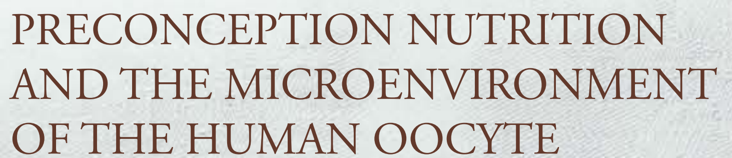

PRECONCEPTION NUTRITION AND THE MICROENVIRONMENT OF THE HUMAN OOCYTE

Proteomic and epidemiologic studies on IVF/ICSI treatment outcomes

Johannes Maarten Twigt

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**PRECONCEPTION NUTRITION
AND THE MICROENVIRONMENT
OF THE HUMAN OOCYTE**

*Proteomic and epidemiologic studies
on IVF/ICSI treatment outcomes*

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
rector magnificus

Prof. Dr. H.G. Schmidt

en volgens besluit van het College voor Promoties.

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Johannes Maarten Twigt
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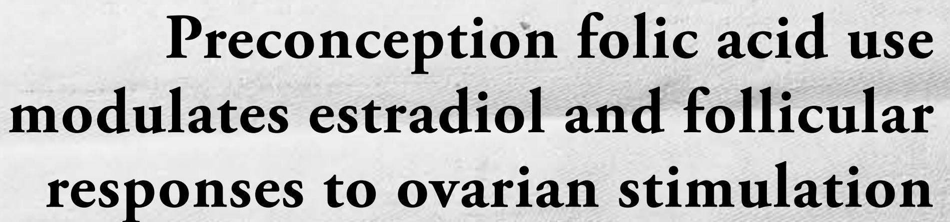
Preconception folic acid use modulates estradiol and follicular responses to ovarian stimulation



I

J.M. Twigt, F. Hammiche, K.D. Sinclair,
N.G.M. Beckers, J.A. Visser, J. Lindemans,
F.H. de Jonge, J.S.E. Laven and R.P.M. Steegers-Theunissen

J Clin Endocrinol Metab, 2011 (2): 322-329

Introduction & Aim

V

Since the first successful application of In Vitro Fertilization (IVF) in 1978[1] and Intracytoplasmic Sperm Injections (ICSI) in 1992,[2] there has been a steady increase in the utilization of these assisted reproduction techniques (ART) to achieve pregnancy in subfertile couples. Initially, the natural menstrual cycle was allowed to recruit the ovarian follicle for IVF. The ovarian follicle is the microenvironment in which the oocyte matures. However, the lack of control of ovulation and the low propensity for a viable embryo and pregnancy after single oocyte fertilization led to the development of Controlled Ovarian Hyperstimulation treatment (COH) to yield more oocytes. The advantage is that COH overcomes the inefficiency of monofollicular ovulation in women and it extends the range of indications for IVF/ICSI treatment.

The principal aim of COH is to stimulate growth and maturation of multiple ovarian follicles, which is achieved through the use of Follicle Stimulating Hormone (FSH) analogues. The administration of a Gonadotropin Releasing Hormone (GnRH) agonist or -antagonist is compulsory to prevent premature ovulation/luteinization of the ovarian follicle, which precludes oocyte retrieval. When sufficient ovarian follicles are mature, a single dose of human Chorionic Gonadotropin (hCG) or recombinant Luteinizing Hormone (rLH) is administered to induce final oocyte maturation, much similar to the surge of LH during the menstrual cycle. Thirty-six hours after hCG/rLH administration, oocytes are retrieved by transvaginal ultrasound guided puncture of the ovarian follicle. The retrieved oocytes are fertilized, and the embryo deemed most viable is transferred to the uterus for implantation.

Currently, the combination of COH and IVF/ICSI is a moderately successful technique to achieve pregnancy in subfertile couples. After six treatment cycles, the cumulative life birth rate is slightly over 50%, which does leave room for improvement.[3] Epidemiologic and experimental studies provide ample suggestions to improve IVF/ICSI treatment success, which range from improving embryo culture conditions and embryo selection to improving patient characteristics (i.e. promoting weight loss, quitting smoking and acquiring healthy nutritional habits). However, also the ovarian response to COH, i.e. the number of growing follicles and amount of steroid hormones produced, affect the chance of IVF/ICSI treatment success and risk of complications.[4, 5]

The ovarian response is a reflection of extrinsic- and intrinsic-ovarian factors that are (non-)permissive for (multiple) follicle growth. Epidemiologic and experimental studies show that such factors can be the result of prevalent genetic-, metabolic- or nutritional and lifestyle factors.[6-8] Indeed, the ovarian response can be predicted with the use of demographic, ultrasound and endocrine markers, which are currently used to decide on an optimal COH strategy. Unfortunately, most of such factors are non-modifiable, with little variability between treatment cycles

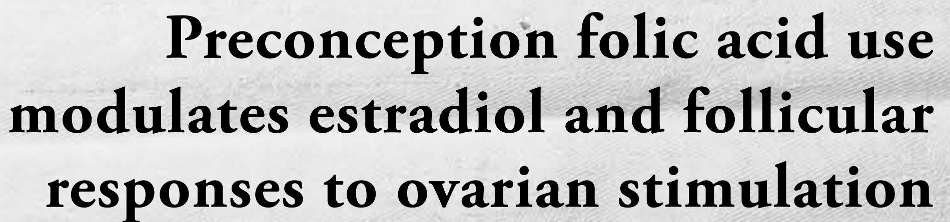
and are therefore not suitable to modify or optimize the ovarian response to COH. Additionally, these factors are not always successful in adequately predicting the ovarian response. Indeed, this will also result from unknown genetic, metabolic and environmental (i.e. lifestyle) factors.[9]

Parallel with the increased demand for assisted reproductive techniques, the prevalence of unhealthy diet consumption is increasing.[10] Given the nature of multiple follicle growth, the relation between oocyte and follicle growth and the modifiable nature of nutrition, nutrition could have a role in the ovarian response to COH and IVF/ICSI treatment outcomes. On a micronutrient level, studies increasingly show the relation between the folate mediated one-carbon pathway and nutrition in the periconception period and reproductive outcomes and offspring health.[11] Unfortunately little attention is given to the preconception period whilst it constitutes an opportunity to improve outcomes of spontaneous as well as ART pregnancies.

The studies in this thesis aim to further outline the role of preconception nutrition, in particular of the folate mediated one-carbon pathway, in the ovarian response to COH and IVF/ICSI treatment outcomes.

The main objectives of this thesis are to investigate the following:
1: What is the role of folate in human reproduction?
2: How is the preconception microenvironment of the human oocyte influenced by folate?
3: What is the influence of preconception nutrition and folate on COH and IVF/ICSI treatment outcomes?

These findings might further promote and support the importance of an optimal preconception nutritional status of couples undergoing COH and IVF/ICSI treatment.



Preconception folic acid use modulates estradiol and follicular responses to ovarian stimulation

II

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N.G.M. Beckers, J.A. Visser, J. Lindemans,
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J Clin Endocrinol Metab, 2011 (2): 322-329

Folate in human reproductive performance

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Vitamins in the prevention of human diseases. W. Hermann and R. Obeid, eds.
2011 Berlin, Walter de Gruyter

INTRODUCTION

The natural B-vitamin folate is mandatory for cellular metabolism, fulfilling a multitude of roles in various processes ranging from cell-cycle regulation, amino acid biosynthesis, DNA nucleotide synthesis and protein processing.[12] Therefore, it is not surprising that folate deficiency potentially results in many derangements in growth and development with implications also for reproduction.[13] Folic acid is a synthetic derivative of folate, which is more resilient against oxidation than naturally occurring folates. By means of the intertwined folate- and methionine-cycle (**Fig. 1**), folates are predominantly utilized in one-carbon metabolism for the synthesis of three out of four DNA-nucleotides (adenine, guanine and thymine) and metabolism of the amino acids, methionine, serine, glycine and cysteine.

Many enzymes involved in folate metabolism require cofactors for normal functioning. Methionine synthase (MTR) is a zinc protein and requires vitamin B12 (cobalamin) as a cofactor and vitamin B6 (pyridoxine) is a cofactor for the transsulphuration pathway. Furthermore, vitamin B2 is needed for adequate synthesis of 5,10-methylenetetrahydrofolate reductase (MTHFR) and zinc is also necessary for adequate uptake of folate from the jejunum.[14, 15]

Humans do not have the ability to synthesize folate. The demand for folate therefore has to be met entirely by dietary intake. Green leafy vegetables, beans or liver are natural sources of folate. Alternatively, in several countries grain, cereal and bread products are increasingly often fortified with folic acid, making these products a rich source of folic acid. Folate is present in food as 5-methyltetrahydrofolate (5-mTHF) with a polyglutamate tail. In the jejunum, 5-mTHF-polyglutamate is hydrolyzed to 5-mTHF-monoglutamate by glutamate carboxypeptidase-II. Because of its low pH optimum, the proton-coupled folate transporter favours folate transport into the enterocytes of the jejunum. Within the enterocytes, 5-mTHF-monoglutamate is converted into 5-mTHF and thereafter released into the circulation.[16] In peripheral tissue, several mechanisms for 5-mTHF uptake exist. Folate receptors (FR) exist in three isoforms, α, β and γ that bind circulating 5-mTHF. The FR-folate complex is internalized by endocytosis and subsequent acidification of the compartment leads to dissociation of 5-mTHF from the receptor. The mechanism of export from the endosome is likely to be facilitated by the proton-coupled folate transporter. FRα is required for the placental transport of 5-mTHF. In peripheral tissue, however, the reduced folate carrier is the most predominant mechanism for 5-mTHF uptake.[16-18]

Intracellular the methyl moiety of 5-mTHF is used by MTR to remethylate homocysteine (Hcy) to methionine and forming tetrahydrofolate (THF). Methionine is further metabolized into S-Adenosylmethionine (SAM) by

methionine adenosyltransferase (MAT). SAM is the ultimate methyl donor for virtually all methylation reactions. After transmethylation of SAM, S-Adenosylhomocysteine (SAH) is formed. By means of a reversible reaction, S-Adenosylhomocysteine Hydrolase (AHCY) hydrolyzes SAH to Hcy and adenosine. A folate independent remethylation pathway for Hcy is present by means of betaine-homocysteine methyltransferase (BHMT) that utilizes a methyl group from betaine to form dimethylglycine and methionine. Approximately 50% of Hcy is metabolized via the remethylation pathway. The remainder is metabolized via the transsulphuration pathway. In this pathway the non-essential amino acid cysteine is required for the synthesis of the endogenous anti-oxidant glutathione. This pathway, however, is tissue specific, with all necessary enzymes only being expressed in the liver, small intestine, kidney and pancreas.[19]

After partition of the methyl group, THF is further metabolized via the folate cycle in several ways (**Fig.1**). (1) THF reacts with formate to form 10-formyl-THF, which either dehydrates to 5,10-methenyl-THF, which is then metabolized to 5,10-methylene-THF (5,10-mTHF), or is allocated to the formation of purines. (2) THF reacts with serine, catalyzed by serine hydroxymethyltransferase (SHMT) to form glycine and 5,10-mTHF. In a reaction catalyzed by MTHFR, 5,10mTHF is reduced to 5-mTHF that can be further conveded into the methionine cycle. MTHFR forms the junction between the methionine and folate cycle, here it is determined whether 5,10-mTHF is utilized for 5-mTHF production or de novo synthesis of thymidylate (dTMP) from deoxyuridylate (dUMP).

A multitude of genes coding for enzymes are required for adequate folate metabolism. Several single nucleotide polymorphisms have been identified which affect the efficiency of one-carbon metabolism. However, only polymorphisms in the MTHFR and methionine synthase reductase (MTRR) enzyme have shown to be of clinical significance in folate metabolism and later pathology. In the gene coding for the MTHFR enzyme a C->T substitution at position 677 results in a thermolabile variant of the enzyme.[20] The frequency of this polymorphism is dependent on geographical location and varies between 0.1-0.5.[21] Homozygotes for this mutation have a 70% reduced MTHFR activity, resulting in hyperhomocysteinemia and DNA hypomethylation.[22, 23] A 66A->G polymorphism in the MTRR gene has an estimated allele frequency of 0.39-0.59 and results in reduced activity of the MTRR enzyme.[21, 24] The product of the MTRR gene maintains MTR in its active state. It should be noted, however, that enzymatic deficiencies due to these polymorphisms are only clinically relevant when nutritional deficiency of cobalamin (MTRR) or folate (MTHFR) are co-existent.

