the diverse intra-follicular sensivity to FSH to be apparent at later stages of follicle development. Subsequently, this may form the base for selection of a dominant follicle within a cohort. In this stage, AMH reduces FSH responsiveness in small antral follicles for further growth to pre-ovular and dominant follicles, and may therefore oppose cyclic recruitment. As more Inhibin relative to Activin is secreted as follicle growth exceeds the ratio Inhibin/Activin secretion shifts in this stage. Activin, BMP 4, BMP 6 and BMP 7 can increase LH-dependent production in antral follicles. As these compounds are secreted by granulosa cells, granulosa-control over the production of androgens as substrate for the synthesis of Estradiol during the pre-ovulatory stage is implied.

The precise role of the TGF-β members in ovulation, luteinization and forming of the corpus luteum are not known although several are likely to be involved. The corpus luteum itself is a post-ovulatory important source of Inhibin A. Activin A may have an impact on the pace of the granulosa cell luteinization.

Ovarian reserve

The size of the acquired follicle pool, which differs significantly among females, has extensive consequences for a woman's reproductive life by both indicating her cumulative pregnancy chance [47] and predicting her age of menopause [48].

The gametes, often referred to as the ovarian reserve, include an un-assessable amount of non-growing follicles and a semi-quantifiable amount of developing follicles. Growing follicles having reached the antral follicle stage with a size exceeding 1-2 mm are measureable [48] by means of sonographic imaging. The level of AMH [1, 49, 50] reflects the number of growing follicles from the stage of secondary oocyte up to the antral follicles of 6-8 mm. These two measures are often referred to as the functional ovarian reserve expressing a woman's fecundity and are linked to spontaneous conception as well as to success after fertility treatment [51, 52]. According to current view, the oocyte pool is non-renewable and of declining size as recruitment from the pool diminishes the pool of follicles in a continuous process. Thus, the magnitude of the starting follicle cohort is important, as this pool vary significantly between individuals with such an enormous range as 35 000 to 2,5 millions pr. ovary at birth [48].

Actually, only a small part of the total ovarian reserve is measurable, due to the fact that total ovarian reserve consists largely of non-growing follicles. As stated, the total ovarian reserve declines with age, which has been the origin of the term "ovarian age". This term is imprecise, as not only age but as much as the size of the original pool and the recruitment rate will decide the number of antral follicles or the ovarian volume which is the measure of ovarian age.

Both epidemiologic studies, as well as experience from ART, display an connection between a rapid decline in fertility and the menopausal transition, which may be due to an initial small ovarian reserve and an early onset of subfertility, which is the case for around 10 % of females [53].

Polycystic ovary syndrome (PCOS)

Polycystic ovary syndrome (PCOS) is perhaps the most striking example in humans where some these fine-tuned mechanisms are not functioning perfectly. The syndrome, associated with polycystic ovaries, anovulation and clinical or biochemical hyperandrogenism, is a phenotypically heterogenic endocrine disorder affecting women of reproductive age with a prevalence of 6-10% [54]. Also obesity, insulin resistance and the metabolic syndrome are related to PCOS [28].

The pathophysiological explanations for follicular abnormalities in PCOS are probably abnormal intra- and extra-ovarian regulation interfering with normal folliculogenesis from initial recruitment, cyclic recruitment and growth of follicles [28]. Polycystic ovaries have several times more primary, secondary and antral follicles compared to non-PCO women [55-57]. Although there is no consensus regarding a explanation of the biological mechanisms behind PCOS, the condition seems to be at least two-factorial [58]. Firstly, the intra-ovarian hyperandrogenism promotes early follicular growth and leads to a 2-5 mm follicle excess. Studies have also revealed a positive correlation between follicle number and serum Tesosterone/Androstendione concentration in PCOS women [59, 60]. Secondly, a low aromatase activity caused by an insufficient FSH stimulation affects the synthesis of Estrogens interfering with selection and growth of a dominant follicle [61]. Insulin resistance, secondary to both genetic and lifestyle factors, is associated with anovulation, but probably not its primary cause [62-64]. Androgens originate from LH-stimulated steroidogenesis in theca interna cells [65] and hyperandrogenism may have both an extra- and intracellular origin. An increased pituitary output of LH secondary to altered GnRh pulse [66] may be reinforced by hyperinsulinism, both triggering an enhanced androgen synthesis [67, 68] and may further deteriorate the regulation of folliculogenesis by disturbing the FSH-to-LH shift that triggers the correct follicular development until ovulation.

Biomarkers of fertility

Traditionally, in counseling of couples seeking help for infertility problems, a work-up including a semen analysis for the male and an endocrine profile consisting of FSH, LH and Estradiol, uterine and tubal factors as well as the number of remaining oocytes (ovarian reserve) in the female normally are performed. However, none of these markers have shown to be good predictors of fertility, and thus they will be useless also in counseling women or couples who want to know their future fertility capacity.

Ultrasonography

Ultrasonography is a useful tool for measurement of ovarian function. Ovarian volume [69], antral follicle count [70] and ultrasound doppler measurement of stromal blood flow [71] has been evaluated as markers. Of these physical methods, antral follicle count has been most investigated, and found to be a better instant and current marker of fertility rather than being able to predict future fertility [72], and antral follicle count has been shown to vary significantly between cycles [73].

Biochemical serum markers

Endocrine biomarkers are easily accessible through a blood test. Generally, a reliable biomarker brings information about the status and changes in physiological processes in the organism at a physical or cellular level. Ideally a biomarker should be easy to use and interpret. Furthermore, a reliable biomarker is characterized by a low degree of diurnal as well as a day-to-day variation allowing a single measurement to be sufficient for clinical management. However, in reality, most biomarkers display

variation in cyclical rhythms which may show daily, monthly or seasonal variation and changes over the span of life [74].

Luteinizing Hormone (LH)

The level of LH is important in ovulation, conception and early pregnancy [75]. Connected to hormonal stimulation for IVF it has been found that both too high as well as too low levels of LH exert detrimental effect on the developing conceptus [76].

Luteinizing Hormone is constituted of two glycoprotein molecules α- and β-subunits, which together constitute a heterodimer forming a functional protein. Luteinizing Hormone, FSH, TSH and hCG all have a common α-subunit containing 92 amino acids while the β-unit is different. The β-subunit of LH is built of 120 amino acids and share for 81% the same amino acids and stimulate the same receptor [77]. The difference in structure of the subunits, decide both bioactivity as well as half-life, which is approximately 20 minutes for LH and more than 24 hours for hCG [78].

The secretion of LH from the anterior pituitary lobe is controlled by pulsatile waves of GnRh from the hypothalamus. According to the "two cell–two gonadotropin hypothesis" (Figure 5), LH together with a synergistic interaction of FSH, is a prerequisite for appropriate steroid production. Luteinizing Hormone receptors at the surface of the granulosa cells control the selection of a dominant follicle and the extinction of all other follicles during the follicular phase.

Follicle Stimulating Hormone (FSH)

Traditionally, the most frequent marker of ovarian reserve both in natural fertility, in infertility work-up and as interpreters of success rate after ART [52] has been "the basal level" of FSH measured in the early follicular phase when it is assumed that a nadir level of Estradiol represent a the lowest feed-back impact on its secretion [79]. However, rising levels of FSH is a late indicator of declining fertility most often noticeable close to the end of a woman's reproductive period and thus not the most reliable marker of fertility capacity.

Anti Müllerian Hormone (AMH)

During the last decade also AMH, produced in granulosa cells from small antral follicles has gained increasing interest as a marker of female fertility capacity [81].

Anti Müllerian Hormone is a homodimetric disulfide-linked glycoproetein with a molecular weight of 140 kD The hormone, a member of the TGF-β family, is exclusively expressed in the gonads and originally named for its role in male sex differentiation [82]. In the male foetus, AMH is produced by the Sertoli cells and induces regression of the Müllerian ducts, the embryonic structure for the female reproductive tract. In the absence of AMH or a non-responding receptor, Müllerian ducts develop to fallopian tubes and uterus, even in the male foetus [83]. In the female foetus, AMH is expressed in the ovary from gestational week 36 [84] long after the Müllerian ducts have lost their sensivity to the hormone [85, 86]. The protein is produced in granulosa cells involved in follicular growth and development and secreted into the follicular fluid and circulation.

The AMH protein in female serum is low at birth followed by initiation of a slight but stable increase by puberty until adulthood and finally ceases to menopause [87]. In follicular fluid, the expression is highest in pre-antral and antral follicles sized 2-9 mm [88], followed by a fading level as the follicle grows through the subsequent stages of follicle development and is lost in the FSH-dependent stages as well as in atretic follicles. It has been shown that oocytes from preantral, late antral and preovulary follicle do up-regulate AMH mRNA levels in granulosa cells, in a fashion dependent upon the developmental stage of the oocyte [89].

