UPPSALA
UNIVERSITET

Digital Comprehensive Summaries of Uppsala Dissertations
from the Faculty of Medicine 987

Folate, Hormones and Infertility

Different factors affecting IVF pregnancy outcome

TIINA MURTO

ACTA
UNIVERSITATIS
UPSALIENSIS
UPPSALA
2014

ISSN 1651-6206
ISBN 978-91-554-8919-9
urn:nbn:se:uu:diva-220476

Dissertation presented at Uppsala University to be publicly examined in Gustavianum, Auditorium Minus, Akademigatan 3, Uppsala, Thursday, 22 May 2014 at 09:15 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in Swedish. Faculty examiner: Associate Professor Sven-Eric Olsson (Institutionen för kliniska vetenskaper, Danderyds sjukhus (KI DS)).

Abstract
Murto, T. 2014. Folate, Hormones and Infertility. Different factors affecting IVF pregnancy outcome. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine* 987. 57 pp. Uppsala: Acta Universitatis Upsaliensis. ISBN 978-91-554-8919-9.

Various hormones have been studied as regards prediction of pregnancy outcome after infertility treatment, but no ideal candidate has been found. Folate and genetic variations in folate metabolism have also been associated with infertility, but it remains unclear how these factors affect IVF pregnancy outcome. It is known that infertility is associated with active folic acid supplement use, but the effect of socioeconomic and lifestyle factors on folic acid supplement use in infertile women has not been well investigated. The overall aim of this work was to obtain information on the prediction of live birth, and to study factors affecting the role of folate and folic acid intake in relation to IVF pregnancy outcome. Infertile women with various infertility diagnoses were studied. Healthy, fertile non-pregnant women were used as controls in three of the studies. Blood samples were taken for assay of eight different hormones, folate and homocysteine, and for genomic DNA extraction. A questionnaire was used to assess background data and use of folic acid supplements. Twenty-four-hour recall interviews were performed for validation of the questionnaire. The studied hormones were not good predictors of live birth. The best predictor was age of the women, together with ovulatory menstrual cycles, and thyroid-stimulating hormone and anti-Müllerian hormone (AMH) status. Well-educated women, high-status employed women, and married and infertile women used the most folic acid supplements. Infertile women had better folate status than fertile women. However, pregnancy outcome after infertility treatment was not dependent on folic acid intake, folate status, genetic variation of 5,10-methylenetetrahydrofolate reductase or socioeconomic status. In conclusion, AMH levels vary less than those of other hormones during the menstrual cycle, and AMH could be used as a predictive marker of live birth together with age and ovulation. Folate might play a minor role in IVF pregnancy outcome, but the importance of folate as regards other health perspectives should not be forgotten.

Keywords: Folate, folic acid supplement, homocysteine, hormones, infertility, IVF, lifestyle factor, MTHFR, predictive value, socioeconomic factor, women

Tiina Murto, Department of Women's and Children's Health, Akademiska sjukhuset, Uppsala University, SE-75185 Uppsala, Sweden.

ISSN 1651-6206
ISBN 978-91-554-8919-9
urn:nbn:se:uu:diva-220476 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-220476)

To my dear family

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I Murto, T., Bjuresten, K., Landgren, B-M., Stavreus-Evers, A. (2013). Predictive value of hormonal parameters for live birth in women with unexplained infertility and male infertility. *Reproductive Biology and Endocrinology* 11(1):61.

II Murto, T., Skoog Svanberg, A., Yngve, A., Nilsson, TK., Altmäe, S., Wånggren, K., Salumets, A., Stavreus-Evers, A. (2014). Folic acid supplementation and IVF pregnancy outcome in women with unexplained infertility. *Reproductive BioMedicine Online*, http://dx.doi.org/10.1016/j.rbmo.2014.01.017 (in press).

III Murto, T., Kallak, TK., Hoas A., Altmäe, S., Salumets, A., Nilsson, TK., Skoog Svanberg, A., Wånggren, K., Yngve, A., Stavreus-Evers, A. Folic acid supplementation and methylenetetrahydrofolate reductase (MTHFR) gene variations in relation to IVF pregnancy outcome. *Submitted*.

IV Murto, T., Skoog Svanberg, A., Yngve, A., Poortvliet, E., Altmäe, S., Salumets, A., Wånggren, K., Stavreus-Evers, A. Socioeconomic and lifestyle factors in relation to folic acid supplement use in infertile and fertile Swedish women. *Manuscript*.

Contents

Abbreviations

AMH	Anti-Müllerian Hormone
ART	Assisted reproductive technology
BMI	Body mass index
CI	Confidence interval
COH	Controlled ovarian hyperstimulation
DNA	Deoxyribonucleic acid
ER	Oestrogen receptor
ET	Embryo transfer
FSH	Follicle-stimulating hormone
GnRH	Gonadotrophin-releasing hormone
hCG	Human chorionic gonadotrophin
Hcy	Homocysteine
HyCoSy	Hysterosalpingo Contrast Sonography
ICSI	Intracytoplasmic sperm injection
IVF	In vitro fertilization
LH	Luteinizing hormone
5-Methyl-THF	5-Methylenetetrahydrofolate
MTHFR	5,10-methylenetetrahydrofolate reductase
NTD	Neural tube defect
OR	Odds ratio
OPU	Ovum pick-up
PCA	Principal component analysis
PGA	Pteroylmonoglutamic acid
PR	Progesterone receptor
PRL	Prolactin
SAM	S-Adenosyl methione
THF	Tetrahydrofolate
TSH	Thyroid-stimulating hormone
χ^2	Chi squared

Introduction

The menstrual cycle

The menstrual cycle is necessary for human reproduction. It prepares a woman's body for pregnancy through a number of synchronized events, regulated by combinational effects of hormones [1,2]. A normal cycle is between 24 and 35 days with an average of 28 days [3]. There are three different phases of the menstrual cycle: follicular phase (menstruation and proliferative phase), ovulation and luteal (or secretory) phase [4] (Figure 1).

During the follicular phase the right amount of follicles will get ready for ovulation as a result of consequential actions of hormones. In the human ovary, this process results in one mature follicle, called the dominant follicle. At mid-cycle estradiol levels rise significantly due to growth of the follicles. This leads to positive feedback and induction of the luteinizing hormone (LH) surge, followed by ovulation of the dominant follicle. Increasing levels of estradiol also affect the endometrium, leading to cell division and growth of the endometrium. After ovulation, the ruptured follicle transforms into a corpus luteum, which continues to differentiate and it produces high amounts of hormones, especially progesterone. In the absence of a pregnancy, one week after ovulation hormone production in the corpus luteum decreases and the corpus luteum itself degenerates a few days later. This leads to degeneration of the endometrium, menstruation and the beginning of a new cycle [4] (Figure 1).

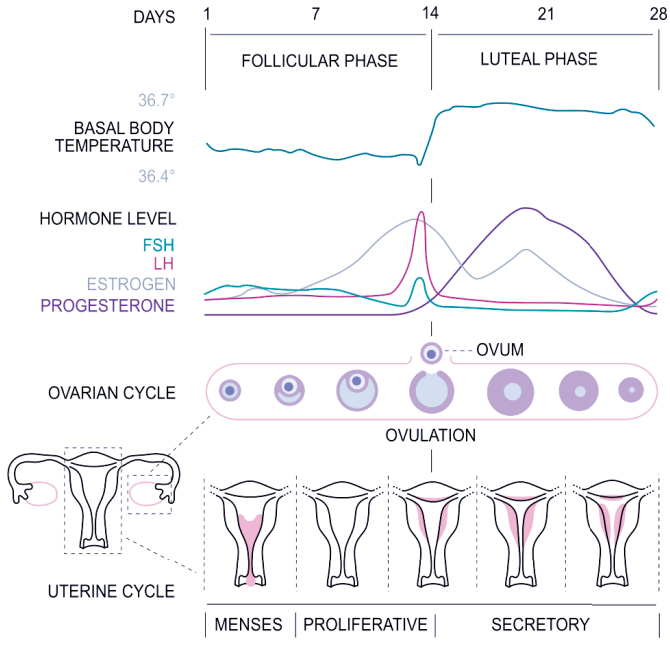

Figure 1. The menstrual cycle. Cycle days, basal body temperature, hormone levels, ovarian cycle and uterine cycle are indicated. (http://en.wikipedia.org/wiki/Menstrual_cycle)

Regulation of the menstrual cycle

FSH and LH

Follicle-stimulating hormone (FSH) and LH are involved in the regulation of ovarian function during the menstrual cycle. FSH stimulates the growth and recruitment of immature follicles in the ovary. Receptors for LH and FSH are present in the ovary and they regulate its function by supporting the production of sex steroid hormones, oestrogens and progestins, as well as folliculogenesis [4].

Oestrogen

Oestrogen is important for oocyte maturation, embryo development, fertilization and the formation of female secondary sex characteristics [5,6]. The action of oestrogen is mediated through binding and activation of oestrogen receptors, ERα and ERβ [6]. The most important form of oestrogen found in body is estradiol [4].

Progesterone

The main roles of progesterone are differentiation of the endometrium, assisting implantation and maintenance of pregnancy [7]. Progesterone exerts its

primary action through binding to intracellular progesterone receptors, PR-A and PR-B [8].

Prolactin

Elevated levels of serum prolactin (PRL) have been associated with various menstrual disorders as a result of the restraining effect on pulsatile gonadotrophin-releasing hormone (GnRH) secretion as well as the inhibition of LH and FSH release [9].

Anti-Müllerian hormone

Anti-Müllerian hormone (AMH) is a dimeric glycoprotein which is produced by the granulosa cells of pre-antral and antral follicles [2,10]. The main role of AMH is to inhibit follicular development from primordial to primary follicular stages [10,11].

Inhibin B

Inhibin B, produced by granulosa cells in antral follicles, is an important marker of follicular growth through regulation of FSH [2,12]. Low serum inhibin B levels have been related to elevated levels of FSH, which has been shown to have an association with decreased oocyte quality and fertility [2].

Thyroid hormones

Thyroid hormones influence the menstrual cycle directly by impact on the ovaries and indirectly through elevated levels of PRL and altered GnRH secretion [13]. Thyroid dysfunction (hypothyroidism and hyperthyroidism) often results in menstrual disturbances [13-15].

Fertility

Fertility is the natural capacity to give life. Building a family has a strong social impact in all cultures worldwide, and fertility is of great importance for both women and men.

Female fertility peaks at the age of 22 years and subsequently decreases until around the age of 38, whereafter it falls more rapidly until menopause, when it ceases [16]. In Sweden the mean age of women giving birth for the first time has increased from 24 years (1973) to more than 28 years (2011). In recent years, an increasing number of women at the age of 35 or later give birth for the first time [17]. Fertility is determined as total fertility rate, defined as the mean number of children born per couple, person or population. In European countries, the fertility rate has continuously decreased since the 1960s. However, in recent years signs of an increasing fertility rate have been noticed [18].

Lifestyle factors and nutritional status have been shown to be related to normal reproductive function [19,20], and, in particular, dietary deficiencies [21] have been associated with reproductive problems and infertility.

Infertility

Infertility is defined as failure to conceive after at least one year of unprotected intercourse, and/or inability to bring a pregnancy to term. The prevalence of infertility is high, affecting approximately one in ten couples worldwide [22]. In 2007 it was estimated that more than 72 million women of reproductive age worldwide were infertile [23].

IVF

The most effective treatment of infertility is in vitro fertilization (IVF), which is one of several forms of treatment included in the common term "assisted reproductive technology" (ART). The first child conceived through IVF was born in 1978 in the UK [22]. Five years later the first child resulting from IVF treatment was born in Sweden [17]. IVF has so far been the most frequent but also the most costly treatment of infertility [17], and it is demanding both mentally and physically for the affected couples [24]. According to the National Board of Health and Welfare (Socialstyrelsen) the number of IVF treatments increased from about 3000 to over 13 000 per year from 1991 to 2008 in Sweden. Approximately 25% of these more than 13 000 treatments resulted in live birth [17].