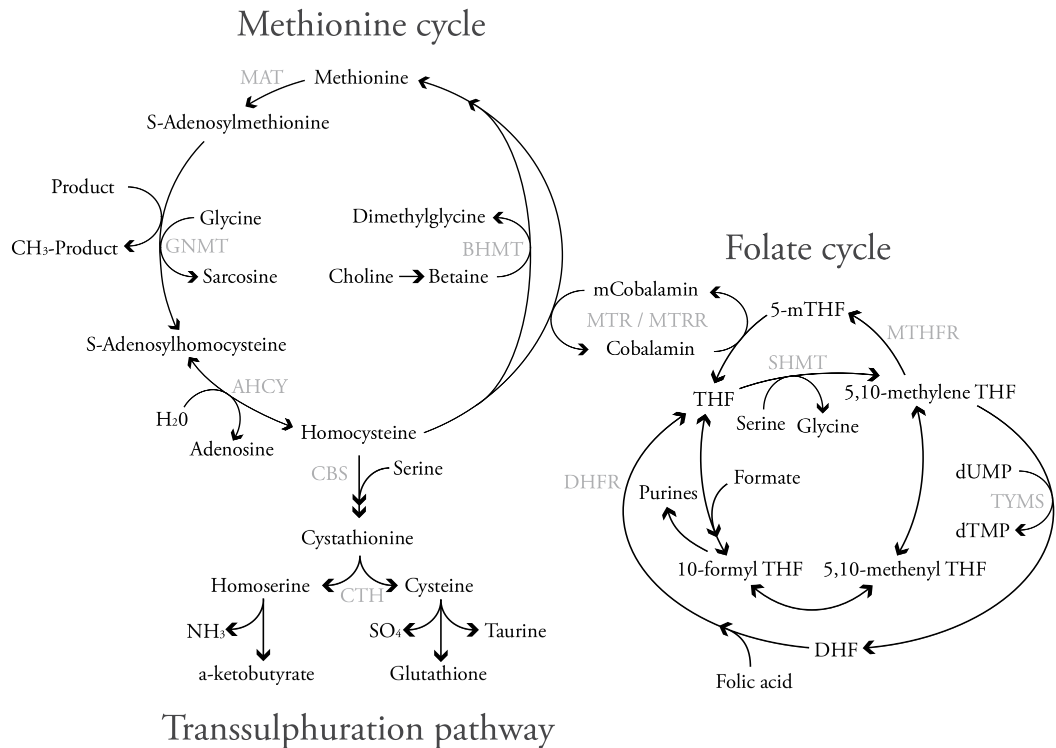


Fig 1. The methionine and folate cycle. Abbreviations: AHCY: S-Adenosylhomocysteine Hydrolase. BHMT: Betaine-Homocysteine Methyltransferase. CBS: Cysthatione-β-Synthase. CTH: Cysthatione-y-Lyase. DHF: Dihydrofolate. dTMP: deoxythymidine monophosphate. dUMP: deoxyuridine monophosphate. GNMT: Glycine-N-Methyltransferase. MAT: Methionine Adenosyltransferase, MTR: Methionine synthase. MTRR: Methionine synthase reductase. SHMT: Serine hydroxymethyltransferase. THF: Tetrahydrofolate. TYMS: Thymidylate synthase.

The pathogenicity of folate deficiency can in theory be elicited in various ways. Due to its nature, especially vulnerable are highly proliferating tissues with a high rate of genomic and epigenomic replication and RNA- and protein synthesis. These processes all depend on the availability of one-carbon groups provided by the folate dependent one-carbon pathway. DNA methylation is an epigenetic mechanism. Methylation of cytosine residues in CG repeats, named CpG-island, exert regulatory properties on gene transcription by interfering with binding of factors necessary for gene transcription. A sufficient folate supply is necessary for adequate DNA methylation, with hypomethylation giving rise to altered gene expression.[25] Folate also influences factors required for the maintenance of DNA integrity. Efficacy of nucleotide excision repair mechanisms and de novo DNA synthesis is modulated by the availability of thymidine triphosphate. Due to folate deficiency increasing amounts of deoxyuridine triphosphate is misincorporated into DNA.[26, 27] Furthermore, folate deficiency alters the gene expression profile and cytosine hypomethylation promotes the deamination of cytosine to uracil, thereafter further stressing uracil specific repair mechanisms and reducing DNA stability.[28]

As portrayed earlier, folate substrate or cofactor deficiency (vitamin B2, -B6 and –B12 and zinc) results in the accumulation of Hcy. Therefore, Hcy levels in blood serum are regarded as a sensitive marker for the functioning of folate metabolism. Despite its necessity, excess Hcy can exert harmful effects in several ways. Reactive Hcy metabolites can form adducts with lysine residues in proteins or can be misincorporated into proteins as a substitute for methionine, thereby potentially affecting protein function.[29] Secondly, it has long been acknowledged that Hcy may disturb the redox balance of the cell by the generation of reactive oxygen species (ROS).[30] An adequate redox balance is required for normal physiology, also in many reproductive processes.[31] An excess of ROS results in oxidative stress, which indiscriminately can affect the functionality of all cellular constituents. Finally, the equilibrium between Hcy and adenosine and SAH is favoured towards SAH and SAH is a potent inhibitor of most SAM-dependent methyltransferases.[32] DNA hypomethylation, therefore, not only occurs in the situation of reduced availability of SAM due to reduced Hcy remethylation but also due to the inhibitory effect of SAH on methyltransferases.[33]

The interdependency of all factors involved in folate metabolism make that it is difficult to discern which is an intermediate and which is an actual effector driving pathologic processes. However, the downstream effects of these pathological processes have in common that they independently affect genomic, epigenomic and proteomic integrity that are essential for normal cellular functioning.

PRECONCEPTION FOLATE IN WOMEN

At birth, women have a resting pool of immature oocytes that have arrested in the diplotene stage of the meiotic prophase I. A single layer of pre-granulosa cells surrounds the oocyte; together this complex is called the primordial follicle. During the process of follicular growth and maturation an antrum is formed which is filled with follicle fluid. The follicle fluid is the microenvironment in which the developing oocyte resides. It is a plasma exudate that reflects follicular cell metabolism. As a result the magnitude of folate or folic acid ingestion affects 5-mTHF and Hcy levels in follicle fluid.[34, 35] Continuously a cohort of follicles initiate the process of maturation, at the end of the monthly menstrual cycle one follicle and its oocyte have reached the maturity suitable for ovulation and consequent fertilization.

An adequate folate balance can potentially reduce the risk or overcome subfertility in women.[36-39] Follicular sensitivity to gonadotropins is in part modulated by proper functioning of the methionine cycle. Low MTHFR activity has been associated with low follicular activity.[40-42] Consequently high Hcy levels are associated with an inadequate high ovarian response to gonadotropins in ewes.[43] Furthermore, raised levels of Hcy in follicle fluid have a detrimental effect on oocyte and embryo quality.[44-46] An adequate redox balance is of influence on follicular development and ovulation, and a disruption of this balance has been shown to induce follicular atresia and decrease sensitivity of the follicle to gonadotropins.[31, 42, 47-49]

PRECONCEPTION FOLATE IN MEN

In men preconception folate status potentially influences semen quality. During the mitotic phase of spermatogenesis methylation marks have to be maintained.[50] Also, the genome and cellular machinery is replicated, adding to the increased demand for one-carbon groups from the folate- and methionine cycle. During mitosis and meiosis new methylation marks are acquired. The pattern of methylation is specific for the differentiation state of the spermatocyte and the entire epigenome is completely established at the pachytene stage of meiosis.[51]

Observational studies indicate that men with low dietary folate intake have a higher frequency of sperm aneuploidy, increased levels of sperm DNA damage and a low sperm density and count, which is associated with a higher rate of subfertility.[52-54] Furthermore, high thiol levels, including Hcy, in the ejaculate have been associated with male subfertility.[46] On the other hand, combined supplementation of folate and zinc sulphate increases total normal sperm count by 76%.[55] Despite its influence on folate metabolism, the MTHFR 677C->T polymorphism is only a risk factor in the setting of insufficient folate intake.[56, 57]

Similar to follicular maturation, ROS are important second messengers during spermatogenesis.[58] Spermatocytes have a high content of polyunsaturated fatty acids, lack DNA repair mechanisms and have no anti-oxidant defence.[59] Therefore, the developing spermatocyte is exceptionally prone to disturbances in the redox balance resulting in excessive oxidative stress, which can arise due to hyperhomocysteinemia. In general, subfertile men have higher levels of ROS in semen, which is associated with lower sperm motility, fertilizing capacity and sperm DNA integrity.[60]

POST CONCEPTION FOLATE IN WOMEN

During embryogenesis, folate dependent processes are required for normal development. As has just been covered, at the point of fertilization the entire epigenome has been attained by both gametes. This pattern is specific for the oocyte and spermatocyte and, excluding imprinted genes, needs to be synchronized. Almost the entire DNA methylation pattern is erased after fertilization. With the exception of imprinted genes the genome is demethylated at the blastocyst stage.[61] During subsequent cleavages from the blastocyst stage onward the DNA is remethylated in a lineage specific manner.[61] The importance of an adequate supply of methyl groups during the periconception period is demonstrated by a phenotype of hypertension, obesity and diabetes in predominantly male offspring exposed to a low methionine diet during early pregnancy.[62, 63] Approximately 8 days post-fertilization placentation begins. Crucial for normal placentation is the invasion of trophoblasts into the endometrial epithelium.[64] Excess levels of Hcy induces trophoblast apoptosis and inhibits trophoblast invasion into endometrial epithelium.[65] As a possible result of excess Hcy, oxidative stress occurs that inhibits normal placentation and alters placental gene expression.[66, 67] In experimental settings folate deficiencies inhibit normal placentation and results in a higher foetal morbidity and mortality.[68, 69] Before circulation is established the embryo resides in a hypoxic environment, during which it is most susceptible to oxidative stress.[70] During embryogenesis, ROS are important second messenger in the regulation of transcription factors.[71] Derangement hereof affects successful embryonic development and can result in miscarriages.[31] This is also reflected by the lower embryo quality after in vitro embryo cultures, were embryos endure higher than normal levels of oxidative stress.[72]

FOLATE AND THE FOETUS

Maternal deficiency of folate during the periconception period is associated with several congenital malformations of the foetus. Generally, congenital

malformations are considered to be of multifactorial origin, being subject to both genetic and environmental influences. Neural crest cells are progenitors of structures most commonly affected by folate deficiencies. Relative to other embryonic progenitor cells, neuroepithelium cells have a high expression of FR, indicating their dependency on a sufficient folate supply.[73] In vitro studies have indicated that neural crest cells are sensitive to fluctuations in folate and Hcy concentrations.[74]

Nutrients necessary for early foetal development are acquired from uterine secretions or, after proper placenta development, from the maternal circulation. Syncytiotrophoblasts form the endothelium of the placental vasculature.[75] On the apical membrane of the syncytiotrophoblast FRα is expressed, which binds circulating 5-mTHF with high affinity. At the basolateral membrane 5-mTHF is transported from the syncytiotrophoblast into the foetal circulation facilitated by the reduced folate carrier. Theoretically this mechanism allows for the build-up of a high concentration gradient, securing foetal 5-mTHF supply, even in a folate deficient environment.[17, 18]

Historically, neural tube defects (NTD) pose the greatest example of congenital malformations due to folate deficiency. A five-time reduction in NTD birth prevalence can be achieved by periconception folic acid supplement use of the mother.[76] Additionally, food folic acid fortification programs have shown a population wide reduction in NTD birth prevalence ranging from 26-50%.[77] Hyperhomocysteinemia in the mother has most widely been drawn on as risk indicator for NTD, with elevated levels being elicited by reduced availability of 5-mTHF due to polymorphisms in genes involved in folate metabolism and uptake or nutritional folate deficiency. Many functional polymorphisms, in genes essential for folate uptake and metabolism have been investigated, nevertheless only the polymorphisms MTRR66A>G and MTHFR677C->T have been shown to increase the birth prevalence of NTD.[76, 78] In vitro studies have indicated that direct interference with the efficiency of methylation reactions increases the birth prevalence of NTD.[79-81] In addition, oxidative stress alters expression of genes necessary for neural tube closure and increase the birth prevalence of NTD.[82, 83] Interestingly, a 70% reduction in NTD birth prevalence can be achieved by strong adherence to a Mediterranean diet alone.[84]

As the most frequently occurring congenital malformation, congenital heart defects (CHD) have been associated with a maternal deficiency of folate intake and/or metabolism. A 6% drop in the birth prevalence of CHD has been noted after the mandatory fortification of food with folic acid.[85] The underlying mechanism of this association is however still lacking. MTHFR deficiency due to the 677C->T polymorphism and oxidative stress has

been associated with CHD in an experimental setting and an excess of Hcy inhibits proper septation of the developing heart.[68, 69, 86, 87] Nevertheless, in the patient population only hyperhomocysteinemia in the mother posed a 4.4 times higher risk of CHD birth prevalence where MTHFR deficiency alone seems not to be a risk factor for CHD in offspring.[88, 89] The common effect of reduced DNA methylation hereof has not been established as an etiologic factor in CHD birth prevalence.