The AMH receptor consists of a single membrane spanning serine threonone kinase receptor called type I and type II. The type II receptor (AMHRII) imparts ligand binding specificity and the type I receptor mediates downstream signalling when activated by the type II receptor. The human gene for AMHRII is located on chromosome 12 and consists of 11 exons spread over more than 8 kb [90]. The AMHRII messenger is expressed by AMHR target organs, the Müllerian duct`s surrounding mesenchyme and the female gonad`s granulosa cells.

The signaling pathway for AMH has been identified in the gonads and the gonadal cell lines. The AMHRII is highly specific. In contrast, the identity of the AMH type I receptor is not clear; three type I receptors of BMPs, Alk2, Alk3 and Alk6 may transduce AMH signals, but none of them have all the characteristics of an AMH type I receptor. Anti Müllerian Hormone activates BMP-specific R-SMADS and reporter genes [91]. A polymorphism in the AMH type II receptor gene has in Dutch women been associated with age at menopause in interaction with parity [92] and also associated with follicular phase Estradiol levels in normo-ovulatory women [93].

The main action of AMH in the follicle is inhibition of initial follicle recruitment and reduction of FSH sensitivity in growing follicles.

Anti Müllerian Hormone has shown to predict the ovarian reserve independently of age and thus assessing ovarian function and dysfunction [94]. However, controversies exist how precise a biomarker AMH is in regard to predict spontaneous conception as well as to predict success after infertility treatment. Remarkably few reports refers AMH to be a predictor of fecundity in women with normal fertility [95], in whom AMH is supposed to be a strong predictor of female fecundability [96]. Most published papers refer studies of infertile couples, where numerous other causes besides ovarian reserve and oocyte quality may represent the actual infertility etiology. In connection to ART, AMH levels are shown to be a precise predictor of poor response, but unrelated to pregnancy outcome [50, 97, 98]. However, also women with negligible serum levels of AMH have been reported to have a fair chance of obtaining a spontaneous and treatment-dependent pregnancy [99].

Serum AMH seems to represent a reliable quantitative measure of the ovarian pool of primordial follicles, however, whether it represents a quality measure is less founded. Diurnal variation in AMH levels has been reported to be significant [100], and a day-to day fluctuation in serum levels of the hormone has been debated. Obviously, many aspects of AMH are still unknown and needs to be further explored. Several studies have recognized the connection between antral follicle count and AMH [81, 101-103]. An inverse correlation between serum AMH and FSH levels has been noted in conditions of abnormal or exhausted follicular development. This is logical; as follicle-count diminishes less granulosa cells are available for production of AMH, which does not imply a direct relationship. The decrease in antral follicles by aging followed by a decrease in both sex steroids and inhibin leading to a rise in serum FSH levels is due to reduced negative feedback.

Studies looking into the relationship between FSH and AMH under hormonal stimulation for IVF have suggested FSH to be a modulator of AMH [104] as a negative association between FSH and AMH serum levels in women undergoing IVF. However, the extension of the FSH-window that occurs during such gonadotropin-stimulation implies real supra-physiological levels of FSH over a prolonged time. This in turn, causes an increased recruitment of antral follicles with FSH-induced accelerated growth that at a certain size looses their AMH expression. Hence, the AMH level must drop until a new cohort of follicles in line reach a stage where the AMH production again increases. For a limited amount of time, the amount of granulosa cells able to produce AMH is reduced.

The serum level of AMH in women using oral contraceptives has been discussed. Some papers refer no change in AMH serum-levels [105-107], while a larger Danish study demonstrates a significant lower AMH level in oral contraceptive users. However, this medication depresses FSH levels by a negative feedback, which, over time, can reduce early follicle recruitment [16, 36]. The oral contraceptive users in

the study referred to did actually show a significantly lower number of follicles, again pointing to the number of granulosa cells as predictive for the levels of AMH.

Apart from the age-related decline in the AMH production, the ovarian production of this hormone is considered to be relatively stable during pregnancy [108]. A few peer-reviewed papers concerning AMH levels in pregnancy have been published, including three cross sectional studies where one reported stable AMH levels without significant changes throughout the entire pregnancy [109], and two revealed falling levels [110, 111]. A prospective longitudinal study reported a significant decline in AMH levels with advancing gestational length [108]. However, measurement of AMH in pregnancy will be blurred by a pregnancy related plasma volume expansion which may reach the magnitude of around 40 % [112]. This increase will inflict a significant dilutive effect upon biochemical blood compounds of the pregnant women despite unchanged production [113].

Studies among non-infertile couples relating female AMH levels to TTP are few, however, a recent published study among women 30-years or older, revealed, in those with low AMH levels, a significantly lower chance of conception within a 6-month time frame [95]. However, the data regarding a predictive role of AMH measurements in relation to TTP in non-infertile couples is still scarce.

Traditionally, markers for hormonal dosage include demographic parameters, such as age, BMI, serum FSH and Inhibin B and ultrasound markers like antral follicle count and ovarian volume. A common problem connected to these markers has been a low sensitivity and specificity. During the last years several papers have focused on AMH as an easy available marker for hormonal dosage in ART. However, there is still limited information regarding the use of AMH in a clinical setup.

AIMS OF THE THESIS

The overall aim of this thesis was to explore a possible time-related variation of AMH and its relation to the gonadotropins; FSH and LH, as central factors in folliculogenesis of spontaneous menstrual cycles as well as during ART.

Specific aims are to investigate were:

- the circadian variation of AMH and gonadotropins and their co-variation to ovarian steroids in normo-ovulatory women (Study I);
- the variation in AMH and gonadotropins in PCOS women in comparison to normo-ovulatory women (Study II);
- the inter- and intra-cyclic variation of AMH related to the number of antral follicles measured by ultrasound (Study III);
- AMH and time to pregnancy in fertile women (Study IV);
- the significance of the mid-follicular phase LH level in patients undergoing IVF (Study V);

MATERIAL AND METHODS

Study design

This thesis consists of four prospective observational studies of normal fertile and PCOS women and one study of women undergoing infertility treatment.

Studies I-III were conducted at Reproductive Medicine Center (RMC), Skåne University Hospital, Malmö, Sweden. Study IV was based on demographic and biological data attained from a previously EU-funded fecundity study in Poland, Ukraine and Greenland (INUENDO). Study V was performed at the Fertility Clinic, Viborg Hospital, Skive, Denmark.

In Study I, AMH was tested in two age groups (above 35 and below 30 years) for variability over 24 hours as well as the endocrine association to gonadotropins and ovarian sex steroids. The study subjects were healthy women, ovulatory and regularly menstruating, non-smokers and had no history of infertility, hormonal medication or any gynecological or chronic diseases. They all presented with a BMI below 30 kg/m^2.

In Study II, young patients below 30 years of age, anovulatory and oligo-menoroic, diagnosed with PCOS based on the Rotterdam criteria, were observed for hormonal variability over 24 hours and the endocrine associations between AMH and gonadotropins/sex-steroids/androgens. The study subjects were all non-smokers and had no history of infertility, hormonal medication, any gynecological or chronic diseases and all had a BMI below 30 kg/m^2. As control group, ten healthy women aged between 20 and 30 years who originally participated in a Study I [100] served as controls.

In Study III, the intra- and inter- cyclic variability of AMH and antral follicle count was tested in two age groups (above 35 and below 30 years) over three menstrual cycles. The study subjects were healthy regularly menstruating women, non-smokers who had no history of infertility, no use of hormonal medication or any gynecological or chronic diseases.

In Study IV, data from the INUENDO study was used. So far, more than 25 peer-reviewed papers have been published from the data- and biobank material originating from this study (www.inuendo.dk). Serum from these women, who all were pregnant at the time for inclusion in the study, was assayed for AMH levels, which were correlated to the waiting time to pregnancy.

Study V was conducted at Skive Fertility Clinic, Denmark. Here, 207 infertility patients undergoing IVF treatment, was assayed for the level of LH day eight of the hormonal stimulation, and the result correlated to the chance of obtaining pregnancy. The women included were aged below 40 years, baseline FSH was below 10 IU/l and they all had a menstrual cycle between 25 and 34 days.

Study subjects

Study I

Study I was performed in 2009. Recruitment was mainly directed towards hospital employees and medical or nursing students by advertisement for healthy non-pregnant women. Potential study subjects answered a standardized questionnaire concerning health, pregnancies, menstrual cycle length and they received oral and written information before signing a consent form and enrolment. In order to elucidate any age-related differences in the longitudinal variations of hormones, the study groups were made up by a group below 30 years of age and the other by women exceeding 35 years.

Eligibility for study subjects included was:

- Regular menstrual period, cycle length 21-35 days
- Body Mass Index below 30 kg/m2
- Non-smoking
- No use of hormonal medication
- Non-pregnant

Demographics of the study population

	All subjects	Group A	Group B
No. of subjects	19	10	9
Age (years), mean (SD)	32 (7,4)	26 (1,7)	39 (3,2)
Age (years), median (range)	32 (22-45)	26 (22-28)	39 (35-45)
Menstrual cycle length, (days), mean (SD)	28,4 (3,5)	30,3 (3,1)	26,3 (2,7)
Menstrual cycle length, (days), median (range)	30 (22-35)	30 (25-35)	28 (22-30)

The study subjects called the research team on the first day of their menstrual bleeding. Blood sampling was initiated on day 2-6 of the menstrual cycle. The circadian profile was performed during a 24-hour period by drawing blood samples every second hour, starting at 8:00 a.m. and continuing until 8:00 a.m. the following day.