The principle of IVF consists of hormonal ovarian stimulation, collection of mature oocytes and fertilization with sperm, incubation of the embryos in the laboratory and embryo transfer into the uterus. During controlled ovarian hyperstimulation (COH) multiple follicles are stimulated to grow and mature by using synthetic FSH, resulting in the possibility to select a high quality embryo(s) for transfer. Ovum pick-up (OPU) is carried out by vaginal ultrasound-guided needle punctures. In conventional IVF, the oocyte is fertilized with motile spermatozoa 4–6 hours after OPU. In intracytoplasmic sperm injection (ICSI), a punctured oocyte is cleaned and a single sperm is injected into it. After fertilization, the embryo is cultivated in an incubator for two, or three, (cleavage stage) to five days (blastocyst stage), and the best quality embryo(s) is selected and transferred into the uterus.

Causes of infertility

The cause of infertility can be a result of female, male, a combination of male and female infertility, or it can remain unexplained [25]. The most common causes of female infertility are ovulation disorders (e.g. oligomenor-

rhoea, amenorrhoea), tubal disorder and endometriosis [26]. Causes of male infertility are in general associated with abnormal results in semen analysis (e.g. oligo- or azoospermia) [27].

A couple is diagnosed as having unexplained infertility after the results of standard infertility tests have been shown to be normal both in the woman and the man, and no reason for infertility has been found [28]. In some cases, women are misdiagnosed as having unexplained infertility although their age indicates a negative impact on ovarian reserve [29]. It has also been suggested that there might be misdiagnoses in some clinics as a result of absence of evaluation of pelvic pathologies using laparoscopy [30], or the fact that women with unexplained infertility might have a subtle form of ovulatory dysfunction which is not diagnosed by way of standard investigations [31].

In many cases infertility has complex aetiologies. It has been suggested that besides clinical diagnosis, lifestyle factors [32,33], some specific genotypes [34] or micronutrient deficiencies [35] may have an association with infertility. Folate is one of the important micronutrients that has been suggested to have an association with infertility [36].

Folate

Folate, water-soluble vitamin B, is necessary for energy production and healthy cell division, and it is also important for the formation of the red blood cells [37]. It is considered to be important for oocyte quality and maturation as well as for implantation and normal continuation of pregnancy [38].

Folate sources

Folates in foods are polyglutamates [39], which can be found especially in green, leafy vegetables, fruit juices, pulses, beans, whole grains, milk and liver [40]. Naturally occurring folates are chemically unstable, as they lose their activity during cooking, processing and storage of food [39,41]. Folic acid (pteroylmonoglutamic acid, PGA) is the synthetic form of folate used in supplements. It is not found naturally in foods. This form has a more stable chemical structure than the natural (dietary) forms, which makes it resistant to heat and light [41]. It also appears to have better bioavailability than naturally occurring folates [42,43]. However, existing data on the bioavailability of food folate is still limited and varies a lot between different studies [44].

Folate metabolism

Folate metabolism consists of two cycles, DNA biosynthesis and methylation. In the gut lumen, natural folates are hydrolysed to the monoglutamate form. During passage through the intestine, folate and folic acid are metabolised to *5-methylenetetrahydrofolate (5-methyl-THF),* the most prevalent form of folate in the circulatory system. The methyl group is catalysed by the key enzyme of folate metabolism, *5,10 -methylenetetrahydrofolate reductase (MTHFR)*, which also links the DNA biosynthesis cycle with the methylation cycle. 5-methyl-THF is an inactive form, which is remethylated to the biologically active form of folate, *tetrahydrofolate (THF)*, by using the vitamin B_{12}-dependent enzyme methionine synthase. THF is a coenzyme, which has its primary roles in metabolic transfer (DNA biosynthesis cycle) and donation of carbon-1-units (methylation cycle).

The methylation cycle has two roles. It guarantees that the cell always has an adequate supply of *S-adenosyl methionine (SAM),* which is an activated form of the essential amino acid methionine. SAM acts as a methyl donor to a many methyltransferases*,* the enzymes which methylate a wide range of products (e.g. lipids, hormones, proteins, DNA). Another role of the methylation cycle is to provide the function of degrading methionine in the liver. Methionine is degraded to homocysteine (Hcy), which can leave the system with the help of the B_6-dependent enzyme cystathionine synthase and be catabolised to sulphate and further to pyruvate. Homocysteine can also be remethylated to methionine with the help of the enzyme methionine synthase [43,45,46]. The most important folate cofactors in the methylation and DNA cycles are shown in Figure 2.

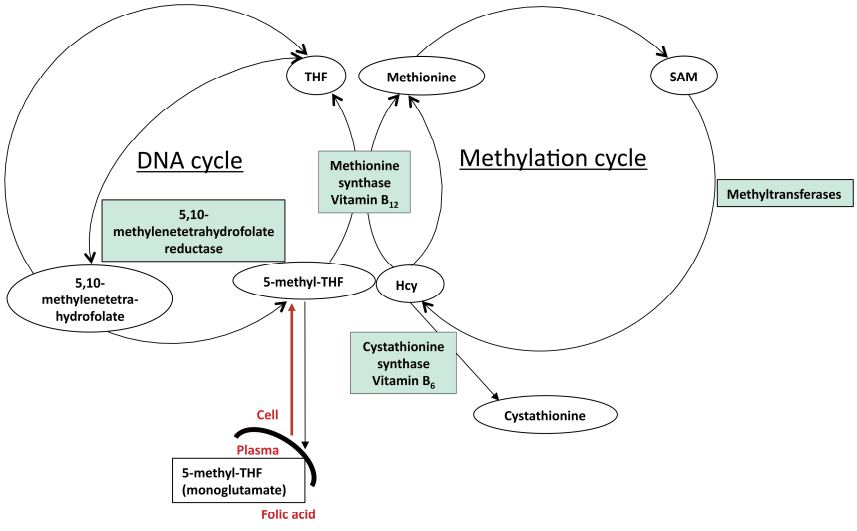

Figure 2. The methylation and DNA cycles, with folate cofactors.

Genetics of folate metabolism

Several variations have been identified in genes involved in folate absorption and metabolism. These polymorphisms may affect the beneficial effect of folates and other B vitamins that a play role in metabolism. The *MTHFR* gene has three commonly known polymorphisms, 677C>T, 1298A>C and 1793G>A that are involved in folate metabolism [47]. The *MTHFR* 677C>T polymorphism seems to be the most influential genetic variation affecting folate metabolism. The homozygous TT genotype of the 677C>T polymorphism is associated with higher plasma homocysteine and lower serum folate levels than the heterozygous (CT) and wild-type (CC) genotypes [48,49]. In contrast, the gene variation 1298A>C has not been shown to alter homocysteine or folate levels in the blood [50,51] and the *MTHFR* polymorphism 1793G>A has been shown to have a decreasing effect on plasma homocysteine [52].

Recommendations, intake and the effect on reproduction

The modern lifestyle, with increased consumption of fast and easily available food with low micronutrient contents, has led to suboptimal nutritional status in many industrialized countries, and in particular a low intake of folic acid has been noticed [53,54]. This has also been shown in Sweden, where women of fertile age do not get adequate levels of folate through diet. According to a recent Swedish dietary survey, women aged 18–44 years had the lowest mean intake of folate, 223–247 μg/day, compared with other age groups [55]. Therefore, there is a government policy to recommend folic acid

supplementation of 400 μg/day for women of reproductive age [56]. Even so, a recent Swedish study revealed that only 20% of women planning pregnancy take folic acid supplements prior to conceiving [57]. Also, international studies have shown that fewer than 50% of such women use preconceptional folic acid supplementation [58-60].

Awareness and use of folic acid supplements has been shown to be poorer in younger women and in women of lower socio-economic and educational status compared with older women of higher socio-economic and educational status [61-65]. In contrast, infertility treatment has been associated with more active folic acid intake [66], but in one study, even though over 80% of infertile women were aware of the benefits of taking folic acid, only half of them were actually taking it [67]. Such studies have highlighted the important role of health professionals as regards recommendations and information. However, in many cases the information has been shown to be poor despite the many promotional campaigns around the world concerning the importance of folic acid [63,66-68]. Folic acid supplementation is commonly recommended at most of the infertility clinics in Sweden, but the policies regarding the given information differ greatly between clinics.

Folate deficiency

Folate deficiency is usually caused by insufficient nutrition or malabsorption, but also smoking, high alcohol consumption, some pharmaceutical compounds and diseases influence folate absorption [37]. Folate levels in blood decrease with inadequate folate intake, and signs of deficiency can easily be identified in a blood sample. As a result of the closely linked metabolisms of folate and the amino acid homocysteine, folate deficiency is also one of the major causes of elevated plasma homocysteine concentrations [46]. Other reasons influencing increasing plasma homocysteine levels are genetic variation (*MTHFR* 677TT-homozygosity), physiological factors (e.g. age) and various diseases (e.g. renal failure, and $B_{12,}$ and B_6 deficiency) [69-71].

Folate deficiency has been linked to several forms of birth defect, in particular neural tube defects (NTDs) [72-75]. Low circulating folate concentrations during pregnancy have also been associated with increased risks of spontaneous abortion [76], preterm delivery, low birth weight and foetal growth retardation [37]. Elevated maternal plasma homocysteine levels have been associated with elevated risks of miscarriage, placental abruption and other pregnancy complications (e.g. pre-eclampsia) [37,77,78].

Folate and infertility

Studies of infertile women have revealed that preconceptional folic acid supplementation increases folate levels and decreases homocysteine levels in follicular fluid [79], and is related to better embryo quality and chance of pregnancy [77]. A reduced risk of ovulatory infertility has also been shown in one study [80].

Polymorphisms in folate-metabolising genes might also be associated with pregnancy outcome after infertility treatment [45]. The *MTHFR* 677TT genotype has been shown to be a risk factor as regards spontaneous abortion, preeclampsia and placental abruption [81]. Conversely, heterozygosity in *MTHFR* 677C>T seem to favour high-quality embryos [45] and an increased chance of pregnancy after fertility treatment [45,82]. However, women with the *MTHFR* 1298CC genotype are less likely than ones with the AA genotype to have a live birth after IVF [82]. A recent study has shown that *MTHFR* 1793G>A heterozygosity is associated with reduced failure after fertility treatment [45]. However, a previous study has also shown no associations between polymorphisms in the *MTHFR* gene and IVF outcome [83].

Problem statement

Today there is no good model to predict pregnancy outcome after infertility treatment. Various hormones and other factors have been studied, with different results, but no ideal model has been found. Folate and several genetic variations in folate metabolism have also been associated with infertility. The studies have shown that infertile women use more folic acid supplements than fertile women, and folic acid supplement use is related to various socioeconomic and lifestyle factors. However, it remains unclear how folic acid intake and the different genetic variations affect pregnancy outcome after infertility treatment. In particular, women with a diagnosis of unexplained infertility are vulnerable in this respect, since there is no clear reason for their infertility and a uniform definition of this diagnosis is lacking. Additionally, studies on women with diagnoses of unexplained infertility are few. The effects of socioeconomic and lifestyle factors on folic acid supplement use in infertile women have also not been well investigated.

Aims

The overall aim of this work was to obtain information on the prediction of live birth, and to study factors affecting the role of folate and folic acid intake in relation to IVF pregnancy outcome.

The specific aims of the following papers were:

I. To study serum hormone levels in relation to the prediction of live birth.

II. To study folic acid supplement use and folate status in relation to IVF pregnancy outcome. Additionally, to determine differences in folic acid supplement use and folate status between women with unexplained infertility and fertile women.

III. To study *MTHFR* 677C>T, 1298A>C and 1793G>A gene variations in infertile women in relation to folic acid intake, folate status and IVF pregnancy outcome. Also, to compare the frequency of gene variations, folic acid intake and folate status in infertile versus fertile women.

IV. To study socioeconomic, lifestyle and dietary factors in relation to folic acid supplement use and folate status in infertile and fertile women. A further aim was to validate the used questionnaire.

