DISSERTATIONES BIOLOGICAE UNIVERSITATIS TARTUENSIS
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**SIGNE ALTMÄE**

# Genomics and transcriptomics of human induced ovarian folliculogenesis

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Institute of Molecular and Cell Biology, University of Tartu, Estonia

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*To my family,*

# TABLE OF CONTENTS

# LIST OF ORIGINAL PUBLICATIONS

I **Altmäe S**, Haller K, Saare M, Peters M, Hovatta O, Stavreus-Evers A, Velthut A, Karro H, Metspalu A, Salumets A. Aromatase gene (*CYP19A1)* variants are essential to controlled ovarian hyperstimulation outcome. Reproductive BioMedicine Online 2009 May;18(5):651–657.

II **Altmäe S**, Haller K, Peters M, Hovatta O, Stavreus-Evers A, Karro H, Metspalu A, Salumets A. Allelic estrogen receptor 1 (ESR1) gene variants predict the outcome of ovarian stimulation in in vitro fertilization. Molecular Human Reproduction 2007 August;13(8):521–6.

III **Altmäe S**, Stavreus-Evers A, Ruiz JR, Laanpere M, Syvänen T, Yngve A, Salumets A, Nilsson TK. Variations in folate pathway genes are associated with unexplained female infertility. Fertility and Sterility 2009 March 24 (epub ahead of print).

IV Laanpere M, **Altmäe S**, Nilsson TK, Salumets A. Folate-metabolizing gene variants and pregnancy outcome of IVF (manuscript submitted to Fertility and Sterility).

V Kõks S, Velthut A, Sarapik A, **Altmäe S**, Reinmaa E, Schalkwyk LC, Fernandes C, Lad HV, Soomets U, Jaakma Ü, Salumets A. The differential transcriptome and ontology profiles of floating and cumulus granulosa cells in stimulated human antral follicles. Molecular Human Reproduction 2009 November 20 (epub ahead of print).



Author's contribution to the articles:

Ref. I Study design, performance of the experiments, participation partly in the analysis of the data, manuscript preparation.

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Ref IV Participation in the study design, participation in performing the experiments, participation in the manuscript preparation.

Ref V Participation in the study design, participation in the data mining, participation in the manuscript preparation.



3

# LIST OF ABBREVIATIONS

| | |
|---|---|
| ADAMTS1 | ADAM metallopeptidase with thrombospondin type 1 motif, 1 |
| AMH | Anti-Mullerian hormone |
| AHR | Aryl hydrocarbon receptor |
| AKR1C1 | Aldo-keto reductase C |
| aOR | Adjusted odds ratio |
| AR | Androgen receptor |
| ART | Assisted reproductive techniques |
| A/T/G/C | Adenine/Thymine/Guanine/Cytosine |
| BDNF | Brain-derived neurotrophic factor |
| BMI | Body mass index |
| BMP15 | Bone morphogenetic factor 15 |
| bp | Base pair |
| cAMP | Cyclic adenosine monophosphate |
| CGB | Chorionic gonadotrophin beta subunit |
| CGCs | Cumulus granulosa cells |
| COH | Controlled ovarian hyperstimulation |
| CTH | Cystathionase |
| CYP1B1 | Aryl hydrocarbon hydroxylase |
| CYP11A1 | Cholesterol monooxygenase |
| CYP17A1 | Steroid 17-alpha-monooxygenase |
| CYP19A1 | Aromatase |
| Del | Deletion |
| DNA | Deoxyribonucleic acid |
| dNTP | Deoxynucleotide triphosphate |
| DUSP6 | Dual specificity phosphatase 6 |
| EGF | Epidermal growth factor |
| EGR1 | Early growth response 1 |
| ER | Estrogen receptor |
| EREG | Epiregulin |
| ERK | Elk-related tyrosine kinase |
| FGCs | Floating granulosa cells |
| FET | Frozen embryo transfer |
| FOLR1 | Folate receptor 1 |
| FSH | Follicle-stimulating hormone |
| FSHB | Follicle-stimulating hormone beta subunit |
| FSHR | Follicle-stimulating hormone receptor |
| GCs | Granulosa cells |
| GDF-9 | Growth differentiation factor-9 |
| GH | Growth hormone |
| GnRH | Gonadotrophin-releasing hormone |
| hCG | Human chorionic gonadotrophin |
| Hcy | Homocysteine |

| | |
|---|---|
| HSD17B1 | Hydroxysteroid (17-beta) dehydrogenase 1 |
| ICSI | Intracytoplasmic sperm injection |
| Ins | Insertion |
| IU | International unit |
| IVF | *In vitro* fertilization |
| LH | Luteinizing hormone |
| LHCGR | LH and hCG receptor |
| LHB | Luteinizing hormone beta subunit |
| mRNA | Messenger ribonucleic acid |
| MGCs | Mural granulosa cells |
| MTHFR | 5,10-methylenetetrahydrofolate reductase |
| NFI | Nuclear factor I |
| NFIB | Nuclear factor I/B |
| OHSS | Ovarian hyperstimulation syndrome |
| OR | Odds ratio |
| PCOS | Polycystic ovary syndrome |
| PCR | Polymerase chain reaction |
| PGD | Preimplantation genetic diagnosis |
| POF | Premature ovarian failure |
| PPi | Pyrophosphate |
| PR | Progesterone receptor |
| RNA | Ribonucleic acid |
| RFLP | Restriction fragment length polymorphism |
| SD | Standard deviation |
| SLC19A1 | Solute carrier family 19, member 1 |
| SNP | Single nucleotide polymorphism |
| TCN2 | Transcobalamin II |
| TGFβ | Transforming growth factor beta |
| WHO | World Health Organization |

# INTRODUCTION

Infertility is an increasing medical and social problem affecting more than 10% of couples of their fertile age (Boivin et al. 2007). In Estonia, accordingly, more than 15 000 infertile couples could be suspected (Part et al. 2007). Even though infertility *per se* may not threaten physical health, it influences the mental and social well-being of couples (The ESHRE Capri Workshop 1996). In addition, infertility contributes to the low birth rate, which is a growing social and national problem in all Europe and also in Estonia.

*In vitro* fertilization (IVF) procedure is the most successful treatment for various causes of infertility. First IVF baby was born in 1978 (Steptoe and Edwards 1978). In Estonia, IVF has been available since 1994 and approximately 1.5% of newborns account for IVF treated couples (Part et al. 2007). IVF procedure consists of three steps – the stimulation of the ovaries (called controlled ovarian hyperstimulation (COH)), fertilisation of the retrieved oocytes and culturing of embryos, and finally the embryo transfer.

The expected outcome of the IVF procedure depends greatly on the effectiveness of COH, where follicle-stimulating hormone (FSH) is used to induce the (poly)folliculogenesis. Sufficient number of mature oocytes is crucial for high pregnancy rates, compensating possible losses during follicular puncture, fertilization, embryo development and implantation, meanwhile hormone overdoses can lead to the life-threatening conditions known as ovarian hyperstimulation syndrome (OHSS). Also many additional factors may influence the IVF outcome, such as patient's age, reason of infertility, and the quality of transferred embryos. Furthermore, there is growing evidence that infertility treatment outcome may be modulated by maternal nutritional status, like B vitamin supplementation for example.

It is well known that the outcome of IVF varies substantially between individual patients and is difficult to predict. Therefore, the identification of markers in patients who will elicit a poor or hyper response to standard treatment would be of great clinical advantage. Indeed, heritable genetic factors have been shown to influence the stimulatory effect of FSH in ovarian stimulation (de Castro et al. 2004, Perez Mayorga et al. 2000, Georgiou et al. 1997). For example, patients with unfavourable FSH receptor genotype need higher doses of FSH to overcome relative ovarian insensitivity (Behre et al. 2005). In addition to the predictive genetic markers of COH and pregnancy outcome, transcriptome of follicular cells that surround the maturing oocyte could be potential marker for IVF success. In fact, a couple of studies have demonstrated the correlation between gene expression profiling of human cumulus granulosa cells with competent embryo and pregnancy outcome.

Identification of these predictive markers of ovarian response to hormonal stimulation, embryo quality and pregnancy outcome in IVF treatment would enable clinicians to individualise ovarian stimulation regimen, minimise the risks of cycle cancellation and ovarian hyperstimulation, select the best embryos and thereby maximise the chance of pregnancy.



4

# 1. REVIEW OF LITERATURE

## 1.1. Infertility

Infertility is a widespread medical and social problem affecting up to 10% of the couples of fertile age (Boivin et al. 2007). Infertility is defined as the inability of a couple to become pregnant in a year without using any contraception (Workshop 2002). It is estimated that over 72 million women worldwide, aged 20–44, are currently infertile, however, only every second couple seeks for infertility medical care (Boivin et al. 2007). The treatment of infertility is psychologically and physiologically demanding for the couple, affecting their quality of life and ability to work. The economical demands on society and also to the couples are high. To reduce these costs, more efficient treatments are of importance.

The reason of infertility in a couple could be caused by the female (over one-third) or by the male factor (over one-third) or by a combination of problems in both partners or is unexplained (approximately one fifth) (The ESHRE Capri Workshop 1996). Female fertility is regulated by a complex coordination and synchronization of interactions in the hypothalamic-pituitary-ovarian axis. Female fertility can be therefore influenced by different diseases or dysfunctions of reproductive tract, neuroendocrine system, and immune system or by any general disease. The major causes of female infertility are disorders in ovulation (mainly polycystic ovary syndrome PCOS), tubal factor infertility, endometriosis and unexplained infertility (Smith et al. 2003). The most prevalent causes for female infertility according to the diagnostic and treatment guidelines by The ESHRE Capri Workshop (Workshop 2002, The ESHRE Capri Workshop1996) are summarised in Table 1. In a case of male factor infertility, it is generally defined by the finding of an abnormal semen analysis (WHO 1999). The classification as unexplained infertility is applied to an infertile couple whose standard investigations (semen analysis, tubal potency, laboratory assessment of ovulation) yield normal results. It could be that unexplained infertility arises from a defect in fertility that cannot be detected with routine methods (for example early endometriosis), or it represents the lower extreme of normal distribution of fertility, 70% of these couples achieve pregnancy in 2 years, while 20–30% remain infertile even after 9 years (Barnea et al. 1985, Templeton and Penney 1982).

**Table 1.** Etiology of female infertility.

| | |
|---|---|
| Anovulatory infertility | |
| | Premature ovarian failure (POF) and early menopause |
| | Polycystic ovary syndrome (PCOS) |
| Tubo-peritoneal infertility | |
| | Tubal factor infertility |
| | Endometriosis |
| Autoimmunity | |
| | POF |
| | Recurrent pregnancy loss |
| | Autoimmunity associated with infertility |
| Uterine abnormalities | |
| | Malformations |
| | Myomas |
| Unexplained infertility | |

## 1.2. *In vitro* fertilization

*In vitro* fertilization (IVF) has been the most promising procedure for infertility treatment, both for female and male factor infertility. More than 900 IVF procedures per million of population a year are performed in Europe and about 1–4% of newborns are IVF-babies (Andersen et al. 2007). IVF procedure consists of three stages: i) ovarian hormonal stimulation, ii) *in vitro* fertilization of the collected oocytes and incubation of the embryos, and iii) embryo transfer into the uterus. Residual good quality embryos not transferred into the uterus are frozen and stored in liquid nitrogen for subsequent use in frozen embryo transfers (FET).

During the first stage of the IVF procedure, known as controlled ovarian hyperstimulation (COH), multiple follicles are triggered to grow and mature by stimulating the ovaries with administration of exogenous FSH following either gonadotrophin-releasing hormone (GnRH) antagonist or agonist protocols. In the next step of the stimulation, cumulus-oocyte cell complexes are retrieved from the ovaries by vaginal ultrasound-guided needle punctures. In a conventional IVF, the punctured cumulus-oocyte complexes are 4–6 hours later fertilised with motile spermatozoa. In intracytoplasmic sperm injection (ICSI) procedure, the punctured oocytes are cleaned from the surrounding cumulus cells and a single sperm is injected into the cytoplasm of the oocyte (Palermo et al. 1996). In general, ICSI is performed in cases of male factor infertility or in selected female factors like morphologic abnormalities of the oocyte, abnormalities of the zona pellucida, limited number of oocytes, or poor fertilization in a previous cycle. In addition, ICSI is undertaken if preimplantation genetic diagnosis (PGD) is planned in case of chromosomal or gene defects (ICSI 2006). Fertilization is routinely assessed 16–18 h after the insemination, and the following embryo cleavage of the normally fertilised oocytes is

evaluated the day after. Different morphological scoring systems for cleaved embryos have been proposed (Milki et al. 2002, Salumets et al. 2003, Salumets et al. 2001), however, an active search for more efficient markers is ongoing (Dominguez et al. 2009, Nagy, Sakkas and Behr 2008).

An uterine embryo transfer is performed 2 or 3 days after the fertilization at the 4–8 cell stage, or at blastocyst stage on day 5 after the fertilization. For most patients, only one or two embryos are transferred in order to avoid multiple pregnancies. Multifetal pregnancies are associated with significantly greater incidence of gestational complications than singletons, with most of these complications being directly related to prematurity (Pinborg 2005). Further, multiple gestations increase the rates of maternal morbidity, including hypertensive disorders (Pinborg et al. 2004b), Caesarean section (Pinborg et al. 2004a) and postpartum hemorrhage (Pinborg et al. 2004b), resulting in increased sick leave and ante- and postpartum hospitalisation (Pinborg et al. 2004b).

Even though the cumulative pregnancy rate per patient can be up to 70%, the success rate per single IVF cycle is still around 30% (Ola and Li 2006). The success of the IVF treatment is primary dependent on the effectiveness of COH. COH can be considered successful if numerous and good-quality oocytes have been obtained from the ovarian aspiration. It has long been noted that the outcome of COH is unpredictably variable between patients. Therefore, the identification of factors that influence the response to standard IVF treatment with FSH would be of great clinical importance.

## 1.3. Human ovary

Human ovary consists of two layers: ovarian cortex and medulla. Cortex is the outer layer, containing ovarian follicles at different developmental stages embedded in a compact cellular connective tissue (Lass et al. 1997). Medulla forms the central part and consists of connective tissue, blood vessels, lymphatic vessels and nerves. As the follicles grow in size they migrate from the cortex to the medulla and thereafter migrate back as the time of ovulation approaches. The functions of the ovary include housing and nutrition of the oocytes and the secretion of hormones and growth factors that are crucial for follicular maturation, embryonal receptivity and embryo implantation.

The oocyte and the surrounding granulosa and theca cells of the follicle form the functional unit of the ovary, where follicle provides a protective cover and a suitable environment for the oocyte. Human ovary holds a decreasing reserve of oocytes from the fetal life until the menopause. At around 20 weeks of gestation, the oocyte quantity reaches its peak of approximately 7 million follicles (Gougeon 1996). At birth the number decreases to one to two million and at the onset of puberty to 300 000 to 400 000 follicles (Gougeon et al. 1994). During the reproductive life, around 400 oocytes ovulate, meanwhile

more than 99% of the oocytes go through atresia and degenerate, and by the time of menopause, there are less than 1000 follicles remained in the ovary (Gougeon et al. 1994). Besides the decrease in follicle number, also the quality of the follicles and the oocytes decline with age, exhibiting structural damage and aneuploidy with an increasing frequency (de Bruin et al. 2004).

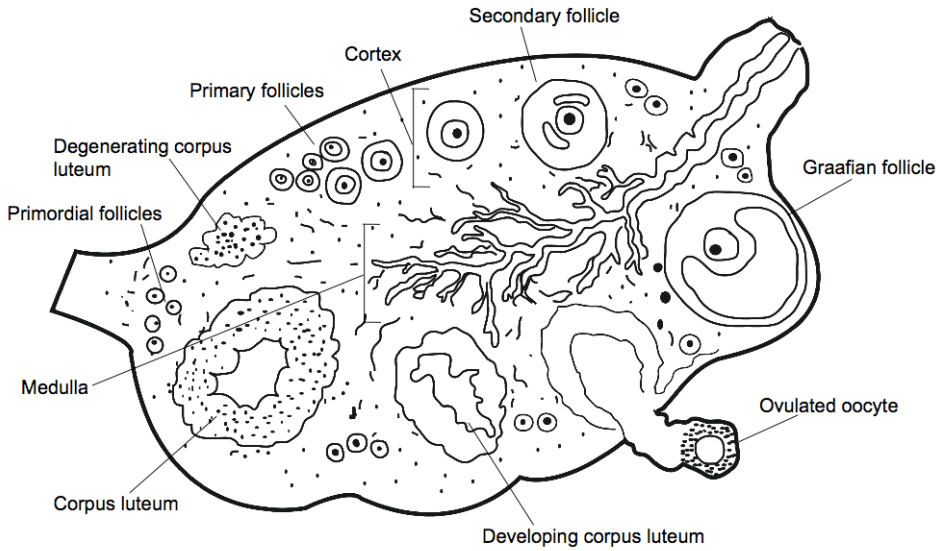


**Figure 1.** Human ovary. The two layers, ovarian cortex and medulla, are indicated, together with follicles in the different developmental stages.

## 1.4. Folliculogenesis

Folliculogenesis is a process of maturation of the ovarian follicle. Folliculogenesis involves the recruitment of the follicle into the growing phase, which is controlled by paracrine and autocrine signals produced in the ovary itself, followed by the proliferation and differentiation of the surrounding granulosa and theca cells, which are regulated in addition to the internal signalling by endocrine signals from outside the ovary. This process is under the primary control of two pituitary hormones, gonadotrophins FSH and luteinizing hormone (LH). During the maturation, the follicle grows and goes through the primordial, primary, secondary and preantral stages before it reaches the antral stage (Figure 2).

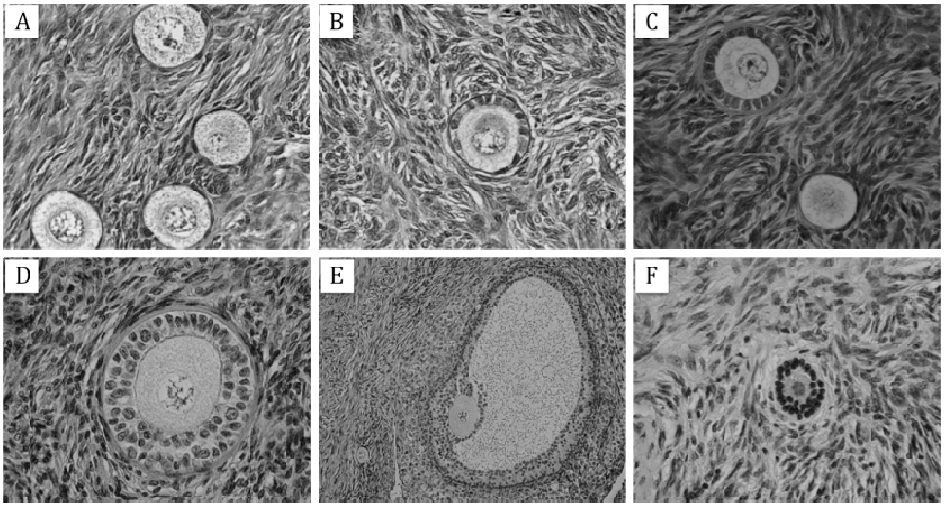


**Figure 2.** Light micrographs of the different follicular stages. A – primordial follicles (magnification 400X), B – a transitional follicle from the primordial to the primary stage (magnification 400X), C – primary follicle (upper) (magnification 400X), D – secondary follicle (magnification 400X), E – antral follicle (magnification 100X), F – atretic follicle (magnification 400X). Photos with permission, by Inger Britt Carlsson.

A resting primordial follicle is surrounded by a single layer of flattened granulosa cells (GCs) (Gosden et al. 2002). During the initial stages of folliculogenesis certain resting primordial follicles start to grow due to the expression of kit-ligand (Packer et al. 1994) and retinoblastoma protein (Bukovsky et al. 1995). This step of the folliculogenesis is considered to be gonadotrophin independent, as primordial follicles do not possess FSH receptors (Speroff and Fritz 2005). The development of primary follicle is the first sign of activation and initial follicular recruitment. At this stage the expression of FSH receptors begins. In human ovaries, it has been demonstrated that FSH receptors are expressed in 1/3 of the primary and two-layer secondary follicles and in all multi-layer follicles (Oktay et al. 1997). During the early growth phase the proliferating GCs grow larger and become cuboidal, providing nutrients and different molecular signals to the oocyte (Wandji et al. 1997). The GCs communicate with each other and with the oocyte via gap junctions, composed mainly by connexins (Eppig 1991). Primary GCs start to secrete mucopolysaccharides forming the zona pellucida, a thick layer of glycoproteins and acid proteoglycans around the oocyte. Further proliferation of GCs and follicular enlargement result in formation of secondary follicle.

Two or more layers of GCs surround secondary follicle. GCs associated with secondary follicle possess FSH, estrogen, and androgen receptors (Speroff and Fritz 2005). FSH binds to FSH receptors on the GC surface, stimulating the proliferation of the GCs, while increasing the number of FSH receptors

expressed on their surface (thereby magnifying its own effects), and stimulating the aromatase enzyme. According to the generally accepted "two cell, two gonadotrophin theory" (Hillier et al. 1994), FSH binding to FSH receptor in the GCs activates cyclic adenosine monophosphate (cAMP) to induce p450 aromatase that converts androgens to estrogens. At the same time as GCs proliferate, theca cells respond to LH. The interaction of LH with its receptor on the surface of the theca cells also activates cAMP that in turn initiates the production of androgens from cholesterol. Androgens are subsequently aromatised in the GCs to estrogens, mainly estradiol-17β (E2) (Erickson and Shimasaki 2001). Estrogens are then released into the follicular fluid and circulation to participate in the further regulation of follicular maturation. In the ovary, estrogens influence the GC proliferation, increase the number of FSH receptors and their sensitivity, and stimulate aromatase activity and thus further estrogen biosynthesis. Androstenedione, at low concentrations, promotes aromatase action and estrogen biosynthesis, but high levels of androgens cause follicular atresia (Speroff and Fritz 2005).

Under the influence of gonadotrophins and growth factors the follicle grows and the surrounding stroma stratifies and differentiates, forming theca interna and theca externa with vessels between the two layers. This enables the follicle to gain a blood supply, resulting a direct exposure to factors circulating in the blood (Reynolds et al. 1992). In the preantral stage, a fluid filled antrum starts to develop. From this point, the GCs proliferate and differentiate to mural GCs (MGCs) in the periphery of the follicle and cumulus GCs (CGCs) closest to the oocyte. The follicular fluid accumulation is limited by the level of FSH present (Eppig 1991), reflecting the steroidogenesis of surrounding GCs and theca cells, containing plasma proteins, proteoglycans, prolactin, inhibin and etc (Speroff and Fritz 2005).

A large fluid-filled antrum is a characteristic feature of the mature follicle, named also Graafian follicle or antral follicle (Figure 3). At the antral stage, under the competition of available FSH, most of the follicles go through atresia, and only a few of them reach the pre-ovulatory phase. The dominant follicle has the advantage of higher rate of GC proliferation, therefore for increased number of FSH receptors, higher aromatisation ability, and increase in estrogen production.

The preovulatory gonadotrophin surge is resulted from the positive feedback action of elevated estrogen on the pituitary gland. It is suggested that also progesterone is involved in the stimulation of ovulation (Zalanyi 2001). Progesterone production starts in the GCs prior to gonadotrophin surge and increases in the corpus luteum after the ovulation. Further, the terminal maturation of the follicle is believed to be stimulated mainly by LH (Sullivan et al. 1999). In the immature follicles LH receptors are expressed in the theca cells, but in antral follicles they are also expressed in GCs (Camp et al. 1991). It has been hypothesised that the maturing follicle continues to develop in the presence of increasing FSH levels because of the capacity to respond to LH

(Sullivan et al. 1999). Following the pre-ovulatory gonadotrophin surge, the dominant follicle releases a mature oocyte that is ready for fertilization (Gougeon 1996).

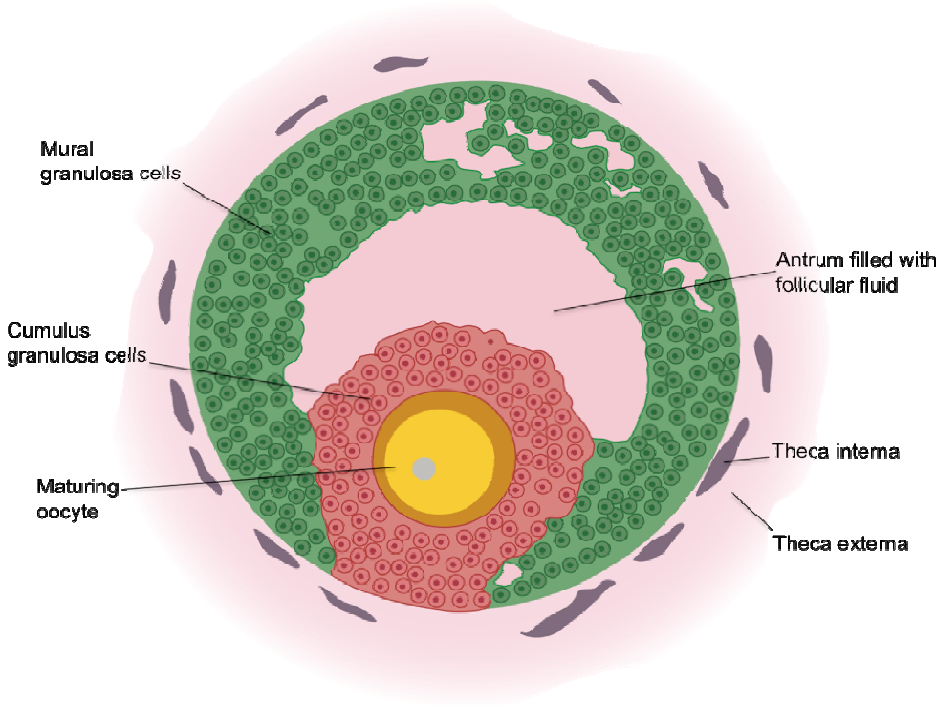


**Figure 3.** Mature Graafian follicle in the ovary. Different granulosa cells surrounding the maturing oocyte together with antrum and thecal layers are indicated.

Besides gonadotrophins and estrogens, androgens, other hormones including progesterone and different growth factors are involved in the intraovarian regulation of folliculogenesis. Many proteins belonging to the transforming growth factor β (TGFβ) family are important as local regulators of follicular development and oocyte maturation, such as bone morphogenetic factor 15 (BMP15), growth differentiation factor-9 (GDF-9), anti-Mullerian hormone (AMH), activins and inhibins (Knight and Glister 2006). Other factors include growth hormone (GH) (Sharara and Nieman 1994), insulin and insulin-like growth factors (Erickson and Shimasaki 2001) and members of the epidermal growth factor (EGF)-family (Shimada et al. 2006). Many factors, however, are still unknown.

## 1.5. Follicular hormonal regulation during the menstrual cycle and IVF

The menstrual cycle consists of a follicular (or proliferative) phase and a luteal (or secretory) phase, separated by ovulation. A normal cycle has approximately the same length in each cycle, from 25 to 35 days with average being 28 days. Ovarian function is under the control of LH and FSH, which bind to their receptors in the ovary and regulate its function by promoting sex steroid production and folliculogenesis (Hillier et al. 1994). The gonadotrophin-dependent growth phase from secondary to preovulatory follicle takes around 85 days, including approximately 14 days from the follicular phase of the menstrual cycle and ending with the ovulation (Gougeon 1986). During this phase the follicles depend on the gonadotrophin (FSH and LH) action.

The hypothalamus secretes pulses of GnRH, which regulates the pituitary gland to produce gonadotrophins in a similar pulsatile pattern. When gonadotrophins act on the theca and GCs, the production of estrogens increases and reaches its maximal level in the preovulatory follicle. Estradiol is the main estrogen synthesised and has dual action in gonadotrophin secretion – at low circulating levels it exerts negative feedback control over FSH and LH production by inhibiting GnRH secretion, meanwhile at high circulating levels positive feedback becomes a dominant force and LH and FSH surge is induced, followed by the ovulation. Growing levels of estradiol also stimulate the growth of the endometrium.

After ovulation, the oocyte moves along the fallopian tube for potential fertilization and the dominant post-ovulatory follicle transforms into the corpus luteum. LH promotes luteinization of mature Graafian follicles and maintains progesterone production from the corpus luteum. High levels of progesterone, in a presence of estrogen, form a negative feedback action that suppresses gonadotrophin secretion. Progesterone is the main hormone that regulates the endometrial maturation for blastocyst implantation during the luteal phase. In the absence of pregnancy, the corpus luteum degenerates, resulting in decrease of circulating steroids, that lead to enhanced secretion of FSH and the initiation of a new cycle (Speroff and Fritz 2005).

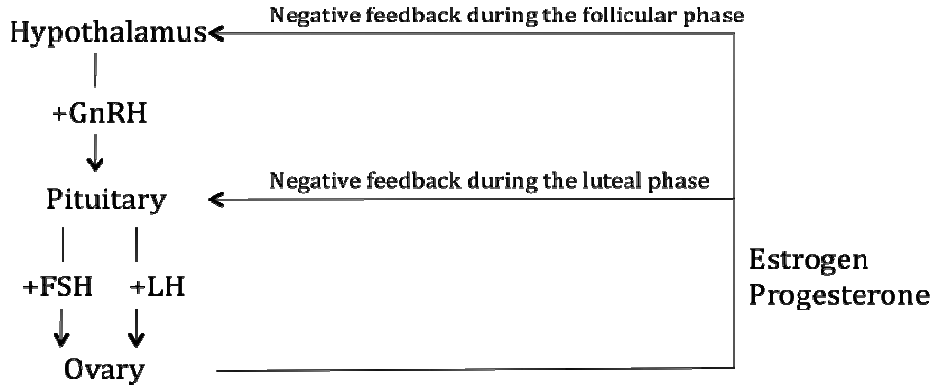


**Figure 4.** Simplified schematic illustration of the hypothalamus-pituitary-ovary axis.

During IVF, the number of mature oocytes is increased with administration of exogenous gonadotrophins. In order to compensate the changes resulted by stimulation, GnRH analogue co-treatment, oral contraceptive pre-treatment, late follicular phase human chorionic gonadotrophin (hCG) and luteal phase progesterone supplementation are usually included (Macklon et al. 2006). Conventional IVF regimens use GnRH agonists or GnRH antagonists to prevent the premature LH rise, premature oocyte maturation, and luteinization by decreasing the release of endogenous LH. When using GnRH agonist, so-called long protocol, the suppression of pituitary function lasts approximately 2 weeks, followed by high doses of exogenous gonadotrophins. With GnRH agonist co-treatment, complete pituitary down-regulation is achieved, overruling the natural follicle recruitment and selection and thereby enabling the growth of many follicles at different developmental stages (Macklon et al. 2006). GnRH antagonist may be administered at any time point of the early to mid-follicular phase of the treatment cycle (Macklon et al. 2006). Therefore, the follicle recruitment and the initial stages of the dominant follicle selection can begin during the natural cycle, the FSH increase in this cycle can be used for secondary follicle recruitment, and thus the exogenous FSH administration can be applied from early to the mid-late follicular phase (Baart et al. 2009). During the normal menstrual cycle, decreasing FSH levels are crucial for dominant follicle selection process from the pool of 20–30 small antral follicles. During the mild stimulation, interference with decreasing FSH enables the development of multiple follicles, meanwhile the follicle recruitment and the initial stage of selection remain unaffected (Macklon et al. 2006). GnRH antagonist protocol enables more physiological approach to COH and has several advantages, like lower gonadotrophin administration and treatment time, as well as no side effects. However, slightly reduced clinical efficacy has been noted (Fauser and Devroey 2005).