Orofacial clefts (OFC) pose a heterogeneous group of malformations ranging from the relatively mild cleft lip to the more severe cleft palate with or without cleft lip. The birth prevalence of OFC has been related to folate deficiency in a multitude of studies.[90] However folate supplementation or MTHFR polymorphisms now seem not to be associated with the incidence of OFC.[88, 90]

DNA hypomethylation can induce meiotic segregation errors which can give rise to trisomy 21 in offspring, causing Down syndrome (DS).[91] This association has led to the investigation of folate deficiency in DS occurrence. Despite a plausible biological mechanism, many contradicting observations have been made and no significant connection between maternal MTHFR deficiency or elevated Hcy levels and DS exists.[92-94]

FOLATE AND PREGNANCY COMPLICATIONS

Between 50-60% of all conceptions fail to survive until the end of the first trimester of pregnancy.[95] The placenta is a highly vascularised organ, therefore hyperhomocysteinemia and its underlying causes have been proposed as risk factors for placenta linked pregnancy complications. Predominant among these are foetal growth restriction, pre-eclampsia, placental abruption and habitual abortion. In experimental studies there have been associations between MTHFR polymorphisms, folate deficiency and pregnancy complications.[68, 69] Nevertheless, experimental findings are not unanimously reflected in clinical findings, where foetal growth restriction, pre-eclampsia and habitual abortion are not associated with the MTHFR 677C->T polymorphism.[96-98] Maternal Hcy status does, however, associate with the occurrence of placenta related pregnancy complications and folic acid use during pregnancy reduced the risk of low birth weight and small for gestational age foetuses.[99-104] In the case of pre-eclampsia though it is not clear whether the hyperhomocysteinemic state is the cause or effect of the pathological process.[105] Only hyperhomocysteinemia and not MTHFR polymorphisms as a risk factor can be explained by the fact that the methylation pathway is restored by folic acid use, which is widely recommend to all pregnant women in the western world.

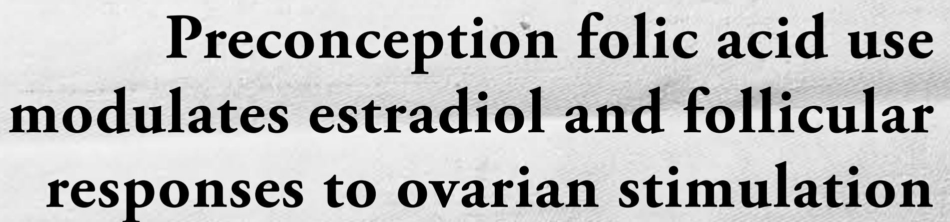
CONCLUSION

Evidence is accumulating on the significance of an adequate folate pathway during human reproduction. Because various essential pathways are affected by a shortage of folate, research into the aetiology, pathophysiology and prevention of adverse reproductive outcome is complicated. This is further complicated by the fact that intervention studies during human pregnancies are nearly impossible, which necessitates inference from in vitro and animal models or observational studies.

Research into single nutrients and gene polymorphisms is a necessity to identify new risk factors and helps to explain underlying biological mechanisms. This type of research, however, is best suited for highly controlled animal- and in vitro studies. In epidemiological studies the rational is to focus on associations between adverse reproductive outcome and biomarkers of the folate- and methylation cycle, i.e., Hcy, SAM, SAH, vitamin B12, and DNA and histone methylation patterns.[106] Besides folate, many cofactors and proper functioning genes are needed for the provision of methylgroups. Because intrinsically biomarkers and methylation patterns of the genome are adjusted for factors influencing these determinants, both measurements are useful to assess the long- and short-term folate status.

Despite growing knowledge on epigenetic mechanisms and intriguing evidence in animals, at present little is known about the relation between adverse reproductive outcome and epigenetics in human. The epigenome is at the crossroad of environmental exposure and gene expression profiles. Therefore, epigenetics pose an interesting field of future research, which can aid in the identification of both genetic and environmental exposures underlying adverse reproductive outcomes associated with methylation cycle deficiencies.

Preconception folic acid use modulates estradiol and follicular responses to ovarian stimulation



III

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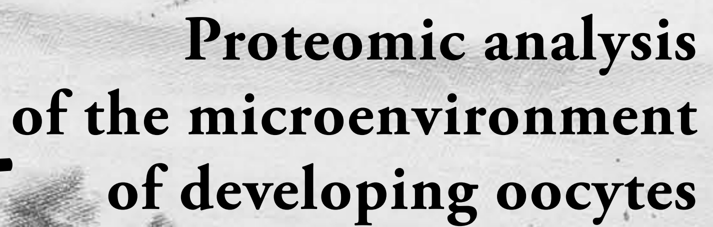


V

Proteomic analysis of the microenvironment of developing oocytes

J.M. Twigt, R.P.M. Steegers-Theunissen, K. Bezstarosti and J. Demmers

Proteomics, 2012 (9): 1463-1471

ABSTRACT

We utilized a setup based on extensive pre-fractionation of proteolytic peptides and nanoflow reversed-phase liquid chromatography mass spectrometry (LC-MS/MS) to identify the (sub)proteome of human follicle fluid. In this in-depth screen, 268 specific proteins were identified, the majority of which are involved in coagulation- and immune response pathways. Our aim is to define a set of follicle fluid protein markers, which could predict oocyte quality.

INTRODUCTION

In Vitro Fertilization (IVF) is an assisted reproduction technology to achieve pregnancy in subfertile couples and animals. The acquired oocytes are fertilized in vitro, yielding embryos that are scored for viability using morphologic criteria. In human, ongoing pregnancy rates per treatment cycle vary between 16-33%.[4] In part, this is due to the inability to select the most viable oocytes and embryos. Therefore, more accurate selection will likely improve success rates after IVF treatment.

Oocytes provide RNA, proteins and cellular machinery for the early zygote, therefore, oocyte quality predicts embryo quality and implantation rates.[107] During ovarian follicle development, a cavity filled with fluid is formed. Follicle fluid comprises the preconception microenvironment in which the oocyte develops. Follicle fluid is a secretory product of granulosa cells and a selective exudate of blood serum.[108] Hence, follicle fluid is a complex mixture of proteins, metabolites and ionic compounds reflecting follicle metabolism and comparable to blood serum.[109-111] Oocyte and embryo quality are associated with the presence and concentration of single biomarkers in follicle fluid, for example folate, cobalamin and homocysteine.[44, 112-114] Finally, the composition of follicle fluid is indicative of the follicular developmental stage.[115, 116]

To select the best oocyte and embryo, it will be helpful if we can objectively assess oocyte quality. Given the complexity of the numerous independent processes involved in oocyte maturation, it is unlikely that a single biomarker can predict oocyte quality.[117] By characterization of the follicle fluid (sub) proteome, a profile of biomarkers associated with oocyte quality could be discovered. This may offer prognostic information aiding the selection of the most viable oocytes and embryo. In the current study, we present a novel approach to analyze the follicle fluid (sub)proteome. We compare two methods based on nanoflow LC-MS/MS combined with peptide and protein fractionation using IEF and SDS-PAGE to uncover a detailed map of the human follicle fluid (sub)proteome.

MATERIALS AND METHODS

Study design

The FOod, Lifestyle and Fertility Outcome study studies the influence of preconception nutrition and lifestyle on fertility and pregnancy outcome and is described in detail before.[44] During oocyte retrieval, we separately collected follicle fluid from the largest follicle in each ovary. The follicle fluid samples were centrifuged for 10 min at 1,700 rpm to remove cells. The samples were frozen without preservatives and stored at -20°C until assayed.

To improve protein coverage, we selected two follicle fluid samples from two follicles of comparable maturity (follicle diameter, 17.3 mm) from two folic acid supplemented women from couples with male factor infertility, which underwent mild GnRH-antagonist supported ovarian stimulation treatment and for who the oocyte from the respective follicle fluid was transferred after fertilization, from which no ongoing pregnancy occurred.

Proteomic analysis

The preparation of the follicle fluid samples and the proteomic analysis according to the two approaches was done as follows:

Removal of abundant proteins: IgGs were removed from the follicle fluid samples by depletion on a protein G Sepharose column. Subsequently, albumin was removed by adding ice cold 95% ethanol to the column eluate and incubated at 4°C for 1 hour. Proteins were collected by centrifugation at 16,000 g for 45 min at 4°C (based on protocols in Fu et al. 2005).[118]

Approach 1, SDS-PAGE: Proteins were separated on a standard SDS-PAGE gel, fixed and stained with Coomassie Blue. The gel lane was divided into 22 slices and further treated according to a standard in-gel digestion protocol.[119]

Approach 2, In-tube gel digestion and prefractionation of proteolytic peptides: A 'tube gel' was prepared by mixing 17 µl acrylamide (40%, 29:1) solution, 2.3 ul 10% ammonium persulfate and 1 µl of TEMED with 50 µL of the follicle fluid sample and allowed to polymerize for 1 hr at RT. The gel piece was cut into pieces and further treated according to a standard in-gel digestion protocol.[119] Proteolytic peptides were extracted from the gel material with 50 µL 30% acetonitrile and dried in a speedvac. 200 µl of Bromophenol Blue containing water and 2 µl of a 10% IPG buffer pH 4-7 (Amersham) were added to the peptide sample and then deposited onto an 11 cm IPG dry strip pH 4-7(Amersham). The strip rehydrated overnight at 4°C. An IPGPhor (Amersham) was used with the following settings: 30 min at 300V, 30 min gradient to 1000V and 5 hrs at 4000V. After the peptide separation, the IPG strip was cut into 0.5 cm slices and peptides from each slice were extracted with 50 µl 2% acetonitrile / 0.1% formic acid.

Mass spectrometry: Nano-flow LC-MS/MS was performed on an 1100 series capillary LC system (Agilent Technologies) coupled to either an LTQ-Orbitrap or an LTQ linear ion trap mass spectrometer (both Thermo) operating in positive mode and equipped with a nanospray source, essentially as described in Bezstarosti et al.[120] Peptide mixtures were trapped on a ReproSil C18 reversed phase column (Dr Maisch GmbH; column dimensions 1.5 cm × 100 µm, packed in-house) at a flow rate of 8 µl/min. Peptide separation was performed on ReproSil C18 reversed phase column

(Dr Maisch GmbH; column dimensions 15 cm × 50 μm, packed in-house) using a linear gradient from 0 to 80% B (A = 0.1% formic acid; B = 80% (v/v) acetonitrile, 0.1% formic acid) in 70 min and at a constant flow rate of 200 nl/min using a splitter. The column eluate was directly sprayed into the ESI source of the mass spectrometer. Mass spectra were acquired in continuum mode; fragmentation of the peptides was performed in data-dependent mode.

Database searching: Tandem mass spectra were extracted, charge state deconvoluted and deisotoped by Mascot Distiller version 2.3. All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version 2.2). Mascot was set up to search the Uniprot database (taxonomy *Homo sapiens*, version 2010_12, 97701 entries) assuming the digestion enzyme trypsin. Mascot was searched with a fragment ion mass tolerance of 0.80 Da and a parent ion tolerance of 15 ppm for Orbitrap spectra and 2.0 Da for ion trap spectra. Iodoacetamide derivative of cysteine was specified in Mascot as a fixed modification. Oxidation of methionine was specified in Mascot as variable modifications.