Material and Methods

Study subjects and design, Study I

This prospective cross-sectional study concerned 71 women with diagnoses of unexplained infertility and male-factor infertility. The women were otherwise healthy and did not use any hormonal medication. They were recruited at their first visit to the Fertility Unit, Karolinska University Hospital Huddinge between 1999 and 2008.

Study subjects and design, Studies II-IV

Infertile women were recruited at the Fertility Unit, Karolinska University Hospital between 2005 and 2007 and at the Centre for Reproduction, Uppsala University Hospital between 2008 and 2010. The women were recruited at their first visit. Healthy, proven fertile, non-pregnant and non-lactating women were randomly collected from the same geographic area as the infertile women.

Study II involved 180 women with unexplained infertility and 188 fertile women. A longitudinal cohort study was performed for investigation of the relationship between folic acid supplement use, folate status and pregnancy outcome in women with unexplained infertility. Additionally, a prospective case-control study was used to compare the use of folic acid supplements and folate status between infertile and fertile women.

In *studies III and IV* prospective observational investigations were performed. In total, 528 women were included, i.e. 340 women with various infertility diagnoses and 188 fertile women.

Infertility diagnosis, ovulation and pregnancy outcome measures

In all studies, laboratory assessments of normal ovulation and the luteal phase were conducted as well as evaluation of tubal patency and semen analysis. Tubal passage was demonstrated by Hysterosalpingo Contrast Sonography (HyCoSy) or if needed laparoscopy was performed to investigate factors such as endometriosis. Semen analyses were based on the criteria of

normality: sperm volume ≥ 2.0 mL, concentration ≥ 20 million/mL, total number of sperm ≥ 40 million/ejaculate, motility and vitality ≥ 50%. The final diagnoses were established after these examinations. A diagnosis of unexplained infertility was chosen when no explanation for infertility was found, the women had normal ovarian function and normal tubal passage and their partners had normal semen samples.

In *study I* ovulation was established by using serum progesterone values of ≥ 32 nmol/L (anovulation < 32 nmol/L) [84]. Live birth was defined as delivery of at least one healthy child, regardless of whether it was spontaneously conceived or conceived after infertility treatment.

In *studies II–IV* live birth was defined as delivery of a child after one infertility treatment cycle. The pregnancy outcome measures are further described in the respective papers. For assessment of pregnancy outcome in relation to folate status, a plasma folate value of 22.5 nmol/L [85] was chosen in connection with folic acid supplement use. Infertile women were divided into two groups according to plasma folate concentration: ≥ 22.5 nmol/L defined folic acid users and < 22.5 nmol/L defined non-users (*studies II and III*).

Assessments

Blood samples

In *study I* hormone profiles for FSH, LH, estradiol, progesterone, PRL, AMH, inhibin B and TSH during one natural menstrual cycle were determined. In *studies II–IV* blood samples were obtained at ovum pick up to determine plasma folate and homocysteine levels, and for genomic DNA extraction (*study III*). Blood samples from the fertile women were collected by a nurse at the research laboratory. The blood samples were centrifuged when collected and stored at – 20 °C to -70 °C until analysis.

All blood samples were analysed using routine methods at the central clinical laboratory at Karolinska University Hospital Huddinge or at Uppsala University Hospital (FSH, LH, estradiol, progesterone, TSH, PRL, p-folate, p-Hcy), or at the research laboratory, Department of Obstetrics and Gynaecology, Uppsala University Hospital (AMH, inhibin B, genotyping).

Genotyping

Blood DNA was extracted from EDTA blood, using a QIAamp DNA Blood Maxi Kit. Genotyping of the *MTHFR* polymorphisms was performed using PCR-based genotyping assays, as described in detail in *study III*.

Questionnaire

In *studies II and III* a questionnaire was used to gather general background data and data on use of dietary supplements. Additionally, in *study IV* information on dietary habits was also assessed. For assessment of general background data, the questionnaire included questions on age, birthplace, marital status, education, employment status as well as physical activity level and perceived subjective health. Self-reported height and weight were also recorded, transformed to BMI (kg/m^2) and categorized as underweight (< 18.5), normal weight (18.5-24.9), overweight (25.0-29.9) and obese (≥ 30) [39]. In addition, smoking habits and alcohol use were recorded. Dietary habits were assessed on the basis of questions regarding breakfast habits, and fruit and vegetable consumption. Beverage intake (including alcoholic drinks) was enquired about in connection with breakfast, lunch, dinner and periods between meals. The intake of dietary folate was estimated from the information on dietary habits and fruit and vegetables ingestion.

A list of supplements, containing 22 dietary preparations widely used in Sweden was included. An open-ended question was also included if the used supplement(s) was not on the list. If taking a folic acid supplement, the participant could self-report the amount (µg) of folic acid they used. In multivitamin supplements the content of folic acid was approximated from the product information.

Three-hundred and forty of 360 (94.4%) infertile women and 188 of 199 (94.5%) fertile women, who agreed to participate in the study, completed the questionnaire.

24-hour recall

In *study IV* a 24-hour recall method was used among 103 women to assess dietary folate intake, and for validation of the questionnaire. This method involve a structured interview of what a person has consumed during the previous 24 hours [86]. Two similarly performed 24-hour recall interviews for each of the participants were carried out, and only data from women participating in both 24- hour recall interviews were included in the analyses (n = 91).

To support estimation of portion sizes and amount of food, the Swedish Food Administrations' portion size model "Matmallen" was used. All information that could possibly facilitate registration of the data was collected, and supplement intake was also reported. When brands were indicated, the producers' internet sites for product information served to help obtain information. The data was entered using StorMATs406 software and the National food database version 02_1 for calculation of nutrient content.

To assess possible under- and over-reporting in the 24-hour recall interviews, the Goldberg cut-off equation [87] was used. Only normal-reported interviews (n = 75) were included in the study to avoiding the possible biases of under- and over-reported results.

Statistical analysis

For statistical analyses SPSS (Statistical Package for the Social Sciences) software was used (IBM® SPSS® Statistics 20.0, IBM Corporation, NY, USA).

The variables are reported as medians and ranges or means ± SD. The χ^2 test or Fisher's Exact Test (for less than five items) were applied for comparisons including categorial variables. The Mann Whitney *U*-test was used for comparisons between two groups. Spearman's and Pearson's correlation analyses were used for correlation. Odd ratios (ORs) and 95% confidence intervals (CIs) were calculated by logistic regression analysis.

The predictive value of ovulation and hormonal analysis was determined by identifying the proportion of women with at least one live birth 5 years or more after inclusion in the study. Calculation of predictive value was performed as previously described [88]. Inhibin B and PRL were excluded from the prediction calculations as a result of the considerable variations in serum concentrations (*study I*). Sample size calculations were carried out by using power calculation (*study II*). Allele frequencies were calculated to investigate deviation from Hardy–Weinberg equilibrium, and Haploview [89] was used for determination of haplotypes from the genotype data. A meta-analysis of four different studies [82,83,90], where one of them was *study III*, was performed to assess IVF pregnancy outcome in relation to *MTHFR* 677 TT versus CC and 1298 CC versus AA polymorphisms (*study III*). The paired *t*-test and Cohen's kappa coefficient were used for comparisons of data from questionnaires and 24-hour recall interviews. Principal component analysis (PCA) was performed for analyses of socioeconomic and lifestyle factors (SIMCA software, Umetrics, Umeå, Sweden) (*study IV*).

Statistical significance was defined as $p < 0.05$ in all studies.

Ethical considerations

The studies were approved by the regional Ethics Committees in Stockholm and Uppsala, Sweden. Written and oral informed consent was obtained from all participants.

Participation in the studies was voluntary and the women could leave whenever they wanted and without giving a reason. Participating or not participat-

ing, or leaving the study did not affect IVF treatment in infertile women. To ensure the participants' anonymity, names and social security numbers were coded to specific ID numbers, which were applied in the used data files. Blood samples from the infertile women were usually collected at the same time as other blood samples were taken or when a venous catheter was put in place, when no additional needle-stick was needed. In one study the infertile women needed to come to the clinic several times for taking the blood samples, which may have been challenging and stressful for women undergoing IVF treatment. The fertile women voluntarily made an appointment for blood sample collection and these samples were taken by a trained nurse.

Results

Study I

No significant differences in hormone values and live birth rates between women with unexplained infertility and women with male-factor infertility were found in this study. The occurrence of ovulation did not affect any of the hormones. A majority of the women in both groups ovulated during the studied cycle.

None of the studied hormones proved to be a good predictor of live birth in the long term in women with unexplained infertility and women with male-factor infertility. The age of the women was seen to have the best predictive value (77%) for future live birth (Table 1). However, when assessing the combination of the three best predictors, i.e. age, ovulation (defined as progesterone ≥ 32 nmol/L) and TSH ≤ 2.5 mIU/L, the predictive value increased to nearly 90% (Table 1). Assay of AMH has increased in popularity in infertility evaluation, and AMH levels are more stable during the menstrual cycle compared with other hormones. Therefore, another combination of three factors, AMH, age and ovulation, was also analysed, which resulted in the second best predictive value of over 80% (Table 1).

Table 1. *The best predictors of live birth in women with unexplained infertility and male-factor infertility.*

	Ovulation [a)]	TSH [b)]	Age [b)]	Ovulation, TSH and age	Ovulation, AMH [b)] and age
Sensitivity %	78.4	94.4	54.1	100.0	83.3
Specificity %	30.3	15.2	81.8	33.3	25.0
Predictive value %	55.8	54.8	76.9	88.2	83.3

a) Ovulation was determined by using the serum progesterone concentration as an indicator, ≥ 32 nmol/L being considered to represent ovulation (Normal).

b) Cut-off values (Normal) were taken to be TSH ≤ 2.5 mIU/L, AMH ≥ 10 pmol/L and age ≤ 32 years.

Study II

The present results indicated that the use of folic acid supplementation was significantly greater among the women with unexplained infertility than among the fertile women. This was seen as regards both multivitamins, and folic acid supplements only. High folic acid intake was also reflected as significantly better folate status in infertile women (Figure 3). However, use of a plasma folate level of ≥ 22.5 nmol/L [85] as a biomarker of folic acid supplement use showed that compliance as regards folic acid intake was remarkably lower than reported in the questionnaires in both groups (infertile: 67.8% vs. 43.3%, fertile: 38.8% vs. 15.4%). Folic acid supplementation or good folate status were not found to have a positive effect on IVF pregnancy outcome in women with unexplained infertility.

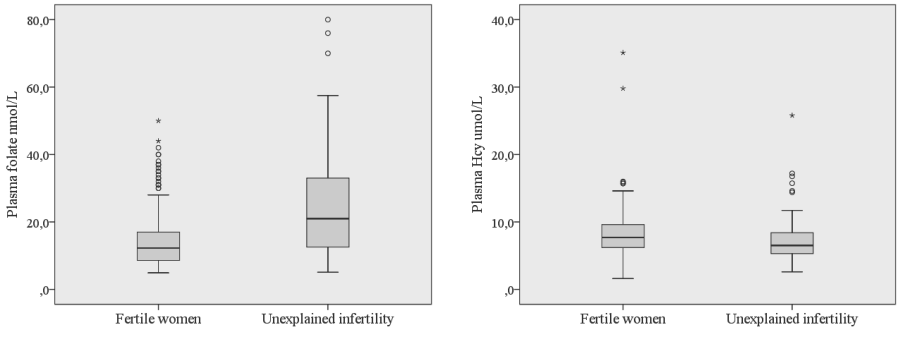

Figure 3. Median plasma folate and homocysteine values in women with unexplained infertility and fertile control women, shown as box plots.

Study III

Genotype frequencies of polymorphisms *MTHFR* 677C>T, 1298A>C and 1793G>A did not differ between infertile and fertile women. As seen in *study II*, the infertile women used significantly more folic acid supplements than fertile women, which was also seen as better folate status in infertile women. However, no association between the studied genotypes and folate status was found.