## 1.6. Transcriptome of human granulosa cells

The bidirectional communication between the oocyte and the surrounding granulosa cells is crucial in folliculogenesis and is needed for an oocyte to achieve competence to sustain fertilization and embryogenesis (Matzuk et al. 2002). Mural GCs, that line the follicle wall, are in close proximity to the theca cells and are proposed to express genes important for follicular rupture (Matzuk et al. 2002). Cumulus GCs, which surround the oocyte during the follicular development and ovulation, are suggested to mediate oocyte development and fertilization, meanwhile in turn being regulated by oocyte factors (Matzuk et al. 2002). Nevertheless, little is known about the complex process that generates a developmentally competent oocyte.

Research on transcripts expressed at a fixed time point enable the determination of the cell function in a distinct biological background, and a focus on large-scale transcriptome studies provide the opportunity for comprehensive analysis of genes and pathways involved in oocyte maturation. The importance of granulosa cell transcriptome on oocyte and embryo developmental potential has been recently shown in IVF patients. During the IVF procedure, the ovarian puncture of follicles gives an ideal opportunity to collect granulosa cells. A number of previous gene expression studies on MGCs or CGCs during IVF have demonstrated the correlation between gene expression profile and ovarian stimulation regimen (Perlman et al. 2006, Grondahl et al. 2009), oocyte fertilization (Anderson et al. 2009), embryo morphology and cleavage rate (Assou et al. 2008, McKenzie et al. 2004, van Montfoort et al. 2008), embryo viability (Feuerstein et al. 2007, Hamel et al. 2008), and embryo competence to establish pregnancy (Anderson et al. 2009). In addition, a study on gene expression profile of mural GCs during IVF showed the differences between normal and poor responders, indicating that the granulosa cell transcriptome predicts the status of ovarian reserve and the competence of GCs in supporting development of the oocyte (Chin et al. 2002). However, a comparative study of gene expression profile between mural GCs and cumulus GCs has not been provided yet.

## 1.7. Genes regulating folliculogenesis

### 1.7.1. Gonadotrophins

Gonadotrophins have the main role in regulating folliculogenesis. FSH and LH are the two principal gonadotrophins; hCG is a third gonadotrophin and is produced by the placenta during pregnancy. FSH and LH are heterodimeric glycoproteins that consist of $\alpha$ and $\beta$ subunits that are non-covalently linked. The two hormones share a common $\alpha$ subunit, while $\beta$ subunit is hormone

specific and includes the receptor-binding domain. The α subunit gene is encoded by a single gene (6q12.219 (Naylor et al. 1983). FSH β subunit is also encoded by a single gene (*FSHB*) (11p13), while LH β/hCG β subunit gene cluster (*LHB/CGB*) is formed of one *LHB* and six *CGB* genes (19q13.32) (Fiddes and Talmadge 1984).

In gonadotrophin genes, only few genetic alterations have been identified, which have been associated with reduction or loss of function. The reason for the low frequency of variations may be their expected effect on reproduction, which eliminates them rapidly from the gene pool. In the α subunit gene, a single amino acid substitution Glu56Ala in human carcinoma is the only genetic variation reported so far, creating protein that fails to associate with the β subunit (Nishimura et al. 1986). Also among *CGB* genes, in *CGB5* gene, only one genetic alteration has been detected, Val79Met substitution, that causes inefficient dimerization with the α subunit (Miller-Lindholm et al. 1999). Six sequence variations in *LHB* gene are known, which lead to normal or slightly decreased activity of the protein, and have been suggested to be associated with female infertility, menstrual disturbances, PCOS and premature ovarian failure (POF) (Arnhold et al. 2009, Elter et al. 1999, Liao et al. 1998, Suganuma et al. 1995, Takahashi et al. 1999).

In contrast to *LHB* gene, where several genetic alterations have been detected, *FSHB* gene is highly conserved (Lamminen et al. 2005). Haplotype analysis in *FSHB* gene has revealed two core haplotypes among Estonians that characterise about 90% of the population (Grigorova et al. 2007). The second most prevalent haplotype detected was suggested to be associated with rapid conception in females (Grigorova et al. 2007). In *FSHB* gene, four inactivating mutations have been identified in females, that result in absent or incomplete pubertal development and infertility (Matthews et al. 1993, Layman et al. 1997). Affected females demonstrate incomplete or absent breast development, low FSH and estradiol, high LH and sterility (Layman et al. 1997, Matthews et al. 1993). The phenotypic symptoms of humans carrying *FSHB* mutations are similar to *FSHB* knock-out female mice, except that estradiol levels are low. FSHB knock-out female mice are infertile due to a block of folliculogenesis at the secondary stage, demonstrating no preovulatory mature follicles, no corpora lutea, lack of estrous cycles, and low levels of FSH (Kumar et al. 1997). The low variability and intolerance of genetic alterations emphasises the important role of FSH in reproduction.

### 1.7.2. LH and FSH receptors

Gonadotrophins exert their actions by binding to distinct cell surface receptors. FSH binds to its receptor FSHR, while LH and hCG bind to the same receptor LHCGR. Genes encoding both receptors are located at chromosome 2p21, *LHCGR* consisting of 11 exons and *FSHR* of 10 exons (Rousseau-Merck et al.

1993). Gonadotrophin receptors belong to the G-protein associated receptor family, composed of seven hydrophobic transmembrane domains. In both *LHCGR* and *FSHR* genes, several activating and inactivating mutations have been determined (Huhtaniemi and Themmen 2005). All activating receptor gene mutations have been identified in the last exon (exon 11 in *LHCGR* and exon 10 in *FSHR*), which encodes a small extracellular extension, the full transmembrane domain and the intracellular C terminal tail (Themmen and Huhtaniemi 2000). Inactivating receptor mutations occur more rarely and exist in homozygous or compound heterozygous states and thus inheriting in a recessive pattern (Themmen and Huhtaniemi 2000).

In addition to mutations, several polymorphisms in *LHCGR* (over 400 SNP according to www.snpper.chip.org database) and in *FSHR* (more than 1080 SNPs, www.snpper.chip.org) have been identified. Polymorphisms 18insLeuGln, Asn291Ser and Ser312Asn in *LHCGR* gene, have been associated with increased receptor activity (Piersma et al. 2006, Piersma et al. 2007, Simoni et al. 2008) and their possible effect in steroid-hormone related disease such as breast cancer has been suggested (Piersma et al. 2007, Powell et al. 2003).

The two most common and well-studied SNPs in *FSHR* gene are Thr307Ala and Asn680Ser, located in the exon 10 in the extracellular domain and intracellular domain, respectively (see Figure 5) (Simoni et al. 1999). These two non-synonymous polymorphisms are in strong linkage disequilibrium (Simoni et al. 1999). A frequency of 307Ala-680Ser genotype has been recently shown to be more prevalent in the ovarian dysfunction group of “poor responders” (Livshyts et al. 2009). Indeed, several studies in COH cycles indicate that polymorphism Asn680Ser is an important factor for determining the prognosis of COH cycles in infertile women undergoing IVF treatment. Asn transition to Ser results in subtle differences in the receptor function as reflected by higher basal FSH levels in the early follicular phase and/or the higher amount of FSH administration needed for effective ovarian stimulation (Behre et al. 2005, de Castro et al. 2004, de Castro et al. 2003, Falconer et al. 2005, Jun et al. 2006, Loutradis et al. 2006, Perez Mayorga et al. 2000, Simoni et al. 2002, Sudo et al. 2002). As women with 680Ser/Ser genotype are described more often to exhibit ovarian resistance, either larger amount of FSH is needed or in worse scenario, the further hormonal stimulation must be cancelled (Behre et al. 2005, de Castro et al. 2003, Perez Mayorga et al. 2000, Sudo et al. 2002). The resistance of FSH action has also been demonstrated in GCs of 680Ser/Ser type women (Greb et al. 2005), and in GCs lower FSHR expression has been associated with poor ovarian response to gonadotrophin stimulation (Cai et al. 2007).

### 1.7.3. Aromatase

Aromatase is one of the key enzymes in the ovarian steroidogenesis, catalysing the final stage of the conversion of androgens, testosterone and androstenedione to estradiol and estrone, respectively (Ryan 1982). Estrogen production depends on the supply of androgens as well as on the expression and activity of the aromatase enzyme. Aromatase activity disappears when follicle enters atresia (Chang et al. 2005). It has been demonstrated that aromatase knock-out mice fail to synthesise endogenous estrogens (Jones et al. 2001) and are infertile because of the follicular disruption and a failure to ovulate (Britt et al. 2001).

Aromatase is encoded by *CYP19A1* gene (15q21.1), spanning over 123 kilo base of genomic DNA and comprising of 10 exons, where the last 9 (II–X) are coding exons (Sebastian and Bulun 2001). The first exon, one of the nine alternate untranslated first exons, regulates the tissue specific expression (Sebastian and Bulun 2001). Mutations in *CYP19A1* gene are infrequent (Zirilli et al. 2008). In patients with aromatase deficiency and thus inadequate conversion of androgens to estrogens rare mutations in *CYP19A1* gene that result in substantial reductions in enzyme activity have been detected (Mullis et al. 1997). Further, a study of common variations in aromatase gene has showed association between an intronic SNP (rs2414096) close to exon 3 and androgen excess in females (Petry et al. 2005).

Over 780 SNPs in the *CYP19A1* gene have been identified according to www.snpper.chip.org. In addition, a tetranucleotide repeat polymorphism comprised of 7 to 13 TTTA repeats in intron 4 has attracted major attention (see Figure 5), since overrepresentation of $(TTTA)_{12}$ allele in breast cancer patients with excessive aromatase activity has been reported (Haiman et al. 2000, Kristensen et al. 1998). Concurrently, women carrying shorter *CYP19* TTTA repeats exhibit lower estrogen values (Haiman et al. 2000, Tworoger et al. 2004). Women with PCOS have been shown to possess more frequently shorter *CYP19A1* alleles of ≤9 TTTA repeats and high serum testosterone and testosterone/estradiol ratio during the early follicular phase of the menstrual cycle (Xita et al. 2008). Fifty nucleotides upstream from $(TTTA)_n$ repeat polymorphism a TCT trinucleotide insertion (Ins) or deletion (Del) variation has been identified (Kurosaki et al. 1997). This 3-bp deletion has been found to co-segregate exclusively with $(TTTA)_7$ repeat site, generating 2 alleles, Del-$(TTTA)_7$ and Ins-$(TTTA)_n$ (Probst-Hensch et al. 1999). With shorter alleles, particularly the Del-$(TTTA)_7$ allele, associations with elevated androgen levels and lower E2/androgen levels have been shown, suggesting lower ovarian aromatase activity (Baghaei et al. 2003). A C/T SNP in 3'-untranslated region of exon 10 (rs10046) has also been reported to be in linkage disequilibrium with TTTA repeat polymorphism, where rs10046 T minor allele and long $(TTTA)_{12}$ allele were associated with elevated aromatase transcript levels in breast cancer tissue (Kristensen et al. 2000). Two previous studies of *CYP19A1* variants and ovarian stimulation outcome found no influence of the rs10046 and ovarian

response to exogenous FSH (de Castro et al. 2004) or the etiology of severe ovarian hyperstimulation syndrome (Binder et al. 2008).

### 1.7.4. Estrogen receptors

Estrogens play important role in female reproduction participating in differentiation of GCs, regulation of FSH/LH secretion in pituitary, and preparation of endometrium for implantation (Speroff and Fritz 2005). Estrogen signalling is mediated by estrogen receptors, which are ligand-activated transcription factors composed of several domains important for hormone binding, DNA binding and activation of transcription (Kuiper et al. 1996). Two estrogen receptors have been identified in humans, ERα (6q25) and ERβ (14q22), encoded by *ESR1* and *ESR2* genes, respectively (Mosselman et al. 1996, Walter et al. 1985). Although these two receptors are different gene products, they are highly similar in their ligand-binding and DNA-binding domains – 58% and 96% conservation of nucleotides, respectively (Jiang and Huhtaniemi 2004).

Estrogens extend the action of FSH on granulosa cells by promoting their proliferation and increasing their expression of FSH receptors (Ireland and Richards 1978). In the ovary, ERα is predominantly expressed in the thecal layer, whereas ERβ is expressed in GCs of growing follicles at all developmental stages (Pelletier and El-Alfy 2000). Both receptors have been knocked out in mice. ERα knock-out mice are cyclic, infertile and have hyperemic ovaries lacking of corpora lutea (Couse and Korach 1999). The ERα knock-out mice have a block in folliculogenesis at the early antral stage, before the increased GC proliferation starts (Hirshfield 1991). ERβ knock-out mice present small ovaries, partially arrested follicular development, increased numbers of primordial, primary and antral follicles and corpora lutea, having compromised fertility with reduced litter size (Krege et al. 1998). It is therefore concluded that in folliculogenesis, the proliferative actions of estrogens are mediated by ERα, meanwhile the differentiation and the anti proliferative effect required for reaching the antral stage require ERβ (Britt and Findlay 2002). In ER genes, a number of sequence variations that may influence the risk of different infertility-related gynaecological disorders and IVF outcome have been identified.

#### 1.7.4.1. ESR1

Recent findings have suggested that genetic variability in *ESR1* gene is involved in the outcome of controlled ovarian stimulation causing a disadvantage to certain patients undergoing IVF treatment (de Castro et al. 2004, Georgiou et al. 1997, Sundarrajan et al. 1999). *ESR1* gene is highly

polymorphic with more than 1650 SNPs identified (www.snpper.chip.org). The most studied variations in *ESR1* are rs2234693 (T/C, defined by restriction enzyme *Pvu*II) and rs9340799 (A/G, defined by restriction enzyme *Xba*I) in intron 1, and $(TA)_n$ dinucleotide repeat polymorphism in the promoter region (see Figure 5). *Pvu*II T/C polymorphism has been associated with decreased pregnancy rates in women undergoing IVF (Georgiou et al. 1997, Sundarrajan et al. 1999). IVF patients carrying *Pvu*II CC genotype demonstrated higher number of follicles, mature oocytes and embryos and the size of the leading follicles were bigger following the COH (Sundarrajan et al. 1999). Patients with *Pvu*II CC genotype showed improved follicular quality, as judged by the mean ratio of follicles to oocytes obtained after FSH stimulation (Georgiou et al. 1997). In addition, *Pvu*II C allele frequency has been found to be lower among poor responders (≤ 3 follicles) compared to normal COH responders (de Castro et al. 2004). Furthermore, *Pvu*II T/C and $(TA)_n$ dinucleotide repeat polymorphism have been associated with susceptibility to endometriosis (Georgiou et al. 1999, Hsieh et al. 2007) and premature ovarian failure (Bretherick et al. 2008, Syrrou et al. 1999).

#### 1.7.4.2. ESR2

Compared to *ESR1* gene with more than 1650 SNPs, only around 300 SNPs have been identified in *ESR2* gene (www.snpper.chip.org). Polymorphisms in *ESR2* gene, *Rsa*I G/A (Val328Val in exon 5) and *Alu*I G/A (nucleotide 1730 in the non-coding end of exon 8) (see Figure 5) have been related to decrease in the serum level of LH, FSH and progesterone in women with ovulatory dysfunctions (Sundarrajan et al. 2001). Further, homozygous *Rsa*I AA and *Alu*I AA genotypes have been suggested to be associated with ovulatory dysfunctions of unknown origin (Sundarrajan et al. 2001). *Alu*I AA has also been proposed to affect the risk of developing endometriosis (Wang et al. 2004), while *Rsa*I AA genotype has been associated with PCOS (Sundarrajan et al. 2001).

### 1.7.5. Progesterone receptor

Progesterone is an important hormone in the complex regulation of normal female reproductive functions. The major physiological role of progesterone in the ovary and uterus is the release of mature oocytes, facilitation of implantation, and maintenance of pregnancy (Graham and Clarke 1997). The production of progesterone takes place in the GCs and starts just prior to the gonadotrophin surge and increases in the corpus luteum (Zalanyi 2001). Progesterone enhances the effect of FSH on GCs by increasing cAMP (Goff et

al. 1979) and inhibits FSH induced estradiol production (Fortune and Vincent 1983, Schreiber et al. 1981).

Progesterone receptor, a member of the steroid receptor superfamily, mediates the physiologic effects of progesterone. The human progesterone receptor is encoded by a single-copy gene *PR* (11q22–23). *PR* uses two separate promoters and translational start sites to produce two isoforms, PR-A and PR-B (Giangrande et al. 2000). Although the two isoforms differ only in that PR-B contains additional 164 amino acids at the amino terminus, they are two functionally distinct transcription factors that mediate their own response genes and physiologic effects, with little overlap (Giangrande et al. 2000, Kastner et al. 1990, Vegeto et al. 1993). The receptors start to be expressed in the large follicles and the expression is increased by the LH surge (Drummond 2006).

Several polymorphic variants in *PR* gene have been described, and over 690 SNPs have been identified (www.snpper.chip.org). A major haplotype block, extending over 70 kilo base, has been identified in *PR* gene that encompasses most reported polymorphisms along the gene area, including 306 bp Alu insertion polymorphism in intron 7 (PROGINS) (see Figure 5) (De Vivo et al. 2002). PROGINS polymorphism is proposed to diminish the response to progesterone (Romano et al. 2007). The Alu insertion has been found to be more prevalent in women with unexplained infertility (Pisarska et al. 2003) and in women with endometriosis (Wieser et al. 2002) than in control individuals. Apart from the haplotype block, two common polymorphisms +44C/T and +331G/A in the promoter area of *PR* have been identified (see Figure 5). In women undergoing IVF treatment, a relationship between +331 A allele and elevated risk for implantation failure has been suggested (Cramer et al. 2003). A allele has also been associated with decreased pregnancy outcomes in women undergoing IVF (Spandorfer et al. 2006). It is proposed that +331 A allele increases transcription of the *PR* gene, which favours the production of the PR-B isoforms, affecting so the PR-A/PR-B ratio (De Vivo et al. 2002). Recently, however, no associations between polymorphisms (H770H, V660L, PROGINS) in *PR* gene and women at risk for recurrent implantation after IVF treatment was reported (Coulam et al. 2008).

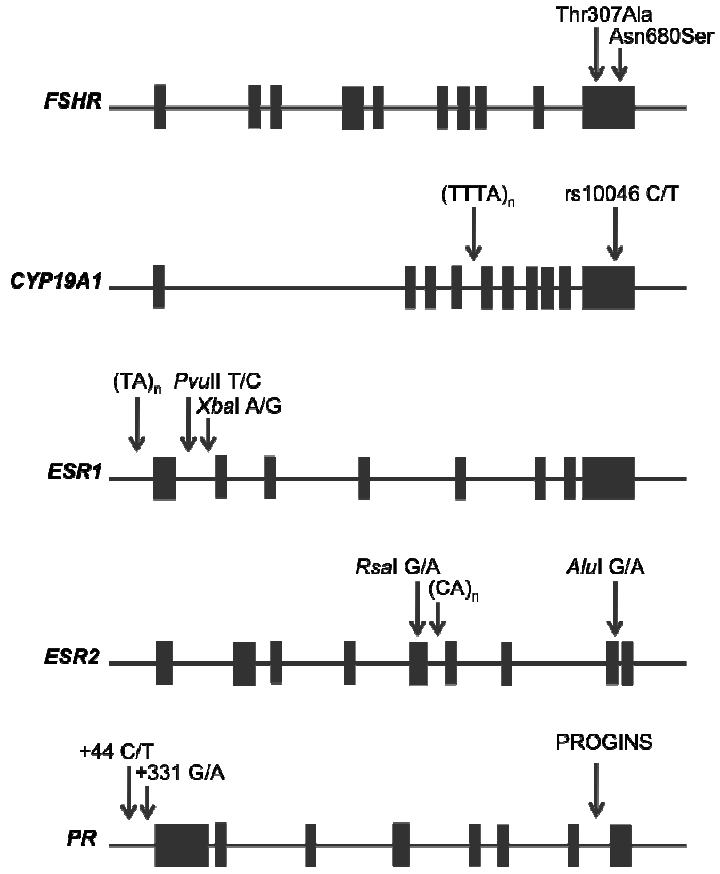


**Figure 5.** The most commonly studied polymorphisms in *FSHR*, *CYP19A1*, *ESR1*, *ESR2* and *PR* genes.

## 1.8. Other factors influencing induced folliculogenesis

Besides sequence variations in genes, numerous factors that influence ovarian response to hormonal stimulation are known. Such predictive parameters include patient's age (Rosenwaks et al. 1995); parameters of ovarian reserve as ovarian volume (Lass et al. 1997, Syrop et al. 1999, Tomas et al. 1997), number of early antral follicles (Chang et al. 1998, Kupesic and Kurjak 2002, Kwee et al. 2007), and ovarian stromal blood flow (Kupesic and Kurjak 2002, Ng et al. 2005); hormonal markers such as serum concentrations of FSH, LH, estradiol, inhibin B, and AMH (Coccia and Rizzello 2008); autoantibodies against FSH (Haller et al. 2007); maternal nutritional status (Tamura and Picciano 2006); and also smoking (Freour et al. 2008).

### 1.8.1. Age

Increasing maternal age is a proven adverse factor in reproductive performance. The age-related decline in fertility is attributable to both, to a decrease in conception rates and an increase in pregnancy loss rates. Declining ovarian reserve has been suggested as a cause of lower live birth rate that occurs after natural conception at around 31 years of age, and at around 35 years in IVF cycles (van Noord-Zaadstra et al. 1991). The number of oocytes in the ovaries declines naturally and progressively through the process of atresia. At the time of birth there are one to two millions oocytes, by the age of 37 years the number of oocytes has decreased to 25 000, ending with less than 1000 at the time of menopause (Faddy et al. 1992, Gougeon et al. 1994). Fertility declines gradually but significantly beginning approximately from 31 years, and decreases more rapidly after age of 37, reflecting primarily a decrease in oocyte quality in association with a gradual increase in the circulating concentrations of early follicular phase FSH (Faddy et al. 1992).

Aging oocytes have been widely suggested to be important factors for the decline in fecundity (Coccia and Rizzello 2008). The relationship between increased maternal age and increased incidence of chromosomal abnormalities, in particular aneuploidies, has been clearly demonstrated (McFadden and Friedman 1997, Sherman et al. 2005). Several mechanisms have been proposed to explain the increased risk of these abnormalities with increased maternal age, such as hormonal imbalances, abnormalities in follicular development resulting from the aging of the somatic cells surrounding the oocyte, and impaired perifollicular microcirculation (Johnson et al. 2006).

In IVF, reduction in the quantity and quality of oocytes because of aging leads to decreased success in achieving pregnancy (Rosenwaks et al. 1995). It is well established that there is a sharp decline in the chance of successful conception in older women using the conventional IVF (Templeton et al. 1996). The influence of woman's age is also evident in ICSI cycles, where with advanced age gradual but significant decrease in viable pregnancy rates occur (Grimbizis et al. 1998). However, the process of aging does not appear to affect the ability of older oocytes to fertilise, as different studies have demonstrated similar fertilization rates between older and younger women (Fitzgerald et al. 1998). Yet, lower implantation and higher spontaneous abortion rates are seen among embryos derived from oocytes obtained from older women (Lim and Tsakok 1997, Romeu et al. 1987).

In addition to reduced quantity and quality of oocytes, age increases the risks of other disorders that may adversely affect fertility, such as uterine fibroids, tubal pathologies, and endometriosis (A committee opinion 2008). Even further, pregnant women at an advanced maternal age have higher risk of pregnancy complications such as primary Caesarean delivery, prolonged and dysfunctional labor, pregnancy associated hypertension, and delivery before 32 weeks of pregnancy (Luke and Brown 2007).

### 1.8.2. Parameters of ovarian reserve

Antral follicle count and ovarian volume are the best markers of ovarian reserve and ovarian response in COH, both of them being assessed by ultrasonography. The antral follicle count is calculated by counting the number of antral follicles with size of 2–10 mm in both ovaries (Scheffer et al. 2002). The pool of antral follicles includes pre-antral and early antral follicles (0.2–2.0 mm) that are gonadotrophin independent, small antral follicles (1.0–6.0 mm) and large antral follicles (> 6.0 mm) that are gonadotrophin dependent (Gougeon 1989). At any time point during the natural cycle, ovaries contain follicles at different developmental size, and antral follicle count has to be done at the early follicular phase (Gougeon 1998).

Another informative marker of ovarian reserve is anti-Mullerian hormone (reviewed in (La Marca et al. 2009). AMH is predominantly produced by the GCs of pre-antral and small antral follicles (Modi et al. 2006). It has been demonstrated that serum AMH levels on day 3 of the natural cycle correlate positively with antral follicle count, negatively with FSH level, and decrease progressively with age (de Vet et al. 2002). Further, AMH is believed to be a superior marker of ovarian response, as several studies have demonstrated association between higher AMH levels and a greater number of retrieved IVF oocytes (summarised in (La Marca et al. 2009)). Also positive correlations between AMH levels and oocyte quality and embryo morphology have been reported (summarised in (La Marca et al. 2009)). Further, as shown recently, circulating AMH levels can predict excessive and poor response to stimulation with exogenous gonadotrophins (Nardo et al. 2009). The relative stability and consistency of AMH serum concentrations and it's low inter- and intra-cycle variability makes AMH a good cycle-independent predictive marker of induced folliculogenesis. However, like other predictive markers, AMH testing and also antral follicle count do not seem to predict accurately the probability of pregnancy after IVF (Broer et al. 2009).

Also basal FSH measurement, in combination with age, is considered as a predictor of IVF outcome (Toner et al. 1991). Basal FSH level reflects the ovarian reserve, as with the ovarian follicle depletion the FSH production increases by impaired negative feedback. In general, basal FSH measurement is considered as a simple and reliable test, however FSH measures vary monthly and lack of clear cut-off point (Coccia and Rizzello 2008).

### 1.8.3. Folate-metabolizing pathway

There is growing evidence that folliculogenesis and thus infertility treatment outcome may be modulated by folate metabolism and thus nutritional status. Folate is an important B vitamin and is believed to be crucial for reproduction (Tamura and Picciano 2006). Folates participate in one-carbon biosynthetic and

epigenetic processes that facilitate the synthesis and methylation of nucleic acids and proteins. Folate is thus needed during periods of rapid cell growth and proliferation, like in oocyte maturation. Indeed, insufficient folate intake has been shown to impair female fertility and fetal viability in several animal models, emphasising the essentiality of folate during mammalian folliculogenesis and fetal development (Mooij et al. 1992). In humans, folate deficiency may cause early spontaneous abortions, birth defects and other adverse pregnancy outcomes (George et al. 2002). Furthermore, regular use of multivitamin supplements including folate has recently been reported to decrease the risk of anovulatory infertility (Chavarro et al. 2008), and preconceptional supplementation with folate and vitamin $B_{12}$ has been found to be associated with lower incidence of miscarriages in women planning pregnancy (Zetterberg 2004). Additionally, it has been demonstrated, that preconception folic acid supplementation increases folate levels and decreases homocysteine levels in follicular fluid (Boxmeer et al. 2008). Figure 6 illustrates the interactions between nutrition, folate metabolism and female fertility.

Dietary or genetically determined folate deficiency may lead to elevate homocysteine (Hcy) concentrations (Jacques et al. 2001). High Hcy concentrations, hyperhomocysteinemia has been associated with several pathologies including pregnancy complications (summarised in (Tamura and Picciano 2006)). In folliculogenesis, hyperhomocysteinemia may activate apoptosis, thereby lead to follicular atresia (Forges et al. 2007). In fact, negative correlations between follicular fluid Hcy concentrations and oocyte maturity (Szymanski and Kazdepka-Zieminska 2003) and *in vitro* embryo quality on culture day 3 have been demonstrated (Ebisch et al. 2006). However, recently a positive correlation between follicular Hcy concentrations and the diameter of the follicle was shown (Boxmeer et al. 2008). Also, IVF outcome seems to be affected by high levels of Hcy, as pregnancy and implantation rates have been shown to be lower, and the abortion rate higher in those women following infertility treatment (Haggarty et al. 2006, Pacchiarotti et al. 2007). Additionally, a recent study on PCOS patients undergoing IVF treatment demonstrated negative correlations between follicular Hcy concentration and fertilization rate and oocyte and embryo quality, indicating further that follicular fluid Hcy may play an important role in the maturation of oocytes and fertilization (Berker et al. 2009).

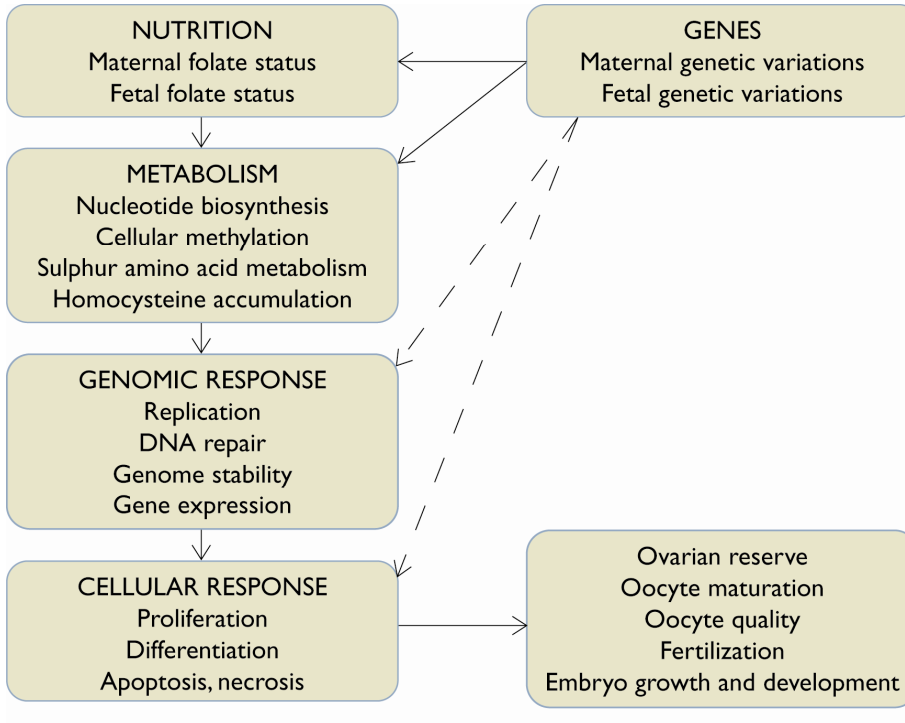


**Figure 6.** Schematic overview of factors affecting folate-metabolizing pathway and subsequently female fertility (Laanpere et al. 2009).

### 1.8.3.1. Genetics of folate-metabolizing pathway

Several variations have been identified in genes involved in the folate absorption and folate-mediated one-carbon metabolism. These polymorphisms my alter the beneficial effect of folates and other B vitamins that play role in the metabolism of methyl groups and change the flux of folate cofactors between methyl donor and nucleotide synthesis (see Figure 7 for folate-metabolizing pathway) (Narayanan et al. 2004). 5,10-methylenetetrahydrofolate reductase (*MTHFR*) gene 677C/T polymorphism seems to be the most influential and prevalent genetic variation affecting the folate metabolism. *MTHFR* gene is involved in the folate methylation cycle, where Hcy is converted to methionine. *MTHFR* 677C/T polymorphism results amino acid change at codon Ala222Val, giving rise to an unstable enzyme with reduced activity (Frosst et al. 1995), which results in the accumulation of Hcy and impaired methylation reactions (Harmon et al. 1996).