Study II

The study was performed through 2011-2012. Recruitment was directed towards non-pregnant patients diagnosed with PCOS based on the Rotterdam criteria and identified through ICD-10 diagnosis code (E28.2) in RMC's electronic medical file system. Information concerning the study was given by letter or at the outpatient clinic. The responding patients answered a standardized questionnaire concerning health, pregnancies and menstrual data. They all received oral and written information before signing a consent form and enrolment.

For comparison to normal menstruating controls, the study subjects from group A (< 30 years) in Study I was chosen as control group.

Eligibility for subjects included in the study group were

- Anovulation, defined as less than 8 bleedings pr. year with interval exceeding 35 days
- Body Mass Index below 30 kg/m^2
- Non-smoking
- Any use of hormonal medication
- No galactorrhea

A total of 16 study subjects were recruited. However, to match the control group of young ovulatory women, eight of these 16 subjects aged 30 years and BMI below 30 kg/m^2 were defined as the study group.

Demographics of the study population

	PCOS	Controls
No. of subjects	8	10
Age (years), mean (SD)	24,6 (3,8)	26 (1,7)
Age (years), median (range)	25 (16-29)	26,1 (22-29)
Menstrual cycle length, (days)(range)	irregular/amenoroic	28,5 (22-35)
Body mass index (kg/m^2), mean (SD)	23,5 (2,9)	21,8 (2,5)

Study III

Enrolment and execution of the study was performed throughout the winter of 2011 and spring/summer of 2012. Recruitment was mainly directed towards hospital employees and medical or nursing students by advertisement directed against healthy non-pregnant women. The potential study subjects answered a standardized questionnaire concerning health, pregnancies and menstrual cycle length and received oral and written information before signing a consent form and enrolment. In order to elucidate any age-related differences in the longitudinal variations of hormones, the study groups were made up by pairing subjects below 30 years and those exceeding 35 years into two separate groups. For BMI, no requirements were set. A total of twenty-seven healthy non-smoking volunteer women, 16 below 30 years and 12 above 35 years fulfilled the inclusion criteria and subsequently participated in the study. One subject in the older study group was excluded from the study due to non-compliance to blood sampling. Another subject completed only two menstrual cycles, but was not excluded from the study. Two participants in the young group had one month's break between cycles two and three. For all the other study subjects, measurements were performed in consecutive cycles.

Eligibility for study subjects included were

- Regular menstrual period, cycle length 21-35 days
- Non-smoking
- No use of hormonal medication

Demographics of the study population

	All subjects	Young group*	Old group**
No. of subjects	27	16	11
Age (years), mean (SD)	33,3 (8,2)	26,9 (1,5)	42,6 (3,5)
Age (years), median (range)	28,3 (24,7-49,7)	26,8 (24,7-29,8)	41,8 (36,9-49,7)
Body mass index, (kg/m^2), mean (SD)	22,5 (16,4-32,5)	22,6 (16,4-29,4)	22,5 (19,3-32,5)

* < 30 years; ** > 35 years

Study subjects called the research team on the first day of their menstrual bleeding for initiation of blood sampling, which started at menstrual cycle day five and continued every fifth day until the next menstrual bleeding. The same procedure was repeated for two more consecutive cycles. A three-dimensional ultrasound for measuring the exact antral follicle count was for all study subjects performed at cycle day five in the three subsequent cycles.

Study IV

The data presented in this study originate from the INUENDO study (www.inuendo.dk) performed in Ukraine, Poland and Greenland between 2002 and 2004. The project, supported by the EU FP5T, aimed to unravel the impact of environmental exposure to xenobiotic compounds with hormone-like actions on human fertility. Data acquisition focused on interviews and collection of biological material in couples visiting antenatal clinics in Warsaw (Poland), Kharkiv (Ukraine) and throughout Greenland. The couples were informed on the objectives of the study by an obstetrician/physician or a midwife and thereafter invited to participate in the study. Both partners were asked to leave blood samples, and all men were asked to leave a semen sample for analysis of WHO-parameters and sperm DNA fragmentation index (DFI) assessed by the Sperm Chromatin Structure Assay (SCSA). In the interviews, information on the waiting Time to Pregnancy (TTP) in the study- subjects was achieved. By 2010, serum-samples from more than 300 participants together with information of gestational length at the time of acquisition of biological material were available for inclusion in the study. Serum samples from the female partners were analyzed for the concentration of AMH. The parameters defined to be relevant were; TTP, Gestational Age at Inclusion (GAI) [114], AMH, sperm concentration and DFI.

Demographics of the study population

	Warsaw	Kharkiv	Greenland
No. of subjects	117	68	143
Female age (years), mean (SD)	28,7 (2,9)	24,2 (4,6)	26,3 (6,0)
Age menarche (years), mean (SD)	13,3 (1,4)	13,2 (1,3)	12,9 (1,2)
Menstrual cycle length (days), mean (SD)	29,2 (3,3)	28,5 (2,5)	28,4 (1,8)
Parity (no), mean (SD)	1,1 (1,1)	1,1 (0,3)	1,9 (1,3)
Body mass index (kg/m^2), mean (SD)	21,2 (2,1)	22,1 (3,7)	24,2 (4,3)

A total of 328 couples could be included in the study due to access to the parameters necessary for statistical analysis according to the aims of the study. All participants had a TTP below 12 months and the pregnancy was obtained spontaneously, which means without use of ART.

Study V

The study was performed in the period from 2000 to 2001 at the Fertility Clinic, Viborg County Hospital, Denmark. Recruitment was performed among patients undergoing IVF treatment with pituitary down-regulation and hormonal stimulation by recombinant FSH. At hormonal stimulation day eight, the serum level of LH was measured and the study subjects divided in groups according to their LH value. Based on the LH serum level, the study subjects were assigned to the following groups;

- LH < 0,5
- LH 0,51-1,0
- LH 1,01- 1,5
- LH > 1,5 IU/l

Eligibility for subjects included in the study group were

- Age below 40 years
- Baseline FSH below 10 IU/l
- Body Mass Index below 30 kg/m^2
- Non-smokers

Demographics of the study population according to LH level stimulation day eight

LH level (IU/l), mean (SD)	**<0,5**	**0,51-1,0**	**1,01-1,5**	**<1,5**
No. of subjects	24	108	38	37
Basal FSH (IU/L), mean (SD)	6,8 (0,33)	6,7 (0,17)	6,4 (0,28)	6,2 (0,11)
Age, mean (SD)	31,4 (0,6)	30,9 (0,4)	30,4 (0,5)	29,8 (0,7)
Body Mass Index (kg/m^2), mean (SD)	24,8 (0,8)	24,5 (0,3)	25,9 (0,7)	25,0 (0,7)

Pituitary down-regulation was performed by the use of a GnRh (0,8 mg. Buserelin) s.c. daily from mid-luteal phase. After having reached down-regulation, the dose was reduced to 0,4 mg daily. At this stage, hormonal stimulation was initiated with a daily individualized dosage between 100 and 375 IU. Final follicular maturation was induced when at least 3 follicles had reached 17 mm. Oocyte retrieval was performed 35 hours later by ultrasound guided trans-vaginal puncture.

Blood sampling

In Study I-III, 10 mL blood was drawn into vacuumed vials containing gel through a heparinized catheter inserted into a forearm vein. Within 2 h, the samples were centrifuged at 2000 g for 10 min, and serum was isolated and stored at -20^{o}C and assayed within in a period of two months.

In Study I, blood sampling was initiated on day two to six of the menstrual cycle. The circadian profile was performed during a 24-hour period by drawing blood samples every second hour, starting at 8:00 a.m. and continuing until 8:00 a.m. on the following day.

In Study II, blood sampling was initiated at a random cycle day. The circadian profile was performed during a 24-hour period by drawing blood samples every second hour, starting at 8:00 a.m. and continuing until 8:00 a.m. the following day.

In Study III, blood sampling began at cycle day five, and continued every fifth day until menstruation. In the successive cycles, the pattern was repeated until three consecutive cycles were completed.

In Study V, blood was drawn and immediately frozen for later analysis of LH.

Assays for biochemical serum markers

In Study I-III, AMH was analyzed using the Immunotech EIA AMH/MIS assay from Beckman–Coulter Inc., Marseille, France [115] The lowest detectable level distinguishable from zero with 95% confidence is 0.7 pmol/l. The total coefficient of variations (CVs) obtained was 25% at 5.7 pmol/l and 12% at 52 pmol/l. For FSH, LH, Progesterone and Estradiol, all samples from one participant were analyzed within the same assay run at a Beckman Access Immunoassay System on a UniCelTMDxI800 from Beckman–Coulter Inc., Brea, CA, USA. The lowest detectable level distinguishable from zero with 95% confidence and total CVs are 0.2 IU/l and <9% for FSH and LH, 0.25 nmol/l and < 14% for Progesterone and 73 pmol/l and <13% for Estradiol.