Neither did the results indicate an association between the studied *MTHFR* polymorphisms and pregnancy outcome in infertile women. A meta-analysis of pregnancy outcome in relation to *MTHFR* 677TT versus CC and 1298CC versus AA polymorphisms was carried out using data from four different studies [82,83,90], one of them being the present study. The results showed that polymorphism 1298 was related to IVF pregnancy outcome, but polymorphism 677 was not (Table 2). In particular, *MTHFR* 1298AA wild-type was seen to be favourable as regards successful IVF pregnancy outcome.

Table 2. *Meta-analysis of pregnancy outcome in relation to MTHFR 677TT vs. CC and 1298CC vs. AA polymorphisms. Data is shown as OR (95% CI).*

	MTHFR 677 TT vs. CC	*MTHFR* 1298 CC vs. AA
Haggarty et al.	0.98 (0.47–2.09)	0.24 (0.08–0.71)
Dobson et al.	1.84 (-0.54–1.75)	0.52 (-1.60–0.28)
Laanpere et al.	0.79 (0.37–1.71)	1.41 (0.61–3.25)
Murto et.al	0.88 (-1.16–0.91)	0.65 (-1.37–0.51)

Study IV

Folic acid supplement use was seen to be related to marital status, educational level and employment status. However, principal component analysis did not show any association between folic acid supplement use and socioeconomic or lifestyle factors. Infertile women had significantly higher mean folic acid daily intake, which was reflected as better folate status. Folic acid supplement use was also related to a greater dietary folate intake in infertile women, but not in fertile women. Socioeconomic and lifestyle factors were not correlated with folate status.

The infertile women were physically more active, smoked less and had better employment status than fertile women. Surprisingly though, they were also more obese (BMI $\geq$ 30 kg/m^2) than the fertile women. However, breakfast habits, used as a lifestyle model, did not differ considerably between infertile and fertile women. The only significant difference was that coffee and tea consumption was lower in infertile women. Socioeconomic and lifestyle factors were not found to be related to IVF outcome.

The validity of the used questionnaire seemed to be good. Only three of eight parameters differed between the questionnaire and the 24-hour recall interview data. However, as regards folic acid supplement use, the results showed only fair agreement.

Discussion

The main finding in the study was that the age of the women was the best predictor of live birth after infertility treatment. High-level folic acid intake, good folate status, and genetic and socioeconomic factors did not show associations with IVF pregnancy outcome.

There are many variables that have been used to predict live birth success and failure after in vitro fertilization, although no good single variable has been found. The age of the woman has been shown to be an important predictor [111,112], which was also shown in this study. In the present study women at the age of 32 or less had the highest chance of a future live birth, while in previous studies the cut-off point for age has been a few years higher [91,92].

The studied hormones did not prove to be good predictors of birth after infertility treatment in long term. Previously it has been suggested that FSH:LH ratio [93], estradiol levels together with age and levels of FSH [94], as well as luteal phase estradiol and progesterone levels [88] could be possible predictors of pregnancy outcome [93]. In addition, increased cycle day three FSH levels together with age have been shown to be associated with a decreased probability of IVF pregnancy [95-97]. In a recent study it was found that mean menstrual cycle length was strongly related to live birth rate in assisted reproduction [98], which is in contrast to the present results, where menstrual cycle length was not related to live birth after IVF.

Recently, AMH has become a popular marker in the field of assisted reproduction [99]. It has been suggested that AMH is a better predictor of live birth after infertility treatment than other previously used markers [100-105]. However, this was not seen in the present study, where AMH had the lowest single predictive value as regards future live birth. This is similar to results reported by Penãrrubia et al [106], who did not find AMH useful in the prediction of IVF pregnancies. On the other hand, the combined predictive value of AMH with age and an ovulatory menstrual cycle (defined as serum progesterone $\geq$ 32 nmol/L) was found to be predictive as regards live birth. Another recent study also revealed an association between AMH plus age in connection with IVF pregnancy outcome [107].

Unexpectedly, the TSH level was found to be one of the three best predictors in the present study. In addition, the combination of age, an ovulatory cycle and a low TSH level turned out to have the best predictive value of live birth in the long term. To our knowledge, TSH has not previously been stud-

ied as a predictive marker of pregnancy outcome in assisted reproduction, which is most probably a result of its controversial role in infertility treatments [108,109], and the significant differences in TSH levels between the different diagnoses [110,111]. This also makes the usefulness of TSH assay somewhat uncertain from a clinical point of view. Consequently, comparing TSH or progesterone levels with those of AMH, it can be seen that the advantage of AMH is that its serum levels remain relatively stable during the menstrual cycle [2,11]. This could make AMH more useful in infertility treatments than other hormones. However, to define the clinical applicability of AMH as a marker of live birth after IVF, further investigations are required.

Folate status or folic acid intake have not been used as predictors of IVF pregnancy outcome, although infertility treatment is one factor associated with high folic acid supplement intake [66], which was also shown in the present study. Therefore, a plasma folate cut-off value of 22.5 nmol/L was selected in connection with IVF pregnancy outcome in women with unexplained infertility and male-factor infertility, to see hypothetically what the predictive value would be. The results showed that plasma folate was a better predictor of pregnancy outcome following infertility treatment (predictive value of 73%) than the previously presented hormones. This raises the question of whether or not folate could be used as a predictor of IVF pregnancy outcome rather than the commonly used hormones. However, it must be remembered that serum folate levels are easily influenced by dietary and/or supplement intake. In fact, folic acid supplement use increases serum folate levels more than do dietary folates [58], owing to the fact that folic acid has a more stable chemical structure and better bioavailability than naturally occurring folates [112]. Additionally, the different folate measurement methods, which are further discussed later in this section, make the definition of a precise cut-off value challenging.

The infertile women used significantly more folic acid supplements and had higher dietary folate intake than fertile women, which was seen as better folate status in infertile women. It has also been shown previously that infertile women use higher amounts of folic acid supplements [77,82], and that supplement use is closely related to a higher intake of dietary vitamins and minerals [68]. Conversely, it has been shown that women who already have children tend not to use folic acid supplements [59,68]. The reason for this might be that these women are not pregnant/planning a future pregnancy, and incentives for the use of folic acid supplementation are lacking. However, an estimated half of all pregnancies are unplanned [113,114], and therefore the importance of folic acid supplementation should be considered by all women of fertile age. As has been highlighted in many previous studies[56,59-61], health professionals have an important role as regards recommendations and infor-

mation on folic acid supplement use both in infertile and fertile women, and this fact should not undervalued.

There are also many socioeconomic and lifestyle factors that have been shown to influence the use of folic acid supplements [61-64]. In particular, increasing age has been associated with an increased folic acid supplement use [63,64,66,68,115,116]. However, this was not confirmed in the present study, where the fertile women were somewhat older than the infertile women, suggesting that folic acid supplement use was related to infertility rather than age. Other socioeconomic and lifestyle factors that have often been related to folic acid supplement use are high educational level, better employment status and western origin [63-65,68]. The results of this study agreed relatively well with those of previous studies, indicating that folic acid supplement users were more often married and had a higher educational level and better employment status than the non-users. In addition, socioeconomic factors have been shown to be associated with folate status [65], although this was not confirmed in this study.

The validity of reported nutrient intake is often crucial and the method of data collection is of importance when investigating dietary supplement intake. This was also shown in this study, where a plasma folate level of ≥ 22.5 nmol/L was used as an indicator of folic acid supplement use. The results indicated lower folic acid supplement use, both in infertile and fertile women, compared with the results from the questionnaire, suggesting that folic acid intake was most likely not on a daily basis in all women who stated this in the questionnaire. A recent Dutch study [77] revealed similar results, although the difference in that study was smaller compared with the results in the present study. Differences between diverse methods were also confirmed when questionnaire data was compared with the data from the 24-hour recall interviews. Calculation of Cohen's kappa coefficient indicated only fair agreement as regards folic acid supplement use.

For infertility treatment as well as public health purposes, it is also important to understand the genetic and nutritional background factors affecting infertility treatment outcome in cases of different infertility diagnoses. However, this has not been well investigated, although genetic variations have been studied in relation to different hormonal parameters during infertility treatment [117]. In addition, genetic variations in the *MTHFR* gene have been shown to be associated with folate status [49-52,64]. In particular, the *MTHFR* 677TT polymorphism is the most common gene variation that has been related to folate status, with decreased serum folate and increased plasma Hcy levels [48,49,118]. The current results did not reveal any correlation between the *MTHFR* gene variations versus folate status. However, good folate status in infertile women might have masked the adverse effects of *MTHFR* gene polymorphisms.

The heterozygote CT genotype of *MTHFR* 677 has been suggested to increase the chance of pregnancy after infertility treatment, although folic acid supplementation among women with different *MTHFR* genotypes has not been shown to increase the success of IVF pregnancy outcome [82,90]. The results of the present study did not indicate that high-level folic acid intake, good folate status or gene variation could assist infertile women in achieving successful pregnancy after infertility treatment. However, the results of a meta-analysis of four studies on pregnancy outcome in relation to the most commonly studied gene variations, *MTHFR* 677TT versus CC and 1298CC versus AA polymorphisms, showed that the *MTHFR* 1298AA gene variation was an advantage as regards IVF pregnancy outcome (Table 2). Therefore, the results suggest that folate might have an inconsequential role in the context of pregnancy outcome, and that other variables in folate metabolism, especially a specific gene variation, could have a more central role.

The results did not indicate any association between socioeconomic or lifestyle factors and IVF outcome, although the infertile women were seen to have a healthier lifestyle and a higher socioeconomic status, which have been shown to have a positive effect on fertility [20]. Unexpected was the fact that the infertile women were more obese than the fertile women, although obesity is known to be negatively associated with fertility [20]. Among these women obesity might be a cause of infertility that could possibly be treated by means of weight loss. This could be especially important for women with a diagnosis of unexplained infertility, who very often have major psychological symptoms as a result of this condition [119]. However, there were no significant differences between different infertility diagnoses as regards pregnancy outcome and the other studied aspects, and therefore no possible enlightenments concerning the diagnosis of unexplained infertility. Obesity also negatively influences folate absorption [120], which should be borne in mind when recommending supplements. The results raise consideration of the need for improved policy concerning lifestyle factors affecting treatment at infertility clinics. Clearly, more active provision of information is needed, as well as more enquiry about lifestyle habits.

Methodological considerations

In the present study IVF outcome was measured as live births, which is an advantage, since very often the outcome is measured only as pregnancy rate. However, successful outcome of infertility treatment is not reached before a live birth [16].

For the long-term prediction of live birth, eight hormones were measured at various times during the menstrual cycle. The blood samples were taken at different times of day, although all samples were taken during the daytime

because of the opening hours of the clinic. The different time points were not taken into account in the analyses, which may be considered as a drawback. However, the studied hormones are not sensitive to external factors, e.g. stress, and the possible effect of taking samples at different time points was also seen to be very small. Convenience for the women was prioritised before these possible aspects.

The study design in *study I* is cross-sectional, which made it easier and faster to perform than longitudinal studies, which would have required a considerably larger sample size. Therefore, a cross-sectional design was chosen for *study I*. However, a cross-sectional design has some limitations as well. The design only allows for comparisons between individuals. It has also been shown to be a less reliable study design compared with longitudinal studies.

The data on height and weight were based on self-reported data from the questionnaire. Self-reported data on height and weight have been shown to have low validity, as a result of underestimation of weight and sometimes overestimation of height [121]. The data analyses were not adjusted according to BMI, which could have led to some bias due to under- or overweight conditions. However, the results when limited to women with normal BMI did not differ from results without this limitation. Additionally, the data on BMI did not influence the validity of the main results, since it was not related to the main research questions.

In this study folate levels were measured in plasma. However, folate is not stable in serum and plasma, and serum/plasma folate levels may be influenced by recent dietary intake [122]. Therefore, plasma Hcy was also measured to ensure as good reliability as possible in the blood samples. Plasma Hcy has been shown to be more stable than plasma folate and its levels are also inversely related to plasma folate concentrations [46,123]. A better option would have been to measure folate in red blood cells, which gives information on long-term folate status, but this method is more expensive and complicated than serum/plasma folate measurement methods [122]. Therefore, this method of measurement was not used. Additionally, in this study the blood cells were also needed for genetic analyses. Another aspect is that fasting plasma folate concentrations have shown increased reliability [124] and the possible effect of dietary/supplement intake could have been minimised. Unfortunately, this was not possible in the present study, which can be seen as a limitation, but the women's comfort was prioritised before all other factors. If taking fasting samples, the women would have had to come for blood sampling in the mornings. This would have meant an additional visit for the most of the infertile women, and the flexibility to decide the most appropriate time for the fertile women would not have been there, which could have led to reduced participation in the study.