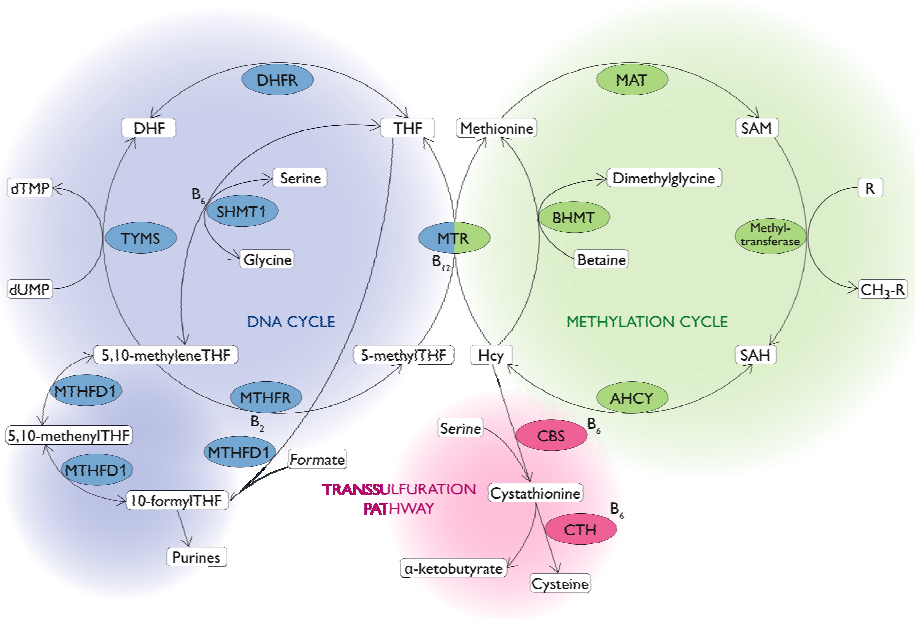


**Figure 7.** Folate-mediated one-carbon metabolism. Folate metabolism can be viewed in two intertwined cycles, DNA biosynthesis (in blue) and methylation cycle (in green). Homocysteine (Hcy) is catabolised in transsulfuration pathway (red). Respective enzyme cofactors, B vitamins $B_2$, $B_6$ and $B_{12}$, are indicated (Laanpere et al. 2009).

Among women undergoing COH, *MTHFR* 677 CC genotype carriers have been shown to require significantly less FSH administration in order to induce ovulation. In addition these patients produce more oocytes and present higher serum estradiol concentrations than 677 TT carriers (Thaler et al. 2006). However, another study detected no correlation between 677C/T variation and COH outcome (Rosen et al. 2007). Instead, they demonstrated association between *MTHFR* 1298A/C polymorphism and COH. *MTHFR* 1298 C allele was associated with higher basal FSH levels, referring to lower ovarian follicular reserve, and with diminished responses to ovarian stimulation demonstrating fewer follicles of >13 mm in size, lower estrogen levels on the day of hCG administration and higher FSH administration during the treatment (Rosen et al. 2007). Further, association between *MTHFR* 667C/T and repeated and otherwise unexplained implantation failures has been proposed, when the prevalence of 677 TT homozygotes was higher in female patients that had experienced at least four consecutive IVF implantation failures despite the transfer of at least three good quality embryos (Azem et al. 2004). A recent study on IVF patients has demonstrated that the *MTHFR* 677 CT heterozygous genotype, rather than the homozygous CC genotype, is associated with

increased chances of having had a previous IVF pregnancy and a live birth in the current IVF cycle. Meanwhile women with *MTHFR* 1298 CC genotype were less likely than ones with AA genotype to produce live birth (Haggarty et al. 2006). However, no associations between polymorphisms in MTHFR gene and IVF outcome have also reported by other studies (Dobson et al. 2007, Martinelli et al. 2003). It should be noted that majority of women undergoing infertility treatment take folate supplements, which could be one explanation for negative/contradicting results. It is commonly known that individuals carrying *MTHFR* 677 T allele have increased plasma Hcy concentrations and with additional folate administration they are able to obtain normal Hcy concentrations (Fohr et al. 2002).

# 2. AIMS OF THE STUDY

The general objective of the present study was to investigate different genetic factors that could affect *in vitro* fertilization treatment outcome in infertile women. Also importance of different polymorphisms in the etiology of female infertility was assessed. In addition, in order to add more understanding to the field of follicular biology in IVF treatment, gene expression profiles of mural granulosa cells and cumulus granulosa cells were analysed.

Accordingly, the study had the following aims:

1. To assess the influence of genetic variations in aromatase gene on ovarian stimulation outcome and the etiology of female infertility among IVF patients.

2. To study the associations between polymorphisms in estrogen receptor ESR1 and ESR2 genes and the COH outcome, as well as the importance of these variants in the etiology of female infertility.

3. To study the influence of polymorphisms in folate pathway-related genes on female infertility and on folate status among infertile women.

4. To examine the effects of polymorphisms in folate-metabolizing pathway on biochemical markers of folate metabolism (folate, vitamin $B_{12}$ and Hcy) in the serum of infertile women and to analyse the associations between genetic and biochemical folate parameters and IVF outcome.

5. To compare the gene expression profiles of mural and cumulus granulosa cells collected during the follicle puncture from recombinant FSH and GnRH antagonist-stimulated female patients undergoing IVF.

# 3. MATERIALS AND METHODS

## 3.1. Subjects, clinical studies and IVF

The studies were approved by the Ethics Committee of the University of Tartu or by the Ethics Committee of Karolinska Institutet, and the informed consent was obtained from every participant. The studies are summarised in the Table 2.

**Estonian IVF patients and IVF (Ref. I, II, IV and V)**

All IVF patients were recruited from Nova Vita Clinic from Estonia during the time period from 2004 to 2008. The study groups were as follows:

In **Study I**, 152 infertile women undergoing IVF or ICSI were recruited, with the mean age of 34.0 ± 4.9 (SD) years. According to their infertility diagnosis, they were classified into 5 groups: tubal factor infertility (n = 67, 44.1%), male factor infertility (n = 48, 31.6%), endometriosis (n = 14, 9.2%), unexplained infertility (n = 14, 9.2%), and infertility due to other reasons such as uterine myomas (n = 9, 5.9%). Women with PCOS were excluded from this study.

**Study II** consisted of 159 normally ovulating infertile women (PCOS patients were not included) undergoing IVF or ICSI, and their mean age was 34.1 ± 4.9 years. Their indications for IVF/ICSI were: tubal factor infertility (n = 71, 44.7%), male factor infertility (n = 49, 30.8%), endometriosis (n = 15, 9.4%), unexplained infertility (n = 15, 9.4%), and infertility due to other reasons (n = 9, 5.7%).

**Study IV** composed of 439 IVF female patients. The mean age of these women was 33.7 ± 4.6 years and their diagnoses of infertility were: tubal factor infertility (n = 169, 38.5%), male factor infertility (n = 111, 25.3%), PCOS (n = 50, 11.4%), endometriosis (n = 35, 8.0%), unexplained infertility (n = 31, 7.1%), and infertility due to other reasons (n = 43, 9.8%). The IVF cycle studied was the first one for 232 women (52.9%), the second for 126 women (28.7%), the third for 42 (9.5%) and the fourth or more for 39 women (8.9%).

In **Study V**, 29 infertile women undergoing ICSI procedure, aged 33.3 ± 4.1 years were included. Reasons for their infertility were: male factor infertility (n = 13), tubal factor infertility (n = 5), PCOS (n = 2), endometriosis (n = 1), and combined causes of infertility (n = 8). Besides male factor infertility in the group of combined causes of infertility, 3 women had tubal occlusions, 2 women had endometriosis, 1 woman had endometriosis and tubal occlusion, 1 woman with PCOS, and 1 woman had autoimmune thyroiditis.

Serum FSH levels were measured in all women (except Study V) between the day 3 and 5 of the spontaneous menstrual cycle using chemiluminescence immunoassay (Immulite 2000® station, Diagnostic Products Corporation, Los Angeles, CA, USA). Transvaginal ultrasound scanning of ovaries was per-

formed prior to initiation of IVF cycle during the first 5 days of the natural menstrual cycle. Ovarian volume was estimated according to the formula: $^1/_2$(A × B × C), where A presents the longitudinal diameter, B the anteroposterior diameter and C the transverse diameter of the ovary (Sample et al. 1977). The number of small antral follicles (4–7 mm) was determined by ultrasound scanning of each ovary in the longitudinal cross-section.

Ovarian hormonal stimulation for IVF was conducted accordingly to the GnRH antagonist (Cetrotide, Merck Serono, Geneva, Switzerland) or agonist (Cetrotide) protocol with the administration of recombinant FSH (Gonal-F, Merck Serono, or Puregon, Schering-Plough, Kenilworth, NJ, USA) for 9–10 days until 1 day before hCG (Ovitrelle, Merck Serono) administration. The COH follow-up included 3–4 ultrasound assessments of endometrium and follicular growth. Daily GnRH antagonist administration (0.25 mg, Cetrotide) was initiated if at least one follicle reached the size of ≥ 14 mm. The GnRH antagonists were given for 4–5 days up to and including the day of hCG administration. Final follicular maturation was achieved using 250 μg of hCG, followed by ovarian puncture 36 h later. Serum estradiol levels were also measured on the day of oocyte retrieval.

A maximum of 2 day embryos were transferred into the uterus, with vaginal progesterone (Lugesteron, Leiras, Turku, Finland) used for luteal support. Pregnancy was confirmed with a positive serum hCG test (≥ 10 IU/l) performed 14 days after the embryo transfer and the clinical pregnancy was recorded by the presence of gestational sac(s) with fetal heartbeat on transvaginal sonography at 6–7 weeks of gestation.

Multiple parameters were assessed to evaluate the efficacy of COH. The total dose of FSH used, the number of follicles punctured at oocyte retrieval, the number of cumulus-oocyte complexes obtained by oocyte retrieval and the number of mature oocytes were counted for all participants. IVF oocytes were evaluated for maturity one day after the insemination by counting the fertilised and unfertilised meiosis II oocytes. ICSI oocytes were considered mature if they reached meiosis II stage by 4 – 6 h after the oocyte retrieval. The total number of embryos included the embryos with two pronuclei and the embryo quality was considered good, when having at least 4 blastomeres and < 20% fragmentation on day 2 after insemination or ICSI.

The following parameters were calculated from the total amount of recombinant FSH (IU) used for ovarian stimulation: i) the amount of FSH used per day, ii) the amount of FSH needed to mature one ovarian puncture follicle, iii) the amount of FSH needed per one punctured oocyte, iv) FSH amount needed to obtain one mature oocyte; v) FSH amount per embryo; and vi) FSH amount per good-quality embryo. Also serum estradiol and the amount of serum estradiol per retrieved follicle were included in the assessment of COH effectiveness.

**Estonian fertile controls (Ref. IV)**
The study group of fertile Estonians consisted of 225 healthy women who have previously had four or more spontaneous pregnancies. The mean age of these women was 43.1 ± 9.1 years, the mean number of pregnancies 5.5 ± 1.6 and the mean number of live births was 3.3 ± 1.2. The genomic DNA of these women was obtained from Estonian Genome Foundation at University of Tartu.

**Swedish women with unexplained infertility (Ref. III)**
The study group consisted of 71 women with unexplained infertility, who attended the Department of Obstetrics and Gynecology, Karolinska University Hospital Huddinge from 2000 to 2007. All these women were of Swedish or Finnish origin. Their mean age was 33.1 ± 3.2 years, the mean body mass index (BMI) was 21.7 ± 2.5 kg/m$^2$, menstrual cycle length 28.4 ± 1.8 days, with 4.7 ± 0.8 days of menses. Unexplained infertility was diagnosed by means of a standard set of tests that included hormonal analyses and at least two analyses of semen from their partner. The women's partners showed normal semen analysis results according to the WHO criteria (WHO 1999). All infertile women had normal ovarian function. Their serum concentration of FSH was lower than 11 IU/L during the early follicular phase (days 2–5) of the natural cycle. All women had serum prolactin concentrations below 20 μg/L, and normal thyroid-stimulating hormone and thyroid hormone serum concentrations. Furthermore, all infertile women showed normal tubal function as demonstrated by hysterosonosalpingography and no recognizable endometriosis according to the disease symptoms, pelvic examination, ultrasonography or diagnostic laparoscopy.

**Swedish controls (Ref. III)**
The control group from Swedish population consisted of 1079 individuals from a cross-sectional population studies (Böttiger et al. 2007a, Böttiger et al. 2007b, Böttiger and Nilsson 2007, Nilsson et al. 2008). The controls were collected from the central Sweden, the same region where the infertile women were recruited.

## 3.2. Analysis of biochemical markers of folate metabolism

Fasting blood samples were analysed for folate, vitamin $B_{12}$ and total Hcy (sum of all Hcy species found in blood, e.g. free or protein-bound). Serum folate concentrations were measured by means of a solid-phase time-resolved fluoroimmunoassay based on a competitive reaction between europium-labelled pteroyl-glutamic acid and sample folate for a limited number of binding sites on folate-binding protein (AutoDelfia Folate; Wallac Oy, Turku, Finland) or with

Chemiluminescent Microparticle Folate Binding Protein assay with ARCHITECT Folate Reagent Kit (Abbott Laboratories, Chicago, IL, USA) and quantified using ARCHITECT *i* Systems (Abbott Laboratories). Vitamin $B_{12}$ concentrations were assessed by means of a fluorometric method with an Abbott IMx autoanalyser (Abbott Laboratories) or with using Chemiluminescent Microparticle Intrinsic Factor assay with ARCHITECT $B_{12}$ Reagent Kit (Abbott Laboratories) and quantified using ARCHITECT *i* Systems (Abbott Laboratories). Hcy was measured in acidified citrate plasma using a fluorescence polarization immunoassay, IMx Homocysteine Reagent Pack, and an IMx Analyzer (Abbott Laboratories). All coefficients of variation were < 7.5%.

## 3.3. Genotyping of polymorphisms

Different genotyping methods were used to analyse 17 polymorphisms in 8 different genes: *CYP19A1* $(TTTA)_n$, *CYP19A1* TCT Ins/Del, *ESR1* $(TA)_n$, *ESR1* *Pvu*II T/C (rs2234693), *ESR1* *Xba*I A/G (rs9340799), *ESR2* $(CA)_n$, *ESR2* *Rsa*I G/A (rs1256049), *MTHFR* 677C/T (rs1801133), *MTHFR* 1298A/C (rs1801131), *MTHFR* 1793G/A (rs2274976), *FOLR1* 1314G/A (rs2071010), *FOLR1* 1816C/delC (rs3833748), *FOLR1* 1841G/A (rs1540087), *FOLR1* 1928C/T (rs9282688), *TCN2* 776C/G (rs1801198), *CTH* 1208G/T (rs1021737), and *SLC19A1* 80G/A (rs1051266).

Genomic DNA was extracted from peripheral EDTA blood using the salting-out method (Aljanabi and Martinez 1997) or QIAamp DNA Blood Maxi kit (Qiagen, Venlo, the Netherlands). Prior to genotyping, polymerase chain reaction (PCR) was performed. Every locus of interest was amplified as one amplicon, except for *CYP19A1* $(TTTA)_n$ and *CYP19A1* TCT Ins/Del, that were amplified together as one amplicon.

**Restriction fragment length polymorphism (RFLP)**

We analysed polymorphisms *ESR1* *Pvu*II T/C, *ESR1* *Xba*I A/G and *ESR2* *Rsa*I G/A using RFLP method. RFLP refers to a variance between samples of homologous DNA molecules as a result of differing locations of restriction sites. In the RFLP analysis, DNA strand is digested with restriction enzyme and the obtained restriction fragments are separated according to their lengths by gel electrophoresis.

**Microsatellite analysis**

Microsatellites are polymorphic loci present in DNA that consist of repeating units of 1–6 base pair (bp). In our study, 3 microsatellites were analysed: *CYP19A1* $(TTTA)_n$ together with *CYP19A1* TCT Ins/Del polymorphism, *ESR1* $(TA)_n$ and *ESR2* $(CA)_n$. In all three variations, one of the PCR primers was fluorescently labelled that enabled the PCR product size measurement using

ABI Prism 377 automated DNA sequencer and Genescan 2.1 software (PE Applied Biosystems, Forster City, CA, USA). Rox 500 (PE Applied Biosystems) was used as an internal size standard.

**Pyrosequencing**
All the polymorphisms studied in the folate-metabolizing pathway genes as *MTHFR* 677C/T, *MTHFR* 1298A/C, *MTHFR* 1793G/A, *FOLR1* 1314G/A, *FOLR1* 1816C/delC, *FOLR1* 1841G/A, *FOLR1* 1928C/T, *TCN2* 776C/G, *CTH* 1208G/T and *SLC19A1* 80G/A, were analysed using pyrosequencing method. Pyrosequencing is a DNA sequencing method, where complementary strand of the template is enzymatically synthesised.

## 3.4. Gene expression analysis from granulosa cells

Mural GCs were obtained from follicular fluid of patients after the ovarian puncture. Additionally to mural GCs, that are shed from the follicular wall into the follicular fluid, a minority of cumulus GCs are also present, and therefore we further use the term of "floating granulosa cells (FGCs)" instead of MGCs. The fluid from all follicles was pooled, centrifuged and the supernatant was removed. The cells were separated on a 50% density gradient of PureSperm 100 (Nidacon, Mölndal, Sweden) and Universal IVF Medium (MediCult, Jyllinge, Denmark), washed in Universal IVF Medium at 37˚C, lysed with Qiagen RNeasy Mini kit lysis buffer (Qiagen, Hilden, Germany), and stored in liquid nitrogen for future use. Cumulus GCs were collected 4 h after the ovarian puncture during oocyte denudation with type IV-S hyaluronidase extracted from bovine testes (Sigma-Aldrich, St-Louis, MO, USA) that was diluted in Sperm Preparation Medium (MediCult). The CGC from all oocytes were pooled and centrifuged, the supernatant was discarded, and the cells were lysed and stored in liquid nitrogen until further use.

Total RNA from collected CGCs and FGCs was extracted using the RNeasy Micro and RNeasy Mini Kit (Qiagen), respectively, according to the manufacturer's instructions. Affymetrix GeneChip Human Gene 1.0 ST Array (consisting of 28 869 transcripts) analysis was performed according to the standard Affymetrix instructions (Affymetrix, Santa Clara, CA, USA). The obtained data were processed using the Affymetrix Microarray Suite 5.0, and the generated cel file data were further analysed with the Affymetrix Expression Console software and the Robust Multichip Analysis, followed by the analyses with the statistical software package R (http://www.r-project.org) and Bioconductor (Ihaka and Gentleman 1996). Finally, gene array data were validated using real-time PCR analysis of selected genes.

## 3.5. Statistical analysis

For statistical analyses different software was used in different studies – the R2.3.1 A Language and Environment (Free Software Foundation, Boston, MA, USA) (Ref. I, II and V); Statistical Package for Social Sciences statistical software (SPSS v. 16.0 for Macintosh; SPSS Inc., Chicago, IL, USA) (Ref. III); Haploview (version 4.1) (Ref. III and IV); and Statistical Analysis System (SAS) (Ref. IV).

Data are presented as mean ± SD, unless otherwise indicated. Nominal variables were analysed by $\chi^2$ tests. Allele frequencies were calculated to investigate the deviation from Hardy-Weinberg equilibrium. Continuous variables were tested for normal distribution and in case of deviation, squared or logarithmic transformation was performed. As all the variables distributed normally (including variables after transformation) analyses were performed using parametric tests. For all analyses, a p value $< 0.05$ was considered statistically significant.

**Table 2.** Summary of the five studies presented in the current thesis.

| Study | Subjects (n) | Studied variables |
|---|---|---|
| I | Estonian IVF patients (152) | *CYP19A1* TCT Ins/Del<br>*CYP19A1* $(TTTA)_n$<br>COH and IVF outcome<br>Female infertility |
| II | Estonian IVF patients (159) | *ESR1 Pvu*II T/C<br>*ESR1 Xba*I A/G<br>*ESR1* $(TA)_n$<br>*ESR2 Rsa*I G/A<br>*ESR2* $(CA)_n$<br>COH and IVF outcome<br>Female infertility |
| III | Swedish infertile women (71)<br>Swedish controls (1079) | *MTHFR* 677C/T<br>*MTHFR* 1298A/C<br>*MTHFR* 1793G/A<br>*FOLR1* 1314G/A<br>*FOLR1* 1816C/delC<br>*FOLR1* 1841G/A<br>*FOLR1* 1928C/T<br>*TCN2* 776C/G<br>*CTH* 1208G/T<br>*SLC19A1* 80G/A<br>Unexplained female infertility<br>Blood folate<br>Blood $B_{12}$<br>Blood Hcy |
| IV | Estonian IVF patients (439)<br>Estonian fertile women (225) | *MTHFR* 677C/T<br>*MTHFR* 1298A/C<br>*MTHFR* 1793G/A<br>*FOLR1* 1314G/A<br>*FOLR1* 1816C/delC<br>*FOLR1* 1841G/A<br>*FOLR1* 1928C/T<br>*TCN2* 776C/G<br>*CTH* 1208G/T<br>*SLC19A1* 80G/A<br>COH and IVF outcome<br>Female infertility<br>Blood folate<br>Blood $B_{12}$<br>Blood Hcy |
| V | Estonian IVF-ICSI patients (29) | Gene expression profile of FGCs<br>Gene expression profile of CGCs |

# 4. RESULTS

## 4.1. Factors influencing COH and pregnancy outcome as well as blood biochemical markers in infertile women (Ref. I, II, III, IV)

### 4.1.1. Woman's age and clinical factors

Woman's age is a major parameter influencing the outcome of IVF treatment. The mean age of the Estonian IVF patients in our groups of male factor infertility, endometriosis and unexplained infertility were similar to the age of women in the reference group with tubal factor infertility. Only women with infertility due to other reasons were significantly older (mean age 39.2 ± 6.7 *vs* 33.6 ± 3.9 years, $p < 0.05$, data from Ref. II) than the reference group.

Among the Estonian IVF patients, the ovarian follicular reserve assessment demonstrated that the mean ovarian volume was 4.9 ± 2.1 $cm^3$, the mean number of early antral follicles 4.5 ± 1.4 and the mean concentration of serum FSH 9.3 ± 5.3 IU/l (data from Ref. I). Following the ovarian stimulation with exogenous FSH administration (data from Ref. II), an average of 1909.1 ± 503.3 IU of FSH was administered for the stimulation. The mean number of obtained follicles per patient was 14.0 ± 6.6, oocytes 12.1 ± 6.6 and mature oocytes 9.9 ± 5.5. After fertilization, an average the women possessed 6.9 ± 4.0 embryos with two pronuclei and out of them 42.1 ± 29.3% developed into good-quality day 2 embryos. In total, the mean dose of FSH that was needed to obtain one follicle was 200.4 ± 206.5 IU, one retrieved oocyte 254.4 ± 255.5 IU, one mature oocyte 324.4 ± 339.7 IU, one embryo with two pronuclei 393.9 ± 342.6 IU, and one good-quality embryo 866.0 ± 696.0 IU. The mean serum estradiol concentration per patient was 4235.0 ± 5090.1 pmol/l and the mean estradiol per follicle was 305.9 ± 294.7 pmol/l.

Linear regression models showed that both, the number of follicles detected on the early menstrual cycle (day 3 – 5) and the age of the woman were important predictors of the total dose of FSH used for ovarian stimulation. With every additional follicle at the early stage of natural cycle 147.8 less units of FSH was needed (regression coefficient $b = –147.8$, $p < 0.05$), and with every additional year 30.8 units more of FSH was needed in COH ($b = 30.8$, $p < 0.05$). In all patients studied, the number of ovarian puncture follicles correlated positively with the follicle count at the early follicular phase of a patient's spontaneous menstrual cycle (Pearson's correlation coefficient $r = 0.55$, $p < 0.05$) and decreased as the women aged ($r = –0.1$, $p < 0.05$). Similarly, the increasing female age was negatively correlated with the number of oocytes obtained ($r = –0.4$, $p < 0.05$), mature oocytes ($r = –0.3$, $p < 0.05$), and embryos with two pronuclei ($r = –0.2$, $p < 0.05$). In addition, as the women



12

aged, the amount of FSH required to obtain one oocyte ($b = 16.2$, $p < 0.05$), a mature oocyte ($b = 16.5$, $p < 0.05$), an embryo with two pronuclei ($b = 19.0$, $p < 0.05$), and a good quality embryo ($b = 30.7$, $p < 0.05$) increased (FSH units that were needed per every additional year are the respective values of b) (data from Ref. II). These obtained results clearly indicated that age is an important factor in the IVF treatment outcome. Therefore all following analyses were adjusted for age. However, among our study group of Estonian infertile women, folate and Hcy concentrations were not associated with IVF patients' age, whereas mean vitamin $B_{12}$ levels appeared to be significantly higher in older women, where with every additional year the women demonstrated 2.681 pmol/l higher $B_{12}$ concentrations ($b = 2.681$, $p < 0.05$).

### 4.1.2. Variations in aromatase gene

In aromatase gene, $(TTTA)_n$ microsatellite polymorphism and TCT trinucleotide insertion/deletion (Ins/Del) were studied in Estonian IVF patients and their influence on IVF outcome was assessed using linear and logistic regression models. We detected negative correlation between *CYP19A1* $(TTTA)_n$ biallelic mean and the amount of FSH used to mature one follicle at the ovarian puncture. Whereby, every added tetranucleotide repeat decreased the FSH dose needed to mature one follicle by 18.38 IU ($b = -18.38$, $p < 0.05$) (Figure 8).

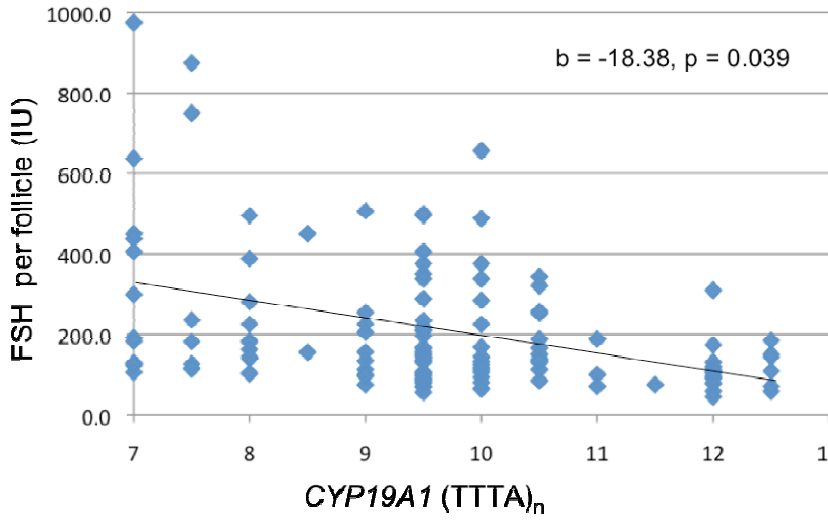


**Figure 8.** Biallelic means of *CYP19A1* $(TTTA)_n$ and the amount of FSH (IU) used to obtain one ovarian puncture follicle at IVF treatment.

Infertile women with Ins/Del and Ins/Ins genotypes needed lower FSH doses to mature one ovarian puncture follicle than women with the Del/Del genotype (180.4 ± 146.7 and 169.8 ± 133.7 IU *vs* 262.3 ± 259.0 IU, $p < 0.05$) and to obtain one mature oocyte (266.6 ± 242.4 and 294.9 ± 272.4 IU *vs* 496.8 ± 568.0 IU, $p < 0.05$) (Figure 9).

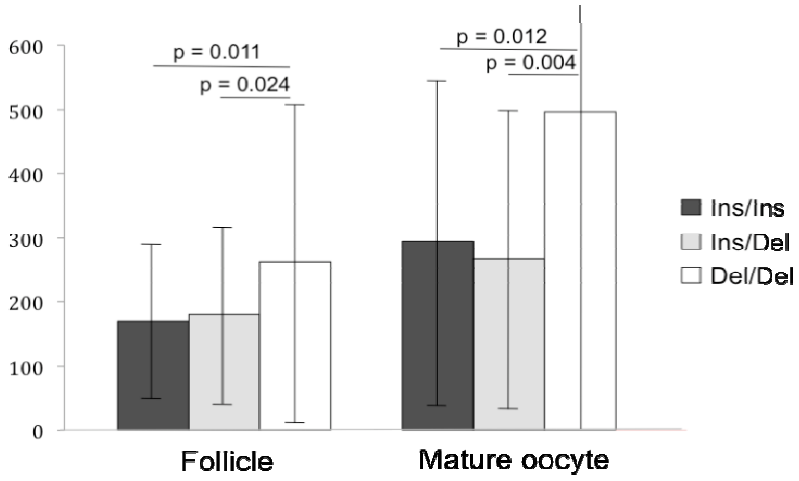


**Figure 9.** *CYP19A1* TCT Ins/Del genotypes and the amount of FSH (IU) used to mature one ovarian puncture follicle and one mature oocyte.

### 4.1.3. Variations in estrogen receptor genes *ESR1* and *ESR2*

Microsatellite polymorphism $(TA)_n$ and SNPs *Pvu*II T/C and *Xba*I A/G in *ESR1* gene, and $(CA)_n$ microsatellite and *Rsa*I G/A polymorphism in *ESR2* gene were studied in Estonian women undergoing IVF procedure and their influence on COH and IVF outcome were evaluated using linear and logistic regression models. A positive correlation between the *ESR1* $(TA)_n$ biallelic mean and the number of follicles matured ($r = 0.5$, $p < 0.05$) as well as the ovarian puncture oocytes obtained ($r = 0.4$, $p < 0.05$) were detected among infertile women (Figure 10).

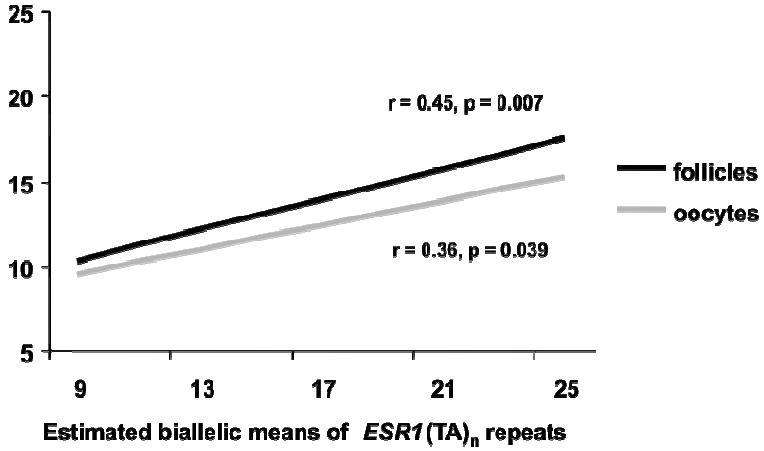


**Figure 10.** Longer *ESR1* $(TA)_n$ microsatellites predict a better outcome of COH, as more follicles and oocytes were obtained in women with longer $(TA)_n$ repeats.