Criteria for protein identification: Scaffold (version Scaffold_2_06_02, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm.[121] Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least one identified peptides (resulting in a 0.1% protein false discovery rate). Protein probabilities were assigned by the Protein Prophet algorithm.[122] Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. We used Ingenuity Pathways Analysis (Ingenuity Systems Inc., Redwood City, CA) to uncover the functionalities of the identified proteins. The data is available in the PRIDE database.[123] The data was converted using PRIDE Converter (http://pride-converter.googlecode.com).[124]

RESULTS AND DISCUSSION

The IEF approach identified 241 proteins; the SDS-PAGE based method 139 proteins (**Table I**). The number of proteins identified in the current study is higher than reported previously and confirms those identified in previous studies (**Fig. 1**), possibly because of the more extensive prefractionation protocol and more sensitive mass spectrometry.[109-111]

The most abundant proteins, e.g. proteins involved in coagulation and immune response pathways, overlap in the different data sets (**Fig. 1**). These same proteins were identified in both human and animal studies, aimed at

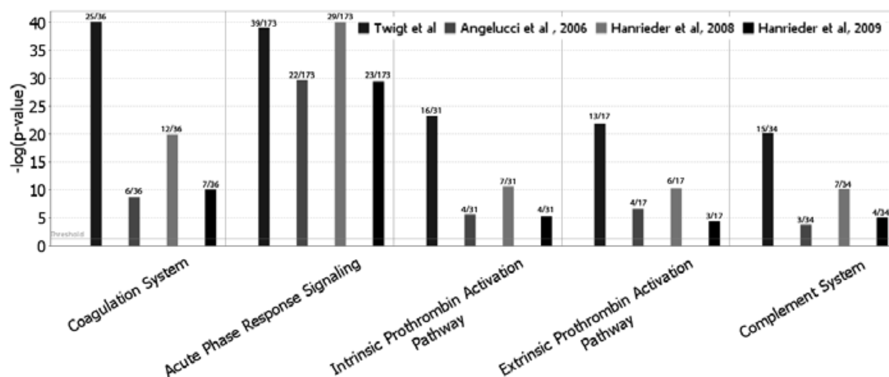


Fig. 1: The five most significant pathways in our follicle fluid screen compared to identifications of these pathways in previous screens, for which these were also among the most abundant pathways. The y-axis displays the –log (p-value) for the respective pathway. The threshold line denotes the cut-off for significance (p-value of 0.05). The ratio on top of the bar reflects the number of identified proteins in the screen relative to the number of proteins in the pathway.

identifying proteins with differential abundances in serum and follicle fluid related to treatment outcomes or follicular developmental stage.[113, 115, 116, 125-127]

Here, coagulation and immune response pathways represent abundant pathways in follicle fluid. The coagulation pathway in follicle fluid is cited to be mainly involved in extracellular matrix remodelling, facilitating follicle growth, ovulation and corpus luteum formation.[128] As part of immune response pathways, the function of the complement system in general is quite well established; its putative role in follicle fluid is nevertheless unclear. Some studies suggest a role during embryo implantation.[113, 129]

The complexity of follicle fluid in terms of the number of proteins and their abundance poses challenges in identifying putative biomarkers. In developing strategies, in which the follicle fluid proteome profile is indicative of oocyte quality, it could be helpful to take into account an exposure for which its effect on oocyte quality is established, such as preconception folic acid use. Folate metabolism is reflected in follicle fluid and folate availability affects oocyte and embryo quality after IVF.[44] Studies in cultured lymphoblasts show that folate affects gene expression and protein profiles in proliferating tissues.[130] We therefore propose that we can use differentially expressed proteins due to folate availability to generate proteome profiles associated with oocyte quality.

In conclusion, we identified a higher number of proteins using the IEF approach compared to the SDS-PAGE approach. This may generally be true for complex protein mixtures in which a small number of highly abundant proteins constitute the majority of the (sub)proteome, as is the case for

follicle fluid. This study confirms previous proteomics screens of follicle fluid (**Fig. 1**) and to our knowledge presents the most detailed proteomic analysis of human monofollicular fluid thus far.[109-111] High resolution, high throughput technologies like mass spectrometry based proteomics enable us to define profiles composed of multiple proteins with various biological functions, which may offer prognostic information that can aid in the selection of the best oocytes and most viable embryo.

A next step in developing a prognostic follicle fluid proteome will have to involve a quantitative approach in which we assess differences in protein abundance levels with regard to oocyte and embryo quality. Focusing on exposures that are known to influence these outcomes can help select differential proteome profiles.

Table I. Identified proteins in follicle fluid using SDS-PAGE and IEF.

Name	Uniprot Accession ID	SDS-PAGE (no. peptides)	IEF (no. peptides)	MW (kDa)
Serum albumin	P02768	38	85	69
Complement C3	P01024	91	114	187
Alpha-1-antitrypsin	P01009	30	37	47
Serotransferrin	P02787	29	70	77
C4B1	Q6U2E9	53	70	193
Basement membrane-specific heparansulfate proteoglycan core protein	P98160	34	117	469
Alpha-2-macroglobulin	P01023	38	60	163
Fibrinogen beta chain	P02675	22	39	56
Ig alpha1-1 chain C region	P01876	10	22	38
Apolipoprotein A-I	P02647	22	35	31
Ig kappa chain C region	P01834	6	14	12
Fibronectin	P02751	28	69	263
Antithrombin-III	P01008	21	28	53
Ig gamma-1 chain C region	P01857	8	20	36
Fibrinogen gamma chain	P02679	19	27	52
Hemopexin	P02790	13	26	52
Fibrinogen alpha chain	P02671	21	27	95
Inter-alpha-trypsin inhibitor heavy chain H4	Q14624	25	34	103
Complement factor H	P08603	15	50	139
Ceruloplasmin	P00450	12	46	122
Inter-alpha-trypsin inhibitor heavy chain H2	P19823	14	20	106
Prothrombin	P00734	11	30	70
Plasminogen	P00747	13	43	91
Haptoglobin	P00738	19	23	45
Protein highly similar to complement factor B	B4E1Z4	28	31	141
Ig gamma-2 chain C region	P01859	2	12	36
Inter-alpha-trypsin inhibitor heavy chain H1	P19827	10	28	101

Table I. Continued from previous page.

Name	Uniprot Accession ID	SDS-PAGE (no. peptides)	IEF (no. peptides)	MW (kDa)
Alpha-1-antichymotrypsin	P01011	16	20	48
Complement C5	P01031	16	38	188
Gelsolin	P06396	17	21	86
Plasma protease C1 Inhibitor	P05155	14	16	55
Angiotensinogen	P01019	9	14	53
Apolipoprotein A-IV	P06727	27	22	45
Kininogen-1	P01042	9	20	72
Heparin cofactor 2	P05546	8	15	57
Clusterin	P10909	11	15	52
Beta-2-glycoprotein 1	P02749	2	15	38
Complement component C6	P13671	6	25	105
Vitamin D-binding protein	P02774	6	22	53
Alpha-2-antiplasmin	P08697	4	14	55
Histidine-richglycoprotein	P04196	8	13	60
Alpha-1B-glycoprotein	P04217	5	14	54
Vitronectin	P04004	8	14	54
Complement component C7	P10643	3	18	94
Immunoglobulin lambda-like polypeptide 5	B9A064	6	6	23
Transthyretin	P02766	6	10	16
Kallistatin	P29622	3	13	49
Complement component C9	P02748	5	16	63
Ig mu chain C region	P01871	8	11	49
Plasma kallikrein	P03952	3	16	71
Insulin-like growth factor-binding protein complex acid labile subunit	P35858	2	16	66
Alpha-2-HS-glycoprotein	C9JV77	2	8	39
Ig heavy chain V-III region BRO	P01766	1	2	13
Coagulation factor XII	P00748	4	10	68
Protein AMBP	P02760	2	12	39

Table I. Continued from previous page.

Name	Uniprot Accession ID	SDS-PAGE (no. peptides)	IEF (no. peptides)	MW (kDa)
Glia-derived nexin	P07093	10	10	44
Complement component C8 beta chain	P07358	1	16	67
Pigment epithelium-derived factor	P36955	10	13	46
Serum paraoxonase/arylesterase 1	P27169	7	11	40
Ig kappa chain V-III region SIE	P01620	4	4	12
Complement component C8 alpha chain	P07357	3	11	65
Ig lambda-2 chain C regions	P0CG05	1	2	11
Complement factor I	P05156	5	14	66
Corticosteroid-binding globulin	P08185	3	7	45
Complement component C8 gamma chain	P07360	7	5	22
Tetranectin	P05452	8	9	23
Lipopolysaccharide-binding protein	P18428	0	5	53
Ig heavy chain V-III region BUT	P01767	1	5	12
Ig delta chain C region	P01880	1	10	42
Thyroxine-binding globulin	P05543	3	9	46
Serum amyloid A-4 protein	P35542	4	2	15
Apolipoprotein E	P02649	9	8	36
Ig lambda chain V-III region LOI	P80748	2	2	12
Plasma serine protease inhibitor	P05154	3	8	46
C4b-binding protein alpha chain	P04003	0	13	67
N-acetylmuramoyl-L-alanine amidase	Q96PD5	0	7	62
Complement factor H-related protein 1	Q03591	2	4	38
Versican core protein	P13611	0	11	373
Ig heavy chain V-III region JON	P01780	0	2	13
Ig heavy chain V-III region TUR	P01779	1	3	12

Table I. Continued from previous page.

Name	Uniprot Accession ID	SDS-PAGE (no. peptides)	IEF (no. peptides)	MW (kDa)
Complement C2	P06681	4	5	83
Ig alpha-2 chain C region	P01877	1	4	37
Vitamin K-dependent protein S	P07225	0	9	75
Carboxypeptidase N catalytic chain	P15169	3	5	52
Ig kappa chain V-II region TEW	P01617	1	5	12
Inter-alpha-trypsin inhibitor heavy chain H3	Q06033	0	12	100
Carboxypeptidase N subunit 2	P22792	1	7	61
Ig gamma-4 chain C region	P01861	0	4	36
Pregnancy zone protein	P20742	1	11	164
Monocyte differentiation antigen CD14	P08571	0	5	40
Ig heavy chain V-III region GAL	P01781	1	6	13
Hepatocyte growth factor-like protein	P26927	0	7	80
Apolipoprotein L1	O14791	5	4	44
Glyceraldehyde-3-phosphate dehydrogenase	P04406	5	5	36
Biotinidase	P43251	0	3	61
Apolipoprotein C-I	P02654	4	2	9
Coagulation factor XIII B chain	P05160	0	10	76
Ig kappa chain V-I region WEA	P01610	1	3	12
Leucine-richalpha-2-glycoprotein	P02750	5	5	38
Ig kappa chain V-IV region Len	P01625	3	2	13
Sex hormone-binding globulin	P04278	4	3	44
Prostatic acid phosphatise	P15309	1	6	45
Afamin	P43652	1	8	69
Ig heavy chain V-III region VH26	P01764	1	2	13
Fibulin-1	P23142	0	9	77

Table I. Continued from previous page.

Name	Uniprot Accession ID	SDS-PAGE (no. peptides)	IEF (no. peptides)	MW (kDa)
Protein Z-dependent protease inhibitor	Q9UK55	0	5	51
Serum amyloid P-component	P02743	6	4	25
Coagulation factor IX	P00740	1	7	52
Haptoglobin-related protein	P00739	1	5	39
Ig heavy chain V-III region CAM	P01768	1	6	14
Ig gamma-3 chain C region	P01860	0	4	41
Coagulation factor V	P12259	0	4	252
Lysozyme C	P61626	2	2	17
Coagulation factor X	P00742	1	7	55
Hyaluronan-binding protein 2	Q14520	1	5	63
Alpha-1-acid glycoprotein 1	P02763	5	5	24
Hemoglobin subunit beta	P68871	3	4	16
Ig kappa chain V-I region EU	P01598	2	3	12
Extracellular matrix protein 1	Q16610	0	9	61
Inhibin alpha chain	P05111	0	4	40
Actin, cytoplasmic 1	P60709	3	5	42
Sulfhydryl oxidase 1	O00391	0	7	83
Vitamin K-dependent protein Z	P22891	0	4	45
Glutathione peroxidase 3	P22352	6	4	26
Insulin-like growth factor-binding protein 3	P17936	1	3	32
Attractin	O75882	0	9	159
Galectin-3-binding protein	Q08380	0	6	65
Immunoglobulin J chain	P01591	3	2	18
Vitamin K-dependent protein C	P04070	1	3	52
Signal peptide, CUB and EGF-like domain-containing protein 1	Q8IWY4	0	7	108
Beta-Ala-His dipeptidase	Q96KN2	0	6	57
Neuronal pentraxin-2	P47972	0	3	47
Carboxypeptidase B2	Q96IY4	0	3	48

Table I. Continued from previous page.

Name	Uniprot Accession ID	SDS-PAGE (no. peptides)	IEF (no. peptides)	MW (kDa)
Collagen alpha-3(VI) chain	12111	0	6	344
Ig kappa chain V-III region B6	P01619	0	3	12
Cadherin-5	P33151	0	2	88
Ig heavy chain V-III region GA	P01769	0	2	13
Apolipoprotein C-III	P02656	2	4	11
Lipoprotein lipase	P06858	2	3	53
Complement factor D	P00746	1	5	27
Tumor necrosis factor-inducible gene 6 protein	P98066	1	4	31
Tubulin alpha-1B chain	P68363	0	6	50
Coagulation factor XIII A chain	P00488	0	4	83
Laminin subunit alpha-1	P25391	0	4	337
Ig heavy chain V-III region TRO	P01762	0	2	13
Complement factor H	F8WDX4	0	2	51
Collagen alpha-1(IV) chain	P02462	0	1	161
Uncharacterizedprotein	F8VWA4	1	3	11
Ig heavy chain V-III region HIL	P01771	0	5	14
Dopamine beta-hydroxylase	P09172	0	4	69
Ig heavy chain V-III region BUR	P01773	0	2	13
Mannosyl-oligosaccharide 1,2-alpha-mannosidase IA	P33908	0	1	73
Apolipoprotein D	P05090	2	1	21
Zinc-alpha-2-glycoprotein	P25311	1	5	34
Heat shockprotein HSP 90-beta	P08238	0	6	83
Tubulin beta chain	P07437	0	4	50
Fetuin-B	Q9UGM5	0	3	42
Plakophilin-2	Q99959	0	2	97
Apolipoprotein A-II	P02652	2	5	11
Metalloproteinase inhibitor 1	P01033	1	5	23
Collagen alpha-1(XV) chain	P39059	1	2	142
Titin	Q8WZ42	0	1	3816

Table I. Continued from previous page.