The questionnaire was used to assess general background, information on dietary habits and use of dietary supplements, especially folic acid. This

method was chosen since it gives relatively widespread information and it is easy to perform. Using a questionnaire has also been shown to be reliable in most cases [125-127], which was also shown in the present study. However, as regards folic acid supplement intake, use of questionnaire data has revealed a risk of overestimated reporting [128]. This was also seen in the present study when comparing the data from the questionnaires with the 24-hour recall interview data or with plasma biomarker data.

In the present study there was 24-hour recall data only from a minority of the study population. The 24-hour recall interviews are extremely time-consuming, which was the reason that the questionnaire and the blood samples were used as the main sources of data in this study. The existing 24-hour recall interview data was used to validate the questionnaire. Only data from two completed 24-hour recall interviews per subject were used, which can be seen to be positive as regards the validity of the study.

Twenty-four-hour recall interviews allow a more specific description of foods and supplement use in the short-term, while questionnaires provide more general long-term information [86]. Therefore, 24-hour recall interview data can be seen as being more reliable than questionnaire data [128]. However, some limitations regarding 24-hour recall interviews have also been perceived. In 24-hour recall interviews people tend to underestimate their daily intake by about 10%, and people who eat less have a tendency to overestimate their intake [86]. In this study both could have happened, since some of the infertile women stated in the interviews and in the questionnaire that they had changed to healthier dietary habits during the time they were trying to become pregnant. However, this possible bias was taken into account in the analyses, where only the normal-reported interviews (as assessed by using the Goldberg cut-off equation [87]) were included. It is preferable that one of the 24-hour recall interviews be performed during the weekend, since it is well known that people most often have different dietary habits during the weekends compared with the weekdays. In this study this was not carried out, which provided us with insufficient data, especially as regards alcohol use. In the questionnaires the women stated that they used approximately three times more alcohol compared with the data from the interviews.

In addition to the above, poor knowledge of the bioavailability of folic acid in supplements and of naturally occurring (dietary) folates [43,44], makes the data regarding folate intake challenging. When evaluating levels of dietary folate, losses of folate by oxidation and during heat treatment [39-41] have to be taken into account. This could not be achieved by way of the methods used in this study. Furthermore, dietary folate intake was estimated from questionnaire data concerning dietary habits and fruit and vegetable ingestion with the help of the national food database. Information on dietary habits, as well as on food folate content in the national food database and information concerning folic acid content in dietary supplements can be questioned as regards validity. The main problem in studies regarding dietary and supple-

ment intake is that well-functioning methods are lacking and further investigations are warranted, especially in the field of micronutrients.

Conclusions

There was no perfect hormonal marker for the prediction of live birth on a long-term basis in infertile women; the best predictor was the age of the woman. In particular, age together with an ovulatory menstrual cycle and the level of AMH could be used as predictors of live birth after infertility treatment.

Infertile women used significantly more folic acid supplements than fertile women, reflected in better folate status in infertile women. Only a few socioeconomic and lifestyle factors were related to folic acid supplement use. Differences in socioeconomic and lifestyle factors were small in comparison of infertile and fertile women.

High-level folic acid intake, good folate status as well as socioeconomic and lifestyle factors in women with different infertility diagnoses were not seen to have a positive effect on IVF pregnancy outcome. The overall significance of *MTHFR* gene variations as regards a positive IVF pregnancy outcome remains unclear as well, but *MTHFR* 1298AA gene variation could be of importance in the context of successful pregnancy outcome.

The questionnaire showed a good validity as regards data on dietary factors. However, using a questionnaire as a data-collecting method as regards folic acid supplement use showed only fair validity and complementary methods, such as 24-hour recall interviews, are recommended.

The importance of folate in health perspectives other than achieving successful pregnancy outcome after infertility treatment should not be forgotten, and therefore healthcare professionals should actively promote the use of folic acid supplements in all women of reproductive age.

Clinical implications

The results of this work indicated that the women at age of 32 or less with ovulatory menstrual cycles and low serum levels of TSH had the highest chance of live birth after infertility treatment. However, the usefulness of TSH in clinical practice is somewhat uncertain as regards its unstable serum levels during the menstrual cycle. Therefore, AMH, which has shown to be more stable during the menstrual cycle, would be more beneficial together with age and ovulatory menstrual cycle in prediction of live birth after IVF. In clinical practice, the age of 32 or less would be challenging age limit, since a majority of women attending infertility treatment are older than 32 years. The women older than 32 years should be treated but also be informed carefully about the fact of the age as regards the chance of pregnancy. It would also be advantageous to measure the serum AMH levels in these women.

Most of the infertile women used folic acid supplements 400-500 μg/day, and they had good folate status as a result of that. However, this did not positively affect the IVF pregnancy outcome. It would not be helpful to recommend these women to use higher amounts of folic acid, but the nurses and doctors should continue the recommendation since folic acid has other important health promoting aspects. Also the supplement use could be followed up during the treatment as regards to improve the compliance of the folic acid supplement use. One of the genetic variations of *MTHFR* gene was shown to be associated with positive IVF pregnancy outcome. Therefore, the genotyping would be valuable to perform in some women when no pregnancy has occurred after several attempts and no reason for the infertility has been found.

Future research

More studies with larger samples are needed to investigate the different factors affecting IVF pregnancy outcome, especially in women with diagnoses of unexplained infertility. Folate in a broader perspective, including other B vitamins and several genetic variations should be included. In addition, studies including also other vitamins and more widely investigated dietary factors would be high of value and interest for future research. Methods of data collection as regards folic acid supplement intake and dietary intake should be considered carefully. It would be beneficial to use both a questionnaire and 24-hour recall interviews in all studied women. Furthermore, socioeconomic and lifestyle factors in relation to dietary supplement use, especially folic acid supplement use, will need additional study in connection with infertile women. Studies on women with diagnoses of unexplained infertility and lifestyle factors, especially BMI, are needed to obtain the better diagnostic for these women.

Summary in Swedish – Sammanfattning på svenska

Ofrivillig barnlöshet är ett vanligt problem som drabbar ungefär vart tionde par i fertil ålder. Det är ungefär lika vanligt att infertiliteten beror på mannen som på kvinnan. Hos ungefär 20 % av alla barnlösa par hittar man ingen förklaring till barnlösheten, och de får diagnosen ”oförklarad barnlöshet”. Oförklarad barnlöshet har ansett som en av de största stressfaktorer man kan drabbas av under livet, oavsett vilken kultur man lever i.

Antalet ofrivilligt barnlösa par ökar, vilket beror på att kvinnor föder sitt första barn allt senare, andelen kvinnor som föder sitt första barn vid 35 års ålder eller senare har ökat kraftigt under de senare åren. Kvinnor är som mest fertila vid 22 års ålder, och därefter sjunker fertiliteten beroende på sjunkande äggstocksfunktion. När detta inträffar och hur snabbt detta går är individuellt.

In vitro fertilisering (IVF) är den bästa behandlingsmetoden vid ofrivillig barnlöshet, men det genomsnittliga graviditetsutfallet efter behandlingen är fortfarande runt 30%. Behandlingen påverkar parets livskvalitet på många sätt. För en stor andel är det krävande både psykiskt och fysiskt och även de ekonomiska konsekvenserna är stora, både för de drabbade paren och för samhället.

Infertila kvinnor kan bli gravida en tid efter fertilitetsbehandling, men idag finns det ingen fungerande metod för att kunna prediktera graviditetsutfallet. Syftet med den första studien var att studera hormonvärden i serum i en slumpmässigt vald naturlig menstruationscykel hos kvinnor med diagnos oförklarad infertilitet och manlig infertilitet i förhållande till att långsiktigt kunna prediktera levande födda barn. I denna studie med 71 kvinnor med diagnos oförklarad infertilitet och manlig infertilitet, togs blodprover under en slumpmässig vald, naturlig menstruation cykel. Serumkoncentrationer av FSH, LH, AMH, inhibin B, östradiol, progesteron, prolaktin och TSH analyserades. Möjligheten att långsiktigt kunna prediktera levande födda barn hos de studerade kvinnorna analyserades genom att använda följande variabler: ovulation definierad som progesteron $\geq$ 32 nmol/L, FSH, FSH:LH kvot, AMH, TSH och ålder. Resultaten visade inga skillnader i hormonvärden eller i antal födda barn mellan kvinnor med diagnos oförklarad infertilitet och manlig infertilitet, och att medianvärdena av hormonnivåerna var inom

referensvärden. Inget av de studerade hormonerna var lämpligt för att långsiktigt kunna prediktera levande födda barn. Det bästa prediktiva värdet hade åldern på kvinnan, följt av ovulation och TSH. Det gjordes en kombinerad beräkning av de tre bästa prediktiva värdena och då ökade det prediktiva värdet till nästan 90%. Mätning av AMH har ökat i popularitet i samband med att prediktera graviditetsutfall efter fertilitetsbehandling, och därför gjordes även i denna studie en kombinerad beräkning av ålder, ovulation och AMH. Den visade att det prediktiva värdet för framtida levande födda barn ökade till över 80%. Utifrån dessa resultat kan vi konkludera att det inte finns några enskilda bra hormonella markörer för att långsiktigt kunna prediktera levande födda barn hos kvinnor med diagnos oförklarad infertilitet och manlig infertilitet, och att den bästa enskilda prediktorn är kvinnans ålder. I synnerhet ålder i kombination med ovulatorisk menstruationscykel och TSH eller AMH är prediktiva för levande födda barn. Fördelen med AMH jämfört med TSH är att serum värdena varierar väldigt lite under menstruationscykeln, vilket gör det till ett användbart verktyg i fertilitetsbehandlingar.

Folsyratillskott används ofta av infertila kvinnor, vilket har visat sig ha en positiv inverkan på folatstatus, men effekten av folsyratillskott på graviditetsutfall hos kvinnor med diagnos oförklarad infertilitet är inte väl undersökt. Syftet med delstudie två var att studera användning av folsyratillskott och folatstatus hos oförklarat infertila kvinnor i förhållande till graviditetsutfall efter IVF, och att studera om det finns skillnader i användning av folsyratillskott och folatstatus mellan oförklarat infertila och fertila kvinnor. I denna studie ingick 188 infertila kvinnor med diagnos oförklarad infertilitet och 180 friska, fertila, icke-gravida kvinnor. Metoderna som användes var blodprover, plasma folat och plasma homocystein, och en enkät, som användes för data för bakgrund och användning av kosttillskott. Resultaten visade att oförklarat infertila kvinnor använde signifikant mera folasyratillskott än vad fertila kvinnor gjorde, samt att de hade betydligt bättre folatstatus jämfört med fertila kvinnor. Däremot visade det sig att compliance för folsyratillskottanvändning var låg i båda grupperna när ett plasma folat cutoff-värde användes som markör för tillskottanvändning. Användning av folsyratillskott och folatstatus visade ingen positiv effekt på graviditetsutfall efter infertilitetsbehandling. Sammanfattningsvis tyder studien att användningen av folsyratillskott inte var lika hög hos alla kvinnor som de har angett i enkäter, och att relativt hög intag av folsyra och bra folatstatus inte är relaterade till IVF-graviditetsutfall hos kvinnor med diagnos oförklarad infertilitet.