Similarly, in patients with the *ESR1 Pvu*II CC genotype more follicles developed after the ovarian stimulation than in patients carrying the TT genotype ($15.8 \pm 6.4$ *vs* $12.9 \pm 6.4$, $p < 0.05$) (Figure 11). Furthermore, the *ESR1 Pvu*II CC genotype tended to be associated with a higher number of obtained oocytes compared to the TT genotype ($13.4 \pm 6.3$ *vs* $11.2 \pm 6.2$, $p = 0.052$) (Figure 11). Meanwhile more FSH was needed to obtain one good quality embryo in women with the wild-type TT genotype than in the TC and CC genotypes (1078.2 ± 703.8 IU *vs* 727.4 ± 649.3 and 838.1 ± 716.5 IU, respectively ($p < 0.05$).

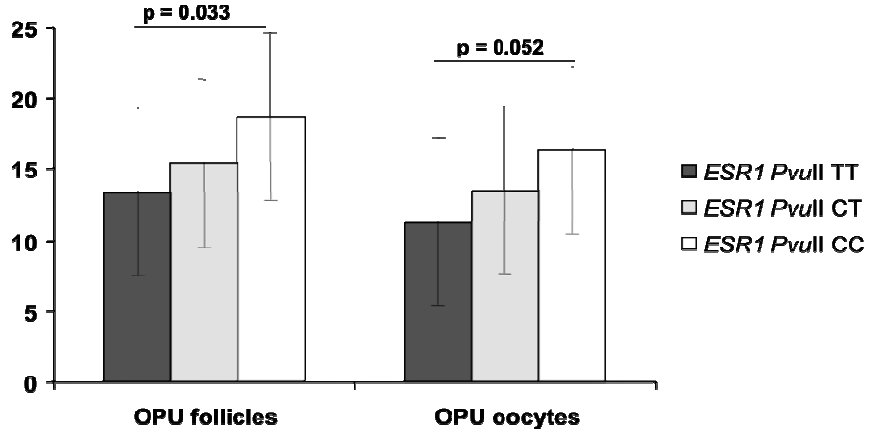


**Figure 11.** *ESR1* PvuII C allele predicts a better outcome of COH, as more follicles and oocytes are retrieved after the ovarian stimulation.

### 4.1.4. Variations in genes involved in folate-metabolizing pathway

Ten polymorphisms in genes involved in folate-metabolizing genes (*MTHFR* 677C/T, *MTHFR* 1298A/C, *MTHFR* 1793G/A, *FOLR1* 1314G/A, *FOLR1* 1816C/delC, *FOLR1* 1841G/A, *FOLR1* 1928C/T, *TCN2* 776C/G, *CTH* 1208G/T, and *SLC19A1* 80G/A) in association with ovarian stimulation and pregnancy outcome were studied among Estonian IVF patients. Logistic regression models were used to estimate the associations between the studied polymorphisms and COH and IVF pregnancy outcomes, and the associations between polymorphisms and the number of previous failed IVF attempts and previous IVF failure percentage. All models were adjusted for patient age, number of ovaries and etiology of infertility; COH outcome analyses were adjusted also for the stimulation protocol and the IVF outcome for the number and quality of transferred embryos.

Several genetic variants demonstrated associations with IVF outcome, which has been summarised in Table 3. Following the ovarian stimulation with FSH administration, estradiol level on the day of ovarian puncture was significantly higher in *FOLR1* 1928C/T heterozygotes compared to the patients with the major CC genotype (4017.69 ± 270.00 *vs* 3809.78 ± 235.36, $p < 0.05$). Also, estradiol level was significantly higher in *SLC19A1* AA minor genotype carriers compared to the GA heterozygotes (4533.94 ± 386.17 *vs* 3617.18 ± 239.88, $p < 0.05$). There was a trend for women with *MTHFR* 1298 CC genotype to require more FSH per oocyte (393.35 ± 59.57) compared to the major homozygous AA (271.29 ± 23.64, $p = 0.058$) and heterozygous AC (271.49 ± 25.24, $p = 0.061$) individuals. Also women heterozygous for *FOLR1* 1314G/A polymorphism required significantly more FSH to obtain one oocyte than patients with the major GG genotype (438.62 ± 49.40 *vs* 262.43 ± 17.41, $p < 0.05$). The proportion of good quality embryos was significantly higher in patients heterozygous for *MTHFR* 677C/T compared to the major CC homozygotes (48.36 ± 2.69% *vs* 41.03 ± 2.27%, $p < 0.05$).

After the embryo transfer, the pregnancy (positive hCG test) as well as clinical pregnancy rates after IVF/ICSI procedure were significantly higher in patients with *MTHFR* 677 heterozygous CT genotype compared to the major CC genotype (adjusted odds ratio, aOR = 1.651, $p < 0.05$ and aOR = 1.865, $p < 0.05$, respectively). Meanwhile, women having heterozygous CdelC and GA genotypes of the linked *FOLR1* polymorphisms 1816C/delC and 1841G/A demonstrated higher risk for pregnancy loss (aOR = 6.017, $p < 0.05$) than major CC – GG homozygotes.

The analysis of previous IVF treatment failure in different genotype groups demonstrated that the mean number of previous failed IVF treatments was significantly higher among *CTH* 1208 TT minor genotype carriers compared to the GT heterozygotes (2.07 ± 0.23 *vs* 1.48 ± 0.12, $p < 0.05$), as well as in

*SLC19A1* GG major genotype carriers compared to the GA heterozygotes (1.93 ± 0.13 *vs* 1.45 ± 0.11, $p < 0.05$). Further, the percentage of previous failed IVF treatments was significantly higher in women with *MTHFR* 1793 GG major homozygous genotype compared to the GA heterozygotes (94.13 ± 1.63% *vs* 79.21 ± 6.20%, $p < 0.05$).

**Table 3.** Heterozygous polymorphisms in folate pathway genes and associations with IVF parameters and biochemical markers of folate metabolism. [a]Heterozygous genotype compared to the major (wild-type) genotype, [b]heterozygous genotype compared to the minor genotype.

| Variation | Phenotype |
|---|---|
| *MTHFR* 677 CT | ↑ good quality embryos[a] |
| | ↑ pregnancy rate[a,b] |
| | ↓ blood folate[a] and ↑ blood folate[b] |
| | ↑ blood Hcy[a] and ↓ blood Hcy[b] |
| *MTHFR* 1298 AC | ↓ FSH per oocyte[b] |
| *MTHFR* 1793 GA | ↓ previous IVF failure[a] |
| | ↑ blood folate[a] |
| *FOLR1* 1314 GA | ↑ FSH per oocyte[a] |
| | ↓ previous IVF failure[a] |
| | ↑ blood folate[a] |
| *FOLR1* 1816-1841 CdelC-GA | ↑ pregnancy loss[a] |
| *FOLR1* 1928 CT | ↑ estrogen at ovarian puncture[a] |
| *TCN2* 776 CG | ↑ blood folate[a] and ↓ blood folate[b] |
| *CTH* 1208 GT | ↑ chance of pregnancy[a] |
| | ↓ previous IVF failure[b] |
| *SCL19A1* 80 GA | ↓ estrogen at ovarian puncture[b] |
| | ↓ previous IVF failure[a] |
| | ↑ blood Hcy[a] |

### 4.1.5. Variations in folate pathway genes and the biochemical markers of the folate metabolism in IVF patients and women with unexplained infertility

The effect of polymorphisms in genes involved in folate-metabolizing genes (*MTHFR* 677C/T, *MTHFR* 1298A/C, *MTHFR* 1793G/A, *FOLR1* 1314G/A, *FOLR1* 1816C/delC, *FOLR1* 1841G/A, *FOLR1* 1928C/T, *TCN2* 776C/G, *CTH* 1208G/T, and *SLC19A1* 80G/A) on blood serum concentrations of folate, vitamin $B_{12}$ and Hcy in Estonian IVF patients and in Swedish women with unexplained infertility were assessed. The difference in mean concentrations of the folate biomarkers between the studied genotypes, and the effect of the biochemical markers on IVF outcome among Estonian IVF patients were analysed using general linear and logistic regression models. Among study

group of unexplained infertile women one-way analysis of variance (ANOVA) was used, and the mean concentrations of folate and $B_{12}$ in genotype subgroups were compared by using Tukey's test, meanwhile the effects of polymorphisms on Hcy concentrations were calculated by using analysis of covariance (ANCOVA) after adjusting for folate and age.

In the Estonian study group of infertile women undergoing IVF, serum folate concentrations were negatively associated with Hcy ($r = –0.435$, $p < 0.05$), noted also among our Swedish women with unexplained infertility ($r = –0.532$, $p < 0.05$). Vitamin $B_{12}$ concentrations were positively associated with folate ($r = 0.229$ $p < 0.05$) and negatively with Hcy concentrations ($r = –0.252$ $p < 0.05$) in Estonian IVF patients.

The analysis of serum folate, $B_{12}$ and Hcy concentrations between studied genotypes in folate-metabolizing pathway genes among Estonians demonstrated that the mean serum folate levels were significantly lower in *MTHFR* 677 CT heterozygous and *MTHFR* 677 TT homozygous subjects when compared to the major CC genotype ($11.65 \pm 0.65$ and $10.72 \pm 1.35$ *vs* $13.71 \pm 0.61$, $p < 0.05$) (Figure 12, see also Table 3). Meanwhile folate concentrations were higher in women with *MTHFR* 1793 GA genotype than in the major GG genotype carriers ($16.82 \pm 1.93$ *vs* $12.33 \pm 0.45$, $p < 0.05$). Also, in folate receptor gene, *FOLR1* 1314 GA heterozygotes showed higher serum folate levels than individuals with the major GG genotype ($15.26 \pm 1.25$ *vs* $12.22 \pm 0.45$, $p < 0.05$). Further, patients with *TCN2* 776 GG genotype had significantly higher serum folate levels than the patients with the major CC genotype ($14.13 \pm 1.00$ *vs* $11.48 \pm 0.76$, $p < 0.05$).

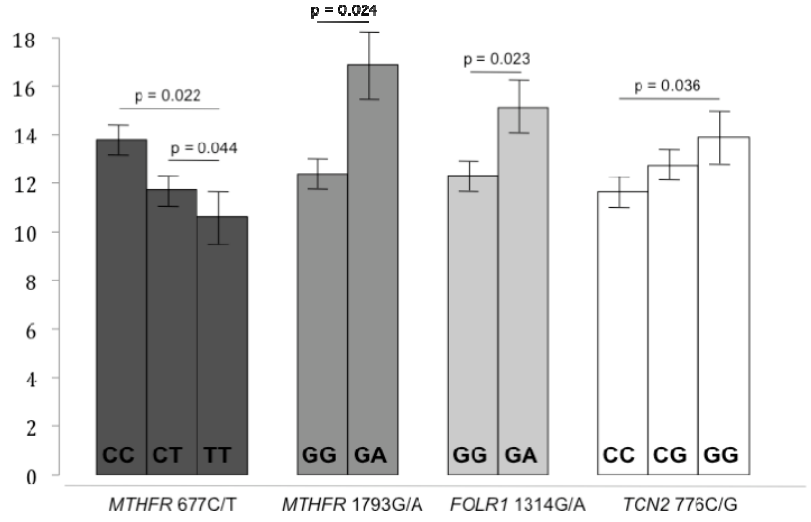


**Figure 12.** Serum folate concentrations differ significantly ($p < 0.05$) between genotypes of *MTHFR* 677C/T, *MTHFR* 1793G/A, *FOLR1* 1314G/A, and *TCN2* 776C/G polymorphisms in the folate-metabolizing pathway genes in Estonian IVF patients.

In our study group of IVF patients, Hcy concentrations were affected by *MTHFR* 677C/T polymorphism as individuals with the minor *MTHFR* 677 TT genotype had significantly higher Hcy concentrations than CC homozygotes (10.91 ± 0.44 *vs* 8.77 ± 0.20, $p < 0.05$) and CT heterozygotes (10.91 ± 0.44 *vs* 9.23 ± 0.22, $p < 0.05$) (Figure 13, see also Table 3). Also, patients carrying one *SLC19A1* 80G/A mutated allele had significantly higher serum Hcy levels compared to the wild-type GG homozygotes (9.57 ± 0.21 *vs* 8.68 ± 0.25, $p < 0.05$).

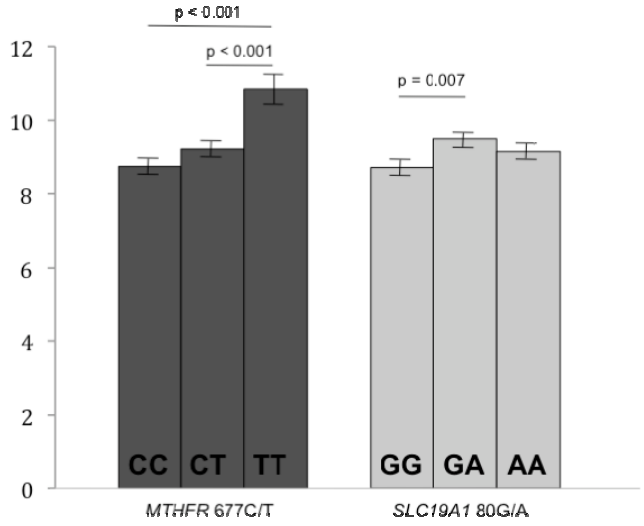


**Figure 13.** Genotypes of *MTHFR* 677C/T and *SLC19A1* 80G/A polymorphisms demonstrate significant differences in Hcy concentrations in infertile women undergoing IVF treatment ($p < 0.05$).

In Swedish infertile women no correlation between the studied polymorphisms in folate pathway genes and serum folate and plasma $B_{12}$ concentrations were detected. Hcy concentrations were affected only by *CTH* 1208G/T polymorphism, where the minor TT genotype had rising effect on homocysteine levels when compared to the GG and GT genotypes (11.50 ± 1.20, 7.88 ± 0.57 and 8.11 ± 0.48, respectively, $p < 0.05$) (Figure 14). Contrary to the expectations, no effect of variations in *MTHFR* gene on homocysteine concentrations were detected, which could be explained by the fact that 83% of women with unexplained infertility were taking folate supplements while recruited into our study.

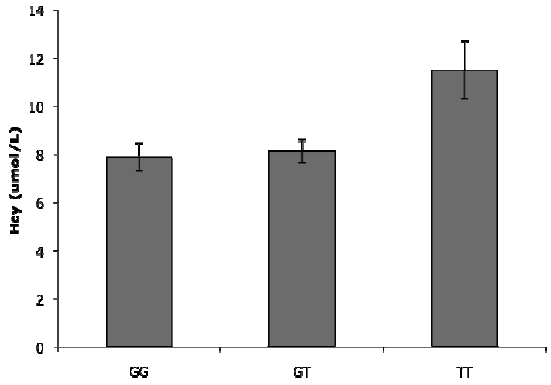


**Figure 14.** *CTH* 1208G/T polymorphism affects Hcy concentrations in the blood. Women with the TT genotype demonstrated significantly higher Hcy values than women with GG and GT genotypes ($p < 0.05$).

## 4.2. Variations and etiology of female infertility (Ref. I, II, III and IV)

### 4.2.1. Aromatase gene

The influence of polymorphisms in aromatase gene, *CYP19A1* $(TTTA)_n$ and *CYP19A1* TCT Ins/Del, on the etiology of infertility among Estonian IVF patients was assessed. Linear regression models were used to examine the associations between $(TTTA)_n$ biallelic means and the cause of female infertility, and logistic regression models were applied for the associations between Ins/Del variation and the etiology of infertility. Patients with endometriosis demonstrated significantly shorter biallelic mean of *CYP19A1* $(TTTA)_n$ repeats when compared to the control group of women with tubal factor infertility ($8.3 \pm 1.1$ *vs* $9.1 \pm 1.4$ repeats, $p < 0.05$) (Figure 15). In the case of *CYP19A1* Ins/Del variation, the presence of Del allele appeared as a genetic risk factor for women with unexplained infertility when compared to the tubal factor infertility group (Del/Del and Ins/Del genotype frequencies of 78.6% in unexplainably infertile women *vs* 49.3% in the reference group, OR = 3.78, $p = 0.056$), and further correction of the model for the early-follicular-phase serum FSH concentrations showed significant association (OR = 4.33, $p < 0.05$).

14

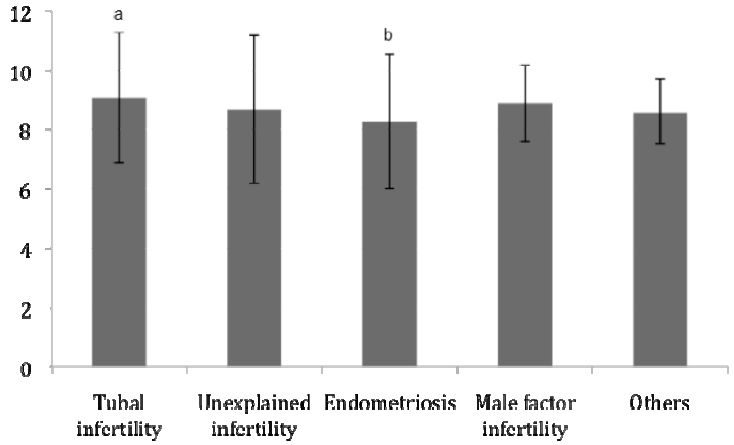


**Figure 15.** Women with endometriosis presented significantly shorter biallelic mean of *CYP19A1* $(TTTA)_n$ microsatellite ($^b$) compared to the reference group with tubal factor infertility ($^a$) ($p < 0.05$).

### 4.2.2. Estrogen receptors *ESR1* and *ESR2*

In order to evaluate the influence of variations in estrogen receptor genes, *ESR1 Pvu*II T/C, *ESR1 Xba*I A/G, *ESR1* $(TA)_n$, *ESR2 Rsa*I G/A, and *ESR2* $(CA)_n$ on the etiology of infertility among Estonian IVF patients, linear regression models and logistic regression analysis were applied. Women with unexplained infertility presented significantly shorter *ESR1* $(TA)_n$ biallelic mean compared to the reference group of women with tubal factor infertility (15.4 ± 3.1 *vs* 17.3 ± 3.0 repeats, $p < 0.05$) (Figure 16).

Among study groups of tubal factor infertility, male factor infertility, endometriosis and infertility due to other reasons all the variations studied in *ESR1* and *ESR2* genes distributed similarly. However, following COH, women with endometriosis tended to have fewer embryos than patients in the reference group of tubal factor infertility (4.7 ± 4.1 *vs* 7.6 ± 4.1, $p = 0.054$). Further, significantly more FSH was needed to mature one follicle in women with endometriosis than in women with tubal factor infertility (371.0 ± 466.8 *vs* 171.5 ± 135.7 IU FSH, $p < 0.05$). Patients with endometriosis needed also more FSH to obtain one oocyte (430.1 ± 511.9 *vs* 225.7 ± 198.3 IU FSH, $p < 0.05$), one mature oocyte (580.3 ± 552.5 *vs* 290.0 ± 366.5 IU FSH, $p < 0.05$), and one embryo with two pronuclei (646.3 ± 702.9 *vs* 342.9 ± 248.1 IU FSH, $p < 0.05$).

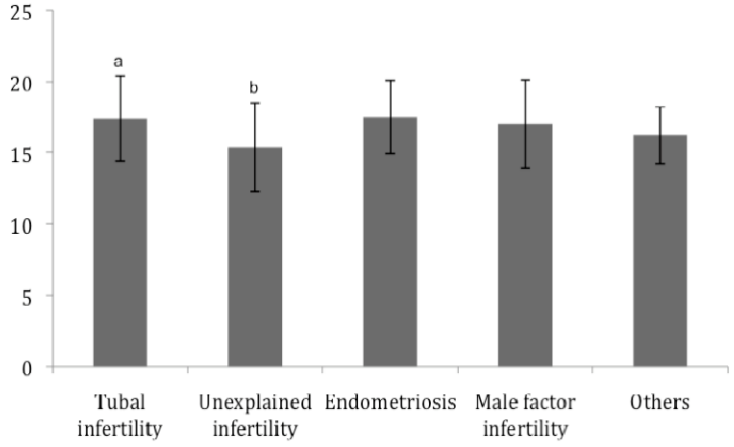


**Figure 16.** Women with unexplained infertility presented significantly shorter biallelic mean of *ESR1* $(TA)_n$ microsatellite ([b]) compared to the reference group of women with tubal factor infertility ([a]) ($p < 0.05$).

### 4.2.3. Folate pathway metabolizing genes

In folate-metabolizing genes ten polymorphisms were analysed, *MTHFR* 677C/T, *MTHFR* 1298A/C, *MTHFR* 1793G/A, *FOLR1* 1314G/A, *FOLR1* 1816C/delC, *FOLR1* 1841G/A, *FOLR1* 1928C/T, *TCN2* 776C/G, *CTH* 1208G/T, and *SLC19A1* 80G/A, and the influence of these variations on the etiology of female infertility was assessed in Estonian and Swedish infertile women. Fisher's exact test was used to estimate the genotype frequencies between Estonian IVF patients and fertile women, while the comparison between Swedish infertile women and controls was accomplished using $\chi^2$ test.

No significant difference in the genotype prevalence of studied folate pathway genes was detected when all Estonian IVF patients were compared to the fertile controls. However, when considering only IVF patients that had experienced ≥ 3 previous failed IVF attempts, significant difference in CTH 1208G/T genotype distribution was detected compared to controls. The GG genotype was present in 41.8% of controls and 50.0% in infertile women with ≥ 3 previous IVF failures, the GT genotype in 47.5 and 27.8%, and the TT genotype in 10.7 and 22.2%, respectively. The odd for an individual carrying CTH 1208 GT genotype to belong to the group of fertile controls rather than to the infertile women with ≥ 3 failed IVF treatments was 3.6 times greater than in case of the TT genotype (OR = 3.567, $p < 0.05$). Also women with SLC19A1 80GA genotype were less likely to belong to the patient group of ≥ 3 failed IVF treatments than women with GG genotype (OR = 0.388, $p > 0.05$).

In Swedish study groups, significant differences in allele frequencies were detected in polymorphisms *MTHFR* 1793G/A, with G allele prevalence of 99.2% in infertile women and 95.3% in controls (OR = 6.842, $p < 0.05$) and in *SLC19A1* 80G/A, with G allele frequency of 59.7% among infertile women and 55.8% in controls (OR = 1.180, $p < 0.05$). A significant difference in genotype distribution between the study groups was noticed in *SLC19A1* 80G/A, where the GG genotype was represented in 32.9% of the controls and 35.7% of the infertile women, the GA genotype in 45.8% and 48.2% and the AA genotype in 21.3% and 16.1%, respectively ($p < 0.05$). The frequencies of the heterozygous and variant homozygous genotypes of the studied polymorphisms are shown in Figure 17. Significant differences in the frequencies of heterozygous genotypes between controls and infertile women were detected as regards of polymorphisms *MTHFR* 677C/T (43.6% and 32.4%, respectively, OR = 1.616, $p < 0.05$) and *MTHFR* 1793G/A (9.1% and 1.4%, respectively, OR = 7.011, $p < 0.05$). A significant difference in the frequencies of minor homozygous genotypes between the study groups was noticed with polymorphism *SLC19A1* 80G/A; AA genotype frequencies being 21.3% in controls and 16.1% in infertile women (OR = 1.417, $p < 0.05$).

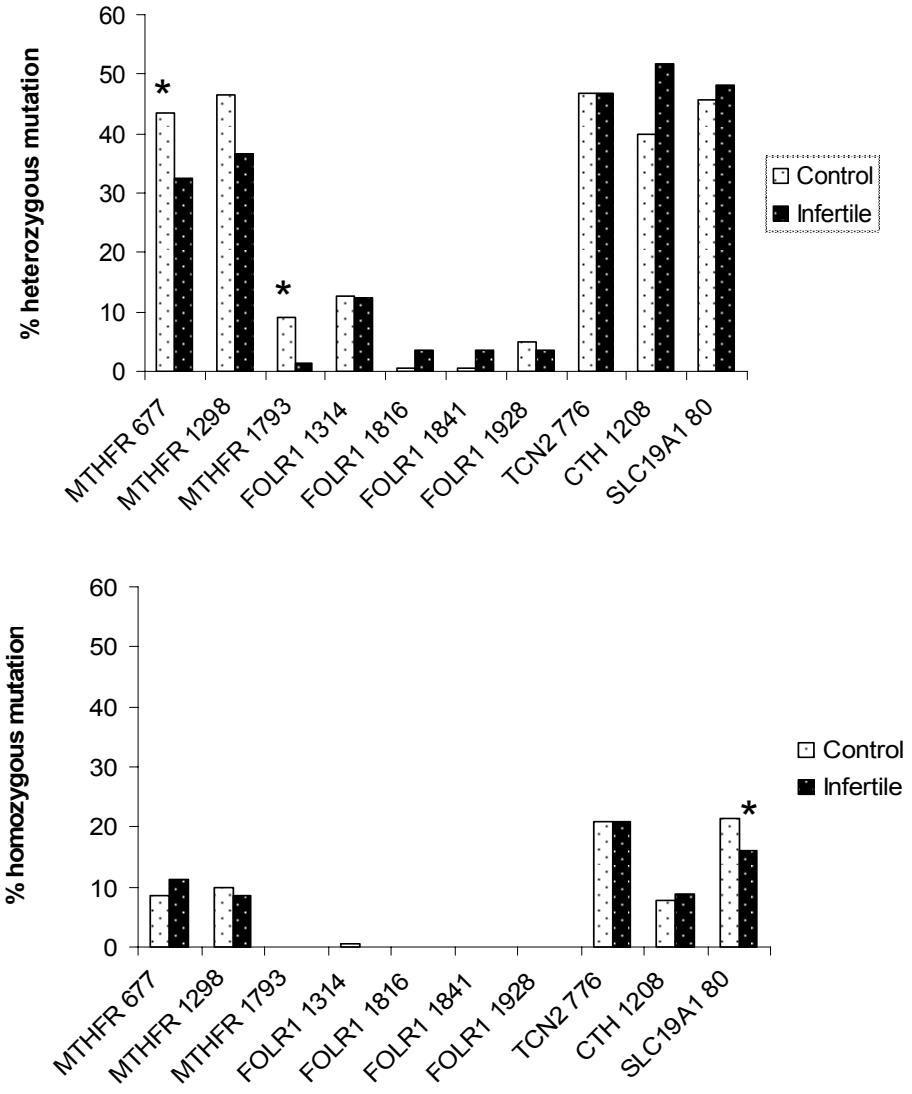


**Figure 17.** Percentages of subjects heterozygous and homozygous for polymorphisms in folate-metabolizing pathway genes among controls from a Swedish population in comparison with women with unexplained infertility. Heterozygosity was compared with both homozygous genotypes. Minor allele homozygosity was compared with heterozygous and major homozygous genotypes. *Statistically significant difference in genotype frequencies between study groups ($p < 0.05$).

## 4.3. Floating and cumulus granulosa cell gene expression profile in IVF patients at the time of follicle puncture (Ref. V)

In mature follicles, granulosa cells divide into two distinct populations of mural GCs that envelope the antrum and cumulus GCs that surround the oocyte. Both cell types, with mural cells in excess, contribute to the floating GCs population in the follicular fluid. We collected FGCs from the follicular fluid in women undergoing IVF-ICSI procedure and CGCs were obtained from the same women after oocyte denudation for micromanipulation. Gene expression analysis of both granulosa cell populations was conducted using the genome-wide Affymetrix transcriptome array. Paired t-test was applied for the array data analysis. The gene expression profile of these two cell groups was considerably different (Figure 18); out of 28 869 transcripts 4 480 were significantly differently expressed (q, corrected p value after multiple testing, $< 0.001$) and 489 of the transcripts demonstrated $\geq$ 2-fold difference in the expression level (222 genes with higher expression level in FGCs and 267 genes in CGCs, respectively). Genes with higher expression in FGCs were involved in immune response, haematological system function and organismal injury, meanwhile genes with elevated expression in CGCs were involved in protein degradation and nervous system function. In both cell populations, transcripts involved in cell-to-cell signalling and interaction pathways were detected.

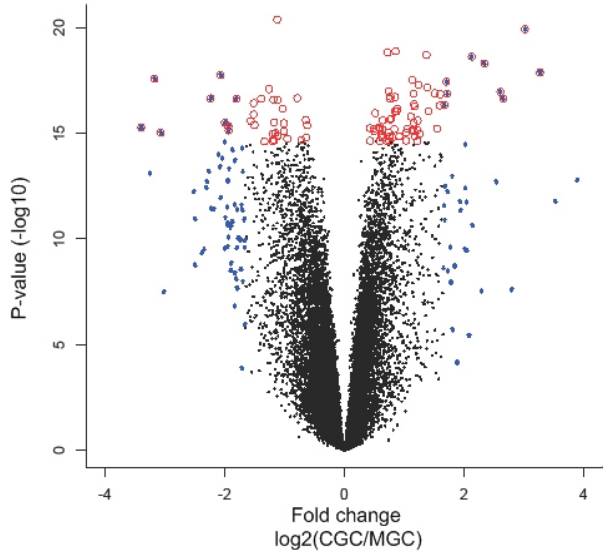


**Figure 18.** Volcano plot depicting all the transcripts grouped by fold difference an p values in gene expression levels between the FGCs and CGCs. Coloured points refer to top 100 transcripts according to fold change (blue diamonds) and p value (red circles).

Based on the gene ontology database and previously published literature we categorised differently expressed transcripts into groups (Table 4). As many interesting genes were under the cut-off value of 2-fold change, we included into the data search genes with ≥ 1.3-fold change. We found different transcripts to be involved in folliculogenesis, ovulation, and oocyte function, as well as in diverse cellular events like steroidogenesis, hormonal signalling, prostaglandin synthesis, cellular adhesion, formation and modulation of the extracellular matrix, cell cycle control, angiogenesis, apoptosis, immune response, retinoid metabolism, cell migration, Wnt signalling, ion binding and transport, G-coupled receptor signalling, transcription control, and metabolism and homeostasis. In FGCs, molecules involved in apoptosis were mostly detected, meanwhile cell-to-cell mediated gap junction proteins and Wnt signalling proteins were up-regulated in the list of CGC specific transcripts. Meanwhile, various transcripts involved in general homeostasis and metabolism were detected in both cell populations. In addition, we identified several novel transcripts that have not been associated with follicular physiology earlier.

**Table 4.** List of significantly differentially expressed transcripts (≥ 1.3-fold) grouped according to their function on the basis of published literature and the gene ontology database. A: Differentially up-regulated genes in floating granulosa cells, B: Differentially up-regulated genes in cumulus granulosa cells. Transcripts that did not have a described function or expression data from the ovaries and did not fall under any available category were listed as "unknown function in ovary".