Sex Hormone-binding Globuline was analyzed by immunometric sandwich assay, intra-assay CV 5,3%, inter-assay CV 8%.

Serum values of total Testosterone and Androstendione were assayed by a competitive immunoassay with luminmetric technique, interassay CV 7%, inter-assay 10%. Free Testosterone concentration, was calculated as recommended by Vermeulen et al. [116].

Ultrasonography

For study III ultrasound analysis was performed using 4D-view™ software, version 9.1 (GE Medical systems, Zipf, Austria) with Sonography-based Automated Volume Calculation (SonoAVC™) software and calculations were performed on multi-planar images showing the ovary in the longitudinal, transverse and coronal planes. SonoAVC software was used to calculate the number and size of antral follicles and average diameter were determined and listed according to their size. The SonoAVC report displays the automated measurements of the mean diameter (relaxed sphere diameter), maximum dimensions (x, y, z diameters) and volume of each object.

Most follicles, as hypo-echoic structures within a relatively hyper-echoic ovarian stroma, can be analyzed using SonoAVC software. However, to ensure that all follicles became recognized the volume of the ovary was finally examined manually in longitudinal and transverse planes to find follicles that had not been detected by the SonoAVC software, or had been incorrectly identified, and thereafter the follicle number was corrected.

The mean diameter and the number of follicles with a diameter of 2.0-8.0 mm were used for statistical analysis.

Interviews

For Study IV, information on TTP was obtained by face-to-face interviews with the participants at a hospital, antenatal clinic or at the residence of the couple. A structured interview questionnaire was used. The following questions were asked:

Leading up to this pregnancy, when was it that you started having sexual intercourse without using any birth control to prevent pregnancy?" Month:_____ Year:_____. We now call this the "STARTING TIME". How long was it from that "STARTING TIME" until you became pregnant? (The date you became pregnant is the date you conceived) How long? Weeks:_____ and/or Months:_____ and/or Years:_____

Ninety-five percent of TTP´s were recorded by these questions.

In four percent where the questions given were left unanswered, TTP were acquired by calculation from the date of the last menstrual period, or if the date of the month was missing the 15th day in the respective month was used. In the remaining cases, where none of the above mentioned methods could be applied, the number of menstrual cycles to pregnancy was used.

Semen collection and analysis

For Study IV, all men were asked to deliver a semen sample. They were instructed to avoid ejaculation for at least 2 days prior to the semen sample collected at home or at the hospital. If collected at home the semen sample was kept close to the body when transported to the laboratory immediately after collection and subsequently analyzed according to the recommendations from WHO (15). Sperm Chromatin Structure Assay was performed at the Section of Toxicology and BiomedicalSciences at ENEA Casaccia Research Centre, Italy (22). Unidentified and coded semen samples (200 μl at –80ºC) were shipped on dry ice. The entire protocol is described elsewhere [117]. Briefly, 1–2 X 10^6 thawed cells were treated with acid–detergent solution (0.08N HCl, 0.15 mol/l NaCl, 0.1% Triton-X 100; pH 1.2) for 30 s and then stained with 6 mg/l of purified Acridine Orange (Molecular Probes, Eugene, Oregon, USA) in a phosphate-citrate buffer, pH 6.0. Cells were analyzed by a FACScan (Becton Dickinson, San Jose, California, USA) equipped with an air-cooled argon ion laser and standard optical filters to detect green and red fluorescence. A total of 10 000 cells were analyzed from each sample. The percentage of abnormal sperm with detectable DFI was calculated from the DFI frequency histogram obtained from the ratio between red and total (red+green) fluorescence intensity (18).

Statistical analysis

For Study I-IV, all statistical analysis was performed using the statistical software SPSS version 17.0 for Windows (SPSS Inc., Chicago, IL). A p-value of less than 0.05 was considered statistically significant.

For Study I-III, mixed model were performed for analyses of repeated measurements of AMH, LH, FSH, Estradiol and Progesterone that were considered to be independent continuous variables (continuous) modeled with Group (A/B) and time (all time points: 8:00 a.m., 10:00 a.m., 12:00 p.m., 2:00 p.m., 4:00 p.m., 6:00 p.m., 8:00 p.m., 10:00 p.m., 12:00 a.m., 2:00 a.m., 4:00 a.m., 6:00 a.m. and 8:00 a.m.) as categorical variables. For AMH, LH, FSH, Estradiol and Progesterone, the analysis was performed with and without the other hormones as continuous covariates. Mixed model analysis allows for evaluation of differences in repeated measurement between patient groups. Compared with more simple statistical methods, mixed model analysis computes the overall mean difference between the groups and the overall time pattern of the variance, and thereby avoids multiple testing at individual time points. Another advantage of this statistical method is that clinically important differences between patient groups under investigation can be adjusted for. Repeated measurements at different time points imply that measurements for the same patient are more similar than those for different patients, i.e. the residuals of the mixed model for repeated measurements within a patient will be correlated. This correlation was assumed to follow an autoregressive structure with one time lag. A random coefficient was kept in the model only if its estimated variance was non-zero. Group-specific circadian variations were estimated as marginal means. The mixed model analysis does also allow for comparison of each single time point with the first value (8:00 a.m. on the first day), and thus compute a significance level for each time point throughout the blood-sampling period.

The maximum relative intra-individual variations in AMH levels (difference between the highest value and the lowest value during the 24-hour period, as percentage of the latter) found in PCOS and control-subjects were compared using the Mann–Whitney test.

For Study IV, the AMH concentrations was expressed as percentile categories (0-25%; 25-50%; 50-75%; 75-100%) for the first, second or third trimester, according to the time of blood sampling in relation to the actual length of pregnancy. These quartiles, based on the 25^{th}, 50^{th} and 75^{th} percentile for each pregnancy trimester were calculated based on the integrated available data from the study and thereafter, in a Cox regression model, used as independent variables in relation to TTP expressed in month. In quartile 1, 16 subjects with AMH levels below detection level were included. As covariates we included the female's age and two indices of semen quality previously shown to be relatively independent male determinants of TTP; DFI as measured by SCSA and sperm concentration.

Mean values for TTP in the different quartiles were tested in a linear regression model with TTP as dependent and AMH-quartiles as fixed factor.

In order to estimate odds-ratio (OR) for TTP in relation to AMH levels, TTP was categorized (lower than/equal to and higher than) by using one of three cut-off levels: TTP =3 months, TTP = 6 months and TTP = 9 months. Here, AMH-quartiles as independent variable were tested in a binary logistic regression model against the TTP-categories with DFI, sperm concentration and female age as covariates.

Relation between female age and AMH-quartiles was tested with univariate analysis of variance, as was the associations between female age and TTP-groups and AMH levels expressed as quartiles, and gestational age.

In study V, statistical differences were computed using analysis of variance, x^2 test or linear regression.

RESULTS

Study I

Circadian variation in AMH

The study aimed to explore an age-related circadian variation in AMH in regularly menstruating and ovulating women. A statistically significant difference in mean AMH levels was found between the young (Figure 6a) and the old groups (Figure 6b) (p=0,011). Anti Müllerian Hormone revealed a significant late night nadir value in both groups confirming a circadian variation in this hormone.

The young group had significantly lower levels of AMH at 6:00 a.m. compared to the first measurement 8:00 a.m., in absolute terms 3,1 pmol/L, 13% in relative values. The older group had nadir values at 2:00, 4:00 and 6:00 a.m. with a maximum absolute difference of 1,7 pmol/L, in relative terms 15%.

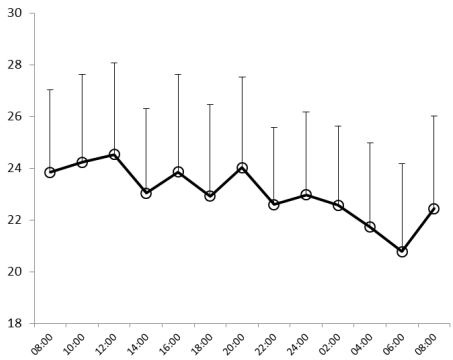

Fig 6a
AMH- young study group

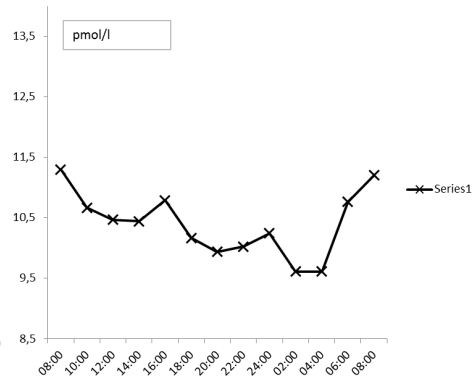

Fig 6b
AMH-old study group

Figure 6a and 6b. The circadian variation of AMH in both study groups

Circadian variation in gonadotropins

Regarding FSH, no difference was seen between the groups. Both young and old groups had late night nadir values.