Name	Uniprot Accession ID	SDS-PAGE (no. peptides)	IEF (no. peptides)	MW (kDa)
Transforming growth factor-beta-induced protein ig-h3	Q15582	0	4	75
Lumican	P51884	0	2	38
Dystonin	Q03001	0	1	861
Peptidyl-prolylcis–trans isomerase B	P23284	1	6	24
Complement C1r subcomponent	P00736	1	3	80
Ig kappa chain V-III region VG(fragment)	P04433	1	2	13
Ig kappa chain V-I region DEE	P01597	1	1	12
Hornerin	Q86YZ3	3	0	282
Thrombospondin-4	P35443	0	5	106
Complement C1r subcomponent-like protein	Q9NZP8	0	5	53
Aggrecan	E7EMK9	0	4	246
Tenascin-X	P22105	0	4	464
Transferrin receptor protein 1	P02786	0	3	85
EGF-containing fibulin-like extracellular matrix protein 1	Q12805	0	3	55
Heat shock cognate 71 kDa protein	P11142	0	2	71
E3 ubiquitin-protein ligase HUWE1	Q7Z6Z7	0	2	482
Plasminogen activator inhibitor 1	P05121	1	3	45
Hemoglobin subunit alpha	P69905	3	1	15
14-3-3 protein theta	P27348	2	1	28
Ig kappa chain V-I region Lay	P01605	1	2	12
Retinol-binding protein 4	P02753	4	0	23
Phosphatidylinositol-glycan-specific phospholipase D	P80108	0	4	92
Coagulation factor XI	P03951	0	3	70
Cholinesterase	P06276	0	2	68
Phospholipid transfer protein	P55058	0	2	55

Table I. Continued from previous page.

Name	Uniprot Accession ID	SDS-PAGE (no. peptides)	IEF (no. peptides)	MW (kDa)
Ig lambda chain V-II region TRO	P01707	0	1	12
Retinoic acid receptor responder protein 2	Q99969	1	2	19
Complement C1q subcomponent subunit B	P02746	1	2	27
Ig kappa chain V-I region HK102 (fragment)	P01602	1	1	13
Alpha-1-acid glycoprotein 2	P19652	0	2	24
Glutathione S-transferase A1	P08263	4	0	26
Peptidyl-prolylcis–trans isomerase A	P62937	0	4	18
C-reactive protein	P02741	2	0	25
78 kDa glucose-regulated protein	P11021	0	3	72
Laminin subunit beta-2	P55268	0	3	196
Apolipoprotein B-100	P04114	0	2	516
Multiple inositol polyphosphate phosphatise	Q9UNW1	0	2	55
Collagen alpha-2(IV) chain	P08572	0	1	168
Endoplasmin	P14625	0	1	92
Angiogenin	P03950	3	1	17
Complement C1q subcomponent subunit C	P02747	2	1	26
Serum amyloid A protein	P02735	1	1	14
Ig lambda chain V-I region HA	P01700	1	2	12
Alpha-enolase	P06733	0	3	47
Complement C1s subcomponent	P09871	0	3	77
Cartilage oligomeric matrix protein	P49747	0	3	83
Ig kappa chain V-I region OU	P01606	0	2	12
Coagulation factor VII	P08709	0	2	52
Ig kappa chain V-III region NG9 (fragment)	P01621	0	2	11

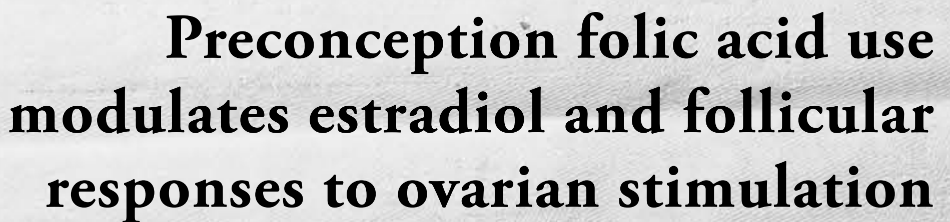
Table I. Continued from previous page.

Name	Uniprot Accession ID	SDS-PAGE (no. peptides)	IEF (no. peptides)	MW (kDa)
Ig kappa chain V-II region RPMI 6410	P06310	0	2	15
Peptidyl-glycine alpha-amidating monooxygenase	P19021	0	2	108
Ectonucleotide pyrophosphatase/ phosphodiesterase family member 2	Q13822	0	1	99
Acyl-CoA synthetase family member 4	Q4L235	0	2	123
Peroxiredoxin-1	Q06830	2	1	22
Protein S100-A8	P05109	1	1	11
Phosphoglycerate kinase 1	P00558	1	2	45
Phosphatidylcholine-sterol acyltransferase	P04180	0	3	50
Complement factor H-related protein 5	Q9BXR6	0	3	64
Hepatocyte growth factor activator	Q04756	0	2	71
von Willebrand factor	P04275	0	2	309
Ig kappa chain V-I region Mev	P01612	0	2	12
Ezrin	P15311	0	2	69
Fattyacid synthase	P49327	0	2	273
Triosephosphate isomerise	P60174	0	2	27
Heat shockprotein HSP 90-alpha	P07900	0	1	85
Sushi repeat-containing protein SRPX	P78539	0	1	52
Plastin-2	P13796	0	1	70
Insulin-like growth factor II	P01344	2	0	20
Ig epsilon chain C region	P01854	0	2	47
Thrombospondin-1	P07996	0	2	129
Retinol-binding protein 1	P09455	0	2	16
L-selectin	P14151	0	2	42
RabGDP dissociation inhibitor beta	P50395	0	2	51

Table I. Continued from previous page.

Name	Uniprot Accession ID	SDS-PAGE (no. peptides)	IEF (no. peptides)	MW (kDa)
Protein kinase C-binding protein NELL2	Q99435	0	2	91
Histone H2A type 1-B/E	P04908	0	2	14
Peroxidasin homolog	Q92626	0	2	165
Protein KIAA0317	O15033	0	1	94
Amyloid beta A4 protein	P05067	0	1	87
Dihydropyrimidinase-related protein 3	Q14195	0	1	62
Desmoglein-2	Q14126	0	1	122
Extracellular sulfatase Sulf-2	Q8IWU5	0	1	100
Ig kappa chain V-I region AU	P01594	0	1	12
Fibrillin-1	P35555	0	1	312
Vasorin	Q6EMK4	0	1	72

Preconception folic acid use modulates estradiol and follicular responses to ovarian stimulation



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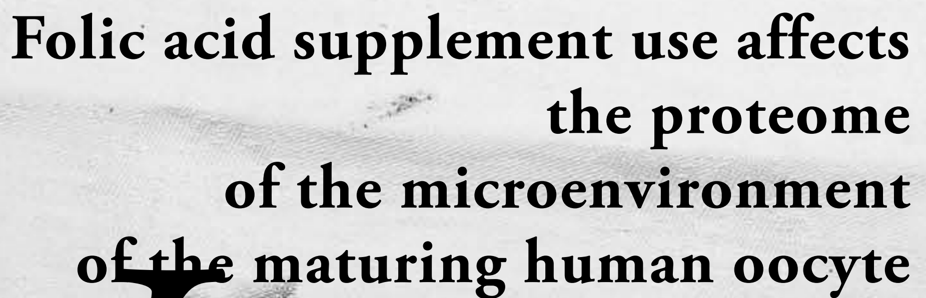

V

Folic acid supplement use affects the proteome of the microenvironment of the maturing human oocyte

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Submitted

ABSTRACT

Background

IVF/ICSI treatment is a moderately successful technique to achieve pregnancy in subfertile couples. Follicle fluid folate, as a substrate of the one-carbon pathway, affects follicle metabolism and oocyte and embryo quality. If we are able to identify new markers for the underlying mechanisms, we might be able to predict oocyte quality and improve IVF/ICSI treatment success rates.

Materials and Methods

In an observational study, we performed qualitative and quantitative proteomic analyses using nanoflow LC-MS/MS and TMT-labelling on 15 monofollicular fluid samples of folic acid supplement users and on 15 samples from nonusers, retrieved from women undergoing IVF/ICSI treatment. The protein data is analyzed using Scaffold proteome software and differential abundances are expressed as Log_2 fold change. Blood samples were obtained before and after treatment for determination of biomarkers of the one-carbon pathway and estradiol. Ovarian follicles were visualised, counted and diameters recorded using transvaginal ultrasound.

Results

We identified 227 unique expressed proteins in follicle fluid. First, C-reactive protein was less abundant in folic acid supplement users (-2.03; p=<0.01). Additionally, apolipoproteins from HDL, most notably A-I (+1.28; p=<0.01) and C-I (+1.11; p=0.016) were more abundant in folic acid supplement users.

Conclusion

Preconception folic acid supplement use seems to suppress the inflammatory pathways in follicle fluid and stimulates the HDL pathway, the preferential source of cholesterol for steroid hormone synthesis, with possible beneficial effects on embryo quality after IVF/ICSI treatment. In vitro studies are needed to further identify the tissue specificity of these pathways through which folate establishes these effects.

INTRODUCTION

In Vitro Fertilization/Intracytoplasmic Sperm Injection (IVF/ICSI) is a moderately successful technique to achieve pregnancy in subfertile couples. The cumulative life birth rate after six IVF/ICSI cycles is approximately 51%.[3] Epidemiologic studies indicate that many factors affect IVF/ICSI treatment success.[131] Therefore, if we can identify biomarkers of underlying pathways that affect oocyte quality, IVF/ICSI treatment may be improved by screening for such markers prior to fertilization and subsequent embryo transfer.

The ovarian follicle is the pre-ovulation microenvironment in which the oocyte matures. Oocyte maturation is a complex process involving many pathways and interactions with the follicular granulosa and theca cells.[132] During follicle development and oocyte maturation, an avascular compartment forms. This compartment is filled with follicle fluid, which is in part an exudate of serum and partially composed of locally produced substances. Follicle fluid contents associate with oocyte quality and provides an overview of metabolic activity of follicular cells and systemic influences on the oocyte microenvironment.[133] Indeed, directly investigating follicular cells could provide a more accurate view of follicle metabolism, but disregards possible harmful systemic influences on the oocyte microenvironment. Follicle fluid is readily available after IVF/ICSI treatment. Therefore, follicle fluid offers the unique potential for the identification of biomarkers for non-invasive screening of oocyte quality.

Many epidemiologic and experimental studies show the influence of the B-vitamin folate on reproductive performance.[11] Apart from associations between maternal folate status and pregnancy outcomes, folate seems to affect several ovarian pathways thereby affecting the number of oocytes retrieved after ovarian stimulation treatment and embryo quality.[44, 46, 134] Folate is a one-carbon donor and affects many biological processes through various pathways of which one-carbon metabolism is of main interest.[130, 135] Through one-carbon metabolism, folate facilitates the synthesis of amino acids, lipids, DNA nucleotides and methylation of various substrates, contributing to genomic integrity and cellular replication.[11] Therefore, especially proliferating tissues, such as the growing ovarian follicle are sensitive to disturbances in one-carbon metabolism.

In this study we use proteomic techniques based on proteolytic peptide fractionation by isoelectric focusing (IEF) and sensitive nanoflow LC-MS/MS in order to further specify the effect of the known influence of folate on oocyte quality and follicle metabolism on a protein level in follicle fluid. This is a first step in identifying biomarkers that affect human oocyte quality after IVF/ICSI treatment.

MATERIALS & METHODS

Study population

The FOod, Lifestyle and Fertility Outcomestudy (FOLFOstudy), designed to study the influence of periconception nutrition and lifestyles on fertility and pregnancy outcome, is described in detail before.[136] In summary, between September 2004 and October 2006, 292 subfertile couples undergoing an IVF/ICSI treatment procedure at the tertiary referral fertility clinic of the Erasmus MC, University Medical Centre in Rotterdam, the Netherlands, were invited to participate. Women diagnosed with endometriosis or hydrosalpinx could not participate, since these conditions detrimentally influence IVF/ICSI treatment outcome. Additionally, couples undergoing oocyte donation could not participate. After the inclusion period, 251 couples participated.