**A**

| FLOATING GRANULOSA CELLS | |
|---|---|
| Functional category | Genes |
| Folliculogenesis/ Cell differentiation | A2M, ANPEP, BHLHB2, BTG2, DUSP6, EPB41L4B, EVI2B, FOSB, ID2, ID4, NR1D1, PLD2, RARRES3, SLC16A7 |
| Ovulation | EGR1, EREG, FGG, NTS, NRIP1, PAI1, |
| Steroidogenesis | ACVR1B, AHR, CYP17A1, CYP11A1, FST, HSD17B1, NR4A1–3, OSBPL6, SCP2 |
| Hormone receptors | ESR1 |
| Prostaglandin synthesis | COX2, PLA2G4A, PTGES, PTGER4 |
| Extra-cellular matrix molecules | FREM2, PTX3, TSG6, |
| Extra-cellular matrix proteases | ADAMTS1, ADAMTS12, CTSL1, MMP9, MMP10, MMP15 |
| Cell cycle | ESCO2, KLF4, PSMB9 |
| Angiogenesis | CALCRL, COL15A1, EDG7, EGR3, ENPP2, FGL2, LYVE1, TRIB1, VCAM1 |
| Apoptosis | AXUD1, EGR2, FRAG1, MSR1, TEK, TNFRSF21, |
| Immune response | CCL20, CD14, CTSC, CYBB, ENPP3, HLA-DPA1, IFI30, IL1B, IL6, IL18, LYZ, MSR1, NFKBIZ, S100A8, SOCS3, TRAF3, TRAM2, TREM1, TRIM22, TLR1,2,3,4,5,8, UCP2 |
| Retinoid metabolism | AOX1, BCDO2, DHRS9, RARRES2 |
| Cell migration | ARHGDIB, DOCK8, PIK3C2B, SHC4 |
| Ion binding and transport | KCNMA1, TMEM16E, TMEM37 |
| G-coupled receptor signalling | GPR109B, RGS18 |
| Transcription factors/mediators | CITED1, HIST2H2BE, NPAS2 |
| Metabolism and homeostasis | ACADSB, ACSS3, AK7, APOA1, GCNT4, GM2A, GK5, HDC, LAPTM5, MBOAT5, MOCOS, MOSC2 |
| Unknown function in ovary | ANKRD22, PGA5, PGA3, PPM1L, PLEK, RBMS3, PPBP, EVI2B, MCC, DEPDC6, PGAP1, EPB41L4B, PHEX |

**B**

| CUMULUS GRANULOSA CELLS | |
|---|---|
| Functional category | Genes |
| Folliculogenesis/ Cell differentiation | ABLIM3, ADM, BEX1, BICC1, DLX5, EPHA5, EPHB1, FABP3, LTBP1, NDRG2 |
| Oocyte development | BDNF, IGFBP5 |
| Steroidogenesis | AKR1C1, CYP1B1, CYP19A1, GAL, GSTA1 |
| Hormone receptors | AR, PGR |
| Prostaglandin synthesis | PLA2G4B, PLA2G10, PTGER2 |
| Adhesion/cell-cell attachment | ASAM, GJA1, GJA5, GJA7 |
| Extra-cellular matrix molecules | CD44, CILP, EPYC, FBLN5, FN1, HAS2, HMCN1, ITGB3, THBS1, TNC, TSPAN7, VCAN |
| Extra-cellular matrix proteases | ADAM12, ADAMTS6, ADAMTS14, CTSK, HTRA1, MMP16, MMP19, TIMP2, TIMP3, |
| Angiogenesis | BMPER, EDIL3, MCAM, SMOC2, |
| Apoptosis | GLIPR1 |
| Immune response | ACE2, ALOX5, DUOX2, IL1R1, IL7R, IL17RB, IL18R1, IL23R, LXN, TXNIP, ULBP1, ULBP3, |
| Retinoid metabolism | LRAT |
| Cell migration | GAP43, NT5E, ST8SIA6 |
| Wnt signalling | DAAM1, NDP |
| Ion binding and transport | CACNA1C, CALB2, GABRA5, GRIN2A, RYR2, SCN9A |
| G-coupled receptor signalling | GPRC5B, GPR56, GPR177, RASD1, RGS4, RGS5 |
| Transcription factors | AFF3, E2F7, FOXG1, KLF12, NFIB, PRB2 |
| Solute carrier family transporters | SLC1A3, SLC7A11, SLC15A1, SLC28A3, SLC38A1, SLC44A5 |
| Metabolism and homeostasis | B3GALT2, EXT1, LIPG, MAOB, PDE5A, PLCXD3, ST6GAL2 |
| Unknown function in ovary | CLSTN2, CORO2A, DTNA, DPY19L4, FBXO32, FRMD5, KRTAP13–2, NEDD4, PEG10, PRB1, RHOBTB3, RIMS2, RPSAP52, STXBP5L |



16

# 5. DISCUSSION

## 5.1. Factors influencing COH and pregnancy outcome in infertile women

Several genetic factors have been identified to influence human induced ovarian folliculogenesis. The variations in genes involved in the follicular maturation and hypothalamic-pituitary-ovarian axis are promising targets for genetic studies. Along with substantial impact on COH variables, these variations may also play important role in the pregnancy outcome of IVF and the pathogenesis of certain forms of female infertility. The main finding of the current thesis was that polymorphisms in genes involved in folliculogenesis influence the ovarian stimulation outcome in IVF treatment in normally ovulating infertile women. In total we analysed 17 polymorphisms in 8 different genes: *CYP19A1* $(TTTA)_n$, *CYP19A1* TCT Ins/Del, *ESR1* $(TA)_n$, *ESR1 Pvu*II T/C, *ESR1 Xba*I A/G, *ESR2* $(CA)_n$, *ESR2 Rsa*I G/A, *MTHFR* 677C/T, *MTHFR* 1298A/C, *MTHFR* 1793G/A, *FOLR1* 1314G/A, *FOLR1* 1816C/delC, *FOLR1* 1841G/A, *FOLR1* 1928C/T, *TCN2* 776C/G, *CTH* 1208G/T, and *SLC19A1* 80G/A.

We demonstrated that women with shorter $(TTTA)_n$ repeats and Del/Del homozygosity in aromatase gene exhibit decreased ovarian FSH-sensitivity during COH, as higher FSH doses were needed to mature ovarian puncture follicles and to obtain mature oocytes. However, we did not detect any association between *CYP19A1* gene polymorphisms and the IVF pregnancy outcome. The functional importance of the linked intronic *CYP19A1* $(TTTA)_n$ and *CYP19A1* Ins/Del genetic markers is not known. However, the long $(TTTA)_{12}$ allele has been associated with elevated aromatase transcript levels in breast cancer tissue (Kristensen et al. 2000). It is known that gene introns contain sequences for transcription and splicing regulation, which may lead to different mRNA levels and isoforms, and result in modified protein activity (Carstens et al. 1998, Gasch et al. 1989). Additionally, the studied variations could be in linkage disequilibrium with other functional gene variants, and thus indirectly modify gene expression and protein function.

Two previous studies have also analysed the effect of *CYP19A1* variants on COH outcome, however no associations were reported between *CYP19A1* C/T SNP (rs10046) and FSH hormone response (de Castro et al. 2004), nor the etiology of severe ovarian hyperstimulation syndrome (Binder et al. 2008). $(TTTA)_n$ microsatellite and C/T SNP are shown to be linked, therefore the published studies apparently contradict our results. However, genotyping C/T SNP in exon 10 only partially predicts the length of $(TTTA)_n$ in intron 4 (Kristensen et al. 2000), and further, different study population and COH regimens could account for the different clinical outcomes. We used intra-cycle GnRH antagonists for the rapid down-regulation of pituitary function, while in the previous study GnRH agonist long protocol was followed (de Castro et al. 2004).

Next finding in the current thesis was the clear association between COH outcome and the genetic variability in *ESR1* gene. The presence of shorter $(TA)_n$ dinucleotide repeat polymorphism and the major *Pvu*II T allele were associated with poorer COH outcome – less follicles matured, less oocytes obtained, and higher FSH doses required to obtain a good quality embryo. Our results are in line with previous studies, where IVF patients with minor CC genotype were associated with improved follicular quality (Georgiou et al. 1997) and higher number of follicles, oocytes and embryos (Sundarrajan et al. 1999). In addition, the minor C allele was lower among poor responders compared to normal COH responders (de Castro et al. 2004). In all these studies COH was performed using a GnRH agonist long protocol, whereas we showed similar results on women using recombinant FSH and GnRH antagonists. Furthermore, we demonstrate that the minor *Pvu*II CC genotype tended to be and the minor *Xba*I GG genotype was associated with higher estrogen levels achieved during COH. Although we and others demonstrate *ESR1* gene variants' role in determining COH outcome, the exact functional importance of these non-coding polymorphisms on *ER* gene/protein function is still unknown. However, it has been suggested that the T allele of the *ESR1 Pvu*II locus might eliminate a functional binding site for the myb family of transcription factors (Herrington et al. 2002).

In the current study, we detected no relationship between *ESR2* gene variants and COH outcome. Our results suggested also no association of *ESR1* and *ESR2* variants with clinical pregnancy outcome, contrary to previous findings (Georgiou et al. 1997, Sundarrajan et al. 1999). Nevertheless, as the polymorphisms in *ESR1* showed an association with the better COH outcome, these variations could have impact on the cumulative pregnancy rate per COH, rather than per single embryo transfer.

We demonstrate also associations between polymorphisms in folate-metabolizing pathway genes with biochemical markers of the folate metabolism and COH outcome, as well as pregnancy outcome. Folate, an important B vitamin, is believed to be essential in reproduction (Tamura and Picciano 2006). Dietary of genetically determined folate deficiency may result in elevated Hcy levels, and both folate deficiency and hyperhomocysteinemia are known risk factors of pregnancy complications and their role in folliculogenesis has been demonstrated (Mooij et al. 1992). Increased plasma Hcy levels have been proposed to affect follicular fluid Hcy levels, playing thus role in the development and quality of oocytes, fertilization and the quality of embryos (Berker et al. 2009). Further, it has been demonstrated that preconception folic acid supplementation alters folate and Hcy levels in follicular fluid (Boxmeer et al. 2008). Among Estonian IVF patients, blood folate, vitamin $B_{12}$ and Hcy did not appear to influence COH and IVF outcome. However, as no follow up on the supplement use on these women was conducted, the final conclusions can not be drawn. Among Swedish infertile women, we detected no effect on plasma Hcy concentrations in relation to any polymorphisms in MTHFR gene.

The majority of these infertile women had been taking folate supplements, therefore the adverse effects of MTHFR gene polymorphisms might have been masked by sufficient folate intake.

For infertility treatment, but also for public health and preventive purposes, it is important to understand the genetic background of factors influencing the balance between low folate status and elevated Hcy concentrations. Knowledge of such factors could facilitate prompt identification and treatment of women who are trying to achieve pregnancy in order to overcome the altering effect of polymorphisms in folate pathway genes.

Interestingly, in the majority cases of the ten studied polymorphisms in the folate pathway the heterozygous individuals rather than the homozygotes appeared to have favourable IVF outcomes, especially women with *MTHFR* 677CT, *MTHFR* 1793 GA and *CTH* 1208 GT genotypes. It is commonly known that individuals carrying the *MTHFR* 677 T allele, particularly TT homozygotes, have increased plasma Hcy concentrations. Also in our IVF patients *MTHFR* 677C/T polymorphism was associated with lower blood folate levels and increased Hcy levels. As the enzyme activity and blood folate levels are the highest and Hcy levels the lowest in the wild-type CC genotype, it has been considered the most advantageous genotype. However, our current findings show that in terms of IVF treatment outcome, the maternal *MTHFR* 677 heterozygous CT genotype was more successful compared to the homozygous CC and TT genotypes in respect of the greater proportion of good quality embryos and increased chance for pregnancy. Also a previous study demonstrated that the CT heterozygous genotype, rather than the homozygous CC genotype, was associated with increased chances of having had a previous IVF pregnancy and a live birth in the current IVF cycle (Haggarty et al. 2006). Furthermore, spontaneously aborted embryos have been shown to exhibit lower frequency of CT genotype compared to the wild-type CC genotype (Bae et al. 2007), meanwhile T allele has been suggested to increase embryo viability in the presence of an adequate folate-containing diet (Reyes-Engel et al. 2002). The reproductive advantage of the *MTHFR* 677 CT genotype could be explained by a favourable balance in the folate metabolism pathway between methyl donor and nucleotide synthesis. The CC genotype is associated with increased DNA hypermethylation reactions (Stern et al. 2000, Friso et al. 2002), while the TT genotype is related to improved DNA biosynthesis (Bagley and Selhub 1998) and decreased deoxyuridine monophosphate misincorporation into DNA (Kapiszewska et al. 2005).

The other studied polymorphisms in *MTHFR* gene, 1298A/C and 1793G/A, also showed positive effect of the heterozygous genotype on fertility – women with 1298 AC (and with AA) genotype had tendency towards increased ovarian responsiveness to FSH stimulation and women with 1793 GA genotype were associated with more successful IVF treatment and with higher serum folate values compared to the wild-type GG genotype. It has been hypothesised that the 1298A/C variation decreases the amount of folate cofactors available for

nucleotide synthesis, influencing thus DNA biosynthesis and increasing apoptosis in the granulosa cells that could lead to the higher FSH doses required in the ovarian stimulation (Rosen et al. 2007). The functional relevance of the 1793G/A polymorphism is not clear, although higher Hcy concentration in association with the wild-type genotype has been reported (Böttiger et al. 2007b).

Also the heterozygous genotype *FOLR1* 1314 GA appeared to be beneficial – heterozygotes demonstrated higher blood folate levels and showed a tendency towards more successful previous IVF treatments when compared to the GG genotype. However, *FOLR1* 1314 GA heterozygotes required higher FSH doses per one retrieved oocyte. The functional significance of this variation is not known, but it has been hypothesised that mutations in the gene could result in a dysfunctional receptor, or reduced receptor expression on the cell surface (Böttiger et al. 2007a). Further, as this polymorphism is located in the 5' untranslated region of the gene, it could alter the gene expression. FOLR1 is a folate receptor and is responsible for the cellular folate uptake. In the female reproductive tract, FOLR1 expression has been detected in the granulosa cells, epithelial cells of the fallopian tube, endometrium and placenta (Weitman et al. 1992). Therefore the altered FOLR1 expression may influence reproductive function through folate availability in these tissues.

Another heterozygous polymorphism in the folate pathway showing favourable IVF results was *CTH* 1208G/T, demonstrating increased chance for pregnancy and lower number of previous IVF failures. In addition, the GT heterozygosity was higher among fertile control group than in IVF patients that did not get pregnant in the current IVF treatment cycle and in IVF patients that had experienced 3 or more previous IVF failures. The functional significance of the Ser403Ile polymorphism in exon 12 of the gene is unknown, however, we found the minor TT genotype to be associated with higher Hcy concentrations in Swedish infertile women. Similar association was reported also in a previous study (Wang et al. 2004). One could hypothesise that as CTH catalyses a reaction producing thiocysteine from cystine, which decomposes to cysteine and hydrogen sulfide, which may act as a regulatory mediator (Lowicka and Beltowski 2007), having an important role in cell proliferation and survival (Yang et al. 2004). Thus, it is possible that highly active CTH, that is effective in cytotoxic Hcy removal, inhibits cell proliferation due to the elevated production of hydrogen sulfide. If the *CTH* 1209G/T polymorphism affects the levels or activity of CTH, a GT heterozygote might exhibit a suitable level of active CTH enzyme.

Taken together, the observed favourable effects of the heterozygous variations in the folate-metabolizing genes on IVF outcome could be explained by the hypothesis that the right balance of donor methyl groups is crucial for the reproductive success, where neither side of the imbalance in the folate metabolism may be unfavourable. Anyhow, a genotype that increases the probability to conceive is more likely to be passed on to the offspring than the

one that does not. Fertility is a heritable polygenic trait, where genetic and environmental factors affect the biological processes in reproduction. Especially nowadays, where the majority of children survive to reproductive age, fertility is considered to be one of the key traits on which natural selection applies (Haggarty et al. 2006).

Besides the sequence variations in the studied genes, we demonstrated that the age of a woman undergoing infertility treatment was an important predictor of the COH outcome. It is generally known that the age is the major negative factor affecting IVF, as the ovarian reserve decreases with aging and the COH results deteriorate requiring higher FSH doses (Kligman and Rosenwaks 2001). We also detected that with the increasing woman's age higher FSH doses were needed to achieve polyfolliculogenesis. In addition, the reduced number of follicles at the early follicular stage of the natural cycle led to fewer follicles after the ovarian stimulation, being in line with the belief of the predictive value of the follicle number in the early follicular phase (Tomas et al. 1997). Furthermore, as expected, our women with infertility due to other reasons were considerably older, demonstrating elevated FSH levels at the early menstrual cycle and higher FSH doses were required for adequate ovarian response.

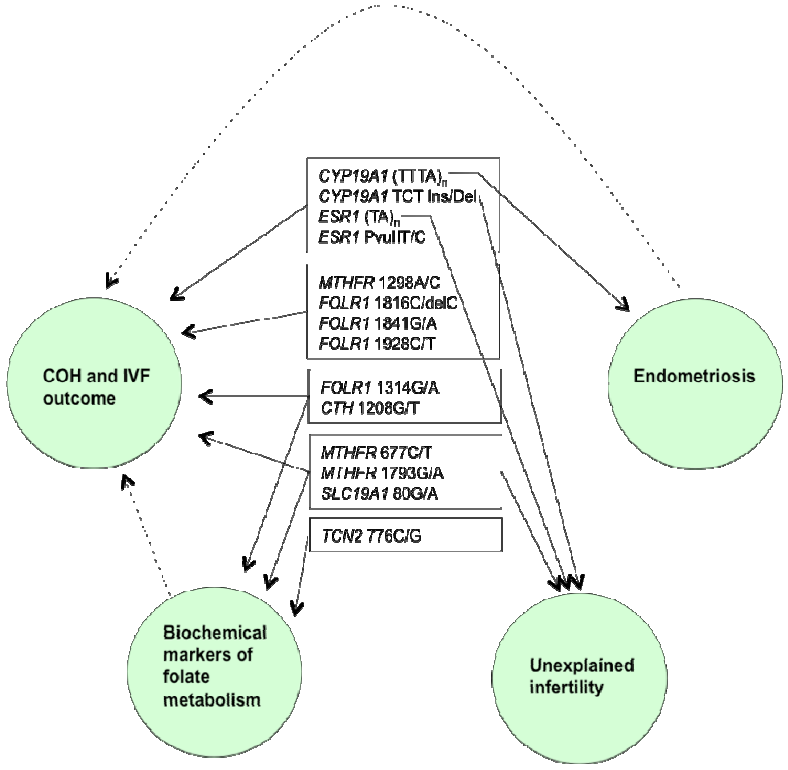


**Figure 19.** Schematic illustration of studied genetic variations in association with female infertility, biochemical markers of folate metabolism and IVF treatment outcome.

## 5.2. Variations and etiology of female infertility

Another important finding in the current thesis was that variations in genes involved in folliculogenesis are potential risk factors for various forms of female infertility. In the aromatase gene, Del variation linked with low-repeat-number $(TTTA)_n$ alleles demonstrated enhanced genetic susceptibility to unexplained infertility and endometriosis. Evidence on the overlapping patient's profiles of folliculogenesis abnormalities, resulting in decreased oocyte fertilization ability in women with endometriosis and unexplained infertility has been provided (Cahill and Hull 2000). Further, despite diagnostic laparoscopy has been included into the routine evaluation of female infertility, endometriosis could have been underestimated due to the non-visible precursor stages of endometriotic lesions, ending up with misdiagnosis of unexplained infertility. An earlier study also showed a dominance of the Del/Del genotype in Japanese endometriosis patients compared to controls (Kado et al. 2002), however, other studies have failed to obtain evidence on the significance of shorter *CYP19A1* TTTA-repeats in the increased risk of endometriosis (Kado et al. 2002, Hur et al. 2007).

In estrogen receptor gene, we showed that women with unexplained infertility presented significantly shorter *ESR1* $(TA)_n$ microsatellite repeats. Women with unexplained infertility are a heterogeneous group of patients, thus our results suggest that variation in the *ESR1* could be one susceptibility factor for unspecified infertility. Interestingly, a lower repeat number of the $(TA)_n$ polymorphism was reported to occur more frequently among women with premature ovarian dysfunction (Bretherick et al. 2008, Syrrou et al. 1999). Also *ESR1 Pvu*II major TT genotype has been associated with premature ovarian failure (Yoon et al. 2009). Further, *Pvu*II T/C and $(TA)_n$ dinucleotide repeat polymorphisms have been associated with susceptibility to endometriosis (Georgiou et al. 1999, Hsieh et al. 2007). In our study, we were unable to show associations between *ESR* gene variants and the etiology of endometriosis. Although the development of endometriosis is estrogen-dependent, the exact pathological mechanisms involved are unknown.

Among the ten studied variations in folate pathway genes, we demonstrated that polymorphisms *MTHFR* 677C/T, *MTHFR* 1793G/A and *SLC19A1* 80G/A may account for infertility in women with otherwise unspecified reason for their infertility. In line with our previous finding of the favourable heterozygous variants in folate-metabolizing pathway genes on infertility treatment outcome, our study group of women with unexplained infertility showed lower prevalence of *MTHFR* 677 CT and *MTHFR* 1793 GA genotypes compared to control individuals. It is evident, that polymorphisms in *MTHFR* gene may affect female infertility. Besides to the link with Hcy concentrations, *MTHFR* gene variants have been suggested to play role in haemostasis (Coulam et al. 2006, Keijzer et al. 2002). Polymorphisms in *MTHFR* gene could alter the required balance between haemorrhage and thrombosis, affecting thereby

embryo implantation process. At the time of implantation, blastocyst interacts with the endometrium and blastocyst-derived syncytiotrophoblasts breach endometrial blood vessels, establishing the primordial uteroplacental circulation. In fact, inherited thrombophilias are associated to implantation failure (Azem et al. 2004, Coulam et al. 2006). Furthermore, in addition to coagulation and fibrinolysis, the genes encoding thrombogenic proteins are shown to be involved in fertilization, embryogenesis and tissue remodelling (Rawlings and Barrett 1993).

We found also *SLC19A1* 80G/A locus to be associated with unexplained female infertility. Major allele G, as well GG and GA genotypes, were more prevalent in infertile women compared to controls. Also Estonian infertile women with the GG genotype were more likely to belong to the group of women with ≥ 3 previous failed IVF treatments. *SLC19A1* gene encodes a protein reduced folate carrier, which is considered to be the major folate transporter in most tissues (Matherly et al. 2007). At the site of embryo implantation and further development, alterations in folic acid concentrations can be crucial for the proliferation of rapidly dividing embryonic and maternal cells. Variation 80G/A introduces amino acid change His27Arg (Chango et al. 2000), however, cellular folate intake has been shown not to be affected *in vitro* by this polymorphism (Whetstine et al. 2001). Nevertheless, higher plasma homocysteine concentrations have been demonstrated in the major GG genotype carriers (Chango et al. 2000). Furthermore, minor allele A has been shown to offer a protective effect against thrombosis (Yates and Lucock 2005). Hypercoagulation and microthrombosis at the implantation site have been hypothesised to cause implantation failure and miscarriage (Azem et al. 2004, Rey et al. 2003). Further, maternal homocysteine concentrations have been associated with defective chorionic villous vascularisation in women with recurrent early pregnancy loss (Nelen et al. 2000). It could be, that among our study group of infertile women, while carrying more frequently the major G allele, the imbalance in coagulation at the implantation site alters throphoblast invasion and thereby embryo implantation. *SLC19A1* 80G/A is an example of an apparently beneficial polymorphism, which is not unusual. Indeed, *MTHFR* 677C/T polymorphism is thought to give some health benefits when folate status is sufficient, as discussed above, and also in protection against some cancers (Ueland et al. 2001).

Eventually, the influence of a single variation to a phenotype may have a weak effect, but it may become evident when coexisting with other polymorphism(s). This has been demonstrated in a previous study, where a polygenic model including *FSHR*, *ESR1* and *ESR2* genes was associated with poor response to FSH stimulation in COH (de Castro et al. 2004). As we demonstrate in our study, using marker-by-marker approach, variations in *CYP19A1*, *ESR1* and in several genes involved in the folate metabolism influence IVF outcome and are involved in the etiology of female infertility. Therefore the next step would be to analyse the effects of different variations in

an integrated complex model among infertile women undergoing IVF, together with patients' age and parameters of ovarian reserve, to design novel, patient-tailored therapeutic approaches for safe ovarian stimulation.

Nevertheless, single genotypes could also be applied in the pharmacogenetic approach to COH, as has recently been demonstrated for patients with unfavourable FSHR genotype using higher initial and total FSH doses to overcome relative ovarian insensitivity (Behre et al. 2005). Furthermore, a previous prospective randomised study has confirmed that individualising the starting FSH dose is important to improve the outcome of IVF treatment in infertile women (Popovic-Todorovic et al. 2003).

## 5.3. Granulosa cell gene expression profiles in IVF patients at the time of follicle puncture

Despite the improvements in the infertility treatment, the pregnancy and live birth rates following IVF and ICSI procedures have stayed low. For instance, subjective morphological parameters are still the main criteria in IVF and ICSI programs for selecting a good-quality embryo(s), which do not truly predict the embryo competence. Knowledge of the communication between different follicular cell types would help to identify new oocyte and embryo viability markers that could be useful in providing important information for improving ovarian stimulation protocols in the infertility treatment programs and thus could help to achieve higher pregnancy outcomes. That was the rationale of our last study presented in the current thesis, where we analysed the transcriptome profiles of floating GCs and cumulus GCs collected during follicle puncture from FSH and GnRH antagonist-stimulated female infertility patients undergoing IVF with ICSI. In fact, a previous study demonstrated that the gene expression profiling of human cumulus cells correlates with embryo potential and pregnancy outcome (Assou et al. 2008).

We detected more than four thousand gene transcripts with differential expression and nearly 500 genes with two or more-fold difference between the two distinct granulosa cell populations, referring to their discrete roles and the complex cellular interplay. These genes were involved in diverse aspects of folliculogenesis and oocyte maturation, steroidogenesis, angiogenesis, extra-cellular matrix remodelling, and immune response, as well as other pathways. It has been hypothesised that during the follicular development, MGCs are essential for estrogen production and follicular rupture, while CGCs are closely related to oocyte development (Anderson et al. 2009).



18

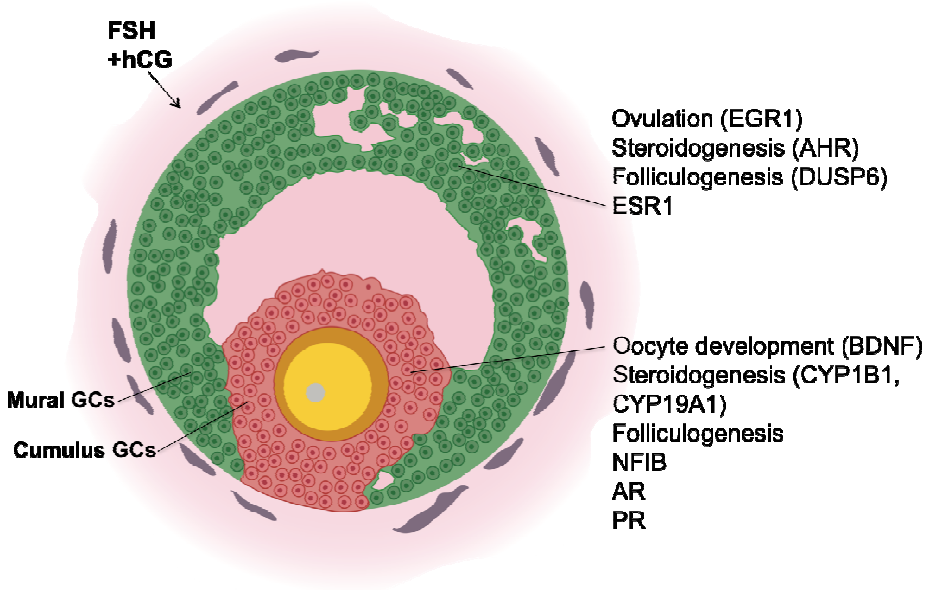


**Figure 20.** Most interesting functional categories together with most interesting genes that were highly expressed in floating GCs and in cumulus GCs are presented. The women underwent GnRH antagonist stimulation protocol, with FSH administration and single injection of hCG.

The ovarian stimulation protocol used during the preparatory phase of IVF includes FSH administration and a single injection of hCG in order to mimic the endogenous LH surge that occurs in natural cycles. Therefore it would be expected that 36 h after the hCG injection, several pathways upstream and downstream of FSHR and LHCGR are active in granulosa cells. We detected no difference in *FSHR* and *LHCGR* expression between FGCs and CGCs. However, we observed up-regulation of the transcription factor *EGR1* that is responsible for *LHCGR* expression in FGCs (Yoshino et al. 2002). Also several mediators of *LHCGR* transcriptional activation, cathepsin L1, *ADAMTS1*, and epiregulin (*EREG*), were up-regulated in FGCs, possibly as a result of LH surge (Doyle et al. 2004, Sekiguchi et al. 2002, Sriraman and Richards 2004). It has been demonstrated that FSH and LH/hCG transmit their action through the activation of kinase complex ERK1/2 (Das et al. 1996, Maizels et al. 2001) and that this pathway is involved in the follicular development, meiotic resumption of oocytes, and cumulus-oocyte complex expansion (Fan et al. 2008, Su et al. 2003). Even though the active *ERK1/2* signalling was detected in both cell types, *DUSP6*, a down-regulator of *ERK1/2* activity, was up-regulated in FGCs. The differential regulation of common pathways in these two closely related cell populations clearly indicates different signalling outcomes in response to identical hormonal stimuli.

One of the most interesting functional categories of the differential gene expression profiles in our study was steroidogenesis. After obtaining the responsiveness to LH and initiation of progesterone production, MGCs in the late antral stage are referred to as luteinised granulosa. As expected, up-regulation of several molecules involved in cholesterol metabolism in FGC was observed, such as *CYP11A1*, *CYP17A1*, *HSD17B1*, and follistatin. We also detected enzymes *AKR1C1* (20αHSD) and *CYP19A1* to be down-regulated in FGCs, both processes have been examined as indicators of luteinization (Niswender et al. 2000, Stocco et al. 2001). Some of the adverse by-products of steroid synthesis pathways could affect the balance of cellular metabolism and survival. In the CGCs, we detected the up-regulation of *CYP1B1*, a member of cytochrome P450 superfamily of enzymes that catalyses the formation of 4-OH-estradiol. This isoform has been considered to be a potential source of reactive oxygen species (Jefcoate et al. 2000) that exert a negative impact on oocyte quality and corpus luteum function (Vidal et al. 2005). *CYP1B1* is a downstream transcript of the aryl hydrocarbon receptor (AHR), a protein involved in the modulation of granulosa cell proliferation via cross-talk with estrogen receptor pathways (Bussmann et al. 2006), which we found up-regulated in FGCs.

A number of hormone receptors that showed differential expression were also observed in our study, such as *ESR1*, androgen receptor (*AR*) and *PR*. *ESR1* was up-regulated in FGCs, which is in accordance with the scarce data available of estrogen receptor expression in follicular cells. Increased *ESR1* expression has been previously described in human MGCs with the development of pre-antral follicle to antral stage (Saunders et al. 2000), and decreasing significantly by corpus luteum formation (van den Driesche et al. 2008). AR and PR on the other hand we up-regulated in CGCs. AR has a proliferation-enhancing role in the presence of mitogens, which depends on the cell distance from the oocyte. This has been demonstrated in cultured porcine granulosa cells, where AR activity was maintained in the cumulus-oocyte complex after antrum enlargement, while MGCs had lost the responsiveness to androgens (Hickey et al. 2004). It has been concluded that the AR-mediated signalling is one way how oocyte might dictate follicular development. *PR* has also shown to be up-regulated in porcine CGC when cultured in LH and FSH-containing medium, resulting in the oocyte meiotic maturation (Shimada and Terada 2002).