Luteinizing Hormone revealed a statistically significant variation in the younger (Figure 7) but not in the older group, with nadir values at 2:00, 4:00 and 6:00 a.m.

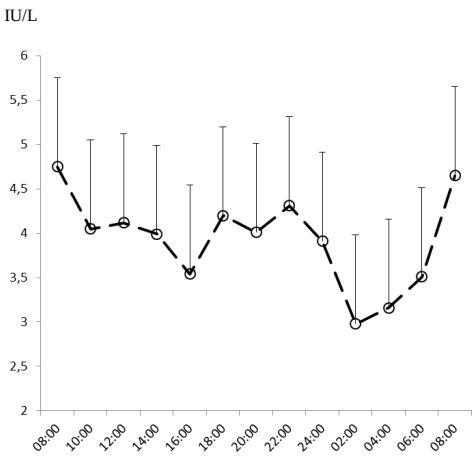

Figure 7. The circadian variation of LH in the young study group

Circadian variation in ovarian derived hormones

Only Progesterone exposed a significant circadian variation, however, no age-related differences were seen. The highest level of Progesterone was found in the first blood sampling at 8:00 a.m., and thereafter the values revealed a continuous fall until a rise back to baseline was seen at late night (Figure 8a and b).

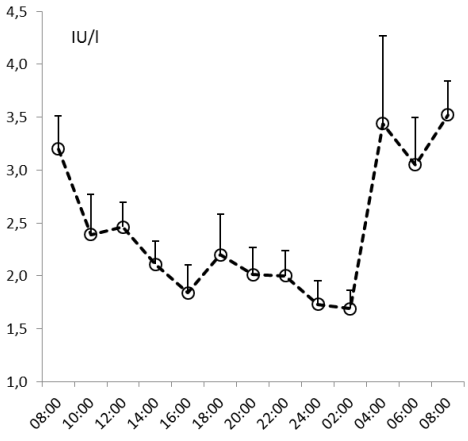

Figure 8a
Progesterone- young study group

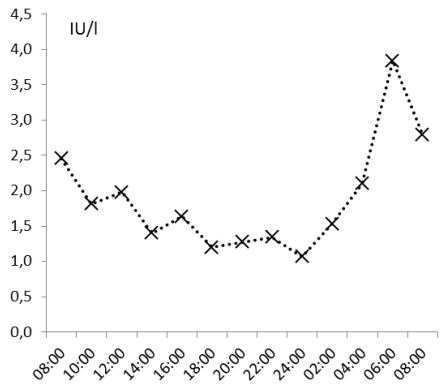

Figure 8b
Progesterone- old study group

Figure 8a and 8b. The circadian variation in Progesterone in both study groups

Co-variation between hormones

A statistically significant co-variation was found between AMH and LH in all study subjects. There were no other co-variations between AMH and the other hormones.

Table I. Serum concentration of AMH, FSH, LH and Progesterone in relation to the time of the day and age group.

Time	**8:00 a.m.**	**10:00 a.m.**	**12:00 p.m.**	**2:00 p.m.**	**4:00 p.m.**	**6:00 p.m.**	**8:00 p.m.**	**10:00 p.m.**	**12:00 a.m.**	**2:00 a.m.**	**4:00 a.m.**	**6:00 a.m.**	**8:00 a.m.**
AMH, Group A, pmol/L mean (SD)	23.8 (10.)	24.2 (11.3)	24.5 (11.8)	23.0 (10.9)	23.9 (12.6)	22.9 (11.8)	24.0 (11.7)	22.6 (10.0)	23.0 (10.7)	22.6 (10.2)	21.7 (10.9)	20.8 * (11.3)	22.4 (12.0)
AMH, Group B, pmol/L mean (SD)	11.3 (3.2)	10.7 (8.8)	10.5 (8.2)	10.4 (8.2)	10.8 (7.9)	10.2 (8.6)	9.9 (7.9)	10.1 (9.1)	10.2 (8.0)	9.6* (8.3)	9.6* (8.0)	10.8* (8.0)	11.2 (7.0)
FSH, Group A, IU/L, mean (SD)	8.8 (3.1)	8.2 (2.6)	8.7 (2.6)	8.4 (2.7)	8.5 (2.3)	8.4 (2.5)	8.6 (2.0)	8.4 (2.3)	8.0 (1.9)	7.2* (1.9)	7.6* (2.4)	7.7* (2.4)	8.3 (2.2)
FSH, Group B, IU/L, mean (SD)	9.7 (2.6)	9.1 (2.7)	9.0 (2.8)	9.1 (2.9)	9.6 (2.9)	9.1 (2.3)	9.6 (3.1)	9.1 (3.1)	8.1* (2.0)	8.3* (3.1)	8.3* (3.0)	7.5* (1.3)	9.2 (3.4)
LH, Group A, IU/L, mean (SD)	4.8 (1.6)	4.1 (1.6)	4.1 (1.5)	4.0 (1.4)	3.8 (1.2)	4.2 (0.9)	4.0 (1.2)	4.3 (1.8)	3.9 (1.7)	3.0* (1.9)	3.2* (1.7)	3.5* (1.7)	4.7 (1.7)
LH, Group B, IU/L, mean (SD)	4.4 (1.3)	4.3 (1.5)	4.4 (1.6)	4.3 (1.7)	4.3 (2.0)	4.5 (1.9)	4.1 (0.9)	4.3 (2.2)	4.1 (2.2)	4.0 (2.5)	4.2 (3.1)	4.9 (3.0)	4.8 (2.3)
Progesterone, Group A, IU/L, mean (SD)	3.15 (1.0)	2.34 (1.2)	2.39 (0.8)	2.23* (0.7)	2.01* (0.8)	2.27 (1.2)	2.0* (0.8)	1.9* (0.8)	1.6* (0.7)	1.61* (0.6)	3.27 (2.6)	3.08 (1.4)	3.59 (1.0)
Progesterone, Group B, IU/L, mean (SD)	2.5 (1.2)	1.8 (1.1)	2.0 (1.4)	1.4* (0.8)	1.61* (1.0)	1.2* (0.8)	1.3* (0.5)	1.3* (0.8)	1.1* (0.8)	1.5* (1.6)	2.1 (1.3)	3.8* (1.5)	2.8 (1.0)

Most noteworthy findings of Study I

Anti Müllerian Hormone displays significant late night nadir values.

Luteinizing Hormone expresses a strong co-variation with AMH. As LH is dependent of the GnRh signaling in the hypothalamic-pituitary axis, this indicates a connection between this axis and AMH.

The gonadotropins, FSH and LH, display a strong positive co-variation to Progesterone. As both FSH and LH, through their receptors at theca and granulosa cells rules the hormone synthesis in the two gonadotropin two cell hormone, this in accordance of the theory of sex steroids production.

Study II

The difference in AMH levels between PCOS-women and controls was highly statistically significant with a mean difference of 37,1 pmol/l, 95 % CI: 31,0; 43,2 pmol/L, (p= 0, 004). In PCOS women, unlike controls, the circadian profile of AMH and LH revealed no statistically significant lower levels at any time in the period of blood sampling (Figure 9a-d). In Progesterone, however, a significant variation in both groups was seen and this was also the case for the androgens (Androstendione and Testosterone).

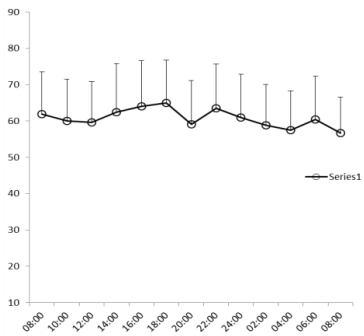

Figure 9a. AMH PCOS patients

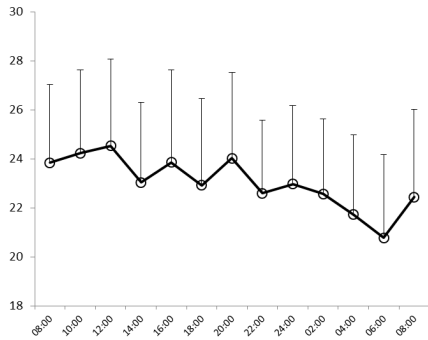

Figure 9b. AMH Controls

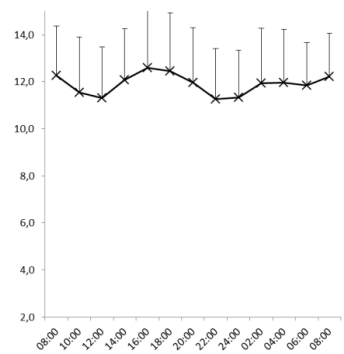

Figure 9c. LH PCOS patients

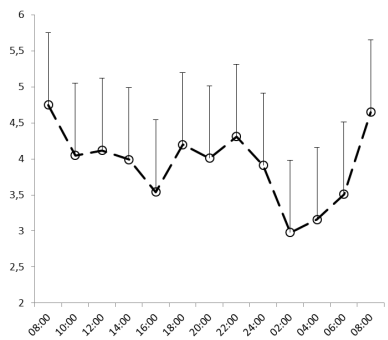

Figure 9d. LH Controls PCOS

Co-variation between hormones

A strong and statistically significant positive co-variation was found between AMH and LH, both in PCOS women and the controls group. Furthermore, in PCOS women, a significant positive co-variation was found between LH and Testosterone, but not between AMH and any of the androgens. Also, LH / FSH and Progesterone co-varied in both groups.