I den tredje studien studerades folsyraintag, folatstatus och graviditetsutfall efter infertilitetsbehandling i förhållande till metylentetrahydrofolatreduktas (*MTHFR*) 677C>T, 1298A>C och 1793G>A genvariationer hos kvinnor med olika infertilitet diagnoser. Dessutom jämfördes användningen av folsy-

ratillskott, folatstatus och frekvensen av de olika genvariationerna mellan kvinnor med olika infertilitet diagnoser och friska, fertila kvinnor. Totalt 528 kvinnor studerades, varav 340 kvinnor med olika infertilitet diagnoser och 188 friska, fertila, icke-gravida kvinnor. Samma metoder användes här som i delstudie två men även genvariationerna analyserades av blodproverna i denna studie. En metaanalys av fyra studier, varav denna studie var en, utfördes för att utvärdera IVF-graviditetsutfall i förhållande till *MTHFR* 677TT vs CC och 1298CC vs AA polymorfismer. Resultaten visade att kvinnor i infertilitetsgruppen använde betydligt mera folsyratillskott och hade bättre folatstatus än fertila kvinnorna, men graviditetsutfall efter fertilitetsbehandling var inte beroende av folsyraintag, folatstatus eller *MTHFR* genvariationer. Resultaten från metaanalysen visade dock att *MTHFR* 1298AA polymorfism var relaterad till graviditetsutfall. Därför konkluderas att användning av folsyratillskott verkar spela mindre roll i samband med IVF-graviditetsutfall, och att andra variabler i folatmetabolismen har större betydelse i detta sammanhang. I synnerhet verkar *MTHFR* 1298AA genvariationen att ha ett positivt samband med framgången för fertilitetsbehandling.

Socioekonomiska och livsstilsfaktorer har visat sig vara relaterade med folsyraintag, men de flesta har studerat gravida kvinnor och studier om infertila kvinnor saknas. Vid studier gällande intag av kosttillskott spelar den använda metoden en viktig roll, eftersom validiteten av det rapporterade intaget kan vara opålitlig vid val av fel metod. Syftet med delstudie fyra var att undersöka socioekonomiska, livsstils- och kostfaktorer i relation till användning av folsyratillskott och folatstatus hos infertila och fertila svenska kvinnor. Dessutom utfördes en sub-analys av 75 kvinnor för att validera den använda enkäten. I denna observationsstudie studerades 340 infertila kvinnor och 188 fertila kvinnor. En enkät användes för att undersöka livsstils- och kostvanor och användning av kosttillskott. 24-timmars kostintervjuer utfördes med 75 kvinnor som validering av den använda enkäten. Blodprover togs för analys av plasma folat och homocystein. Resultaten visade att användning av folsyratillskott var relaterad till civilstånd, utbildningsnivå och sysselsättningsstatus. Infertila kvinnor hade signifikant högre folsyraintag från tillskott vilket också återspeglades som en bättre folatstatus hos dessa kvinnor. Folsyratillskottanvändning var också relaterad till högre folatintag från kosten hos infertila kvinnor men inte hos fertila kvinnor. Infertila kvinnor var fysiskt mer aktiva, rökte mindre och hade bättre sysselsättningsstatus, men samtidigt var de också mer obesa än fertila kvinnor. Socioekonomiska och livsstilsfaktorer visade sig inte vara relaterade till folatstatus eller graviditetsutfall efter IVF-behandling. Validiteten av den använda enkäten visade sig vara relativt bra gällande olika kostfaktorer, men i förhållande till användning av folsyratillskott visade enkätsvaren endast en svag överenskommelse med 24-timmars kostintervjusvaren. Sammanfattningsvis kan man säga att gifta kvinnor med högre utbildning och bättre sysselsättningsstatus

använder mest folsyratillskott. Endast enstaka socioekonomiska och livsstilsfaktorer skiljer sig mellan infertila och fertila kvinnor, och dessa faktorer påverkar inte graviditetsutfallet efter infertilitetsbehandling. Andra datainsamlingsmetoder en enkät rekommenderas för studier gällande folsyratillskottanvändning.

Acknowledgements

This work was carried out at the Department of Women's and Children's Health, Uppsala University, Sweden. Many people have made this thesis possible and I would like to express my gratitude to everyone who helped, supported and encouraged me during all these years. In particular I wish to thank the following persons:

All the women who participated voluntarily in this research, you made this work possible!

Anneli Stavreus-Evers, my main supervisor; I am deep grateful to you for introducing me to this interesting field of research. Thank you for always believing in me and for giving me strength and support during these "roller coaster" years. Your way of handling many things at the same time without too much stress is impressive; things will work out in the end and stressing out does not help – that I have learned☺. I am very happy for our years together, which have included laughs, tears and endless discussions in your car or on Skype. Thank you also for giving me the freedom to work in different countries, close to my dearest!

Agneta Skoog Svanberg, my co-supervisor and my midwife colleague; I am so pleased that I have had you in our team. Your endless support and encouragement have meant so much to me during these years – you have always listened to me and been available even at a distance –thank you so much! I really admire your capability and strength, as well as your ambitious journey from ski guide to deputy head, as you wrote in your presentation recently.

Agneta Yngve, my previous co-supervisor, thank you for introducing me to the interesting field of folates and for your knowledge of nutrition.

Britt-Marie Landgren, co-author and my unofficial mentor; you have taught me a lot, not only the hormones of the menstrual cycle, but also to see the forest from the trees, and "the ketchup effect"☺. It has been a great pleasure to get to know you and work with you!

Ove Axelsson and *Jan Gustafsson*, former and current head of the Department of Women's and Children's Health, for support and the opportunity to carry out my doctoral studies at the Department.

Matts Olovsson, head of our research group, for your encouragement and always friendly attitude and smiley face when meeting you.

Andres Salumets, *Torbjörn Nilsson* and *Signe Altmäe*, for your good co-authorship, knowledge of genetics, and always kind help when the genetics went over my understanding. *Signe*; Thank you also for your support and encouragement during the years, but most of all thank you for your friendship!

Kerstin Bjuresten and all the staff at the Fertility Unit at Karolinska University Hospital Huddinge. *Kjell Wånggren*, *Anne-Li Mååg, Maria Eriksson* and all the staff at the Centre for Reproduction at Uppsala University Hospital – for your prodigious and valuable help with recruiting the patients.
Kerstin and Kjell; Thank you also for the good co-authorship.

Gudrun Abascal, Ingela Wiklund and Harald Almström, for the support and funding from the clinic during the time I was working at BB Stockholm AB.

Eric Poortvliet, for your SPSS skills and all the help with 24h recalls.

Nick Bolton, for your skilful language revision and always friendly attitude.

The administrative staff; *Malin Ghanem, Susanne Löberg, Kerstin Ståhlberg, Erika Anundsson, Hans Lindgren and Martin Selinus* – for your kindness, patience and help with papers, computer problems etc. etc.

All the members in the PhD network, for the fruitful discussions and pleasant meetings, although those were way too few due to my "flapping" lifestyle☺.

All the current and former staff at the research lab, it is always so nice to come to Uppsala and have great company at lunches and coffee breaks. The friendly and helpful environment at the lab is unique☺. In particular, I want to thank: *Malin Helmestam*, we really have walked this line together as you wrote in your thesis. You managed to be PhD one year before me, but I "won" with the second birth ;-) Thank you for all these years, I really will miss you! *Helena Volgsten*, you are such a warm and good person. It has been a joy to get to know you. It feels that I have followed your steps and gathered strength from our discussions to finalize this thesis. Thank you! *Theodora Kunovac Kallak*, your kindness and help with so many things have

been so valuable. Thank you also for the supportive "WhatsApp" discussions!

Susanna "Sanna" Kugelberg, Pia Liljeroth, Anna-Karin Klint Carlander and Ellinor Östensson, my dear friends and PhD/PhD student "buddies"; thank you for your peer support and all the countless discussions during the years, but most of all thank you for your friendship – that means a lot more than any research ever! Also, a special thanks to *Pia* for all the Swedish language reviews.

My all "civilian" friends, you have heard stories about folates and my struggling PhD project so many times, not always understanding but still supporting me. Thank you for the discussions about life, dinners, wine, laughs and the nice memories we share together☺. In addition, a special thanks to *Eftihia "Effie" Bengtsson, Karolina Höög, Jessica Dundeberg and Kitty Andersson* for the good "hotel" – services during the years I was "homeless" in Sweden!

Parent-in-laws, *aunts and cousins*, for your encouraging interest and support.

Hilkka "Hilu" Näse and Katriina Murto and families, my "sister" and sister-in-law; I am so grateful to have two dear "sisters" who always make me realize the real importance of life! Thank you also for always being there for me, but most importantly, thank you for just being you! Hopefully now we will have more time for "godmother"-dinners☺.

My mother, Seija, and father, Pertti; without your help and support this thesis would have been almost impossible to finish. You have always believed, supported and encouraged me with my decisions. You are always ready to help, even at short notice, with different things; moving between countries, babysitting, food and laundry service etc. etc. I may have sometimes forgotten to tell you how much you and your help mean to me. KIITOS ÄITI & ISKI, olette rakkaita ja apunne on korvaamaton!

Vili, my beloved husband and my best friend; thank you for your endless love, support and patience. This road has not been easy for you either; you have reviewed my presentations, articles and thesis, fixed my computer problems in the middle of the night and listened patiently while I told you of the ups and downs of my research. I promise I won't write any articles during the coming sailing season, let's just sail and enjoy it☺. I love you!

Alda and Klara, my dear daughters, for your love and all the joy you bring. You make me realize what is most important in life; play, laugh and live, not

only work! My life has totally changed after you two and is fulfilled with unconditional love! Äiti rakastaa teitä näääään paljon ja maailman eniten!!

*

The studies in this thesis were supported by BB Stockholm AB, The Family Planning Foundation and Födelsefonden, Uppsala, Sweden, The Regional Research Foundation, Örebro, Sweden, a Pampers Scholarship, Sweden and R&D grants from Praktikertjänst AB, Stockholm, Sweden.

References

1. Hawkins SM, Matzuk MM. The menstrual cycle: basic biology. Ann N Y Acad Sci. 2008;1135:10-18.
2. Broekmans FJ, Soules MR, Fauser BC. Ovarian aging: mechanisms and clinical consequences. Endocr Rev. Aug 2009;30(5):465-493.
3. Lebovic DI, Gordon JD, Taylor RN. Reproductive Endocrinology & Infertility 1st ed. Virginia: Scrub Hill Press, Inc.; 2005.
4. Speroff L, Fritz MA. Clinical Gynecologic Endocrinology and Infertility. 8th ed. Philadelphia, PA.: Lippincott Williams & Wilkins; 2011.
5. Sundarrajan C, Liao W, Roy AC, Ng SC. Association of oestrogen receptor gene polymorphisms with outcome of ovarian stimulation in patients undergoing IVF. Mol Hum Reprod. Sep 1999;5(9):797-802.
6. Sundarrajan C, Liao WX, Roy AC, Ng SC. Association between estrogen receptor-beta gene polymorphisms and ovulatory dysfunctions in patients with menstrual disorders. J Clin Endocrinol Metab. Jan 2001;86(1):135-139.
7. Graham JD, Clarke CL. Physiological action of progesterone in target tissues. Endocr Rev. Aug 1997;18(4):502-519.
8. Scarpin KM, Graham JD, Mote PA, Clarke CL. Progesterone action in human tissues: regulation by progesterone receptor (PR) isoform expression, nuclear positioning and coregulator expression. Nucl Recept Signal. 2009;7:e009.
9. Kostrzak A, Warenik-Szymankiewicz A, Meczekalski B. The role of serum PRL bioactivity evaluation in hyperprolactinaemic women with different menstrual disorders. Gynecol Endocrinol. 2009;25(12):799-806.
10. La Marca A, Broekmans FJ, Volpe A, Fauser BC, Macklon NS. Anti-Mullerian hormone (AMH): what do we still need to know? Hum Reprod. Sep 2009;24(9):2264-2275.
11. Baird DT, Collins J, Egozcue J, et al. Fertility and ageing. Hum Reprod Update. May-Jun 2005;11(3):261-276.
12. Luisi S, Florio P, Reis FM, Petraglia F. Inhibins in female and male reproductive physiology: role in gametogenesis, conception, implantation and early pregnancy. Hum Reprod Update. Mar-Apr 2005;11(2):123-135.