Two recent studies have proposed a number of genes that are expressed in cumulus GCs as markers for competent embryo and pregnancy outcome, including nuclear factor I/B (*NFIB*) (Assou et al. 2008) and brain-derived neurotrophic factor (*BDNF*) (Anderson et al. 2009), which we detected highly expressed in CGCs. Expression of *NFIB* in human cumulus cells has been associated with embryo potential and successful pregnancy, however, lower *NFIB* expression has been found to be more beneficial than higher expression in CGCs (Assou et al. 2008). *NFIB* belongs to the nuclear factor I (NFI) family and acts as a transcription factor. NFI proteins have been shown to have role in

the regulation of tissue-specific gene expression during mammalian embryogenesis (Steele-Perkins et al. 2005). As the literature lacks of the information about *NFIB* expression in MGCs, it could be only speculated that the higher transcript expression in CGCs, as we observed in our study, is needed for folliculogenesis. Or it could be that the obtained high expression levels in CGCs refer to the lower embryo potential, however, we don't have a comparison group of other CGCs for examining this hypothesis.

Another interesting molecule, *BDNF*, was also higher expressed in CGCs than in FGCs. *BDNF* has been detected in the human follicular fluid, secreted by CGCs (Seifer et al. 2002). In mural and cumulus granulosa cell culture, *BDNF* has found to be secreted from cumulus but not from MGCs (Seifer et al. 2002) and a recent immunohistochemical staining supports that *BDNF* is expressed predominantly from cumulus granulosa cells (Seifer et al. 2002). Further, a study in ovarian granulosa cumulus cells has found BDNF expression to be regulated by cAMP, and also that LH seems to be a regulator of BDNF through the cAMP signalling pathway (Feng et al. 2003). *BDNF* belongs to the neurotrophin family of proteins that activate the high-affinity TrkB receptor and the pan-neurotrophin low-affinity receptor p75 (Barbacid 1994). In the mouse ovary, BDNF has been reported to be important for the development of early follicles (Paredes et al. 2004). Additionally, a recent study on mice demonstrated ovarian BDNF to be crucial for first polar body extrusion, and as well for cytoplasmic maturation of the oocyte, which are important for the oocyte development into preimplantation embryo (Kawamura et al. 2005). It is concluded that BDNF is essential for female fertility, however, lower expression in the CGCs has been associated with better oocyte maturation and subsequent embryo development than higher expression (Anderson et al. 2009). It is unclear why also BDNF should be lower in CGCs, as proposed, but it may reflect the time point at which the cumulus was recovered as expression of some cumulus genes can be temporary (Yoshino et al. 2006), or the high gene expression in our study refers to the reduced quality of the mature oocyte. However, also here we don't have another cell group of CGCs for comparison for drawing this conclusion.

The conclusion has been drawn that cumulus function may reflect and determine oocyte function, and further the subsequent embryo development potential (Anderson et al. 2009). In addition to the few previous CGC gene expression studies, our study is the first to describe the unique transcriptomes of FGC and CGC of stimulated human pre-ovulatory follicles required to decipher the complicated cellular network at the studied time-point of follicular maturation. In addition to providing new insights for the basic science in the field of follicular biology, the obtained results could also be used for identifying non-invasive markers for oocyte and embryo competence, offering a novel approach towards improved embryo selection and IVF pregnancy success.

# CONCLUSIONS

Nowadays, the utilization of assisted reproductive technology has improved the prospects of infertility treatment. Regardless of constant improvement of pregnancy rate in *in vitro* fertilization (IVF), the success rates are still around 30% per cycle (Andersen et al. 2007). The expected outcome of the IVF treatment depends greatly on the effectiveness of controlled ovarian hyper-stimulation (COH), where follicle-stimulating horomone (FSH) is used to induce the folliculogenesis, as well as on the quality of oocytes. The response to the FSH stimulation varies substantially among individuals and is difficult to predict. Several markers have been proposed, but the search for optimal markers that could predict COH outcome and also a good-quality oocyte, enabling thereby better IVF treatment outcome, is ongoing.

The knowledge that the current thesis adds into the field of assisted reproduction is:

- Polymorphisms in genes involved in folliculogenesis influence the COH and IVF outcome in infertility treatment.

We demonstrate that women with shorter *CYP19A1* $(TTTA)_n$ repeats and *CYP19A1* TCT Del/Del homozygosity in aromatase gene and women with shorter $(TA)_n$ repeats and *Pvu*II T allele in *ESR1* gene exhibit decreased ovarian FSH-sensitivity during COH. In addition, heterozygous genotypes of *MTHFR* 677C/T, *MTHFR* 1793 G/A, *FOLR1* 1314G/A and *CTH* 1208G/T variations in the folate pathway genes predict favourable IVF outcomes, meanwhile polymorphisms *MTHFR* 677C/T, *FOLR1* 1314G/A, *TCN2* 776C/G, *SLC19A1* 80G/A influence the concentration of the biochemical markers of the folate metabolism (folate and Hcy) in the blood, which also may alter female reproductive functions.

- Polymorphisms in genes involved in follicular development could be potential genetic risk factors for female infertility.

We show that women carrying *CYP19A1* TCT Del variation linked with low-repeat-number $(TTTA)_n$ alleles in aromatase gene have enhanced genetic susceptibility to endometriosis and unexplained infertility, and shorter $(TA)_n$ microsatellite repeats in *ESR1* gene could be a potential genetic risk factor for unexplained female infertility. Further, polymorphisms *MTHFR* 677C/T, *MTHFR* 1793G/A and *SLC19A1* 80G/A may account for infertility in women with otherwise unspecified reason for their infertility.



19

- We are the first to describe the unique gene expression profile of floating granulosa cells and cumulus granulosa cells of stimulated human pre-ovulatory follicles. This detailed understanding of the communication between different follicular cell types would help to improve the COH protocols and in addition would help to identify new oocyte and embryo viability markers for improving pregnancy outcomes following IVF treatment.

In conclusion, the results of the current thesis demonstrate that the genetic variation in genes involved in folliculogenesis influences the stimulatory effect of FSH used in ovarian stimulation in IVF patients and are associated with etiology of female infertility. The knowledge of the individual's genetic background would enable to predict the FSH doses needed for optimal ovarian stimulation in IVF treatment in order to avoid the poor response or hyper-response to the hormonal ovarian stimulation. Further, the data of the granulosa cell gene expression add information to the process of hormonal stimulation in IVF, which could be applied in improving IVF treatment protocols and embryo selection. These findings, together with previous studies have great importance for future development in infertility treatment, allowing to individualise the patient's COH protocols and to make thereby IVF procedure safer and more effective.

# REFERENCES


(1996) Infertility revisited: the state of the art today and tomorrow. European Society for Human Reproduction and Embryology. *Hum Reprod,* 11**,** 1779–807.

(2006) Intracytoplasmic sperm injection (ICSI). *Fertil Steril,* 86**,** S122.

(2008) Age-related fertility decline: a committee opinion. *Fertil Steril,* 90**,** S154–5.

Aljanabi, S. M. & I. Martinez (1997) Universal and rapid salt-extraction of high quality genomic DNA for PCR-based techniques. *Nucleic Acids Res,* 25**,** 4692–3.

Andersen, A. N., V. Goossens, L. Gianaroli, R. Felberbaum, J. de Mouzon & K. G. Nygren (2007) Assisted reproductive technology in Europe, 2003. Results generated from European registers by ESHRE. *Hum Reprod,* 22**,** 1513–25.

Anderson, R. A., R. Sciorio, H. Kinnell, R. A. Bayne, K. J. Thong, P. A. de Sousa & S. Pickering (2009) Cumulus gene expression as a predictor of human oocyte fertilisation, embryo development and competence to establish a pregnancy. *Reproduction,* 138**,** 629–37.

Arnhold, I. J., A. Lofrano-Porto & A. C. Latronico (2009) Inactivating mutations of luteinizing hormone beta-subunit or luteinizing hormone receptor cause oligo-amenorrhea and infertility in women. *Horm Res,* 71**,** 75–82.

Assou, S., D. Haouzi, K. Mahmoud, A. Aouacheria, Y. Guillemin, V. Pantesco, T. Reme, H. Dechaud, J. De Vos & S. Hamamah (2008) A non-invasive test for assessing embryo potential by gene expression profiles of human cumulus cells: a proof of concept study. *Mol Hum Reprod,* 14**,** 711–9.

Azem, F., A. Many, I. Ben Ami, I. Yovel, A. Amit, J. B. Lessing & M. J. Kupferminc (2004) Increased rates of thrombophilia in women with repeated IVF failures. *Hum Reprod,* 19**,** 368–70.

Baart, E. B., N. S. Macklon & B. J. Fauser (2009) Ovarian stimulation and embryo quality. *Reprod Biomed Online,* 18 Suppl 2**,** 45–50.

Bae, J., S. J. Shin, S. H. Cha, D. H. Choi, S. Lee & N. K. Kim (2007) Prevalent genotypes of methylenetetrahydrofolate reductase (MTHFR C677T and A1298C) in spontaneously aborted embryos. *Fertil Steril,* 87**,** 351–5.

Baghaei, F., R. Rosmond, L. Westberg, M. Hellstrand, E. Eriksson, G. Holm & P. Bjorntorp (2003) The CYP19 gene and associations with androgens and abdominal obesity in premenopausal women. *Obes Res,* 11**,** 578–85.

Bagley, P. J. & J. Selhub (1998) A common mutation in the methylenetetrahydrofolate reductase gene is associated with an accumulation of formylated tetrahydrofolates in red blood cells. *Proc Natl Acad Sci U S A,* 95**,** 13217–20.

Barbacid, M. (1994) The Trk family of neurotrophin receptors. *J Neurobiol,* 25**,** 1386–403.

Barnea, E. R., T. R. Holford & D. R. McInnes (1985) Long-term prognosis of infertile couples with normal basic investigations: a life-table analysis. *Obstet Gynecol,* 66**,** 24–6.

Behre, H. M., R. R. Greb, A. Mempel, B. Sonntag, L. Kiesel, P. Kaltwasser, E. Seliger, F. Ropke, J. Gromoll, E. Nieschlag & M. Simoni (2005) Significance of a common single nucleotide polymorphism in exon 10 of the follicle-stimulating hormone (FSH) receptor gene for the ovarian response to FSH: a pharmacogenetic approach to controlled ovarian hyperstimulation. *Pharmacogenet Genomics,* 15**,** 451–6.

Berker, B., C. Kaya, R. Aytac & H. Satiroglu (2009) Homocysteine concentrations in follicular fluid are associated with poor oocyte and embryo qualities in polycystic

ovary syndrome patients undergoing assisted reproduction. *Hum Reprod,* 24**,** 2293–302.

Binder, H., R. Dittrich, I. Hager, A. Muller, S. Oeser, M. W. Beckmann, M. Hamori, P. A. Fasching & R. Strick (2008) Association of FSH receptor and CYP19A1 gene variations with sterility and ovarian hyperstimulation syndrome. *Reproduction,* 135**,** 107–16.

Boivin, J., L. Bunting, J. A. Collins & K. G. Nygren (2007) International estimates of infertility prevalence and treatment-seeking: potential need and demand for infertility medical care. *Hum Reprod,* 22**,** 1506–12.

Böttiger, A. K., N. O. Hagnelius & T. K. Nilsson (2007a) Mutations in exons 2 and 3 of the FOLR1 gene in demented and non-demented elderly subjects. *Int J Mol Med,* 20**,** 653–62.

Böttiger, A. K., A. Hurtig-Wennlof, M. Sjostrom, A. Yngve & T. K. Nilsson (2007b) Association of total plasma homocysteine with methylenetetrahydrofolate reductase genotypes 677C>T, 1298A>C, and 1793G>A and the corresponding haplotypes in Swedish children and adolescents. *Int J Mol Med,* 19**,** 659–65.

Böttiger, A. K. & T. K. Nilsson (2007) Pyrosequencing assay for genotyping of the Transcobalamin II 776C>G polymorphism. *Scand J Clin Lab Invest,* 67**,** 247–51.

Boxmeer, J. C., R. M. Brouns, J. Lindemans, E. A. Steegers, E. Martini, N. S. Macklon & R. P. Steegers-Theunissen (2008) Preconception folic acid treatment affects the microenvironment of the maturing oocyte in humans. *Fertil Steril,* 89**,** 1766–70.

Bretherick, K. L., C. W. Hanna, L. M. Currie, M. R. Fluker, G. L. Hammond & W. P. Robinson (2008) Estrogen receptor alpha gene polymorphisms are associated with idiopathic premature ovarian failure. *Fertil Steril,* 89**,** 318–24.

Britt, K. L., A. E. Drummond, M. Dyson, N. G. Wreford, M. E. Jones, E. R. Simpson & J. K. Findlay (2001) The ovarian phenotype of the aromatase knockout (ArKO) mouse. *J Steroid Biochem Mol Biol,* 79**,** 181–5.

Britt, K. L. & J. K. Findlay (2002) Estrogen actions in the ovary revisited. *J Endocrinol,* 175**,** 269–76.

Broer, S. L., B. W. Mol, D. Hendriks & F. J. Broekmans (2009) The role of anti-mullerian hormone in prediction of outcome after IVF: comparison with the antral follicle count. *Fertil Steril,* 91**,** 705–14.

Bukovsky, A., M. R. Caudle, J. A. Keenan, J. Wimalasena, J. S. Foster & S. E. Van Meter (1995) Quantitative evaluation of the cell cycle-related retinoblastoma protein and localization of Thy-1 differentiation protein and macrophages during follicular development and atresia, and in human corpora lutea. *Biol Reprod,* 52**,** 776–92.

Bussmann, U. A., L. E. Bussmann & J. L. Baranao (2006) An aryl hydrocarbon receptor agonist amplifies the mitogenic actions of estradiol in granulosa cells: evidence of involvement of the cognate receptors. *Biol Reprod,* 74**,** 417–26.

Cahill, D. J. & M. G. Hull (2000) Pituitary-ovarian dysfunction and endometriosis. *Hum Reprod Update,* 6**,** 56–66.

Cai, J., H. Y. Lou, M. Y. Dong, X. E. Lu, Y. M. Zhu, H. J. Gao & H. F. Huang (2007) Poor ovarian response to gonadotropin stimulation is associated with low expression of follicle-stimulating hormone receptor in granulosa cells. *Fertil Steril,* 87**,** 1350–6.

Camp, T. A., J. O. Rahal & K. E. Mayo (1991) Cellular localization and hormonal regulation of follicle-stimulating hormone and luteinizing hormone receptor messenger RNAs in the rat ovary. *Mol Endocrinol,* 5**,** 1405–17.

Carstens, R. P., W. L. McKeehan & M. A. Garcia-Blanco (1998) An intronic sequence element mediates both activation and repression of rat fibroblast growth factor receptor 2 pre-mRNA splicing. *Mol Cell Biol,* 18**,** 2205–17.

Chang, M. Y., C. H. Chiang, T. T. Hsieh, Y. K. Soong & K. H. Hsu (1998) Use of the antral follicle count to predict the outcome of assisted reproductive technologies. *Fertil Steril,* 69**,** 505–10.

Chang, S. Y., H. Y. Kang, K. C. Lan, C. Y. Chang, F. J. Huang, M. Y. Tsai & K. E. Huang (2005) Expression of steroid receptors, their cofactors, and aromatase in human luteinized granulosa cells after controlled ovarian hyperstimulation. *Fertil Steril,* 83 Suppl 1**,** 1241–7.

Chango, A., N. Emery-Fillon, G. P. de Courcy, D. Lambert, M. Pfister, D. S. Rosenblatt & J. P. Nicolas (2000) A polymorphism (80G–>A) in the reduced folate carrier gene and its associations with folate status and homocysteinemia. *Mol Genet Metab,* 70**,** 310–5.

Chavarro, J. E., J. W. Rich-Edwards, B. A. Rosner & W. C. Willett (2008) Use of multivitamins, intake of B vitamins, and risk of ovulatory infertility. *Fertil Steril,* 89**,** 668–76.

Chin, K. V., D. B. Seifer, B. Feng, Y. Lin & W. C. Shih (2002) DNA microarray analysis of the expression profiles of luteinized granulosa cells as a function of ovarian reserve. *Fertil Steril,* 77**,** 1214–8.

Coccia, M. E. & F. Rizzello (2008) Ovarian reserve. *Ann N Y Acad Sci,* 1127**,** 27–30.

Coulam, C. B., R. S. Jeyendran, L. A. Fishel & R. Roussev (2006) Multiple thrombophilic gene mutations are risk factors for implantation failure. *Reprod Biomed Online,* 12**,** 322–7.

Coulam, C. B., R. S. Jeyendran & R. Roussev (2008) Association of progesterone receptor polymorphisms with recurrent implantation failure after in vitro fertilization and embryo transfer. *J Assist Reprod Genet,* 25**,** 119–22.

Couse, J. F. & K. S. Korach (1999) Reproductive phenotypes in the estrogen receptor-alpha knockout mouse. *Ann Endocrinol (Paris),* 60**,** 143–8.

Cramer, D. W., M. D. Hornstein, P. McShane, R. D. Powers, P. J. Lescault, A. F. Vitonis & I. De Vivo (2003) Human progesterone receptor polymorphisms and implantation failure during in vitro fertilization. *Am J Obstet Gynecol,* 189**,** 1085–92.

Das, S., E. T. Maizels, D. DeManno, E. St Clair, S. A. Adam & M. Hunzicker-Dunn (1996) A stimulatory role of cyclic adenosine 3',5'-monophosphate in follicle-stimulating hormone-activated mitogen-activated protein kinase signaling pathway in rat ovarian granulosa cells. *Endocrinology,* 137**,** 967–74.

de Bruin, J. P., M. Dorland, E. R. Spek, G. Posthuma, M. van Haaften, C. W. Looman & E. R. te Velde (2004) Age-related changes in the ultrastructure of the resting follicle pool in human ovaries. *Biol Reprod,* 70**,** 419–24.

de Castro, F., F. J. Moron, L. Montoro, J. J. Galan, D. P. Hernandez, E. S. Padilla, R. Ramirez-Lorca, L. M. Real & A. Ruiz (2004) Human controlled ovarian hyperstimulation outcome is a polygenic trait. *Pharmacogenetics,* 14**,** 285–93.

de Castro, F., R. Ruiz, L. Montoro, D. Perez-Hernandez, E. Sanchez-Casas Padilla, L. M. Real & A. Ruiz (2003) Role of follicle-stimulating hormone receptor Ser680Asn polymorphism in the efficacy of follicle-stimulating hormone. *Fertil Steril,* 80**,** 571–6.

de Vet, A., J. S. Laven, F. H. de Jong, A. P. Themmen & B. C. Fauser (2002) Antimullerian hormone serum levels: a putative marker for ovarian aging. *Fertil Steril,* 77**,** 357–62.



20

De Vivo, I., G. S. Huggins, S. E. Hankinson, P. J. Lescault, M. Boezen, G. A. Colditz & D. J. Hunter (2002) A functional polymorphism in the promoter of the progesterone receptor gene associated with endometrial cancer risk. *Proc Natl Acad Sci U S A,* 99**,** 12263–8.

Dobson, A. T., R. M. Davis, M. P. Rosen, S. Shen, P. F. Rinaudo, J. Chan & M. I. Cedars (2007) Methylenetetrahydrofolate reductase C677T and A1298C variants do not affect ongoing pregnancy rates following IVF. *Hum Reprod,* 22**,** 450–6.

Dominguez, F., A. Pellicer & C. Simon (2009) The human embryo proteome. *Reprod Sci,* 16**,** 188–90.

Doyle, K. M., D. L. Russell, V. Sriraman & J. S. Richards (2004) Coordinate transcription of the ADAMTS-1 gene by luteinizing hormone and progesterone receptor. *Mol Endocrinol,* 18**,** 2463–78.

Drummond, A. E. (2006) The role of steroids in follicular growth. *Reprod Biol Endocrinol,* 4**,** 16.

Ebisch, I. M., W. H. Peters, C. M. Thomas, A. M. Wetzels, P. G. Peer & R. P. Steegers-Theunissen (2006) Homocysteine, glutathione and related thiols affect fertility parameters in the (sub)fertile couple. *Hum Reprod,* 21**,** 1725–33.

Elter, K., C. T. Erel, N. Cine, U. Ozbek, B. Hacihanefioglu & E. Ertungealp (1999) Role of the mutations Trp8 => Arg and Ile15 => Thr of the human luteinizing hormone beta-subunit in women with polycystic ovary syndrome. *Fertil Steril,* 71**,** 425–30.

Eppig, J. J. (1991) Intercommunication between mammalian oocytes and companion somatic cells. *Bioessays,* 13**,** 569–74.

Erickson, G. F. & S. Shimasaki (2001) The physiology of folliculogenesis: the role of novel growth factors. *Fertil Steril,* 76**,** 943–9.

Faddy, M. J., R. G. Gosden, A. Gougeon, S. J. Richardson & J. F. Nelson (1992) Accelerated disappearance of ovarian follicles in mid-life: implications for forecasting menopause. *Hum Reprod,* 7**,** 1342–6.

Falconer, H., E. Andersson, A. Aanesen & G. Fried (2005) Follicle-stimulating hormone receptor polymorphisms in a population of infertile women. *Acta Obstet Gynecol Scand,* 84**,** 806–11.

Fan, H. Y., M. Shimada, Z. Liu, N. Cahill, N. Noma, Y. Wu, J. Gossen & J. S. Richards (2008) Selective expression of KrasG12D in granulosa cells of the mouse ovary causes defects in follicle development and ovulation. *Development,* 135**,** 2127–37.

Fauser, B. C. & P. Devroey (2005) Why is the clinical acceptance of gonadotropin-releasing hormone antagonist cotreatment during ovarian hyperstimulation for in vitro fertilization so slow? *Fertil Steril,* 83**,** 1607–11.

Feng, B., S. Chen, R. M. Shelden & D. B. Seifer (2003) Effect of gonadotropins on brain-derived neurotrophic factor secretion by human follicular cumulus cells. *Fertil Steril,* 80**,** 658–9.

Feuerstein, P., V. Cadoret, R. Dalbies-Tran, F. Guerif, R. Bidault & D. Royere (2007) Gene expression in human cumulus cells: one approach to oocyte competence. *Hum Reprod,* 22**,** 3069–77.

Fiddes, J. C. & K. Talmadge (1984) Structure, expression, and evolution of the genes for the human glycoprotein hormones. *Recent Prog Horm Res,* 40**,** 43–78.

Fitzgerald, C., A. E. Zimon & E. E. Jones (1998) Aging and reproductive potential in women. *Yale J Biol Med,* 71**,** 367–81.

Fohr, I. P., R. Prinz-Langenohl, A. Bronstrup, A. M. Bohlmann, H. Nau, H. K. Berthold & K. Pietrzik (2002) 5,10-Methylenetetrahydrofolate reductase genotype determines

the plasma homocysteine-lowering effect of supplementation with 5-methyltetrahydrofolate or folic acid in healthy young women. *Am J Clin Nutr,* 75**,** 275–82.
Forges, T., P. Monnier-Barbarino, J. M. Alberto, R. M. Gueant-Rodriguez, J. L. Daval & J. L. Gueant (2007) Impact of folate and homocysteine metabolism on human reproductive health. *Hum Reprod Update,* 13**,** 225–38.
Fortune, J. E. & S. E. Vincent (1983) Progesterone inhibits the induction of aromatase activity in rat granulosa cells in vitro. *Biol Reprod,* 28**,** 1078–89.
Freour, T., D. Masson, S. Mirallie, M. Jean, K. Bach, T. Dejoie & P. Barriere (2008) Active smoking compromises IVF outcome and affects ovarian reserve. *Reprod Biomed Online,* 16**,** 96–102.
Friso, S., S. W. Choi, D. Girelli, J. B. Mason, G. G. Dolnikowski, P. J. Bagley, O. Olivieri, P. F. Jacques, I. H. Rosenberg, R. Corrocher & J. Selhub (2002) A common mutation in the 5,10-methylenetetrahydrofolate reductase gene affects genomic DNA methylation through an interaction with folate status. *Proc Natl Acad Sci U S A,* 99**,** 5606–11.
Frosst, P., H. J. Blom, R. Milos, P. Goyette, C. A. Sheppard, R. G. Matthews, G. J. Boers, M. den Heijer, L. A. Kluijtmans, L. P. van den Heuvel & et al. (1995) A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. *Nat Genet,* 10**,** 111–3.
Gasch, A., U. Hinz & R. Renkawitz-Pohl (1989) Intron and upstream sequences regulate expression of the Drosophila beta 3-tubulin gene in the visceral and somatic musculature, respectively. *Proc Natl Acad Sci U S A,* 86**,** 3215–8.
George, L., J. L. Mills, A. L. Johansson, A. Nordmark, B. Olander, F. Granath & S. Cnattingius (2002) Plasma folate levels and risk of spontaneous abortion. *JAMA,* 288**,** 1867–73.
Georgiou, I., M. Konstantelli, M. Syrrou, I. E. Messinis & D. E. Lolis (1997) Oestrogen receptor gene polymorphisms and ovarian stimulation for in-vitro fertilization. *Hum Reprod,* 12**,** 1430–3.
Georgiou, I., M. Syrrou, I. Bouba, N. Dalkalitsis, M. Paschopoulos, I. Navrozoglou & D. Lolis (1999) Association of estrogen receptor gene polymorphisms with endometriosis. *Fertil Steril,* 72**,** 164–6.
Giangrande, P. H., E. A. Kimbrel, D. P. Edwards & D. P. McDonnell (2000) The opposing transcriptional activities of the two isoforms of the human progesterone receptor are due to differential cofactor binding. *Mol Cell Biol,* 20**,** 3102–15.
Goff, A. K., P. C. Leung & D. T. Armstrong (1979) Stimulatory action of follicle-stimulating hormone and androgens on the responsiveness of rat granulosa cells to gonadotropins in vitro. *Endocrinology,* 104**,** 1124–9.
Gosden, R. G., J. Mullan, H. M. Picton, H. Yin & S. L. Tan (2002) Current perspective on primordial follicle cryopreservation and culture for reproductive medicine. *Hum Reprod Update,* 8**,** 105–10.
Gougeon, A. (1986) Dynamics of follicular growth in the human: a model from preliminary results. *Hum Reprod,* 1**,** 81–7.
Gougeon, A. (1989) Some aspects of the dynamics of ovarian follicular growth in the human. *Acta Eur Fertil,* 20**,** 185–92.
Gougeon, A. (1996) Regulation of ovarian follicular development in primates: facts and hypotheses. *Endocr Rev,* 17**,** 121–55.
Gougeon, A. (1998) Ovarian follicular growth in humans: ovarian ageing and population of growing follicles. *Maturitas,* 30**,** 137–42.

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

Graham, J. D. & C. L. Clarke (1997) Physiological action of progesterone in target tissues. *Endocr Rev,* 18**,** 502–19.

Greb, R. R., K. Grieshaber, J. Gromoll, B. Sonntag, E. Nieschlag, L. Kiesel & M. Simoni (2005) A common single nucleotide polymorphism in exon 10 of the human follicle stimulating hormone receptor is a major determinant of length and hormonal dynamics of the menstrual cycle. *J Clin Endocrinol Metab,* 90**,** 4866–72.

Grigorova, M., K. Rull & M. Laan (2007) Haplotype structure of FSHB, the beta-subunit gene for fertility-associated follicle-stimulating hormone: possible influence of balancing selection. *Ann Hum Genet,* 71**,** 18–28.

Grimbizis, G., M. Vandervorst, M. Camus, H. Tournaye, A. Van Steirteghem & P. Devroey (1998) Intracytoplasmic sperm injection, results in women older than 39, according to age and the number of embryos replaced in selective or non-selective transfers. *Hum Reprod,* 13**,** 884–9.

Grondahl, M. L., R. Borup, Y. B. Lee, V. Myrhoj, H. Meinertz & S. Sorensen (2009) Differences in gene expression of granulosa cells from women undergoing controlled ovarian hyperstimulation with either recombinant follicle-stimulating hormone or highly purified human menopausal gonadotropin. *Fertil Steril,* 91**,** 1820–30.

Haggarty, P., H. McCallum, H. McBain, K. Andrews, S. Duthie, G. McNeill, A. Templeton, N. Haites, D. Campbell & S. Bhattacharya (2006) Effect of B vitamins and genetics on success of in-vitro fertilisation: prospective cohort study. *Lancet,* 367**,** 1513–9.

Haiman, C. A., S. E. Hankinson, D. Spiegelman, I. De Vivo, G. A. Colditz, W. C. Willett, F. E. Speizer & D. J. Hunter (2000) A tetranucleotide repeat polymorphism in CYP19 and breast cancer risk. *Int J Cancer,* 87**,** 204–10.

Haller, K., A. Salumets, M. Grigorova, I. Talja, L. Salur, M. C. Bene, M. Laan & R. Uibo (2007) Putative predictors of antibodies against follicle-stimulating hormone in female infertility: a study based on in vitro fertilization patients. *Am J Reprod Immunol,* 57**,** 193–200.

Hamel, M., I. Dufort, C. Robert, C. Gravel, M. C. Leveille, A. Leader & M. A. Sirard (2008) Identification of differentially expressed markers in human follicular cells associated with competent oocytes. *Hum Reprod,* 23**,** 1118–27.

Harmon, D. L., J. V. Woodside, J. W. Yarnell, D. McMaster, I. S. Young, E. E. McCrum, K. F. Gey, A. S. Whitehead & A. E. Evans (1996) The common 'thermolabile' variant of methylene tetrahydrofolate reductase is a major determinant of mild hyperhomocysteinaemia. *QJM,* 89**,** 571–7.

Herrington, D. M., T. D. Howard, G. A. Hawkins, D. M. Reboussin, J. Xu, S. L. Zheng, K. B. Brosnihan, D. A. Meyers & E. R. Bleecker (2002) Estrogen-receptor polymorphisms and effects of estrogen replacement on high-density lipoprotein cholesterol in women with coronary disease. *N Engl J Med,* 346**,** 967–74.

Hickey, T. E., D. L. Marrocco, R. B. Gilchrist, R. J. Norman & D. T. Armstrong (2004) Interactions between androgen and growth factors in granulosa cell subtypes of porcine antral follicles. *Biol Reprod,* 71**,** 45–52.

Hillier, S. G., P. F. Whitelaw & C. D. Smyth (1994) Follicular oestrogen synthesis: the 'two-cell, two-gonadotrophin' model revisited. *Mol Cell Endocrinol,* 100**,** 51–4.