At the start of treatment all couples completed a questionnaire regarding nutrition, lifestyle, medication and disease history. We collected blood samples from all couples on cycle day (CD) 2, before treatment commenced. On the day of hCG administration serum was collected from women only.

From this observational cohort we selected n=30 monofollicular samples on outcome (not pregnant) and folate concentrations in follicle fluid. Therefore, the evaluation of folic acid supplement use was observational, not assigned. Based on Brouwer et al., we selected 15 samples with a follicle fluid folate concentration below 22.5 nmol/L (no folic acid supplement use, nonusers) and 15 samples of women with a folate concentration equal to or above 22.5 nmol/L (folic acid supplement use, users).[137] All monofollicular samples are from the oocyte that yielded the transferred embryo, but did not result in pregnancy.

The Dutch Central Committee for Human Research (CCMO) and the Medical Ethical and Institutional Review Board of the Erasmus MC, University Medical Centre in Rotterdam, the Netherlands, approved the study protocol. Participants provided written informed consent before participation and obtained materials and questionnaires were processed anonymously.

IVF procedure

All women started ovarian stimulation treatment with daily injections of 150 IU recombinant follicle stimulating hormone (rFSH) s.c. on CD2 (Puregon®, MSD Oss, Oss, the Netherlands, or Gonal-F®, Merck-Serono, Schiphol-Rijk, the Netherlands). Administration of daily s.c. GnRH-antagonist (Orgalutran®, MSD Oss, Oss, the Netherlands, or Cetrotide®, Merck-Serono, Schiphol-Rijk, the Netherlands) was started when at least one follicle was 14 mm. To induce final oocyte maturation, a single dose of 5000 or 10 000 IU hCG s.c. (Pregnyl®, MSD Oss, Oss, the Netherlands) was administered as soon as the largest follicle

reached a diameter of 18 mm and at least one additional follicle of 15 mm was observed. Oocyte retrieval was carried out 35 h after hCG injection by transvaginal ultrasound-guided aspiration of follicles. Luteal phase supplementation of 600 mg/day micronized progesterone intravaginally was started on the evening following oocyte pick-up and continued for 12 days thereafter. On day 3 after oocyte pick-up, a maximum of two embryos were transferred.

Sample collection and analysis

During oocyte retrieval, monofollicular fluid from the leading follicle of each ovary was aspirated and collected separately. These were then centrifuged for 10 min at 1,700 rpm to separate red blood cells (RBC), leucocytes and follicular cells. The samples were frozen without preservatives and stored at -20°C. Venous blood samples were drawn from each woman on CD2 before the first injection of rFSH and at the day of hCG administration. For determination of folate, cobalamin and hormones, venous blood samples were drawn into dry vacutainer tubes and allowed to clot. After centrifugation at 2,000 x g, serum was collected before being assayed. Serum and monofollicular fluid samples from each patient were analyzed using an immunoelectrochemoluminescence assay (Roche Modular E170, Roche Diagnostics GmbH, Mannheim, Germany). Total protein concentrations in monofollicular fluid were determined photometrically on a Hitachi 917 (Roche Diagnostics GmbH). Serum concentrations of FSH were measured by luminescence-based immunometric assay (Immulite 2000, Diagnostic Products Corporation (DPC), Los Angeles, CA, USA), and estradiol was determined using coated tube radioimmunoassay from the same supplier. For the determination of plasma total homocysteine (tHcy) and pyridoxine in whole blood, venous blood samples were drawn into EDTA containing vacutainer tubes. The EDTA-blood samples were placed on ice and within 1 hour, plasma was separated by centrifugation. Total homocysteine in EDTA plasma and monofollicular fluid and pyridoxine as pyridoxal-5-phosphate in whole blood was determined using HPLC with reversed phase separation and fluorescence detection. For the determination of RBC folate, 100 ml blood from one EDTA tube was hemolyzed with 2 ml freshly prepared ascorbic acid (0.05 g ascorbic acid in 25 ml AD) directly after blood sampling. Subsequently, the hematocrit of the EDTA blood was determined on a Sysmex XE-2100 (Groffin Meyvis, Etten-Leur, The Netherlands). The hemolysate was centrifuged for 10 min at 2000g shortly before the folate measurement. The folate concentration in the hemolysate was calculated in RBC folate using the following formula: (nmol hemolysate folate X 21) – (nmol/l serum folate X (1 – hematocrit))/hematocrit = nmol/l RBC folate. Inter-assay coefficients of variation for folate were 4.5% at 13

nmol/L and 5.7% at 23 nmol/L; for cobalamin 3.6% at 258 pmol/L and 2.2% at 832 pmol/L; for tHcy 4.8% at 14.6 mmol/L and 3.3% at 34.2 mmol/L; for total protein 1.5% at 55 g/l and 1.3% at 84 g/l; for FSH < 5.8%; and for estradiol, <8.8%. The detection limit for folate was 1.36 nmol/L, for cobalamin 22 pmol/L, for pyridoxine 5 nmol/L; for tHcy 4 mmol/L, for total protein 0.1 g/l, for FSH 0.1 U/L and for estradiol 10 pmol/L.

Statistical analysis

Given the skewed distribution of the variables, measures of location and spread are depicted as Median and Interquartile Range (IQR), respectively.

Monofollicular fluid total protein concentrations vary with follicle maturity.[115, 116] Therefore, concentrations of tHcy and B-vitamins in monofollicular fluid are expressed in concentration per gram of protein.

Comparison of the two groups was done using the Mann-Whitney-U test. Proportions were compared using a chi-square test.

A p-value <0.05 was considered statistically significant. All statistical analyses were done using SPSS 17.0 for Windows software (SPSS Inc., Chicago, IL, USA).

Sample preparation and mass spectrometric analysis

IgGs were removed by depletion on a protein G Sepharose column. Albumin was removed by adding ice cold 95% ethanol to the column eluate, the resulting mixture was incubated at 4°C for 1 hr. Proteins were collected by centrifugation at 16,000 x g for 45 min at 4°C. A 'tube gel' was prepared by mixing 17 µL acrylamide (40%, 29:1) solution, 2.3 ul 10% ammonium persulfate and 1 µL of TEMED with 50 µL of the follicle fluid sample and allowed to polymerize for 1 hr at RT. The 'in-tube' gel block was cut into pieces and further treated according to a standard in-gel digestion protocol.[138] Extracted proteolytic peptides were labelled with TMT 6-plex labelling reagents (Thermo Scientific) allowing for peptide quantitation. Peptides were mixed at the 6-plex level and further fractionated by either reversed phase chromatography combined with elution at high pH, by isoelectric focusing on a 3100 Offgel fractionator (Agilent) or by HILIC chromatography. Each time, 18-22 fractions were collected and further analyzed by nanoflow LC-MS/MS. Nanoflow LC-MS/MS was performed on an 1100 series capillary LC system (Agilent Technologies) coupled to an LTQ-Orbitrap mass spectrometer (Thermo) operating in positive mode and equipped with a nanospray source. Peptide mixtures were trapped on a ReproSil C18 reversed phase column (Dr Maisch GmbH; column dimensions 1.5 cm × 100 µm, packed in-house) at a flow rate of 8 µL/min. Peptide separation was performed on ReproSil C18 reversed phase column (Dr Maisch GmbH; column

dimensions 15 cm × 50 μm, packed in-house) using a linear gradient from 0 to 80% B (A = 0.1 % formic acid; B = 80% (v/v) acetonitrile, 0.1 % formic acid) in 70 min and at a constant flow rate of 200 nL/min using a splitter. The column eluent was directly sprayed into the ESI source of the mass spectrometer. Mass spectra were acquired in continuum mode; fragmentation of the peptides was performed in data-dependent mode. Peak lists were automatically created from raw data files using the Mascot Distiller software (version 2.3; MatrixScience). The Mascot search algorithm (version 2.2, MatrixScience) was used for searching against the IPI database (IPI_human_20100507.fasta). The peptide tolerance was set to 10 ppm and the fragment ion tolerance was set to 0.8 Da. A maximum number of 2 missed cleavages by trypsin were allowed and carbamidomethylated cysteine and oxidized methionine were set as fixed and variable modifications, respectively. The Mascot score cut-off value for a positive protein hit was set to 60. Individual peptide MS/MS spectra with Mascot scores below 40 were checked manually and either interpreted as valid identifications or discarded. Typical contaminants, also present in immunopurifications using beads coated with pre-immune serum or antibodies directed against irrelevant proteins were omitted from the table. Each follicle fluid sample was analyzed at least two-times to reduce measurement error.

Scaffold (version Scaffold_2_06_02, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm.[121] Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least one identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm.[122] Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

RESULTS

Study population

Except for Body Mass Index (BMI), baseline characteristics were comparable between folic acid supplement users and nonusers. The BMI in users is slightly lower (**Table I**)

Biochemical parameters

As a result of our selection criteria, there were differences in biomarker concentration in serum between users and nonusers prior to treatment (**Table II**). Users had higher levels of serum folate, RBC folate, cobalamin and

Table I. Baseline characteristics of women undergoing ovarian stimulation treatment according to follicle fluid folate concentration (n=30).

	Folic acid use (≥22.5 nmol/L) (n=15)	**Non folic acid use (<22.5 nmol/L) (n=15)**	**p**
Age (years), median (IQR)	36.0 (35.0-40.0)	35.0 (32.0-38.0)	ns
Body Mass Index (kg/m^2), median (IQR)	22.0 (20.0-24.0)	23.0 (23.0-25.0)	0.048
Ethnicity, % (n)			0.06
Dutch	57.1 (8)	93.3 (14)	
Non-Dutch European	14.3 (2)	0 (0)	
Non-European	28.6 (4)	6.7 (1)	
Education, % (n)			ns
Low	6.7 (1)	20.0 (3)	
Intermediate	33.3 (5)	26.7 (4)	
High	60.0 (9)	53.3 (8)	
Fertilization procedure, % (n)			ns
IVF	73.3 (11)	86.7 (13)	
ICSI	26.7 (4)	13.3 (2)	
Cause of subfertility, % (n)			ns
Male factor	46.7 (7)	20.0 (3)	
Female factor	13.3 (2)	40.0 (6)	
Combined	0 (0)	6.7 (1)	
Unknown	40.0 (6)	33.3 (5)	
Smoking (yes), % (n)	0 (0)	1 (6.7)	ns
Duration of subfertility (months), median (IQR)	33.0 (14.0-56.0)	28.0 (12.0-50.0)	ns

pyridoxine. Additionally, tHcy levels were lower in these women (**Table II**). Biomarker concentrations after treatment are similar to those before treatment and show a comparable profile, with vitamin levels higher and tHcy levels lower in users (**Table II**).

We observed comparable differences in follicle fluid biomarker levels (**Table II**). Users had higher follicle fluid levels of folate, cobalamin and pyridoxine; tHcy levels were lower in users. Total protein levels were comparable between users and nonusers.

Table II. Biochemical markers in blood serum and follicle fluid of women undergoing ovarian stimulation treatment according to follicle fluid folate concentrations (n=30).

	Folic acid use (≥22.5 nmol/L) (n=15)	Non folic acid use (<22.5 nmol/L) (n=15)	p
Follicles (n)	6 (2-7)	7 (5-8)	ns
Follicle diameter (mm)	20.5 (18.6-22.6)	20.0 (17.3-21.0)	ns
Baseline serum			
FSH (U/L)	8.3 (7.3-11.1)	8.5 (7.1-9.8)	ns
Estradiol (pmol/L)	149.5 (131.8-208.3)	133.0 (111.0-180.0)	ns
Cobalamin (pmol/L)	376.0 (288.0-447.0)	256.0 (210.0-325.0)	0.015
Pyridoxine (nmol/L)	97.0 (77.5-121.5)	66.0 (51.0-75.0)	<0.01
Folate (nmol/L)	36.8 (31.6-40.6)	15.4 (12.7-17.7)	<0.001
RBC folate (nmol/L)	1,618 (1,250-1,831)	937 (718-1,245)	0.02
Homocysteine (µmol/L)	7.6 (6.9-8.1)	11.2 (9.0-14.4)	<0.001
hCG-day serum			
Estradiol (pmol/L)	1871 (1022-2981)	1868 (1381-3697)	ns
Cobalamin (pmol/L)	389.5 (285.8-419.8)	244.0 (185.0-337.0)	<.0.01
Pyridoxine (nmol/L)	102.5 (66.5-129.5)	63.0 (50.0-87.0)	0.012
Folate (nmol/L)	39.9 (33.5-44.0)	15.4 (12.3-21.7)	<0.001
RBC folate (nmol/L)	1,899 (1,290-2,133)	911 (781-1,189)	<0.001
Homocysteine (µmol/L)	7.2 (6.8-8.5)	10.8 (8.8-14.4)	<0.001
Follicle fluid			
Cobalamin / total protein (pmol/g)	4.18 (3.54-5.33)	2.86 (2.34-3.96)	0.02
Pyridoxine / total protein (nmol/g)	2.12 (1.37-3.04)	1.14 (0.69-1.56)	0.02
Folate / total protein (nmol/g)	36.1 (31.8-40.4)	17.0 (11.1-19.6)	<0.001
Homocysteine / total protein (µmol/g)	0.10 (0.085-0.11)	0.15 (0.12-0.21)	<0.001
Total protein (g/L)	55.0 (53.0-61.0)	58.0 (55.0-60.0)	ns

Variables are depicted Median (Interquartile Range)

Follicle diameter and the number of visualized follicles at oocyte pickup were comparable between users and nonusers (**Table II**).