13. Poppe K, Velkeniers B, Glinoer D. Thyroid disease and female reproduction. Clin Endocrinol (Oxf). Mar 2007;66(3):309-321.
14. Redmond GP. Thyroid dysfunction and women's reproductive health. Thyroid. 2004;14 Suppl 1:S5-15.
15. Krassas GE. Thyroid disease and female reproduction. Fertil Steril. Dec 2000;74(6):1063-1070.
16. Homan GF, Davies M, Norman R. The impact of lifestyle factors on reproductive performance in the general population and those undergoing infertility treatment: a review. Hum Reprod Update. May-Jun 2007;13(3):209-223.
17. Socialstyrelsen. Graviditeter, förlossningar och nyfödda barn. Medicinska födelseregistret 1973–2011. Assisterad befruktning 1991–2010. Stockholm2013.
18. Fertility statistics. European Commission; 2011. http://epp.eurostat.ec.europa.eu/statistics_explained/index.php/Fertility_statistics. Accessed 2012-10-29.
19. Hassan MA, Killick SR. Negative lifestyle is associated with a significant reduction in fecundity. Fertil Steril. Feb 2004;81(2):384-392.
20. Sharma R, Biedenharn KR, Fedor JM, Agarwal A. Lifestyle factors and reproductive health: taking control of your fertility. Reprod Biol Endocrinol. 2013;11:66.
21. Isaksson R, Tiitinen A. Present concept of unexplained infertility. Gynecol Endocrinol. May 2004;18(5):278-290.
22. Vayena E, Rowe PJ, Griffin PD, Eds. Current practises and controversies in assisted reproduction. Geneva: WHO;2002.
23. Boivin J, Bunting L, Collins JA, Nygren KG. International estimates of infertility prevalence and treatment-seeking: potential need and demand for infertility medical care. Hum Reprod. Jun 2007;22(6):1506-1512.
24. Eugster A, Vingerhoets AJ. Psychological aspects of in vitro fertilization: a review. Soc Sci Med. Mar 1999;48(5):575-589.
25. Infertility revisited: the state of the art today and tomorrow. The ESHRE Capri Workshop. European Society for Human Reproduction and Embryology. Hum Reprod. Aug 1996;11(8):1779-1807.
26. Smith S, Pfeifer SM, Collins JA. Diagnosis and management of female infertility. JAMA. Oct 1 2003;290(13):1767-1770.
27. Tournaye H. Evidence-based management of male subfertility. Curr Opin Obstet Gynecol. Jun 2006;18(3):253-259.
28. Hart R. Unexplained infertility, endometriosis, and fibroids. Bmj. Sep 27 2003;327(7417):721-724.
29. Maheshwari A, Hamilton M, Bhattacharya S. Effect of female age on the diagnostic categories of infertility. Hum Reprod. Mar 2008;23(3):538-542.

30. Moayeri SE, Lee HC, Lathi RB, Westphal LM, Milki AA, Garber AM. Laparoscopy in women with unexplained infertility: a cost-effectiveness analysis. Fertil Steril. Aug 2009;92(2):471-480.
31. Blacker CM, Ginsburg KA, Leach RE, Randolph J, Moghissi KS. Unexplained infertility: evaluation of the luteal phase; results of the National Center for Infertility Research at Michigan. Fertil Steril. Mar 1997;67(3):437-442.
32. Domar AD, Conboy L, Denardo-Roney J, Rooney KL. Lifestyle behaviors in women undergoing in vitro fertilization: a prospective study. Fertil Steril. Mar 2012;97(3):697-701 e691.
33. Nelson SM, Fleming R. Obesity and reproduction: impact and interventions. Curr Opin Obstet Gynecol. Aug 2007;19(4):384-389.
34. Shah K, Sivapalan G, Gibbons N, Tempest H, Griffin DK. The genetic basis of infertility. Reproduction. Jul 2003;126(1):13-25.
35. Cetin I, Berti C, Calabrese S. Role of micronutrients in the periconceptional period. Hum Reprod Update. Jan-Feb 2010;16(1):80-95.
36. Tamura T, Picciano MF. Folate and human reproduction. Am J Clin Nutr. May 2006;83(5):993-1016.
37. Scholl TO, Johnson WG. Folic acid: influence on the outcome of pregnancy. Am J Clin Nutr. May 2000;71(5 Suppl):1295S-1303S.
38. Ebisch IM, Thomas CM, Peters WH, Braat DD, Steegers-Theunissen RP. The importance of folate, zinc and antioxidants in the pathogenesis and prevention of subfertility. Hum Reprod Update. Mar-Apr 2007;13(2):163-174.
39. Nordic Nutrition Recommendations 2012. Part 1. Summary, principles and use. 5th ed. Copenhagen: Norden; 2013.
40. Whitney E, Rolfes S. Understanding Nutrition. 10th ed. Belmont: Thompson Learning; 2005.
41. Scott JM. Folate and vitamin B12. Proc Nutr Soc. May 1999;58(2):441-448.
42. Sanderson P, McNulty H, Mastroiacovo P, et al. Folate bioavailability: UK Food Standards Agency workshop report. The British journal of nutrition. Aug 2003;90(2):473-479.
43. Gibney M, Margetts B, Kearney J, Arab L, eds. Public Health Nutrition. Oxford Blackwell Publishing; 2004. The Nutrition Society textbook series.
44. Ohrvik VE, Witthoft CM. Human folate bioavailability. Nutrients. Apr 2011;3(4):475-490.
45. Laanpere M, Altmäe S, Stavreus-Evers A, Nilsson TK, Yngve A, Salumets A. Folate-mediated one-carbon metabolism and its effect on female fertility and pregnancy viability. Nutr Rev. Feb 2010;68(2):99-113.

46. Scott JM. Reduced folate status is common and increases disease risk. It can be corrected by daily ingestion of supplements or fortification. Novartis Found Symp. 2007;282:105-117; discussion 117-122, 212-108.
47. Rady PL, Szucs S, Grady J, et al. Genetic polymorphisms of methylenetetrahydrofolate reductase (MTHFR) and methionine synthase reductase (MTRR) in ethnic populations in Texas; a report of a novel MTHFR polymorphic site, G1793A. Am J Med Genet. Jan 15 2002;107(2):162-168.
48. Nelen WL, Blom HJ, Thomas CM, Steegers EA, Boers GH, Eskes TK. Methylenetetrahydrofolate reductase polymorphism affects the change in homocysteine and folate concentrations resulting from low dose folic acid supplementation in women with unexplained recurrent miscarriages. J Nutr. Aug 1998;128(8):1336-1341.
49. Zetterberg H. Methylenetetrahydrofolate reductase and transcobalamin genetic polymorphisms in human spontaneous abortion: biological and clinical implications. Reprod Biol Endocrinol. Feb 17 2004;2:7.
50. van der Put NM, Gabreels F, Stevens EM, et al. A second common mutation in the methylenetetrahydrofolate reductase gene: an additional risk factor for neural-tube defects? Am J Hum Genet. May 1998;62(5):1044-1051.
51. Weisberg I, Tran P, Christensen B, Sibani S, Rozen R. A second genetic polymorphism in methylenetetrahydrofolate reductase (MTHFR) associated with decreased enzyme activity. Mol Genet Metab. Jul 1998;64(3):169-172.
52. Böttiger AK, Hurtig-Wennlof A, Sjöstrom M, Yngve A, Nilsson TK. 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. Apr 2007;19(4):659-665.
53. Hoeft B, Weber P, Eggersdorfer M. Micronutrients - a global perspective on intake, health benefits and economics. Int J Vitam Nutr Res. Oct 2012;82(5):316-320.
54. Troesch B, Hoeft B, McBurney M, Eggersdorfer M, Weber P. Dietary surveys indicate vitamin intakes below recommendations are common in representative Western countries. The British journal of nutrition. Aug 2012;108(4):692-698.
55. Amcoff E, Edberg A, Enghardt Barbieri H, et al. Riksmaten – vuxna 2010–11. Livsmedels- och näringsintag bland vuxna i Sverige. Resultat från matvaneundersökning utförd 2010–11. Uppsala: Livsmedelsverket;2012.
56. Folic acid: an update on scientific developments The European Food Safety Authority (EFSA); 2010.

57. Tyden T, Stern J, Nydahl M, et al. Pregnancy planning in Sweden--a pilot study among 270 women attending antenatal clinics. Acta Obstet Gynecol Scand. Apr 2011;90(4):408-412.
58. Elkin AC, Higham J. Folic acid supplements are more effective than increased dietary folate intake in elevating serum folate levels. BJOG. Feb 2000;107(2):285-289.
59. Forster DA, Wills G, Denning A, Bolger M. The use of folic acid and other vitamins before and during pregnancy in a group of women in Melbourne, Australia. Midwifery. Apr 2009;25(2):134-146.
60. Lane IR. Preventing neural tube defects with folic acid: nearly 20 years on, the majority of women remain unprotected. J Obstet Gynaecol. Oct 2011;31(7):581-585.
61. Binns C, Scott J, Nwafor N, Graham K, Oddy W, Lee A. Which mothers take folic acid and folate containing foods? Asia Pac J Clin Nutr. 2006;15(3):335-340.
62. Cleves MA, Hobbs CA, Collins HB, Andrews N, Smith LN, Robbins JM. Folic acid use by women receiving routine gynecologic care. Obstet Gynecol. Apr 2004;103(4):746-753.
63. Timmermans S, Jaddoe VW, Mackenbach JP, Hofman A, Steegers-Theunissen RP, Steegers EA. Determinants of folic acid use in early pregnancy in a multi-ethnic urban population in The Netherlands: the Generation R study. Prev Med. Oct 2008;47(4):427-432.
64. Baraka MA, Steurbaut S, Leemans L, et al. Determinants of folic acid use in a multi-ethnic population of pregnant women: a cross-sectional study. J Perinat Med. Nov 2011;39(6):685-692.
65. Gall S, Seal J, Taylor R, Dwyer T, Venn A. Folate status and socio-demographic predictors of folate status, among a national cohort of women aged 26-36 in Australia, 2004-2006. Australian and New Zealand journal of public health. Oct 2012;36(5):421-426.
66. McGuire M, Cleary B, Sahm L, Murphy DJ. Prevalence and predictors of periconceptional folic acid uptake--prospective cohort study in an Irish urban obstetric population. Hum Reprod. Feb 2010;25(2):535-543.
67. Frishman GN, Spurrell TP, Heber WW. Folic acid. Preconception knowledge and use by infertile women. J Reprod Med. Dec 2001;46(12):1025-1030.
68. Pouchieu C, Levy R, Faure C, et al. Socioeconomic, lifestyle and dietary factors associated with dietary supplement use during pregnancy. PloS one. 2013;8(8):e70733.
69. Selhub J. Homocysteine metabolism. Annu Rev Nutr. 1999;19:217-246.
70. Selhub J, Jacques PF, Wilson PW, Rush D, Rosenberg IH. Vitamin status and intake as primary determinants of homocysteinemia in an elderly population. Jama. Dec 8 1993;270(22):2693-2698.