Most noteworthy findings of Study II

In PCOS-women, both AMH and LH showed a different circadian profile compared to normo-ovulatory women. The higher level of LH indicates an increased activity in GnRh signaling which triggers pituitary secretion of the hormone. A significant co-variation exists between AMH and LH, and between LH and Testosterone, but not between AMH and Testosterone. This finding indicates a close relationship between LH and AMH, maybe with LH as the controller of AMH.

Study III

To calculate the variation in AMH levels in the course of three menstrual cycles, the first measurement at cycle day five in the first menstrual cycle monitored was chosen as baseline.

The overall variability in mean concentration revealed a substantial and statistically significant variation ($p < 0.001$). In absolute terms, the mean maximum difference in AMH level was calculated to be 13,4 pmol/L, range 3,9-33 pol/L and in relative terms mean 87,4%, range 50,0 -147%.

In the young study-subjects below 30 years of age, a significant intra-cyclic variability was also found. Here, cycle day 10 and 15 had significant higher AMH levels compared to baseline. For study subjects with cycles exceeding 30 days, the level of AMH at cycle day 30 was significantly lower. This variation was calculated to be statistically significant. In the older group, no such variability could be found.

As expected, the amount of antral follicles measured cycle day 5, was significant lower in the older group compared to the younger, a median number of 10 versus 25 follicles in the youngest group.

A statistically significant positive correlation was found between AMH level, both mean cycle-level and cycle day five level, and antral follicles sized 2-8 mm, rho 0,84, $p < 0.01$ (Figure 12). For follicles exceeding 8 mm, a negative correlation to AMH level was found.

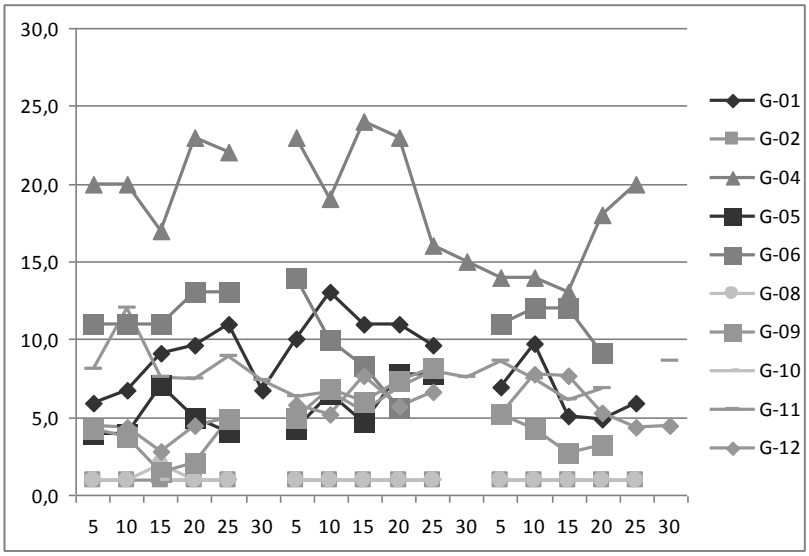

Figure 10. Longitudinal AMH-measurements in women > 35 years

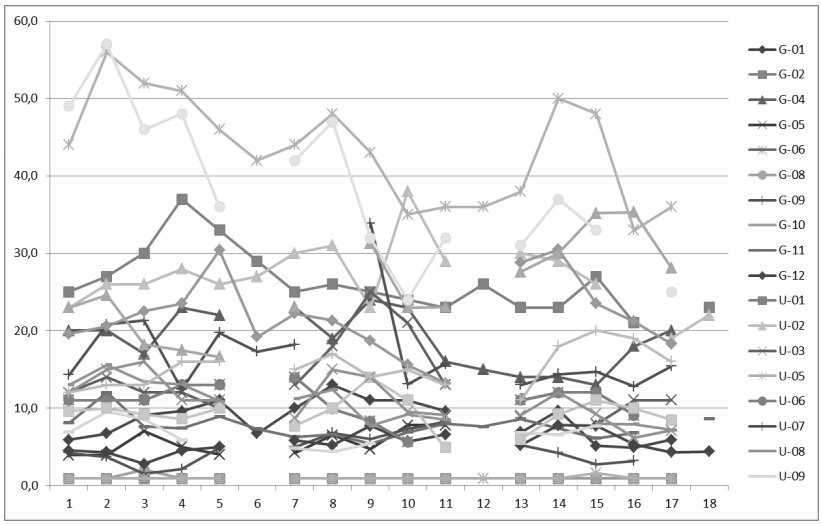

Figure 11. Longitudinal AMH-measurements in women < 30 years

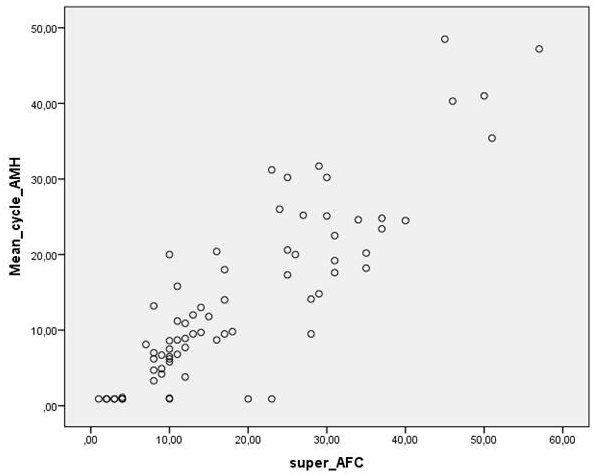

Figure 12. Scatterplot of the correlation between mean-cycle AMH level and the number of antral follicles cycle-day five

Most noteworthy findings in Study III

The major finding of the study was a substantially and statistically significant overall intra-individual variation in AMH levels seen over three menstrual cycles. A difference in AMH-variability between age groups was found, as only the younger group revealed an overall statistically significant variation in AMH level.

Another important finding of this study was the relatively strong correlation between AMH levels and ultrasound determined number of antral follicles, this association being most pronounced for follicles with a diameter of less than 6 mm.

Study IV

On behalf of the cross sectional design in this study, with blood samples collected in all trimesters of the pregnancy, the normal plasma gestational length related plasma volume expansion had to be taken into consideration. Actually, as expected due to plasma volume changes during pregnancy, AMH concentrations demonstrated a continuous statistical significant decrease from the first to the third trimester of pregnancy.

The Cox-regression analysis disclosed a difference in TTP of 1,2 months between the quartiles with the highest versus the lowest AMH level, 95% CI: -2.25;-0,11. The women belonging to the quartile with the lowest AMH levels had an odds-ratio (OR) of 9,3 for experiencing a TTP above nine months compared to the subjects in the quartile with the highest AMH levels.

Women in the quartile with the highest AMH levels had a significantly lower age compared to those in the quartile with the lowest AMH values (Fig 13).

In 18 samples, AMH concentration was below detection level. In these subjects, TTP was in average six months.