The follicle fluid proteome

The proteomics screen identified 227 unique expressed proteins (**Supplementary Table**). Proteins with at least a $\log_2$ fold change of 0.5 in users relative to nonusers are shown in **Table III**. The inflammatory marker C-reactive protein (CRP) is less abundant in users. Several proteins are more abundant in users such as the apolipoproteins (Apo). The increase in Apo CII, Apo AI, Apo CI and Apo CIII abundance are most profound. Apo-E, however, is less abundant in users (**Supplementary Table**)

Table III. Differentially abundant proteins in follicle fluid of folic acid supplement users relative to non folic acid users (n=30).

Protein	MW (kDa)	IPI identifier	Median Log_2 fold change	IQR	p
Up regulated					
Apolipoprotein C-II	11	IPI00021856	1.28	(1.11-1.45)	ns
Apolipoprotein A-I	31	IPI00021841	1.28	(0.92-1.64)	<0.01
Apolipoprotein C-I	9	IPI00021855	1.11	(0.73-1.49)	0.016
Apolipoprotein C-III	11	IPI00021857	0.73	(0.65-0.81)	ns
Plasma Kallikrein	71	IPI00654888	0.67	(0.52-0.82)	0.085
Interleukin-1 receptor accessory protein	65	IPI00031789	0.65	(0.47-0.82)	ns
Apolipoprotein M	21	IPI00030739	0.62	(0.53-0.70)	ns
Farnesyl pyrophosphate synthetase like-4	40	IPI00382869	0.56	(0.56-0.57)	ns
C4b-binding protein alpha chain	67	IPI00021727	0.53	(0.37-0.68)	ns
Histidine-rich glycoprotein	60	IPI00022371	0.51	(0.36-0.65)	ns
Down regulated					
Low affinity IgG Fc region receptor III-A	29	IPI00218834	-0.50	(-0.93-0.057)	ns
Small proline-rich protein 3	18	IPI00082931	-0.93	(-1.22- -0.65)	ns
C-reactive protein	25	IPI00022389	-2.03	(-2.26- -1.80)	<0.01

DISCUSSION

In this study, we show significant changes in monofollicular fluid protein abundance associated with follicle fluid folate concentrations of women who underwent IVF/ICSI treatment. Women who were folic acid supplement users had lower CRP levels in follicle fluid, an acute phase response protein that serves as a marker of inflammation. Additionally, users have higher Apo levels. Apo's are constituents of cholesterol transport complexes, and cholesterol is the substrate for steroid hormone production.

These results should be considered within the context of the strengths and limitations of this study. The FOLFO study is a prospective periconception observational study specifically designed to address the influence of food and lifestyle factors on IVF/ICSI treatment outcomes. From this cohort we randomly selected follicle fluid samples with knowledge only on folate status and treatment outcome, with only BMI marginally different between the two groups. Oocyte quality between the women can be considered comparable, because all oocytes yielded embryos suitable for transfer. Given the observational nature of the study, we cannot exclude that folic acid supplement use is a proxy for other factors, such as adequate nutrition, high education and a healthy lifestyle. Nevertheless, the major difference between users and nonusers are folate and associated B-vitamin levels, suggesting that the observed effects in the follicle fluid proteome are indeed due to changes in one-carbon metabolism. The follicle fluid proteome is conditional on follicle maturity (i.e. size).[115, 116] We have investigated the association between folate and the follicle fluid proteome in monofollicular fluids from only mature follicles comparable in size using a validated and reproducible technique.[139] This excludes the possibility that differential protein abundances are due to differences in follicle size or the number of (im)mature follicles in a punctuate, but also allows to associate identifications in the follicle fluid to a specific oocyte.[115, 116]

A higher CRP abundance in nonusers suggests a more pronounced inflammatory process. Indeed, other studies are contradicting on the relation between folate and CRP levels.[140, 141] In this study it is unclear whether the elevated CRP levels in follicle fluid of nonusers is due to local or peripheral changes in expression, and what mechanism resulted in CRP elevation. Indeed, BMI is slightly higher in nonusers, which also associates with CRP levels.[142] Elevated estradiol levels, due to ovarian stimulation treatment or oral contraceptive use, also increase CRP levels.[142, 143] Despite that estradiol levels after stimulation treatment were comparable in our sample, given previous evidence for the effect of folate on the ovarian response, the higher CRP abundance in nonusers may also reflect a higher oestrogen exposure during the treatment cycle in these women.[134, 144] Nevertheless, regardless the source of CRP, the elevated concentration is reflected in follicle fluid of nonusers and can therefore exert influence on oocyte and follicle development.

A number of Apo's, most notably AI and CI, are more abundant in follicle fluid of users. Apo's are proteins that associate with triglycerides and cholesterol(-esters) to form lipoproteins, such as High Density Lipoprotein (HDL) and Low Density Lipoprotein (LDL). While Apo-AI is the principal Apo of HDL, all here identified differentially abundant Apo's can associate with HDL.[145] Although other lipoproteins can be detected, studies show that HDL is the principal lipoprotein in follicle fluid and HDL is the preferential source of cholesterol(-esters) for steroid hormone synthesis in the ovary.[146-148] Uptake of cholesterol from HDL in the ovary does not involve internalization and degradation of the lipoprotein particle.[148] Therefore, the source of increased Apo levels in follicle fluid remains elusive. Indeed, granulosa cells synthesize some Apo's.[149] Nevertheless, knockout models for cystathione β-synthase and methylenetetrahydrofolatereductase, crucial enzymes in one-carbon metabolism; show decreased liver Apo-AI production and circulating HDL levels.[150, 151] In human, biomarkers of compromised folate metabolism associate with lower circulating HDL levels.[150, 152] Regardless, animal and human clinical studies highlight the importance of HDL cholesterol metabolism in reproduction. Both hypercholesterolemia and decreasing HDL levels were negatively associated with oocyte and embryo quality.[153, 154] Finally, knockout models for HDL metabolizing enzymes result in a subfertile phenotype.[153]

Two cytokeratins (**Supplementary Table**) appear markedly less abundant in samples from folic acid supplement users. In human, granulosa cells loose cytokeratin expression during maturation, not expressing cytokeratins once they near luteinisation.[155] Thus, lower cytokeratin abundance in follicle fluid from folic acid supplement users would suggest the presence of more mature granulosa cells. This statement is supported by previous research on the effects of folate metabolism on ovarian follicle growth.[134, 144] Nevertheless, given the issues with keratin identification in proteomic studies, i.e. the sequence homology between the different keratins and the high risk of keratin contamination during sample processing, we cannot draw strong conclusions on the differential abundance of cytokeratins between users and nonusers.[156]

Several mechanisms can underlie changes in the proteome of follicle fluid. First, not only folate concentrations are higher in serum and follicle fluid of users, but also the concentrations of two cofactors of one-carbon metabolism, cobalamin and pyridoxine. Also, tHcy is lower in both serum and follicle fluid of users. Resulting inhibition of one-carbon metabolism through one-carbon donor or cofactor shortage or elevated tHcy levels affects many processes.[29] Inhibition of one-carbon metabolism can result in diminished DNA nucleotide, amino acid, phospholipid synthesis and oxidative stress.[11] Accordingly, also epigenetic mechanism, such as DNA methylation, necessary

for normal growth and development are affected, resulting in altered gene expression profiles.[157] Finally, pyridoxine is involved in over 180 enzymatic reactions.[158] Therefore, in vitro experiments are needed to pinpoint the possible independent effects of these cofactors and derivatives of one-carbon metabolism on the follicle fluid proteome.

High resolution, high throughput technologies like mass spectrometry based proteomics of follicle fluid is a useful tool and medium to identify mechanisms through which one-carbon metabolism affects follicle metabolism. One-carbon metabolism is involved in and likely establishes its effects through numerous different pathways. Findings by Bliek et al. support this statement, were folate supplementation of culture media affected both mRNA and protein expression in lymphoblasts.[130, 135] Given this complexity, -omics technologies, such as proteomics, can identify different independent and related processes and provide a view of the net effect of folate on ovarian follicle metabolism. Although we detect different protein abundances in follicle fluid of users and nonusers, we can only speculate on the origin of these differences. Most likely, both changes in follicle metabolism and peripheral tissues, resulting in different serum concentrations underlie our observations. Optimizing techniques to specifically isolate the different subtypes of follicular cells from monofollicular fluid, without culturing, could prove helpful in identifying the origin of the identified changes. Also, even though the oocyte is transcriptionally active, little is known about the contribution of the oocyte to the follicle fluid composition.[159] Regardless, changes in protein abundance conditional on folate levels can provide information on possible mechanisms through which folate affects follicle metabolism and thereby reproductive capacity and outcome.

CONCLUSION

In this study, we identified 227 unique expressed proteins in follicle fluid, some of which are differentially abundant conditional on folic acid supplement use. Most likely, folate establishes its effect on the follicle microenvironment through changes in intra-ovarian pathways and peripheral tissues. Of special interest is the suggestion that follicle fluid folate influences follicle maturation, as suggested by cytokeratin abundance, a hypothesis that requires further testing. Additionally, folate possibly affects follicle metabolism and oocyte maturation through extra-ovarian mechanisms by exerting effects on Apo synthesis in the liver.

In vitro studies using granulosa cells can help further elucidate the mechanism through which one-carbon metabolism affects intra-ovarian metabolism and can address the role of the availability of several cofactors and derivatives as biomarkers of this pathway

Supplementary Table. Relative abundance of proteins in follicle fluid, conditional on folic acid supplement use.

Name	Accession No	MW (kDa)	Folic acid supplement use (Log2 fold change)	p
Hemopexin	IPI00022488	52	-0.2	0.51
Vitamin D-binding protein precursor	IPI00555812	53	-0.1	0.93
Complement component 4B preproprotein	IPI00418163	193	-0.1	0.97
Alpha-2-HS-glycoprotein	IPI00022431	47	0	0.91
Ceruloplasmin	IPI00017601	122	0	0.91
Complement factor B	IPI00019591	141	-0.3	0.87
Isoform 1 of Complement factor H	IPI00029739	139	0	0.97
Antithrombin-III	IPI00032179	53	0.1	0.77
Isoform HMW of Kininogen-1	IPI00032328	72	0.2	0.94
Basement membrane-specific heparan sulfate proteoglycan core protein	IPI00024284	469	-0.3	0.36
Plasminogen	IPI00019580	91	0.2	0.6
Inter-alpha (Globulin) inhibitor H2, isoform CRA-a	IPI00305461	107	0.2	0.53
Beta-2-glycoprotein 1	IPI00298828	38	-0.1	0.82
Complement C5	IPI00032291	188	0	0.99
Prothrombin (Fragment)	IPI00019568	70	0	0.85
Isoform 1 of Inter-alpha-trypsin inhibitor heavy chain H4	IPI00896419	103	-0.1	0.87
Alpha-1-Antichymotrypsin	IPI00550991	51	-0.3	0.42
Isoform 1 of Fibronectin	IPI00022418	263	0.1	0.76
Apolipoprotein A-IV	IPI00304273	45	0	1
Alpha-1B-glycoprotein	IPI00022895	54	0	0.94
Isoform 1 of Gelsolin	IPI00026314	86	0.1	0.89
Afamin	IPI00019943	69	0.1	0.82
Complement component 6 precursor	IPI00879709	106	0	0.92
Inter-alpha-trypsin inhibitor heavy chain H1	IPI00292530	101	0.2	0.67

Supplementary Table. Continued from previous page.