71. Austin RC, Lentz SR, Werstuck GH. Role of hyperhomocysteinemia in endothelial dysfunction and atherothrombotic disease. Cell Death Differ. Jul 2004;11 Suppl 1:S56-64.
72. Scott J. Folic acid consumption throughout pregnancy: differentiation between trimesters. Ann Nutr Metab. 2011;59(1):46-49.
73. Safi J, Joyeux L, Chalouhi GE. Periconceptional Folate Deficiency and Implications in Neural Tube Defects. J Pregnancy. 2012:295083.
74. Molloy AM, Scott JM. Folates and prevention of disease. Public Health Nutr. Apr 2001;4(2B):601-609.
75. Wilcox AJ, Lie RT, Solvoll K, et al. Folic acid supplements and risk of facial clefts: national population based case-control study. BMJ. Mar 3 2007;334(7591):464.
76. George L, Mills JL, Johansson AL, et al. Plasma folate levels and risk of spontaneous abortion. Jama. Oct 16 2002;288(15):1867-1873.
77. Boxmeer JC, Macklon NS, Lindemans J, et al. IVF outcomes are associated with biomarkers of the homocysteine pathway in monofollicular fluid. Hum Reprod. May 2009;24(5):1059-1066.
78. Nelen WL, Blom HJ, Steegers EA, den Heijer M, Thomas CM, Eskes TK. Homocysteine and folate levels as risk factors for recurrent early pregnancy loss. Obstet Gynecol. Apr 2000;95(4):519-524.
79. Boxmeer JC, Brouns RM, Lindemans J, et al. Preconception folic acid treatment affects the microenvironment of the maturing oocyte in humans. Fertil Steril. Jun 2008;89(6):1766-1770.
80. Chavarro JE, Rich-Edwards JW, Rosner BA, Willett WC. Use of multivitamins, intake of B vitamins, and risk of ovulatory infertility. Fertil Steril. Mar 2008;89(3):668-676.
81. Ray JG, Laskin CA. Folic acid and homocyst(e)ine metabolic defects and the risk of placental abruption, pre-eclampsia and spontaneous pregnancy loss: A systematic review. Placenta. Sep 1999;20(7):519-529.
82. Haggarty P, McCallum H, McBain H, et al. Effect of B vitamins and genetics on success of in-vitro fertilisation: prospective cohort study. Lancet. May 6 2006;367(9521):1513-1519.
83. Dobson AT, Davis RM, Rosen MP, et al. Methylenetetrahydrofolate reductase C677T and A1298C variants do not affect ongoing pregnancy rates following IVF. Hum Reprod. Feb 2007;22(2):450-456.
84. Landgren BM, Unden AL, Diczfalusy E. Hormonal profile of the cycle in 68 normally menstruating women. Acta Endocrinol (Copenh). May 1980;94(1):89-98.

85. Brouwer IA, van Dusseldorp M, Thomas CM, et al. Low-dose folic acid supplementation decreases plasma homocysteine concentrations: a randomized trial. Am J Clin Nutr. Jan 1999;69(1):99-104.
86. Willett W. Nutritional epidemiology. 2nd ed. New York: Oxford University Press; 1998.
87. Goldberg GR, Black AE, Jebb SA, et al. Critical evaluation of energy intake data using fundamental principles of energy physiology: 1. Derivation of cut-off limits to identify under-recording. Eur J Clin Nutr. Dec 1991;45(12):569-581.
88. Csemiczky G, Wramsby H, Landgren BM. Luteal phase oestradiol and progesterone levels are stronger predictors than follicular phase follicle stimulating hormone for the outcome of in-vitro fertilization treatment in women with tubal infertility. Hum Reprod. Nov 1996;11(11):2396-2399.
89. Barrett JC. Haploview: Visualization and analysis of SNP genotype data. Cold Spring Harbor protocols. Oct 2009;2009(10):pdb ip71.
90. Laanpere M, Altmäe S, Kaart T, Stavreus-Evers A, Nilsson TK, Salumets A. Folate-metabolizing gene variants and pregnancy outcome of IVF. Reprod Biomed Online. Jun 2011;22(6):603-614.
91. Chuang CC, Chen CD, Chao KH, Chen SU, Ho HN, Yang YS. Age is a better predictor of pregnancy potential than basal follicle-stimulating hormone levels in women undergoing in vitro fertilization. Fertil Steril. Jan 2003;79(1):63-68.
92. Piette C, de Mouzon J, Bachelot A, Spira A. In-vitro fertilization: influence of women's age on pregnancy rates. Hum Reprod. Jan 1990;5(1):56-59.
93. Seckin B, Turkcapar F, Ozaksit G. Elevated day 3 FSH/LH ratio: a marker to predict IVF outcome in young and older women. J Assist Reprod Genet. Mar 2011;29(3):231-236.
94. Srouji SS, Mark A, Levine Z, Betensky RA, Hornstein MD, Ginsburg ES. Predicting in vitro fertilization live birth using stimulation day 6 estradiol, age, and follicle-stimulating hormone. Fertil Steril. Sep 2005;84(3):795-797.
95. Scott RT, Toner JP, Muasher SJ, Oehninger S, Robinson S, Rosenwaks Z. Follicle-stimulating hormone levels on cycle day 3 are predictive of in vitro fertilization outcome. Fertil Steril. Apr 1989;51(4):651-654.
96. Toner JP, Philput CB, Jones GS, Muasher SJ. Basal follicle-stimulating hormone level is a better predictor of in vitro fertilization performance than age. Fertil Steril. Apr 1991;55(4):784-791.
97. Padilla SL, Garcia JE. Effect of maternal age and number of in vitro fertilization procedures on pregnancy outcome. Fertil Steril. Aug 1989;52(2):270-273.

98. Brodin T, Bergh T, Berglund L, Hadziosmanovic N, Holte J. Menstrual cycle length is an age-independent marker of female fertility: results from 6271 treatment cycles of in vitro fertilization. Fertil Steril. Nov 2008;90(5):1656-1661.
99. La Marca A, Sighinolfi G, Radi D, et al. Anti-Mullerian hormone (AMH) as a predictive marker in assisted reproductive technology (ART). Hum Reprod Update. Mar-Apr 2010;16(2):113-130.
100. Lee TH, Liu CH, Huang CC, Hsieh KC, Lin PM, Lee MS. Impact of female age and male infertility on ovarian reserve markers to predict outcome of assisted reproduction technology cycles. Reprod Biol Endocrinol. 2009;7:100.
101. Wunder DM, Guibourdenche J, Birkhauser MH, Bersinger NA. Anti-Mullerian hormone and inhibin B as predictors of pregnancy after treatment by in vitro fertilization/intracytoplasmic sperm injection. Fertil Steril. Dec 2008;90(6):2203-2210.
102. Nelson SM, Yates RW, Fleming R. Serum anti-Mullerian hormone and FSH: prediction of live birth and extremes of response in stimulated cycles--implications for individualization of therapy. Hum Reprod. Sep 2007;22(9):2414-2421.
103. Dehghani-Firouzabadi R, Tayebi N, Asgharnia M. Serum level of anti-mullerian hormone in early follicular phase as a predictor of ovarian reserve and pregnancy outcome in assisted reproductive technology cycles. Arch Iran Med. Jul 2008;11(4):371-376.
104. Brodin T, Hadziosmanovic N, Berglund L, Olovsson M, Holte J. Antimullerian hormone levels are strongly associated with live-birth rates after assisted reproduction. J Clin Endocrinol Metab. Mar 2013;98(3):1107-1114.
105. Wu CH, Chen YC, Wu HH, Yang JG, Chang YJ, Tsai HD. Serum anti-Mullerian hormone predicts ovarian response and cycle outcome in IVF patients. J Assist Reprod Genet. Jul 2009;26(7):383-389.
106. Penarrubia J, Fabregues F, Manau D, et al. Basal and stimulation day 5 anti-Mullerian hormone serum concentrations as predictors of ovarian response and pregnancy in assisted reproductive technology cycles stimulated with gonadotropin-releasing hormone agonist--gonadotropin treatment. Hum Reprod. Apr 2005;20(4):915-922.
107. Wang JG, Douglas NC, Nakhuda GS, et al. The association between anti-Mullerian hormone and IVF pregnancy outcomes is influenced by age. Reprod Biomed Online. Dec 2010;21(6):757-761.
108. Laufer MR, Floor AE, Parsons KE, Kuntz KM, Barbieri RL, Friedman AJ. Evaluation of hormonal testing in the screening for in vitro fertilization (IVF) of women with tubal factor infertility. J Assist Reprod Genet. Feb 1995;12(2):93-96.

109. Zollner U, Lanig K, Steck T, Dietl J. Assessment of endocrine status in patients undergoing in-vitro fertilization treatment. Is it necessary? Arch Gynecol Obstet. Mar 2001;265(1):16-20.
110. Cramer DW, Sluss PM, Powers RD, et al. Serum prolactin and TSH in an in vitro fertilization population: is there a link between fertilization and thyroid function? J Assist Reprod Genet. Jun 2003;20(6):210-215.
111. Arojoki M, Jokimaa V, Juuti A, Koskinen P, Irjala K, Anttila L. Hypothyroidism among infertile women in Finland. Gynecol Endocrinol. Apr 2000;14(2):127-131.
112. McNulty H, Pentieva K. Folate bioavailability. Proc Nutr Soc. Nov 2004;63(4):529-536.
113. Font-Ribera L, Perez G, Salvador J, Borrell C. Socioeconomic inequalities in unintended pregnancy and abortion decision. Journal of urban health : bulletin of the New York Academy of Medicine. Jan 2008;85(1):125-135.
114. Finer LB, Henshaw SK. Disparities in rates of unintended pregnancy in the United States, 1994 and 2001. Perspectives on sexual and reproductive health. Jun 2006;38(2):90-96.
115. Nilsen RM, Vollset SE, Rasmussen SA, Ueland PM, Daltveit AK. Folic acid and multivitamin supplement use and risk of placental abruption: a population-based registry study. Am J Epidemiol. Apr 1 2008;167(7):867-874.
116. Arkkola T, Uusitalo U, Pietikainen M, et al. Dietary intake and use of dietary supplements in relation to demographic variables among pregnant Finnish women. The British journal of nutrition. Nov 2006;96(5):913-920.
117. Rosen MP, Shen S, McCulloch CE, Rinaudo PF, Cedars MI, Dobson AT. Methylenetetrahydrofolate reductase (MTHFR) is associated with ovarian follicular activity. Fertil Steril. Sep 2007;88(3):632-638.
118. Ashfield-Watt PA, Pullin CH, Whiting JM, et al. Methylenetetrahydrofolate reductase 677C-->T genotype modulates homocysteine responses to a folate-rich diet or a low-dose folic acid supplement: a randomized controlled trial. Am J Clin Nutr. Jul 2002;76(1):180-186.
119. Dhaliwal LK, Gupta KR, Gopalan S, Kulhara P. Psychological aspects of infertility due to various causes--prospective study. Int J Fertil Womens Med. Jan-Feb 2004;49(1):44-48.
120. da Silva VR, Hausman DB, Kauwell GP, et al. Obesity affects short-term folate pharmacokinetics in women of childbearing age. International journal of obesity. Apr 9 2013.
121. Villanueva EV. The validity of self-reported weight in US adults: a population based cross-sectional study. BMC Public Health. 2001;1:11.

122. Piyathilake CJ, Robinson CB, Cornwell P. A practical approach to red blood cell folate analysis. Analytical chemistry insights. 2007;2:107-110.
123. Refsum H, Smith AD, Ueland PM, et al. Facts and recommendations about total homocysteine determinations: an expert opinion. Clin Chem. Jan 2004;50(1):3-32.
124. De Bruyn E, Gulbis B, Cotton F. Serum and red blood cell folate testing for folate deficiency: new features? European journal of haematology. Dec 18 2013.
125. Messerer M, Wolk A. Sensitivity and specificity of self-reported use of dietary supplements. Eur J Clin Nutr. Dec 2004;58(12):1669-1671.
126. Ishihara J, Sobue T, Yamamoto S, Sasaki S, Akabane M, Tsugane S. Validity and reproducibility of a self-administered questionnaire to determine dietary supplement users among Japanese. Eur J Clin Nutr. May 2001;55(5):360-365.
127. da Silva NF, Sichieri R, Pereira RA, da Silva RM, Ferreira MG. Reproducibility, relative validity and calibration of a food frequency questionnaire for adults. Cadernos de saude publica. Sep 2013;29(9):1783-1794.
128. Yen J, Zoumas-Morse C, Pakiz B, Rock CL. Folate intake assessment: validation of a new approach. J Am Diet Assoc. Aug 2003;103(8):991-1000; discussion 1000.

Acta Universitatis Upsaliensis

Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine 987

Editor: The Dean of the Faculty of Medicine

A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title "Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine".)

Distribution: publications.uu.se
urn:nbn:se:uu:diva-220476

ACTA
UNIVERSITATIS
UPSALIENSIS
UPPSALA
2014