Hirshfield, A. N. (1991) Development of follicles in the mammalian ovary. *Int Rev Cytol,* 124**,** 43–101.
Hsieh, Y. Y., Y. K. Wang, C. C. Chang & C. S. Lin (2007) Estrogen receptor alpha-351 XbaI*G and -397 PvuII*C-related genotypes and alleles are associated with higher susceptibilities of endometriosis and leiomyoma. *Mol Hum Reprod,* 13**,** 117–22.
Huhtaniemi, I. T. & A. P. Themmen (2005) Mutations in human gonadotropin and gonadotropin-receptor genes. *Endocrine,* 26**,** 207–17.
Hur, S. E., S. Lee, J. Y. Lee, H. S. Moon, H. L. Kim & H. W. Chung (2007) Polymorphisms and haplotypes of the gene encoding the estrogen-metabolizing CYP19 gene in Korean women: no association with advanced-stage endometriosis. *J Hum Genet,* 52**,** 703–11.
Ihaka, R. & R. Gentleman (1996) R: A language for data analysis and graphics. *Journal of Computational and Graphical Statistics,* 5**,** 299–314.
Ireland, J. J. & J. S. Richards (1978) Acute effects of estradiol and follicle-stimulating hormone on specific binding of human [125I]iodofollicle-stimulating hormone to rat ovarian granulosa cells in vivo and in vitro. *Endocrinology,* 102**,** 876–83.
Jacques, P. F., A. G. Bostom, P. W. Wilson, S. Rich, I. H. Rosenberg & J. Selhub (2001) Determinants of plasma total homocysteine concentration in the Framingham Offspring cohort. *Am J Clin Nutr,* 73**,** 613–21.
Jefcoate, C. R., J. G. Liehr, R. J. Santen, T. R. Sutter, J. D. Yager, W. Yue, S. J. Santner, R. Tekmal, L. Demers, R. Pauley, F. Naftolin, G. Mor & L. Berstein (2000) Tissue-specific synthesis and oxidative metabolism of estrogens. *J Natl Cancer Inst Monogr***,** 95–112.
Jiang, M. & I. Huhtaniemi (2004) Polymorphisms in androgen and estrogen receptor genes: effects on male aging. *Exp Gerontol,* 39**,** 1603–11.
Johnson, N. P., E. M. Bagrie, A. Coomarasamy, S. Bhattacharya, A. N. Shelling, S. Jessop, C. Farquhar & K. S. Khan (2006) Ovarian reserve tests for predicting fertility outcomes for assisted reproductive technology: the International Systematic Collaboration of Ovarian Reserve Evaluation protocol for a systematic review of ovarian reserve test accuracy. *BJOG,* 113**,** 1472–80.
Jones, M. E., A. W. Thorburn, K. L. Britt, K. N. Hewitt, M. L. Misso, N. G. Wreford, J. Proietto, O. K. Oz, B. J. Leury, K. M. Robertson, S. Yao & E. R. Simpson (2001) Aromatase-deficient (ArKO) mice accumulate excess adipose tissue. *J Steroid Biochem Mol Biol,* 79**,** 3–9.
Jun, J. K., J. S. Yoon, S. Y. Ku, Y. M. Choi, K. R. Hwang, S. Y. Park, G. H. Lee, W. D. Lee, S. H. Kim, J. G. Kim & S. Y. Moon (2006) Follicle-stimulating hormone receptor gene polymorphism and ovarian responses to controlled ovarian hyperstimulation for IVF-ET. *J Hum Genet,* 51**,** 665–70.
Kado, N., J. Kitawaki, H. Obayashi, H. Ishihara, H. Koshiba, I. Kusuki, K. Tsukamoto, G. Hasegawa, N. Nakamura, T. Yoshikawa & H. Honjo (2002) Association of the CYP17 gene and CYP19 gene polymorphisms with risk of endometriosis in Japanese women. *Hum Reprod,* 17**,** 897–902.
Kapiszewska, M., M. Kalemba, U. Wojciech & T. Milewicz (2005) Uracil misincorporation into DNA of leukocytes of young women with positive folate balance depends on plasma vitamin B12 concentrations and methylenetetrahydrofolate reductase polymorphisms. A pilot study. *J Nutr Biochem,* 16**,** 467–78.
Kastner, P., A. Krust, B. Turcotte, U. Stropp, L. Tora, H. Gronemeyer & P. Chambon (1990) Two distinct estrogen-regulated promoters generate transcripts encoding the



21

two functionally different human progesterone receptor forms A and B. *EMBO J,* 9**,** 1603–14.

Kawamura, K., N. Kawamura, S. M. Mulders, M. D. Sollewijn Gelpke & A. J. Hsueh (2005) Ovarian brain-derived neurotrophic factor (BDNF) promotes the development of oocytes into preimplantation embryos. *Proc Natl Acad Sci U S A,* 102**,** 9206–11.

Keijzer, M. B., M. den Heijer, H. J. Blom, G. M. Bos, H. P. Willems, W. B. Gerrits & F. R. Rosendaal (2002) Interaction between hyperhomocysteinemia, mutated methylenetetrahydrofolatereductase (MTHFR) and inherited thrombophilic factors in recurrent venous thrombosis. *Thromb Haemost,* 88**,** 723–8.

Kligman, I. & Z. Rosenwaks (2001) Differentiating clinical profiles: predicting good responders, poor responders, and hyperresponders. *Fertil Steril,* 76**,** 1185–90.

Knight, P. G. & C. Glister (2006) TGF-beta superfamily members and ovarian follicle development. *Reproduction,* 132**,** 191–206.

Krege, J. H., J. B. Hodgin, J. F. Couse, E. Enmark, M. Warner, J. F. Mahler, M. Sar, K. S. Korach, J. A. Gustafsson & O. Smithies (1998) Generation and reproductive phenotypes of mice lacking estrogen receptor beta. *Proc Natl Acad Sci U S A,* 95**,** 15677–82.

Kristensen, V. N., T. I. Andersen, A. Lindblom, B. Erikstein, P. Magnus & A. L. Borresen-Dale (1998) A rare CYP19 (aromatase) variant may increase the risk of breast cancer. *Pharmacogenetics,* 8**,** 43–8.

Kristensen, V. N., N. Harada, N. Yoshimura, E. Haraldsen, P. E. Lonning, B. Erikstein, R. Karesen, T. Kristensen & A. L. Borresen-Dale (2000) Genetic variants of CYP19 (aromatase) and breast cancer risk. *Oncogene,* 19**,** 1329–33.

Kuiper, G. G., E. Enmark, M. Pelto-Huikko, S. Nilsson & J. A. Gustafsson (1996) Cloning of a novel receptor expressed in rat prostate and ovary. *Proc Natl Acad Sci U S A,* 93**,** 5925–30.

Kumar, T. R., Y. Wang, N. Lu & M. M. Matzuk (1997) Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility. *Nat Genet,* 15**,** 201–4.

Kupesic, S. & A. Kurjak (2002) Predictors of IVF outcome by three-dimensional ultrasound. *Hum Reprod,* 17**,** 950–5.

Kurosaki, K., H. Saitoh, H. Oota, Y. Watanabe, M. Kiuchi & S. Ueda (1997) Combined polymorphism associated with a 3-bp deletion in the 5'-flanking region of a tetrameric short tandem repeat at the CYP19 locus. *Nihon Hoigaku Zasshi,* 51**,** 191–5.

Kwee, J., M. E. Elting, R. Schats, J. McDonnell & C. B. Lambalk (2007) Ovarian volume and antral follicle count for the prediction of low and hyper responders with in vitro fertilization. *Reprod Biol Endocrinol,* 5**,** 9.

La Marca, A., F. J. Broekmans, A. Volpe, B. C. Fauser & N. S. Macklon (2009) Anti-Mullerian hormone (AMH): what do we still need to know? *Hum Reprod,* 24**,** 2264–75.

Laanpere, M., S. Altmäe, A. Stavreus-Evers, T. K. Nilsson, A. Yngve & A. Salumets (2009) Folate-mediated one-carbon metabolism and its effect on female fertility and pregnancy viability. *Nutrition Reviews,* (in press).

Lamminen, T., P. Jokinen, M. Jiang, P. Pakarinen, H. Simonsen & I. Huhtaniemi (2005) Human FSH beta subunit gene is highly conserved. *Mol Hum Reprod,* 11**,** 601–5.

Lass, A., R. Silye, D. C. Abrams, T. Krausz, O. Hovatta, R. Margara & R. M. Winston (1997) Follicular density in ovarian biopsy of infertile women: a novel method to assess ovarian reserve. *Hum Reprod,* 12**,** 1028–31.

Layman, L. C., E. J. Lee, D. B. Peak, A. B. Namnoum, K. V. Vu, B. L. van Lingen, M. R. Gray, P. G. McDonough, R. H. Reindollar & J. L. Jameson (1997) Delayed puberty and hypogonadism caused by mutations in the follicle-stimulating hormone beta-subunit gene. *N Engl J Med,* 337**,** 607–11.

Liao, W. X., A. C. Roy, C. Chan, S. Arulkumaran & S. S. Ratnam (1998) A new molecular variant of luteinizing hormone associated with female infertility. *Fertil Steril,* 69**,** 102–6.

Lim, A. S. & M. F. Tsakok (1997) Age-related decline in fertility: a link to degenerative oocytes? *Fertil Steril,* 68**,** 265–71.

Livshyts, G., S. Podlesnaja, S. Kravchenko, I. Sudoma & L. Livshits (2009) A distribution of two SNPs in exon 10 of the FSHR gene among the women with a diminished ovarian reserve in Ukraine. *J Assist Reprod Genet,* 26**,** 29–34.

Loutradis, D., E. Patsoula, V. Minas, G. A. Koussidis, A. Antsaklis, S. Michalas & A. Makrigiannakis (2006) FSH receptor gene polymorphisms have a role for different ovarian response to stimulation in patients entering IVF/ICSI-ET programs. *J Assist Reprod Genet,* 23**,** 177–84.

Lowicka, E. & J. Beltowski (2007) Hydrogen sulfide (H2S) – the third gas of interest for pharmacologists. *Pharmacol Rep,* 59**,** 4–24.

Luke, B. & M. B. Brown (2007) Maternal risk factors for potential maltreatment deaths among healthy singleton and twin infants. *Twin Res Hum Genet,* 10**,** 778–85.

Macklon, N. S., R. L. Stouffer, L. C. Giudice & B. C. Fauser (2006) The science behind 25 years of ovarian stimulation for in vitro fertilization. *Endocr Rev,* 27**,** 170–207.

Maizels, E. T., A. Mukherjee, G. Sithanandam, C. A. Peters, J. Cottom, K. E. Mayo & M. Hunzicker-Dunn (2001) Developmental regulation of mitogen-activated protein kinase-activated kinases-2 and -3 (MAPKAPK-2/-3) in vivo during corpus luteum formation in the rat. *Mol Endocrinol,* 15**,** 716–33.

Martinelli, I., E. Taioli, G. Ragni, P. Levi-Setti, S. M. Passamonti, T. Battaglioli, C. Lodigiani & P. M. Mannucci (2003) Embryo implantation after assisted reproductive procedures and maternal thrombophilia. *Haematologica,* 88**,** 789–93.

Matherly, L. H., Z. Hou & Y. Deng (2007) Human reduced folate carrier: translation of basic biology to cancer etiology and therapy. *Cancer Metastasis Rev,* 26**,** 111–28.

Matthews, C. H., S. Borgato, P. Beck-Peccoz, M. Adams, Y. Tone, G. Gambino, S. Casagrande, G. Tedeschini, A. Benedetti & V. K. Chatterjee (1993) Primary amenorrhoea and infertility due to a mutation in the beta-subunit of follicle-stimulating hormone. *Nat Genet,* 5**,** 83–6.

Matzuk, M. M., K. H. Burns, M. M. Viveiros & J. J. Eppig (2002) Intercellular communication in the mammalian ovary: oocytes carry the conversation. *Science,* 296**,** 2178–80.

McFadden, D. E. & J. M. Friedman (1997) Chromosome abnormalities in human beings. *Mutat Res,* 396**,** 129–40.

McKenzie, L. J., S. A. Pangas, S. A. Carson, E. Kovanci, P. Cisneros, J. E. Buster, P. Amato & M. M. Matzuk (2004) Human cumulus granulosa cell gene expression: a predictor of fertilization and embryo selection in women undergoing IVF. *Hum Reprod,* 19**,** 2869–74.

Milki, A. A., M. D. Hinckley, J. Gebhardt, D. Dasig, L. M. Westphal & B. Behr (2002) Accuracy of day 3 criteria for selecting the best embryos. *Fertil Steril,* 77**,** 1191–5.

Miller-Lindholm, A. K., E. Bedows, C. F. Bartels, J. Ramey, V. Maclin & R. W. Ruddon (1999) A naturally occurring genetic variant in the human chorionic gonadotropin-beta gene 5 is assembly inefficient. *Endocrinology,* 140**,** 3496–506.

Modi, D., D. Bhartiya & C. Puri (2006) Developmental expression and cellular distribution of Mullerian inhibiting substance in the primate ovary. *Reproduction,* 132**,** 443–53.

Mooij, P. N., M. G. Wouters, C. M. Thomas, W. H. Doesburg & T. K. Eskes (1992) Disturbed reproductive performance in extreme folic acid deficient golden hamsters. *Eur J Obstet Gynecol Reprod Biol,* 43**,** 71–5.

Mosselman, S., J. Polman & R. Dijkema (1996) ER beta: identification and characterization of a novel human estrogen receptor. *FEBS Lett,* 392**,** 49–53.

Mullis, P. E., N. Yoshimura, B. Kuhlmann, K. Lippuner, P. Jaeger & H. Harada (1997) Aromatase deficiency in a female who is compound heterozygote for two new point mutations in the P450arom gene: impact of estrogens on hypergonadotropic hypogonadism, multicystic ovaries, and bone densitometry in childhood. *J Clin Endocrinol Metab,* 82**,** 1739–45.

Nagy, Z. P., D. Sakkas & B. Behr (2008) Symposium: innovative techniques in human embryo viability assessment. Non-invasive assessment of embryo viability by metabolomic profiling of culture media ('metabolomics'). *Reprod Biomed Online,* 17**,** 502–7.

Narayanan, S., J. McConnell, J. Little, L. Sharp, C. J. Piyathilake, H. Powers, G. Basten & S. J. Duthie (2004) Associations between two common variants C677T and A1298C in the methylenetetrahydrofolate reductase gene and measures of folate metabolism and DNA stability (strand breaks, misincorporated uracil, and DNA methylation status) in human lymphocytes in vivo. *Cancer Epidemiol Biomarkers Prev,* 13**,** 1436–43.

Nardo, L. G., T. A. Gelbaya, H. Wilkinson, S. A. Roberts, A. Yates, P. Pemberton & I. Laing (2009) Circulating basal anti-Mullerian hormone levels as predictor of ovarian response in women undergoing ovarian stimulation for in vitro fertilization. *Fertil Steril,* 92**,** 1586–93.

Naylor, S. L., W. W. Chin, H. M. Goodman, P. A. Lalley, K. H. Grzeschik & A. Y. Sakaguchi (1983) Chromosome assignment of genes encoding the alpha and beta subunits of glycoprotein hormones in man and mouse. *Somatic Cell Genet,* 9**,** 757–70.

Nelen, W. L., J. Bulten, E. A. Steegers, H. J. Blom, A. G. Hanselaar & T. K. Eskes (2000) Maternal homocysteine and chorionic vascularization in recurrent early pregnancy loss. *Hum Reprod,* 15**,** 954–60.

Ng, E. H., O. S. Tang, C. C. Chan & P. C. Ho (2005) Ovarian stromal blood flow in the prediction of ovarian response during in vitro fertilization treatment. *Hum Reprod,* 20**,** 3147–51.

Nilsson, T. K., Z. M. Lof-Ohlin & A. K. Bottiger (2008) Genotyping of the reduced folate carrier-1 c.80G>A polymorphism by pyrosequencing technology: Importance of PCR and pre-PCR optimization. *Scand J Clin Lab Invest,* 68**,** 166–70.

Nishimura, R., J. Shin, I. Ji, C. R. Middaugh, W. Kruggel, R. V. Lewis & T. H. Ji (1986) A single amino acid substitution in an ectopic alpha subunit of a human carcinoma choriogonadotropin. *J Biol Chem,* 261**,** 10475–7.

Niswender, G. D., J. L. Juengel, P. J. Silva, M. K. Rollyson & E. W. McIntush (2000) Mechanisms controlling the function and life span of the corpus luteum. *Physiol Rev,* 80**,** 1–29.

Oktay, K., D. Briggs & R. G. Gosden (1997) Ontogeny of follicle-stimulating hormone receptor gene expression in isolated human ovarian follicles. *J Clin Endocrinol Metab,* 82**,** 3748–51.

Ola, B. & T. C. Li (2006) Implantation failure following in-vitro fertilization. *Curr Opin Obstet Gynecol,* 18**,** 440–5.
Pacchiarotti, A., M. A. Mohamed, G. Micara, A. Linari, D. Tranquilli, S. B. Espinola & C. Aragona (2007) The possible role of hyperhomocysteinemia on IVF outcome. *J Assist Reprod Genet,* 24**,** 459–62.
Packer, A. I., Y. C. Hsu, P. Besmer & R. F. Bachvarova (1994) The ligand of the c-kit receptor promotes oocyte growth. *Dev Biol,* 161**,** 194–205.
Palermo, G. D., J. Cohen & Z. Rosenwaks (1996) Intracytoplasmic sperm injection: a powerful tool to overcome fertilization failure. *Fertil Steril,* 65**,** 899–908.
Paredes, A., C. Romero, G. A. Dissen, T. M. DeChiara, L. Reichardt, A. Cornea, S. R. Ojeda & B. Xu (2004) TrkB receptors are required for follicular growth and oocyte survival in the mammalian ovary. *Dev Biol,* 267**,** 430–49.
Part, K., M. Laanpere, K. Rahu, K. Haldre, M. Rahu & H. Karro. 2007. *Eesti Naise Tervis: seksuall- ja reproduktiivtervis, tervisekäitumine, hoiakud ja terviseteenuste kasutamine*. Tartu: Tartu Ülikooli Naistekliinik.
Pelletier, G. & M. El-Alfy (2000) Immunocytochemical localization of estrogen receptors alpha and beta in the human reproductive organs. *J Clin Endocrinol Metab,* 85**,** 4835–40.
Perez Mayorga, M., J. Gromoll, H. M. Behre, C. Gassner, E. Nieschlag & M. Simoni (2000) Ovarian response to follicle-stimulating hormone (FSH) stimulation depends on the FSH receptor genotype. *J Clin Endocrinol Metab,* 85**,** 3365–9.
Perlman, S., T. Bouquin, B. van den Hazel, T. H. Jensen, H. T. Schambye, S. Knudsen & J. S. Okkels (2006) Transcriptome analysis of FSH and FSH variant stimulation in granulosa cells from IVM patients reveals novel regulated genes. *Mol Hum Reprod,* 12**,** 135–44.
Petry, C. J., K. K. Ong, K. F. Michelmore, S. Artigas, D. L. Wingate, A. H. Balen, F. de Zegher, L. Ibanez & D. B. Dunger (2005) Association of aromatase (CYP 19) gene variation with features of hyperandrogenism in two populations of young women. *Hum Reprod,* 20**,** 1837–43.
Piersma, D., E. M. Berns, M. Verhoef-Post, A. G. Uitterlinden, I. Braakman, H. A. Pols & A. P. Themmen (2006) A common polymorphism renders the luteinizing hormone receptor protein more active by improving signal peptide function and predicts adverse outcome in breast cancer patients. *J Clin Endocrinol Metab,* 91**,** 1470–6.
Piersma, D., M. Verhoef-Post, M. P. Look, A. G. Uitterlinden, H. A. Pols, E. M. Berns & A. P. Themmen (2007) Polymorphic variations in exon 10 of the luteinizing hormone receptor: functional consequences and associations with breast cancer. *Mol Cell Endocrinol,* 276**,** 63–70.
Pinborg, A. (2005) IVF/ICSI twin pregnancies: risks and prevention. *Hum Reprod Update,* 11**,** 575–93.
Pinborg, A., A. Loft & A. Nyboe Andersen (2004a) Neonatal outcome in a Danish national cohort of 8602 children born after in vitro fertilization or intracytoplasmic sperm injection: the role of twin pregnancy. *Acta Obstet Gynecol Scand,* 83**,** 1071–8.
Pinborg, A., A. Loft, L. Schmidt, J. Langhoff-Roos & A. N. Andersen (2004b) Maternal risks and perinatal outcome in a Danish national cohort of 1005 twin pregnancies: the role of in vitro fertilization. *Acta Obstet Gynecol Scand,* 83**,** 75–84.
Pisarska, M. D., S. A. Carson, P. R. Casson, X. Tong, J. E. Buster & D. G. Kieback (2003) A mutated progesterone receptor allele is more prevalent in unexplained infertility. *Fertil Steril,* 80**,** 651–3.



22

Popovic-Todorovic, B., A. Loft, H. E. Bredkjaeer, S. Bangsboll, I. K. Nielsen & A. N. Andersen (2003) A prospective randomized clinical trial comparing an individual dose of recombinant FSH based on predictive factors versus a 'standard' dose of 150 IU/day in 'standard' patients undergoing IVF/ICSI treatment. *Hum Reprod,* 18**,** 2275–82.

Powell, B. L., D. Piersma, M. E. Kevenaar, I. L. van Staveren, A. P. Themmen, B. J. Iacopetta & E. M. Berns (2003) Luteinizing hormone signaling and breast cancer: polymorphisms and age of onset. *J Clin Endocrinol Metab,* 88**,** 1653–7.

Probst-Hensch, N. M., S. A. Ingles, A. T. Diep, R. W. Haile, F. Z. Stanczyk, L. N. Kolonel & B. E. Henderson (1999) Aromatase and breast cancer susceptibility. *Endocr Relat Cancer,* 6**,** 165–73.

Rawlings, N. D. & A. J. Barrett (1993) Evolutionary families of peptidases. *Biochem J,* 290 ( Pt 1)**,** 205–18.

Rey, E., S. R. Kahn, M. David & I. Shrier (2003) Thrombophilic disorders and fetal loss: a meta-analysis. *Lancet,* 361**,** 901–8.

Reyes-Engel, A., E. Munoz, M. J. Gaitan, E. Fabre, M. Gallo, J. L. Dieguez, M. Ruiz & M. Morell (2002) Implications on human fertility of the 677C-->T and 1298A-->C polymorphisms of the MTHFR gene: consequences of a possible genetic selection. *Mol Hum Reprod,* 8**,** 952–7.

Reynolds, L. P., S. D. Killilea & D. A. Redmer (1992) Angiogenesis in the female reproductive system. *FASEB J,* 6**,** 886–92.

Romano, A., B. Delvoux, D. C. Fischer & P. Groothuis (2007) The PROGINS polymorphism of the human progesterone receptor diminishes the response to progesterone. *J Mol Endocrinol,* 38**,** 331–50.

Romeu, A., S. J. Muasher, A. A. Acosta, L. L. Veeck, J. Diaz, G. S. Jones, H. W. Jones, Jr. & Z. Rosenwaks (1987) Results of in vitro fertilization attempts in women 40 years of age and older: the Norfolk experience. *Fertil Steril,* 47**,** 130–6.

Rosen, M. P., S. Shen, C. E. McCulloch, P. F. Rinaudo, M. I. Cedars & A. T. Dobson (2007) Methylenetetrahydrofolate reductase (MTHFR) is associated with ovarian follicular activity. *Fertil Steril,* 88**,** 632–8.

Rosenwaks, Z., O. K. Davis & M. A. Damario (1995) The role of maternal age in assisted reproduction. *Hum Reprod,* 10 Suppl 1**,** 165–73.

Rousseau-Merck, M. F., M. Atger, H. Loosfelt, E. Milgrom & R. Berger (1993) The chromosomal localization of the human follicle-stimulating hormone receptor gene (FSHR) on 2p21–p16 is similar to that of the luteinizing hormone receptor gene. *Genomics,* 15**,** 222–4.

Ryan, K. J. (1982) Biochemistry of aromatase: significance to female reproductive physiology. *Cancer Res,* 42**,** 3342s–3344s.

Salumets, A., C. Hyden-Granskog, S. Makinen, A. M. Suikkari, A. Tiitinen & T. Tuuri (2003) Early cleavage predicts the viability of human embryos in elective single embryo transfer procedures. *Hum Reprod,* 18**,** 821–5.

Salumets, A., C. Hyden-Granskog, A. M. Suikkari, A. Tiitinen & T. Tuuri (2001) The predictive value of pronuclear morphology of zygotes in the assessment of human embryo quality. *Hum Reprod,* 16**,** 2177–81.

Sample, W. F., B. M. Lippe & M. T. Gyepes (1977) Gray-scale ultrasonography of the normal female pelvis. *Radiology,* 125**,** 477–83.

Saunders, P. T., M. R. Millar, K. Williams, S. Macpherson, D. Harkiss, R. A. Anderson, B. Orr, N. P. Groome, G. Scobie & H. M. Fraser (2000) Differential expression of

estrogen receptor-alpha and -beta and androgen receptor in the ovaries of marmosets and humans. *Biol Reprod,* 63**,** 1098–105.
Scheffer, G. J., F. J. Broekmans, L. F. Bancsi, J. D. Habbema, C. W. Looman & E. R. Te Velde (2002) Quantitative transvaginal two- and three-dimensional sonography of the ovaries: reproducibility of antral follicle counts. *Ultrasound Obstet Gynecol,* 20**,** 270–5.
Schreiber, J. R., K. Nakamura & G. F. Erickson (1981) Progestins inhibit FSH-stimulated granulosa estrogen production at a post-cAMP site. *Mol Cell Endocrinol,* 21**,** 161–70.
Sebastian, S. & S. E. Bulun (2001) A highly complex organization of the regulatory region of the human CYP19 (aromatase) gene revealed by the Human Genome Project. *J Clin Endocrinol Metab,* 86**,** 4600–2.
Seifer, D. B., B. Feng, R. M. Shelden, S. Chen & C. F. Dreyfus (2002) Brain-derived neurotrophic factor: a novel human ovarian follicular protein. *J Clin Endocrinol Metab,* 87**,** 655–9.
Sekiguchi, T., T. Mizutani, K. Yamada, T. Yazawa, H. Kawata, M. Yoshino, T. Kajitani, T. Kameda, T. Minegishi & K. Miyamoto (2002) Transcriptional regulation of the epiregulin gene in the rat ovary. *Endocrinology,* 143**,** 4718–29.
Sharara, F. I. & L. K. Nieman (1994) Identification and cellular localization of growth hormone receptor gene expression in the human ovary. *J Clin Endocrinol Metab,* 79**,** 670–2.
Sherman, S. L., S. B. Freeman, E. G. Allen & N. E. Lamb (2005) Risk factors for nondisjunction of trisomy 21. *Cytogenet Genome Res,* 111**,** 273–80.
Shimada, M., I. Hernandez-Gonzalez, I. Gonzalez-Robayna & J. S. Richards (2006) Paracrine and autocrine regulation of epidermal growth factor-like factors in cumulus oocyte complexes and granulosa cells: key roles for prostaglandin synthase 2 and progesterone receptor. *Mol Endocrinol,* 20**,** 1352–65.
Shimada, M. & T. Terada (2002) FSH and LH induce progesterone production and progesterone receptor synthesis in cumulus cells: a requirement for meiotic resumption in porcine oocytes. *Mol Hum Reprod,* 8**,** 612–8.
Simoni, M., J. Gromoll, W. Hoppner, A. Kamischke, T. Krafft, D. Stahle & E. Nieschlag (1999) Mutational analysis of the follicle-stimulating hormone (FSH) receptor in normal and infertile men: identification and characterization of two discrete FSH receptor isoforms. *J Clin Endocrinol Metab,* 84**,** 751–5.
Simoni, M., E. Nieschlag & J. Gromoll (2002) Isoforms and single nucleotide polymorphisms of the FSH receptor gene: implications for human reproduction. *Hum Reprod Update,* 8**,** 413–21.
Simoni, M., F. Tuttelmann, C. Michel, Y. Bockenfeld, E. Nieschlag & J. Gromoll (2008) Polymorphisms of the luteinizing hormone/chorionic gonadotropin receptor gene: association with maldescended testes and male infertility. *Pharmacogenet Genomics,* 18**,** 193–200.
Smith, S., S. M. Pfeifer & J. A. Collins (2003) Diagnosis and management of female infertility. *JAMA,* 290**,** 1767–70.
Spandorfer, S. D., N. Normand, K. Dragisic, Z. Rosenwaks & S. S. Witkin (2006) A G–>A polymorphism at position +331 in the progesterone receptor gene is strongly associated with IVF outcome. *Fertil Steril,* 86**,** s3–4.
Speroff, L. & M. A. Fritz. 2005. *Clinical Gynecologic Endocrinology and Infertility*. Philadelphia: Lippincott Williams & Wilkins.

Sriraman, V. & J. S. Richards (2004) Cathepsin L gene expression and promoter activation in rodent granulosa cells. *Endocrinology,* 145**,** 582–91.

Steele-Perkins, G., C. Plachez, K. G. Butz, G. Yang, C. J. Bachurski, S. L. Kinsman, E. D. Litwack, L. J. Richards & R. M. Gronostajski (2005) The transcription factor gene Nfib is essential for both lung maturation and brain development. *Mol Cell Biol,* 25**,** 685–98.

Steptoe, P. C. & R. G. Edwards (1978) Birth after the reimplantation of a human embryo. *Lancet,* 2**,** 366.

Stern, L. L., J. B. Mason, J. Selhub & S. W. Choi (2000) Genomic DNA hypomethylation, a characteristic of most cancers, is present in peripheral leukocytes of individuals who are homozygous for the C677T polymorphism in the methylenetetrahydrofolate reductase gene. *Cancer Epidemiol Biomarkers Prev,* 9**,** 849–53.

Stocco, C. O., J. Chedrese & R. P. Deis (2001) Luteal expression of cytochrome P450 side-chain cleavage, steroidogenic acute regulatory protein, 3beta-hydroxysteroid dehydrogenase, and 20alpha-hydroxysteroid dehydrogenase genes in late pregnant rats: effect of luteinizing hormone and RU486. *Biol Reprod,* 65**,** 1114–9.

Su, Y. Q., J. M. Denegre, K. Wigglesworth, F. L. Pendola, M. J. O'Brien & J. J. Eppig (2003) Oocyte-dependent activation of mitogen-activated protein kinase (ERK1/2) in cumulus cells is required for the maturation of the mouse oocyte-cumulus cell complex. *Dev Biol,* 263**,** 126–38.

Sudo, S., M. Kudo, S. Wada, O. Sato, A. J. Hsueh & S. Fujimoto (2002) Genetic and functional analyses of polymorphisms in the human FSH receptor gene. *Mol Hum Reprod,* 8**,** 893–9.

Suganuma, N., K. Furui, M. Furuhashi, Y. Asada, F. Kikkawa & Y. Tomoda (1995) Screening of the mutations in luteinizing hormone beta-subunit in patients with menstrual disorders. *Fertil Steril,* 63**,** 989–95.

Sullivan, M. W., A. Stewart-Akers, J. S. Krasnow, S. L. Berga & A. J. Zeleznik (1999) Ovarian responses in women to recombinant follicle-stimulating hormone and luteinizing hormone (LH): a role for LH in the final stages of follicular maturation. *J Clin Endocrinol Metab,* 84**,** 228–32.

Sundarrajan, C., W. Liao, A. C. Roy & S. C. Ng (1999) Association of oestrogen receptor gene polymorphisms with outcome of ovarian stimulation in patients undergoing IVF. *Mol Hum Reprod,* 5**,** 797–802.

Sundarrajan, C., W. X. Liao, A. C. Roy & S. C. Ng (2001) Association between estrogen receptor-beta gene polymorphisms and ovulatory dysfunctions in patients with menstrual disorders. *J Clin Endocrinol Metab,* 86**,** 135–9.

Syrop, C. H., J. D. Dawson, K. J. Husman, A. E. Sparks & B. J. Van Voorhis (1999) Ovarian volume may predict assisted reproductive outcomes better than follicle stimulating hormone concentration on day 3. *Hum Reprod,* 14**,** 1752–6.

Syrrou, M., I. Georgiou, P. C. Patsalis, I. Bouba, G. Adonakis & G. N. Pagoulatos (1999) Fragile X premutations and (TA)n estrogen receptor polymorphism in women with ovarian dysfunction. *Am J Med Genet,* 84**,** 306–8.