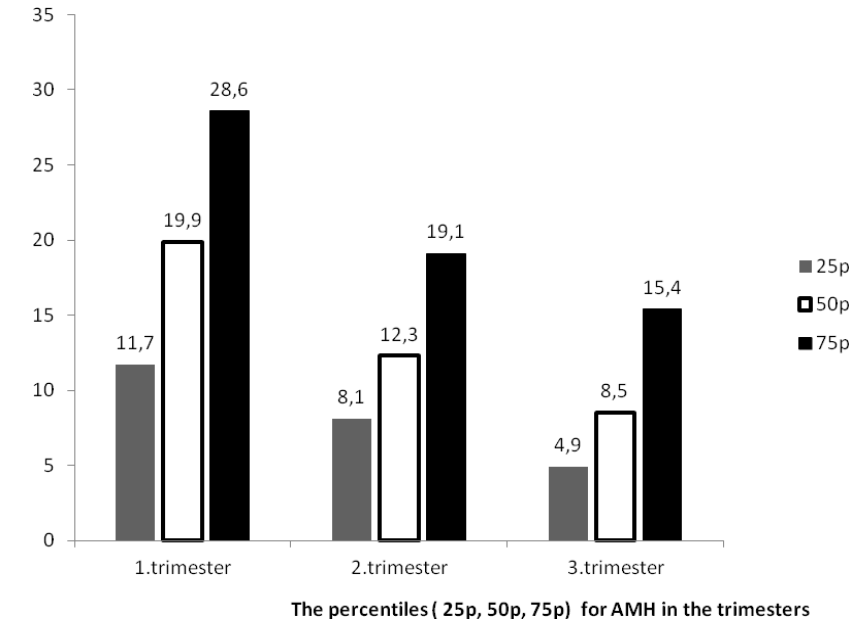

Figure 13. The 25th, 50th and 75th percentile in pregnancy trimester 1-3

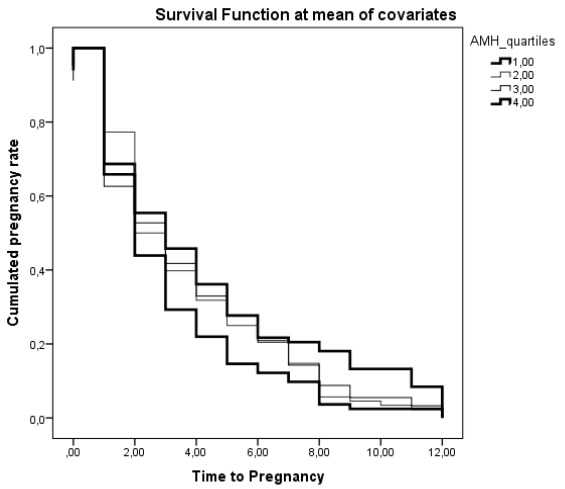

Figure 14. Survival analysis of TTP in the 4 quartiles of the AMH level

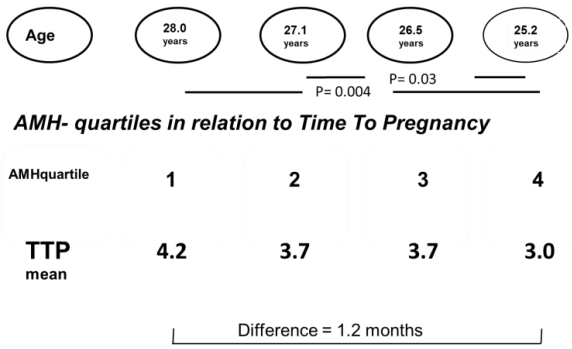

Figure 15. The Time to Pregnancy (TTP) and age of the study subjects in the four quartiles of AMH level

Most noteworthy findings of Study IV
During pregnancy, the concentration of AMH is declining. Thus the concentration of AMH in pregnancy is dependent on when in pregnancy the blood sample is taken. The level of AMH in pregnant women is associated with the duration in TTP. A considerable overlap exists between the low and the high AMH groups question the value of AMH as a predictor of fertility *in vivo.*

Study V

The patients were divided into four groups according to the LH level cycle day eight of the hormonal stimulation. The consumption of exogenous FSH revealed a statistically significant inverse correlation to the LH level at day 8 of the stimulation (r = 0,22). By comparing LH levels between the groups with lowest level with the highest, the lowest group had a 28% higher FSH consumption during hormone stimulation ($p < 0.002$). Also the duration of the hormone stimulation revealed the same pattern (r= 0.28, $p> 0.001$).

The Estradiol level showed a significant positive correlation to the LH level day 8. The number of oocytes harvested was positively correlated to the LH level, as the number of harvested oocytes was highest in the group possessing the highest LH level with a non-significant difference of 1,7 oocytes to the lowest group.

Clinical pregnancy rate was lowest in the group with highest LH levels. Also implantation as well as live birth rates exposed the same pattern.

Most noteworthy findings in Study V
During hormonal stimulation for multifollicuar development in connection to IVF, a positive correlation was found between the follicular Estradiol secretion and the midfollicular LH level, which on the other hand was inversely correlated to the exogenous gonadotropins consumption and duration of hormonal stimulation. This highlights the pivotal role of LH in the steroidgenesis of growing follicles. However, oocyte quality expressed by clinical pregnancy and implantation rate seems affected by the LH level as the groups with the highest levels of LH had the poorest pregnancy outcome.

GENERAL DISCUSSION

In the general discussion integrating the five papers of this thesis, focus has been applied on the biological fundaments as well as clinical implications of the research presented. The strength and weaknesses of each study have been addressed in the papers constituting the dissertation.

Variation of AMH

Circadian variation

In Study I-II, the circadian variation of AMH was studied in women with regular ovulatory cycles and in women diagnosed with PCOS. Among the normal cycling women, a significant circadian variation was found with late night decrease in serum AMH, which however, appears not clinically relevant. This contrasted to the findings for the PCOS women where AMH levels were found to be significantly higher but without any circadian variation.

Circadian rhythms are endogenous biological rhythms in 24-hour phases, well known in mammals in connection to the reproductive functions [118, 119]. Both a central as well as a peripheral circadian pacemaker seems to be active. A central clock acts the Suprachiasmatic Nucleus (SCN) in the hypothalamus and at a cellular level clock genes seems to be active. Such genes have been found in central nervous tissue like the hypothalamus and the pituitary as well as in peripheral endocrine organs like the adrenal, thyroid gland and gonads [120]. Also hypothalamic neurons secreting GnRh express circadian rhythms of clock gene origin [121] linking the hypothalamic pituitary ovarian axis to the clock-function. Functionally, environmental signals are being processed by the brain and the SCN are sent as downstream signals to endocrine organs controlling the hormone secretion [122]. The rhythmic secretion of LH [123] works like this, and lesions of the SCN have in female rats shown to disturb LH secretion and ovulation [124].

In the neuroendocrine control of the reproductive function a pulsatile GnRH-secretion is essential. Compared to normal menstruating women, in PCOS women the GnRh signalling is different, which is mirrored in the gonadotropin-secretion [125-128]. This is in accordance with our own findings of a 3-fold higher AMH-level in PCOS women compared to controls, significantly higher LH and lower FSH levels. The most reliable explanation for this difference is an altered neuroendocrine function with an GnRH-pulse probably under the influence of positive feed-back effects from ovarian steroids and androgens, resulting in excess LH and low FSH levels [127].

In diagnosing PCOS, high serum levels for AMH have actually been suggested as additional diagnostic criteria for the syndrome [129]. Together with antral follicle count, AMH is actually so closely linked to hyperandrogenism and anovulation that it may replace serum androgen assessment for diagnosing these conditions.

Inter- and intracyclic variation

In study III a significant variability of AMH in the course of three menstrual cycles were seen. In women with a high ovarian reserve the variation in absolute terms showed an average of 13,4 pmol/L and in relative terms an average of 87,4 %.

The high variation of AMH, inter- as well as intracyclic is, from a biologic point of view, logic. At any given time, the level of AMH mirrors the number of AMH producing granulosa cells in secondary oocytes up to the stages of small antral follicles. The issue of an inter-cycle variation in AMH levels is discussed. According to the traditional view, follicular development is situated to the follicular phase [114, 130, 131] while the effects of Progesterone have been anticipated as inhibitory leading to follicular quiescence in the luteal phase [132, 133]. A prerequisite for AMH to be stable over time would imply a

constant number of developing follicles growing from the primordial pool via its intermittent stages, which seems biologically unlikely. Actually, both in animals and humans, the antral follicle count has been found to display substantially intercyclic variability [73, 134] and by longitudinal ultrasonic measurement found to emerge in waves [135-137]. This is also in accordance with histological studies [138]. The existence of wave patterns in follicular recruitment and antral follicle formation could significantly affect the serum level of AMH and endocrine changes in steroid hormones associated to these wave patterns would be likely to occur, similarly to analogous observations in domestic animals [139, 140]. Thus, important from both clinical and biological point of view, there is a lack of proper data on possible inter-cycle variation in serum concentration of AMH.

The clinical significance of our findings refers to several studies reporting cut-off levels for different aspects of AMH as a fertility marker. Both cut-off levels of AMH as a predictor of natural conception and response of hormonal stimulation and outcome of ART have been suggested [97, 141, 142]. Several longitudinal measurements of AMH in our study subjects, both in the high and low range, crosses published cut-off values, some of them several times.

In Study II, a significant intracyclic variation of AMH was shown preovulary raised AMH-levels, and the change from follicular to luteal phase has been shown by others [143]. Added to the finding of a significant intercyclic variation, the relevance of one single AMH measurement can be questioned as sufficient for judging the ovarian reserve. This is especially important in very low serum concentration, as serial measurements may unravel a dynamic appearance of AMH-producing antral follicels. Both in counseling of patients prior to ART and a proper timing of controlled ovarian stimulation may result in a reconsidered prognosis for treatment-related conception.

AMH as a fertility marker

Role of AMH in natural conception

The strength of AMH as a fertility marker of natural conception has been demonstrated in Study IV.