Szymanski, W. & A. Kazdepka-Zieminska (2003) Effect of homocysteine concentration in follicular fluid on a degree of oocyte maturity. *Ginekol Pol,* 74**,** 1392–6.

Takahashi, K., T. Ozaki, M. Okada, H. Kurioka, H. Kanasaki & K. Miyazaki (1999) Increased prevalence of luteinizing hormone beta-subunit variant in patients with premature ovarian failure. *Fertil Steril,* 71**,** 96–101.

Tamura, T. & M. F. Picciano (2006) Folate and human reproduction. *Am J Clin Nutr,* 83**,** 993–1016.

Templeton, A., J. K. Morris & W. Parslow (1996) Factors that affect outcome of in-vitro fertilisation treatment. *Lancet,* 348**,** 1402–6.
Templeton, A. A. & G. C. Penney (1982) The incidence, characteristics, and prognosis of patients whose infertility is unexplained. *Fertil Steril,* 37**,** 175–82.
Thaler, C. J., H. Budiman, H. Ruebsamen, D. Nagel & P. Lohse (2006) Effects of the common 677C>T mutation of the 5,10-methylenetetrahydrofolate reductase (MTHFR) gene on ovarian responsiveness to recombinant follicle-stimulating hormone. *Am J Reprod Immunol,* 55**,** 251–8.
Themmen, A. P. N. & I. T. Huhtaniemi (2000) Mutations of gonadotropins and gonadotropin receptors: elucidating the physiology and pathophysiology of pituitary-gonadal function. *Endocr Rev,* 21**,** 551–83.
Tomas, C., S. Nuojua-Huttunen & H. Martikainen (1997) Pretreatment transvaginal ultrasound examination predicts ovarian responsiveness to gonadotrophins in in-vitro fertilization. *Hum Reprod,* 12**,** 220–3.
Toner, J. P., C. B. Philput, G. S. Jones & S. J. Muasher (1991) Basal follicle-stimulating hormone level is a better predictor of in vitro fertilization performance than age. *Fertil Steril,* 55**,** 784–91.
Tworoger, S. S., J. Chubak, E. J. Aiello, C. M. Ulrich, C. Atkinson, J. D. Potter, Y. Yasui, P. L. Stapleton, J. W. Lampe, F. M. Farin, F. Z. Stanczyk & A. McTiernan (2004) Association of CYP17, CYP19, CYP1B1, and COMT polymorphisms with serum and urinary sex hormone concentrations in postmenopausal women. *Cancer Epidemiol Biomarkers Prev,* 13**,** 94–101.
Ueland, P. M., S. Hustad, J. Schneede, H. Refsum & S. E. Vollset (2001) Biological and clinical implications of the MTHFR C677T polymorphism. *Trends Pharmacol Sci,* 22**,** 195–201.
van den Driesche, S., V. M. Smith, M. Myers & W. C. Duncan (2008) Expression and regulation of oestrogen receptors in the human corpus luteum. *Reproduction,* 135**,** 509–17.
van Montfoort, A. P., J. P. Geraedts, J. C. Dumoulin, A. P. Stassen, J. L. Evers & T. A. Ayoubi (2008) Differential gene expression in cumulus cells as a prognostic indicator of embryo viability: a microarray analysis. *Mol Hum Reprod,* 14**,** 157–68.
van Noord-Zaadstra, B. M., C. W. Looman, H. Alsbach, J. D. Habbema, E. R. te Velde & J. Karbaat (1991) Delaying childbearing: effect of age on fecundity and outcome of pregnancy. *BMJ,* 302**,** 1361–5.
Vegeto, E., M. M. Shahbaz, D. X. Wen, M. E. Goldman, B. W. O'Malley & D. P. McDonnell (1993) Human progesterone receptor A form is a cell- and promoter-specific repressor of human progesterone receptor B function. *Mol Endocrinol,* 7**,** 1244–55.
Vidal, J. D., C. A. Vandevoort, C. B. Marcus, N. R. Lazarewicz & A. J. Conley (2005) 2,3,7,8-tetrachlorodibenzo-p-dioxin induces CYP1B1 expression in human luteinized granulosa cells. *Arch Biochem Biophys,* 439**,** 53–60.
Walter, P., S. Green, G. Greene, A. Krust, J. M. Bornert, J. M. Jeltsch, A. Staub, E. Jensen, G. Scrace, M. Waterfield & et al. (1985) Cloning of the human estrogen receptor cDNA. *Proc Natl Acad Sci U S A,* 82**,** 7889–93.
Wandji, S. A., V. Srsen, P. W. Nathanielsz, J. J. Eppig & J. E. Fortune (1997) Initiation of growth of baboon primordial follicles in vitro. *Hum Reprod,* 12**,** 1993–2001.
Wang, Z., S. Yoshida, K. Negoro, S. Kennedy, D. Barlow & T. Maruo (2004) Polymorphisms in the estrogen receptor beta gene but not estrogen receptor alpha



23

gene affect the risk of developing endometriosis in a Japanese population. *Fertil Steril,* 81**,** 1650–6.

Weitman, S. D., A. G. Weinberg, L. R. Coney, V. R. Zurawski, D. S. Jennings & B. A. Kamen (1992) Cellular localization of the folate receptor: potential role in drug toxicity and folate homeostasis. *Cancer Res,* 52**,** 6708–11.

Whetstine, J. R., A. J. Gifford, T. Witt, X. Y. Liu, R. M. Flatley, M. Norris, M. Haber, J. W. Taub, Y. Ravindranath & L. H. Matherly (2001) Single nucleotide polymorphisms in the human reduced folate carrier: characterization of a high-frequency G/A variant at position 80 and transport properties of the His(27) and Arg(27) carriers. *Clin Cancer Res,* 7**,** 3416–22.

WHO. 1999. *Laboratory manual for the examination of human semen and sperm-cervical mucus interaction*. Cambridge: Cambridge University Press.

Wieser, F., C. Schneeberger, D. Tong, C. Tempfer, J. C. Huber & R. Wenzl (2002) PROGINS receptor gene polymorphism is associated with endometriosis. *Fertil Steril,* 77**,** 309–12.

Workshop, T. E. C. (2002) Physiopathological determinants of human infertility. *Hum Reprod Update,* 8**,** 435–47.

Xita, N., I. Georgiou, L. Lazaros, V. Psofaki, G. Kolios & A. Tsatsoulis (2008) The synergistic effect of sex hormone-binding globulin and aromatase genes on polycystic ovary syndrome phenotype. *Eur J Endocrinol,* 158**,** 861–5.

Yang, G., K. Cao, L. Wu & R. Wang (2004) Cystathionine gamma-lyase over-expression inhibits cell proliferation via a H2S-dependent modulation of ERK1/2 phosphorylation and p21Cip/WAK-1. *J Biol Chem,* 279**,** 49199–205.

Yates, Z. & M. Lucock (2005) G80A reduced folate carrier SNP modulates cellular uptake of folate and affords protection against thrombosis via a non homocysteine related mechanism. *Life Sci,* 77**,** 2735–42.

Yoon, S. H., S. Y. Ku, E. G. Min, B. M. Kang, S. T. Oh & Y. M. Choi (2009) Estrogen receptor alpha gene polymorphisms in patients with idiopathic premature ovarian failure. *Fertil Steril,* September, Supplement**,** S95.

Yoshino, M., T. Mizutani, K. Yamada, M. Tsuchiya, T. Minegishi, T. Yazawa, H. Kawata, T. Sekiguchi, T. Kajitani & K. Miyamoto (2002) Early growth response gene-1 regulates the expression of the rat luteinizing hormone receptor gene. *Biol Reprod,* 66**,** 1813–9.

Yoshino, O., H. E. McMahon, S. Sharma & S. Shimasaki (2006) A unique preovulatory expression pattern plays a key role in the physiological functions of BMP-15 in the mouse. *Proc Natl Acad Sci U S A,* 103**,** 10678–83.

Zalanyi, S. (2001) Progesterone and ovulation. *Eur J Obstet Gynecol Reprod Biol,* 98**,** 152–9.

Zetterberg, H. (2004) Methylenetetrahydrofolate reductase and transcobalamin genetic polymorphisms in human spontaneous abortion: biological and clinical implications. *Reprod Biol Endocrinol,* 2**,** 7.

Zirilli, L., V. Rochira, C. Diazzi, G. Caffagni & C. Carani (2008) Human models of aromatase deficiency. *J Steroid Biochem Mol Biol,* 109**,** 212–8.

# SUMMARY IN ESTONIAN

## Stimuleeritud ovariaalne follikulogenees: geneetiliste faktorite uuring

Viljatus on levinud meditsiiniline probleem, millega puutub kokku kuni 10% reproduktiivses eas paaridest. Viljatuse e. infertiilsuse all mõistetakse olukorda, kus paaril ei ole õnnestunud vähemalt ühe aasta jooksul rasestuda. Viljatuse põhjused võivad olla nii naise- kui ka mehepoolsed. Mehepoolse viljatusega kaasnevad patoloogilised muutused seemnerakkude kvaliteedis. Naise viljatust võivad aga põhjustada mitmed günekoloogilised haigused, põletikud, geneetilised haigused ning hormonaalsed ja autoimmunoloogilised probleemid. Levinuimaks viljatuse põhjuseks naistel Eestis on tubaarne viljatus, sagedased on ka endometrioos ning polütsüstiliste munasarjade sündroom (PCOS). Harvem on naise viljatuse põhjuseks enneaegne munasarjade puudulikkus (POF) või jääb konkreetne lastetuse põhjus selgusetuks, s.t. esineb seletamatu põhjusega infertiilsus.

Lastetuse ravis saavutatakse kõige paremaid tulemusi kehavälise viljastamise e. IVF-meetodil. IVF-protseduur koosneb kolmest etapist: i) ettevalmistavast ravietapist; ii) munarakkude viljastamisest ja iii) embrüote kultiveerimisest ning siirdamisest. IVF-protseduuri ettevalmistava ravietapi eesmärgiks on paljude folliikulite arengu stimuleerimine e. kontrollitud ovariaalne hüperstimulatsioon (COH). Tänapäeval kasutatakse munarakkude arengu stimuleerimisel gonadotropiine vabastava hormooni (GnRH) agoniste või antagoniste koos folliikuleid stimuleeriva hormooniga (FSH) ning munarakkude lõplik küpsemine indutseeritakse inimese koorioni gonadotropiiniga (hCG). Munasarjade punktsioonil saadud munarakud viljastatakse ning 2 – 5 päeva vanustest *in vitro* embrüotest valitakse emakasse siirdamiseks 1 – 3 embrüot. Keskmiselt rasestub IVF-protseduuri järgselt siiski ainult 30% naistest. IVF-protseduuri edukus sõltub erinevatest kliinilistest faktoritest; ennekõike naise vanusest, viljatuse põhjusest, COH-i tulemuslikkusest (munarakkude arv ja kvaliteet) ning emakasse siiratud embrüote arengupotentsiaalist.

IVF-i hormonaalne stimulatsioon on edukas, kui punktsioonil saadakse piisavalt ning hea kvaliteediga munarakke. COH-i edukust hinnatakse kasutatud FSH koguse, folliikulite ja munarakkude arvu ning kvaliteedi alustel. COH-i edukus sõltub erinevatest faktoritest. Üheks peamiseks faktoriks on naise vanus; naise vananedes ovariaalne reserv ning stimulatsiooni tulemuslikkus halvenevad. COH-i edukusel on samuti väga suur tähtsus naise geneetilistel faktoritel, ennekõike polümorfismidel (variatsioonidel) follikulogeneesi mõjutavates geenides. Näiteks esineb FSH retseptori geeni 10. eksonis polümorfism (A2039G), mis kodeerib Asn680Ser aminohappelist muutust. Ser/Ser genotüübi korral on IVF-protseduurides stimulatsiooniks kasutatava FSH kogused kõrgemad ning esineb sagedamini stimulatsiooni katkestamisi munasarjade resistentsuse tõttu. Lisaks võivad COH-i tulemuslikkust mõjutada teiste

follikulogeneesi regulatsioonis oluliste geenide variatsioonid. Östrogeeni retseptori alfa (*ESR1*) geeni 1. intronis on näiteks leitud T/C variatsioon, mis põhjustab *Pvu*II restriktsioonisaidi tekkimist või kadumist. *Pvu*II TT genotüübiga patsientidel on saadud IVF-tsüklis vähem munarakke kui TC või CC genotüüpidega patsientidel. Lisaks on TT genotüübiga IVF patsientide rasestumise tõenäosus madalam. Samas on jäänud paljude kandidaatgeenide olulisus COH-i ja IVF-i tulemuslikkuse määramisel veel selgusetuks.

IVF-protseduuris siiratakse 4–8 rakulisi embrüoid. Siiratud embrüote kvaliteeti hinnatakse mikroskoopiliselt embrüo morfoloogiliste parameetrite (rakkude jagunemise kiirus ja kuju) alusel, mis siiski ei kajastada embrüo tegelikku arengupotentsiaali. Embrüote selektsioonis on viimasel ajal kasutatud nii embrüo rakkude geneetilisi uuringuid kui ka embrüo rakkude ja embrüot ümbritsevate follikulaarsete rakkude geeniekspressiooni uuringuid. Üheks oluliseks läbimurdeks geeniekspressiooni analüüsil on olnud geeniekspressiooni kiipide kasutusele võtmine. Embrüote selektsiooni uudse lahedusena on välja pakutud munarakku ja embrüot ümbritsevate follikulaarsete rakkude geeniekspressiooni analüüsi mikrokiipide meetodil. Samas on vastava meetodi arengut piiranud embrüot ümbritsevate rakkude geeniekspressiooni mustri ebapiisav tundmine. Folliikli rakkude geeniekspressiooni mustri analüüs võimaldaks teoreetiliselt emakasse siirdamiseks välja valida kõige parema arengupotentsiaaliga embrüoid.

Käesoleva doktoritöö eesmärgiks oli analüüsida indutseeritud ovariaalset follikulogeneesi mõjutavate geneetiliste faktorite – geenide variatsioonide ja geeniekspressiooni mustri – mõju IVF tulemuslikkusele ning viljatuse patogeneesile. Töö raames genotüpiseeriti viljatutel naistel 17 erinevat polümorfismi 8 erinevas geenis – *CYP19A1* $(TTTA)_n$, *CYP19A1* TCT Ins/Del, *ESR1* $(TA)_n$, *ESR1 Pvu*II T/C, *ESR1 Xba*I A/G, *ESR2* $(CA)_n$, *ESR2 Rsa*I G/A, *MTHFR* 677C/T, *MTHFR* 1298A/C, *MTHFR* 1793G/A, *FOLR1* 1314G/A, *FOLR1* 1816C/delC, *FOLR1* 1841G/A, *FOLR1* 1928C/T, *TCN2* 776C/G, *CTH* 1208G/T ja *SLC19A1* 80G/A. Analüüsiti nimetatud geenivariatsioonide seoseid naise viljatuse põhjuste, COH-i tulemuslikkuse ja rasestumise tõenäosusega IVF-protseduuris. Lisaks hinnati folaadi metabolismi biokeemiliste markerite (seerumi folaat, vitamiin $B_{12}$ ja homotsüsteiin) seoseid folaadi metabolismi raja geenide variatsioonide, viljatuse patogeneesi ja IVF tulemuslikkusega. Folliikli granuloosa rakkude geeniekspressiooni analüüsi käigus võrreldi IVF-patsientidel erinevate funktsionaalsete rakupopulatsioonide (muraalne ja kumuluse granuloosa rakkude) geeniekspressiooni mustrit.

Uurimistöö olulisemad tulemused on järgnevad:

- Viljatud naised, kellel esinevad lühemad *CYP19A1* $(TTTA)_n$ mikrosatelliitsed alleelid ja TCT Del/Del homosügootsus aromataasi geenis ning patsiendid, kellel esinevad lühemad $(TA)_n$ kordused ning *Pvu*II T alleel ESR1 geenis on vähem tundlikumad munasarjade hormonaalses stimulatsioonis FSH toime suhtes. Lisaks on MTHFR 677C/T, MTHFR 1793G/A,

FOLR1 1314G/A ja CTH 1208G/T heterosügootsetel indiviididel paremad IVF ravitulemused. Lisaks mõjutavad MTHFR 677C/T, FOLR1 1314G/A, TCN2 776C/G ja SLC19A1 80G/A variatsioonid folaadi metabolismi ning seerumi biokeemiliste markereite tasemeid, mis omakorda aga mõjutavad naise viljakust.

- Aromataasi geeni TCT Del variatsioon ning lühemad $(TTTA)_n$ kordused assotsieeruvad endometrioosi ning seletamatu põhjusega viljatuse kujunemisega. Samuti võivad lühemad $(TA)_n$ kordused *ESR1* geenis ning MTHFR 677C/T, MTHFR 1793G/A ja SLC19A1 80G/A variatsioonid olla seletamatu põhjusega naise viljatuse riskifaktoriks.
- Folliikuli muraalse ja kumuluse granuloosa rakkude geeniekspressiooni analüüs tõi esile rakupopulatsiooni-spetsiifilise geeniekspressiooni mustri, kus erinevad geenid, mis osalevad follikulogeneesis, hormonaalses signaalirajas, raku adhesioonis, angiogeneesis, apoptoosis, ekstratsellulaarse maatriksi moduleerimises, rakutsükli kontrollis, immuunvastuses, transkriptsiooni kontrollis jt. olid erinevalt reguleeritud. Saadud informatsiooni on võimalik rakendada IVF ravi ovariaalsete hormonaalsete stimulatsiooniskeemide täiustamisel ning embrüote mitteinvasiivsete selektsioonimeetodite arendamisel.

Kokkuvõtteks, käesoleva uurimistöö tulemused näitavad, et geneetilised variatsioonid follikulogeneesiga seotud geenides mõjutavad hormonaalse stimulatsiooni tulemuslikkust ning rasedustulemusi IVF ravis ning samuti on seotud viljatuse etioloogiaga. Uuringu tulemustel on tähtis mõju lastuse ravi edasisele arengule, kuna geneetilise tausta arvestamine hormonaalse stimulatsiooni läbiviimisel võimaldab muuta viljatuse ravi ohutumaks ning edukamaks. Lisaks aitavad uurimistöö tulemused granuloosa rakkude geeniekspressiooni analüüsil parandada IVF ravi hormonaalset stimulatsiooni, embrüote selektsiooni ja rasestumise tõenäosust.



24

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## Publications


1. Gomez-Gallego F, Santiago C, González-Freire M, Yvert T, Muniesa CA, Serratosa L, **Altmäe S**, Ruiz JR, Lucia A.The C allele of the AGT Met235Thr polymoprhism is associated with power sports performance. Appl Physiol Nutr Metab. 2009 Dec;34(6):1108-11.
2. Gomez-Gallego F, Ruiz JR, Buxens A, **Altmäe S**, Artieda M, Santiago C, Gonzalez-Freire M, Verde Z, Arteta D, Martinez A, Tejedor D, Lao JI, Arenas J, Lucia A. Are elite-endurance athletes genetically predisposed to lower disease risk? Physiol Genomics. 2009 Dec 22 (Epub ahead of print).
3. **Altmäe S**, Martínez-Conejero JA, Salumets A, Simón C, Horcajadas JA, Stavreus-Evers A. Endometrial gene expression analysis at the time of embryo implantation in women with unexplained infertility. Mol Hum Reprod. 2009 Nov 22 (epub ahead of print).
4. Laanpere M, **Altmäe S**, Stavreus-Evers A, Nilsson TK, Yngve A and Salumets A. Folate-mediated one-carbon metabolism and its effect on female fertility and pregnancy viability. Nutrition Reviews (in press).
5. Kõks S, Velthut A, Sarapik A, **Altmäe S**, Reinmaa E, Schalkwyk LC, Fernandes C, Lad HV, Soomets U, Jaakma Ü, Salumets A. The differential transcriptome and ontology profiles of floating and cumulus granulose cells in stimulated human antral follicles. Mol Hum Reprod. 2009 Nov 20 (epub ahead of print).
6. Gomez-Gallego F, Santiago C), González-Freire M, Yvert T, Muniesa CA, Serratosa L, **Altmäe S**, Ruiz JR, and Lucia A. The C allele of the AGT Met235Thr polymoprhism is associated with power sports performance. Applied Physiology, Nutrition and Metabolism (in press).
7. Santiago C, Rodríguez-Romo G, Gómez-Gallego F, González-Freire M, Yvert T, Verde Z, Naclerio F, **Altmäe S**, Esteve-Lanao J, Ruiz JR, Lucia A. Is there an association between ACTN3 R577X polymorphism and muscle power phenotypes in young, non-athletic adults? Scand J Med Sci Sports. 2009 Oct 5. (epub ahead of print).
8. Santiago C, Ruiz JR, Buxens A, Artieda M, Arteta D, Gonzalez-Freire M, Rodríguez-Romo G, **Altmäe S**, Lao JI, Gómez-Gallego F, Lucia A. Trp64Arg polymorphism in ADRB3 gene is associated with elite endurance performance. Br J Sports Med. 2009 Jun 23. (epub ahead of print)
9. **Altmäe S**, Stavreus-Evers A, Ruiz JR, Laanpere M, Syvänen T, Yngve A, Salumets A, Nilsson TK. Variations in folate pathway genes are associated with unexplained female infertility. Fertil Steril. 2009 Mar 24. (epub ahead of print)
10. **Altmäe S**, Haller K, Saare M, Peters M, Hovatta O, Stavreus-Evers A, Velthut A, Karro H, Metspalu A, Salumets A. Aromatase gene (*CYP19A1)* variants, female infertility and ovarian stimulation outcome: a preliminary report. Reproductive BioMedicine Online 2009 May;18(5):651–7.
11. Aghajanova L, **Altmäe S**, Bjuresten K, Hovatta O, Landgren B-M, Stavreus-Evers A. Disturbances in the LIF pathway in the endometrium among women with unexplained infertility. Fertil Steril. 2009 Jun; 91(6):2602–10.
12. Aghajanova L, Bjuresten K, **Altmäe S**, Landgren B-M, Stavreus-Evers A. HB-EGF but not amphiregulin or their receptors HER1 and HER4 is altered in endometrium of women with unexplained infertility. Reprod Sci. 2008 May;15(5):484–92.

13. Merisalu A, Punab M, **Altmäe S**, Haller K, Tiido T, Peters M, Salumets A. The contribution of genetic variations of aryl hydrocarbon receptor pathway genes to male infertility. Fertil Steril. 2007 Oct;88(4):854–9.
14. **Altmäe, S**, Haller, K, Saare, M, Peters, M, Karro, H, Metspalu, A, Salumets, A. (2007). Aromataasi CYP19 geeni (TTTA)n variatsioon mõjutab FSH-stimulatsiooni tulemuslikkust lastetuse ravis. Eesti Arst, 86(9), 685–686.
15. **Altmäe S**, Haller K, Peters M, Hovatta O, Stavreus-Evers A, Karro H, Metspalu A, Salumets A. Allelic estrogen receptor 1 (ESR1) gene variants predict the outcome of ovarian stimulation in in vitro fertilization. Mol Hum Reprod. 2007 Aug;13(8):521–6.
16. Kõks S, Nikopensius T, Koido K, Maron E, **Altmäe S**, Heinaste E, Vabrit K, Tammekivi V, Hallast P, Kurg A, Shlik J, Vasar V, Metspalu A, Vasar E. Analysis of SNP profiles in patients with major depressive disorder. Int. J. Neuro-psychopharmacol., 2006; 9(2): 167–174.
17. Merisalu, A, Punab, M, Peters, M, **Altmäe, S**, Haller, K, Tiido, T, Giwercman, A, Metspalu, A, Salumets, A. (2006). Arüül-süsivesinike retseptori repressori Ala185 alleel soodustab mehepoolse viljatuse kujunemist. Eesti Arst, 85(9), 637–638.
18. **Altmäe S**, Runina A, Kuningas M, Kahre T, Peters M, Ehrenberg A, Karro H, Salumets A, Metspalu A. Loote reesusstaatuse mitteinvasiivne diagnostika – prenataalse diagnostika uus võimalus Eestis. Eesti Arst 2005; 84 (12): 847–852
19. Koido K, Kõks S, Nikopensius T, Maron E, **Altmäe S**, Heinaste E, Vabrit K, Tammekivi V, Hallast P, Kurg A, Shlik J, Vasar V, Metspalu A, Vasar E Polymorphisms in wolframin (WFS1) gene are possibly related to increased risk for mood disorders. Int. J. Neuropsychopharmacol., 2005, 8(2): 235–244.
20. Maron E, Nikopensius T, Kõks S, **Altmäe S**, Heinaste E, Vabrit K, Tammekivi V, Hallast P, Koido K, Kurg A, Metspalu A, Vasar E, Vasar V, Shlik J. Association study of 90 candidate gene polymorphisms in panic disorder. Psychiatric Genet., 2005, 15(1):17–24.

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## Teadusartiklid

1. Gomez-Gallego F, Santiago C, González-Freire M, Yvert T, Muniesa CA, Serratosa L, **Altmäe S**, Ruiz JR, Lucia A.The C allele of the AGT Met235Thr polymoprhism is associated with power sports performance. Appl Physiol Nutr Metab. 2009 Dec;34(6):1108-11.
2. Gomez-Gallego F, Ruiz JR, Buxens A, **Altmäe S**, Artieda M, Santiago C, Gonzalez-Freire M, Verde Z, Arteta D, Martinez A, Tejedor D, Lao JI, Arenas J, Lucia A. Are elite-endurance athletes genetically predisposed to lower disease risk? Physiol Genomics. 2009 Dec 22 (Epub ahead of print).
3. **Altmäe S**, Martínez-Conejero JA, Salumets A, Simón C, Horcajadas JA, Stavreus-Evers A. Endometrial gene expression analysis at the time of embryo implantation in women with unexplained infertility. Mol Hum Reprod. 2009 Nov 22 (epub ahead of print).
4. Laanpere M, **Altmäe S**, Stavreus-Evers A, Nilsson TK, Yngve A and Salumets A. Folate-mediated one-carbon metabolism and its effect on female fertility and pregnancy viability. Nutrition Reviews (in press).
5. Kõks S, Velthut A, Sarapik A, **Altmäe S**, Reinmaa E, Schalkwyk LC, Fernandes C, Lad HV, Soomets U, Jaakma Ü, Salumets A. The differential transcriptome and ontology profiles of floating and cumulus granulose cells in stimulated human antral follicles. Mol Hum Reprod. 2009 Nov 20 (epub ahead of print).
6. Gomez-Gallego F, Santiago C), González-Freire M, Yvert T, Muniesa CA, Serratosa L, **Altmäe S**, Ruiz JR, and Lucia A. The C allele of the AGT Met235Thr polymoprhism is associated with power sports performance. Applied Physiology, Nutrition and Metabolism (in press).
7. Santiago C, Rodríguez-Romo G, Gómez-Gallego F, González-Freire M, Yvert T, Verde Z, Naclerio F, **Altmäe S**, Esteve-Lanao J, Ruiz JR, Lucia A. Is there an association between ACTN3 R577X polymorphism and muscle power phenotypes in young, non-athletic adults? Scand J Med Sci Sports. 2009 Oct 5. (epub ahead of print).
8. Santiago C, Ruiz JR, Buxens A, Artieda M, Arteta D, Gonzalez-Freire M, Rodríguez-Romo G, **Altmäe S**, Lao JI, Gómez-Gallego F, Lucia A. Trp64Arg polymorphism in ADRB3 gene is associated with elite endurance performance. Br J Sports Med. 2009 Jun 23. (epub ahead of print)
9. **Altmäe S**, Stavreus-Evers A, Ruiz JR, Laanpere M, Syvänen T, Yngve A, Salumets A, Nilsson TK. Variations in folate pathway genes are associated with unexplained female infertility. Fertil Steril. 2009 Mar 24. (epub ahead of print)
10. **Altmäe S**, Haller K, Saare M, Peters M, Hovatta O, Stavreus-Evers A, Velthut A, Karro H, Metspalu A, Salumets A. Aromatase gene (*CYP19A1)* variants, female infertility and ovarian stimulation outcome: a preliminary report. Reproductive BioMedicine Online 2009 May;18(5):651–7.
11. Aghajanova L, **Altmäe S**, Bjuresten K, Hovatta O, Landgren B-M, Stavreus-Evers A. Disturbances in the LIF pathway in the endometrium among women with unexplained infertility. Fertil Steril. 2009 Jun; 91(6):2602–10.
12. Aghajanova L, Bjuresten K, **Altmäe S**, Landgren B-M, Stavreus-Evers A. HB-EGF but not amphiregulin or their receptors HER1 and HER4 is altered in endometrium of women with unexplained infertility. Reprod Sci. 2008 May;15(5):484–92.

13. Merisalu A, Punab M, **Altmäe S**, Haller K, Tiido T, Peters M, Salumets A. The contribution of genetic variations of aryl hydrocarbon receptor pathway genes to male infertility. Fertil Steril. 2007 Oct;88(4):854–9.
14. **Altmäe, S**, Haller, K, Saare, M, Peters, M, Karro, H, Metspalu, A, Salumets, A. (2007). Aromataasi CYP19 geeni (TTTA)n variatsioon mõjutab FSH-stimulatsiooni tulemuslikkust lastetuse ravis. Eesti Arst, 86(9), 685–686.
15. **Altmäe S**, Haller K, Peters M, Hovatta O, Stavreus-Evers A, Karro H, Metspalu A, Salumets A. Allelic estrogen receptor 1 (ESR1) gene variants predict the outcome of ovarian stimulation in in vitro fertilization. Mol Hum Reprod. 2007 Aug;13(8):521–6.
16. Kõks S, Nikopensius T, Koido K, Maron E, **Altmäe S**, Heinaste E, Vabrit K, Tammekivi V, Hallast P, Kurg A, Shlik J, Vasar V, Metspalu A, Vasar E. Analysis of SNP profiles in patients with major depressive disorder. Int. J. Neuro-psychopharmacol., 2006; 9(2): 167–174.
17. Merisalu, A, Punab, M, Peters, M, **Altmäe, S**, Haller, K, Tiido, T, Giwercman, A, Metspalu, A, Salumets, A. (2006). Arüül-süsivesinike retseptori repressori Ala185 alleel soodustab mehepoolse viljatuse kujunemist. Eesti Arst, 85(9), 637–638.
18. **Altmäe S**, Runina A, Kuningas M, Kahre T, Peters M, Ehrenberg A, Karro H, Salumets A, Metspalu A. Loote reesusstaatuse mitteinvasiivne diagnostika – pre-nataalse diagnostika uus võimalus Eestis. Eesti Arst 2005; 84 (12): 847–852
19. Koido K, Kõks S, Nikopensius T, Maron E, **Altmäe S**, Heinaste E, Vabrit K, Tammekivi V, Hallast P, Kurg A, Shlik J, Vasar V, Metspalu A, Vasar E. Polymorphisms in wolframin (WFS1) gene are possibly related to increased risk for mood disorders. Int. J. Neuropsychopharmacol., 2005, 8(2): 235–244.
20. Maron E, Nikopensius T, Kõks S, **Altmäe S**, Heinaste E, Vabrit K, Tammekivi V, Hallast P, Koido K, Kurg A, Metspalu A, Vasar E, Vasar V, Shlik J. Association study of 90 candidate gene polymorphisms in panic disorder. Psychiatric Genet., 2005, 15(1):17–24.

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