Here, the size of the ovarian reserve correlates to both quantitative and qualitative aspects of the female gamete, and is, especially in women with very high or low levels of AMH, able to predict a difference in TTP. Few other papers have been published on the issue of the ovarian reserve assessed by AMH and its relation to natural conception. In one of the publications, a cohort of women in later stages of their reproductive life (30–42 years) was studied. Here a significant reduced fecundity was found in subjects with very low AMH levels [95]. In another recent study of young females who discontinued contraception due to wish of pregnancy, surprisingly, high levels of AMH but not low levels was found predictable for a reduced fecundability [144]. Although the authors had adjusted for irregular menstrual cycles; most likely their study subjects had a phenotype for PCOS. Few studies have looked into the fertility potential of regular cycling PCOS-women, however, reduced fecundability has been reported to be connected to reduced levels of Progesterone during the luteal phase [145]. In the referred study, low levels were not predicting reduced fecundity [28]. In our study a considerable overlap between the low and the high AMH groups was found concerning the number of cycles necessary to obtain conception. Moreover, women with non-detectable AMH levels were found to have conceived with an average TTP of 6 months. As AMH expresses both qualitative as quantitative aspects of the female gamete, one can assume that the qualitative aspect of the ovarian reserve in the young study-subjects with non-detectable AMH presented in Study IV may compensate for the quantity. This would be in accordance with the few studies published on AMH-levels and natural conception [95, 144]. The clinical relevance of non-detectable AMH-level, especially in young women, does not necessarily eradicate any prognosis of conception and should be followed by repeated testing. One of the basal questions to be discussed in this thesis was if AMH could be seen as such a robust marker for the prediction of natural conception that pregnancy could be postponed without increasing the risk of infertility. According to previous knowledge

[95] as well as the data from the present thesis, the answers to these questions still depend on female age (Study IV). Young women with high ovarian reserve may postpone pregnancy some years without reducing their fertility to a critical extent. However, quantity cannot compensate for lost quality. In addition, subfertility is not solely dependent on the female ovarian reserve as numerous factors other than the size of the ovarian reserve are crucial for obtaining conception. This leads to an appropriate timing of infertility investigations to be initiated due to recommendations that couples, which have not conceived after one year of regular unprotected sexual intercourse, should seek clinical investigation including assessment of ovulation and semen analysis.

Role of AMH in ART

The third and last question the optimal biomarker should be able to answer was, in case of subfertility, which treatment would be the optimal for a given couple. The strength of AMH as a fertility marker in ART has not been a matter of our own investigation. However, a large amount of studies has been published connected to this issue, with diverse results especially connected to the ability to predict treatment success [141, 146, 147]. However, in the issue of stimulation response and oocyte yield, AMH seems to be a good predictor helping physicians in the field of fertility to better individualize treatment and stimulation strategies [148].

Co-variation between LH and AMH

In study I and II, a statistically significant co-variation between LH and AMH throughout 24 hours was confirmed. As the level of AMH is an expression of the number of growing follicles, LH must logically be connected to maturation of follicles which association is reported in PCOS women [58]. Luteinizing Hormone itself is mastered by the GnRh and the pace of the GnRh pulses is crucial both in normal physiologic as well as in pathological conditions [66]. A too high amplitude will cause a hyper-secretion of LH and hence disturbances both in steroid synthesis, secretion of AMH and folliculogenesis, as shown by the findings from PCOS patients reported in Study II. This study illustrates the consequences of a too high LH stimulation as the androgen level is significantly higher in PCOS women compared to normo ovulatory controls, as well as the impact on the follicle excess. According to studies of monkeys, the major androgens effect on folliculogenesis have been found to be an up-regulation of FSH receptors on granulosa cells [149] and an increase in antral follicle count [150].

Synthesis of androgens from cholesterol in theca cells is controlled by LH, serving as a “gate-keeper” for steroid synthesis in the theca and granulosa cells, a coordinated activity described by the term “two-cell two gonadotropin” concept [151, 152]. Actually, in PCOS women a strong and significant correlation was found between LH and the androgen level. Also, LH was correlated to AMH level, but AMH and androgen level was not correlated. Interestingly, this will place LH in the center as a possible controlling factor of both compounds.

Very small amounts of LH seems to be sufficient for the initiation and sustainment of steroid production, as less than 1% of the LH receptors has to be engaged by LH to deliver steroid hormones [153]. This is in accordance with the findings from Study V on infertile women undergoing ART with GnRh down-regulation and hormonal stimulation. The residual level of LH after downregulation and desensibilization of the pituitary combined with recombinant FSH stimulation, exert a crucial impact on the duration of the hormonal stimulation as well as the cumulated amount of FSH used. Also, the serum level of Estradiol correlates with LH, which highlights the pivotal role of LH in co-operation with FSH in controlling steroidogenesis in preovulatory follicles. Interestingly, this study (Study V) reveals the best outcome with a LH level in the middle range as the oocyte quality seems to be affected by a high LH level under hormone stimulation for IVF. This effect could be seen on the implantation and baby-take-home rate (Study V). The factors accounting for the effect is not obvious, however, LH exerts its effect not only in

growing follicles but also in extragonadal tissue like the endometrium, the fallopian tubes and in the oocyte [154]. The precise role of these extragonadal receptors has never been defined, however, at these sites a high LH level may effect implantation and early embryo development.

CONCLUDING REMARKS

The clinical relevance of the studies constituting this dissertation has been to expose the substantially variation over time in AMH. The degree of variation may question the role of AMH as a stable marker of ovarian reserve. Furthermore, this thesis indicates a close relationship between AMH and the hypothalamic-pituitary-ovarian axis, both in normal ovulatory women as well as in anovulatory PCOS women.

The AMH-level is closely related to LH. As LH also controls androgens initiating follicle recruitment, and at the same time correlates with AMH retarding recruitment, these findings may place LH in a central position controlling and "economizing" the "household" of primordial follicles comprising the ovarian reserve.

In a long perspective, the understanding of the signaling in this axis could open up for therapy to normalize its activity as a treatment for the anovulatory aspects of PCOS.

To conclude, in pregnant women, the AMH level correlates to the waiting time for conception. A time-related variation exists for AMH, especially between menstrual cycles within the same individual, in which has to be taken into consideration when used clinically. The slight decrease in serum AMH levels during the late night appears, however, not to be of clinically relevance. Anti Müllerian Hormone should be used as an additional criterion in the diagnosis of PCOS. A single measurement of AMH is not sufficient for a precise judgment of the ovarian reserve, especially in case of very low serum levels.

Although this thesis brings about new knowledge to the field, it is obvious that in order to obtain a deeper understanding of the biology behind AMH and LH-variations as well as come closer to whether and how useable they are as predictors of fertility *in vivo* and *in vitro* further studies are needed. The findings from the many studies, suggesting AMH to be a robust fertility marker, should be externally validated by applying the data to new population cohorts.

FUTURE PERSPECTIVES

In the present studies, the natural biologic variation in AMH during the follicular phase has been explored. A natural step further, in order to gain more insight into the biology of AMH in connection to LH and the hypothalamic-pituitary-ovarian axis, will be to study the circadian variation of these hormones also in the luteal phase. At this stage of the menstrual cycle, it will be of interest to study the connection between LH and the secretion of Progesterone due to the latter hormones' crucial impact on implantation and maintenance of the early pregnancy. However, a possibility exists that such studies may unravel secretion pattern explaining problems connected to spontaneous conceptions.

Also in IVF-patients treated with a GnRh agonist and subsequent hormonal stimulation, the longitudinal LH-secretion pattern will be of interest to explore. The possibility to gain knowledge in secretion pattern connected to oocyte competence and pregnancy rate would be imminent.

In the studies of PCOS-women, we have only reported circadian variations in anovulatory normal weighted patients representing only on phenotype of the syndrome. At the Reproductive Medicine Centre in Malmö, nine more women fulfilling PCOS-criteria have had their circadian profile explored. However, data is not published. A natural continuation will therefore be to proceed to recruit new PCOS women of different phenotypes in a sufficient number to be able to examine differences between them, which would add to the understanding of this heterogenic syndrome.

Time to pregnancy is an interesting parameter for the study of fecundity over time. However, a longitudinally observation of a cohort of couples attempting to conceive is laborious and expensive. A substantially more efficient approach would be to study pregnant women, and to explore the connection of TTP to ovarian reserve markers like AMH as reported in this thesis. While here data had to be based on one measurement at different gestational pregnancy length, a new study should recruit pregnant women in early pregnancy and follow the level of AMH in each trimester of the pregnancy. This would help to identify factors influencing the level of AMH throughout the pregnancy, and explore the strength of AMH as a marker of fertility in a fertile population.

Another aspect relevant to the findings of the present studies is whether an increase in the "androgen priming" would impact on folliculogenesis measured by AMH. Experiments have suggested that ovarian follicular recruitment and growth may be increased by increased androgen stimulation of folliculogenesis, which may be exerted both by androgen supplementation as well as use of an aromatase inhibitor. Here again, the covariation between LH, androgens and AMH could be a measure of such an effect.

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