Name	Accession No	MW (kDa)	Folic acid supplement use (Log2 fold change)	p
Angiotensinogen	IPI00032220	53	0	0.95
Apolipoprotein A-I	IPI00021841	31	1.3	0.0011*
Complement C3 (Fragment)	IPI00783987	187	-0.3	0.34
Isoform 1 of Clusterin	IPI00291262	52	-0.1	0.82
Complement component C7	IPI00296608	94	0.3	0.7
Histidine-rich glycoprotein	IPI00022371	60	0.5	0.54
Vitronectin	IPI00298971	54	0	0.96
Complement component C9	IPI00022395	63	-0.3	0.51
Serpin peptidase inhibitor, clade D (Heparin cofactor), member 1	IPI00292950	60	-0.2	0.77
Protein AMBP	IPI00022426	39	0.1	0.83
Complement component C8 alpha chain	IPI00011252	65	-0.1	0.9
55 kDa protein	IPI00029863	55	0.1	0.88
Complement factor I	IPI00291867	66	-0.2	0.78
Isoform 2 of Attractin	IPI00162735	141	0	0.93
Lumican	IPI00020986	38	-0.1	0.89
Apolipoprotein A-II	IPI00021854	11	0.3	0.63
Complement component C8 beta chain	IPI00294395	67	0	0.95
Coagulation factor XII	IPI00019581	68	0	0.91
Isoform 1 of Inter-alpha-trypsin inhibitor heavy chain H3	IPI00028413	100	0.2	0.7
Plasma kallikrein	IPI00654888	71	0.7	0.085
Pigment epithelium-derived factor	IPI00006114	46	-0.1	0.77
Retinol-binding protein 4	IPI00022420	23	0	1
Kallistatin	IPI00328609	49	0	0.99
Isoform 1 of Coagulation factor XI	IPI00008556	70	0.1	0.76
Coagulation factor IX	IPI00296176	52	-0.1	0.87
Plasma protease C1 inhibitor	IPI00291866	55	0.2	0.7
Thyroxine-binding globulin	IPI00292946	46	-0.1	0.9

Supplementary Table. Continued from previous page.

Name	Accession No	MW (kDa)	Folic acid supplement use (Log2 fold change)	p
Insulin-like growth factor-binding protein complex acid labile subunit	IPI00020996	66	0	0.95
Isoform 1 of Glia-derived nexin	IPI00009890	44	-0.3	0.54
Hepatocyte growth factor-like protein	IPI00292218	80	0	0.99
Vitamin K-dependent protein S	IPI00294004	75	0.1	0.89
Complement C1r subcomponent	IPI00296165	82	0.1	0.81
Isoform 1 of Extracellular matrix protein 1	IPI00003351	61	0.1	0.85
Complement component C8 gamma chain	IPI00011261	22	-0.1	0.94
Carboxypeptidase N subunit 2	IPI00479116	61	0.3	0.65
Hyaluronan-binding protein 2	IPI00746623	63	0	0.86
Tetranectin	IPI00009028	23	0.1	0.92
Complement C1s subcomponent	IPI00017696	77	0.1	0.81
Coagulation factor XIII B chain	IPI00007240	76	0.1	0.8
Isoform 1 of Carboxypeptidase B2	IPI00329775	48	0	0.98
Coagulation factor X	IPI00019576	55	0.1	0.93
Complement factor H-related protein 1	IPI00011264	38	-0.4	0.71
Corticosteroid-binding globulin	IPI00027482	45	0.1	0.89
Insulin-like growth factor-binding protein 3	IPI00018305	32	-0.1	0.82
Isoform 1 of N-acetylmuramoyl-L-alanine amidase	IPI00163207	62	0.1	0.97
Leucine-rich alpha-2-glycoprotein	IPI00022417	38	-0.3	0.51
Isoform 1 of Phosphatidylinositol-glycan-specific phospholipase D	IPI00299503	92	0.2	0.64
Protein Z-dependent protease inhibitor	IPI00007199	55	0.1	0.92

Supplementary Table. Continued from previous page.

Name	Accession No	MW (kDa)	Folic acid supplement use (Log2 fold change)	p
Isoform 1 of Fibrinogen alpha chain	IPI00021885	95	-0.1	0.9
Serum amyloid P-component	IPI00022391	25	-0.4	0.37
Isoform 1 of Sex hormone-binding globulin	IPI00023019	44	0.3	0.62
Serum paraoxonase/arylesterase 1	IPI00218732	40	0	0.99
Isoform LMW of Kininogen-1	IPI00215894	48	0	0.95
Complement C1r subcomponent-like protein	IPI00009793	53	Reference Missing	
Fetuin-B	IPI00743766	42	0.1	0.85
Biotinidase	IPI00744685	62	0	0.99
Beta-Ala-His dipeptidase	IPI00064667	57	0.3	0.64
Isoform V0 of Versican core protein	IPI00009802	373	-0.1	0.88
Carboxypeptidase N catalytic chain	IPI00010295	52	0.3	0.78
Plasma serine protease inhibitor	IPI00007221	46	-0.3	0.53
Properdin	IPI00021364	51	0	0.99
Actin, cytoplasmic 1	IPI00021439	42	0.2	0.91
Monocyte differentiation antigen CD14	IPI00029260	40	0	0.94
Zinc-alpha-2-glycoprotein	IPI00166729	34	0.3	0.71
Isoform 2 of Prostatic acid phosphatase	IPI00289983	48	0.3	0.66
Thrombospondin-4	IPI00328550	106	0.3	0.77
Complement factor H-related 5	IPI00006543	67	0.1	0.94
Glutathione S-transferase A1	IPI00657682	26	0.4	0.63
Butyrylcholinesterase, isoform CRA_b	IPI00025864	73	0.1	0.76
Selectin L precursor	IPI00218795	44	-0.1	0.86
Complement C2 (Fragment)	IPI00303963	83	-0.1	0.93
Selenoprotein P	IPI00029061	43	0.1	0.93
C4b-binding protein alpha chain	IPI00021727	67	0.5	0.57

Supplementary Table. Continued from previous page.

Name	Accession No	MW (kDa)	Folic acid supplement use (Log2 fold change)	p
Metalloproteinase inhibitor 1	IPI00032292	23	0	0.99
Isoform Long of Complement factor H-related protein 2	IPI00006154	31	-0.2	0.8
Apolipoprotein E	IPI00021842	36	-0.4	0.56
Small proline-rich protein 3	IPI00082931	18	-0.9	0.2
Insulin-like growth factor-binding protein 7	IPI00016915	29	0	0.96
Complement C1q subcomponent subunit C	IPI00022394	26	0.2	0.83
Isoform 1 of Sulfhydryl oxidase 1	IPI00003590	83	0	0.9
Transforming growth factor-beta-induced protein ig-h3	IPI00018219	75	0.1	0.93
Apolipoprotein C-I	IPI00021855	9	1.1	0.016*
Lipoprotein lipase	IPI00027847	53	-0.5	0.47
Keratin, type II cytoskeletal 1	IPI00220327	66	-2.2	0.054
Lysozyme C	IPI00019038	17	-0.1	0.87
Isoform 1 of Pregnancy zone protein	IPI00025426	164	-0.4	0.51
Neuronal pentraxin-2	IPI00026946	47	-0.3	0.7
Hepatocyte growth factor activator	IPI00029193	71	0	0.97
Tumor necrosis factor-inducible gene 6 protein	IPI00303341	31	-0.1	0.83
Mannosyl-oligosaccharide 1,2-alpha-mannosidase IA	IPI00844511	73	-0.2	0.65
Isoform 1 of C-reactive protein	IPI00022389	25	-2	0.0095*
Isoform 12 of CD44 antigen	IPI00297160	39	-0.1	0.89
Isoform 2 of Calsyntenin-1 (Fragment)	IPI00007257	109	-0.2	0.73
Glutathione peroxidase 3	IPI00026199	26	0.1	0.93
Complement C1q subcomponent subunit B	IPI00477992	27	0.2	0.9
Angiogenin	IPI00008554	17	0	0.99

Supplementary Table. Continued from previous page.

Name	Accession No	MW (kDa)	Folic acid supplement use (Log2 fold change)	p
Secreted protein, acidic, cysteine-rich (Osteonectin), isoform CRA_a	IPI00014572	36	-0.1	0.85
Transthyretin	IPI00022432	16	0.2	0.81
Plastin-2	IPI00010471	70	0	0.98
Complement factor D preproprotein	IPI00165972	28	0	0.95
Cysteine-rich secretory protein 3	IPI00942117	28	Reference Missing	
Isoform 1 of EGF-containing fibulin-like extracellular matrix protein 1	IPI00029658	55	0.3	0.7
Lipopolysaccharide-binding protein	IPI00032311	53	-0.2	0.78
Lymphatic vessel endothelial hyaluronic acid receptor 1	IPI00290856	35	0	0.78
Beta-2-microglobulin	IPI00004656	14	-0.2	0.82
Dopamine beta-hydroxylase	IPI00171678	69	0.5	0.58
Isoform C of Fibulin-1	IPI00296537	74	0.2	0.83
Isoform 1 of Vitamin K-dependent protein Z	IPI00027843	45	0.3	0.83
Isoform 1 of Vascular cell adhesion protein 1	IPI00018136	81	0	0.99
COL6A3 protein	IPI00072917	322	0.1	0.84
Isoform 1 of Cartilage acidic protein 1	IPI00451624	71	0.3	0.61
Inhibin beta A chain	IPI00028670	47	-0.2	0.67
Gamma-glutamyl hydrolase	IPI00023728	36	-0.3	0.79
Retinoic acid receptor responder protein 2	IPI00019176	19	0	0.95
Inhibin alpha chain	IPI00007080	40	0	0.99
Isoform 1 of Ectonucleotide pyrophosphatase/ phosphodiesterase family member 2	IPI00156171	99	0	0.96

Supplementary Table. Continued from previous page.

Name	Accession No	MW (kDa)	Folic acid supplement use (Log2 fold change)	p
Isoform 1 of Follistatin	IPI00021081	38	-0.2	0.82
Insulin-like growth factor-binding protein 2 precursor	IPI00297284	35	0	0.97
Coagulation factor XIII A chain	IPI00297550	83	0.4	0.79
Vitamin K-dependent protein C	IPI00021817	52	0	0.95
Isoform 1 of Insulin-like growth factor II	IPI00001611	20	0	0.98
Cystatin-C	IPI00032293	16	0	0.96
Prostaglandin-H2 D-isomerase	IPI00013179	21	-0.2	0.76
Phosphatidylcholine-sterol acyltransferase	IPI00022331	50	0.2	0.75
Putative uncharacterized protein ALB	IPI00022434	72	0	0.98
Isoform 1 of Peptidase inhibitor 16	IPI00301143	49	0.1	0.93
Isoform 1 of Ficolin-3	IPI00293925	33	0	0.93
Apolipoprotein B-100	IPI00022229	516	0.1	0.83
Isoform 1 of Interleukin-1 receptor accessory protein	IPI00031789	65	0.6	0.28
HSPA5 protein	IPI00003362	72	0.1	0.97
Signal peptide, CUB and EGF-like domain-containing protein 1	IPI00217435	108	-0.3	0.9
Isoform 5 of Peptidyl-glycine alpha-amidating monooxygenase	IPI00177543	108	0	0.99
Out at first protein homolog	IPI00328703	31	0	0.97
Cadherin-5	IPI00012792	88	0	0.97
Isoform alpha-enolase of Alpha-enolase	IPI00465248	47	0.4	0.36
Follistatin-related protein 1	IPI00029723	35	-0.2	0.81
Amphiregulin	IPI00012023	28	0	0.97
Isoform Sap-mu-0 of Proactivator polypeptide	IPI00012503	58	-0.1	0.89

Supplementary Table. Continued from previous page.

Name	Accession No	MW (kDa)	Folic acid supplement use (Log2 fold change)	p
Muscle type neuropilin 1	IPI00165438	72	0.2	0.73
Intercellular adhesion molecule 2	IPI00009477	31	0	0.95
Isoform 1 of Mast/stem cell growth factor receptor	IPI00022296	110	0	0.98
Fructose-bisphosphate aldolase A	IPI00465439	39	0.3	0.66
von Willebrand factor	IPI00023014	309	0	0.97
Insulin-like growth factor-binding protein 5	IPI00029236	31	-0.1	0.96
Aminopeptidase N	IPI00221224	110	0.1	0.93
Di-N-acetylchitobiase	IPI00007778	44	0	0.98
Isoform 1 of Metallothionein-1G	IPI00008752	6	0.3	0.85
Vasorin	IPI00395488	72	0	0.98
Dipeptidyl peptidase 4	IPI00018953	88	0	0.87
Insulin-like growth factor IA	IPI00001610	17	0	0.95
Ribonuclease pancreatic	IPI00014048	18	-0.1	0.95
Isoform 2 of Neural cell adhesion molecule L1-like protein	IPI00299059	137	0.3	0.57
Procollagen C-endopeptidase enhancer 1	IPI00299738	48	0	1
Isoform 1 of Multiple inositol polyphosphate phosphatase 1	IPI00293748	55	0	0.97
Intercellular adhesion molecule 1	IPI00008494	58	0	0.99
Thrombospondin-1	IPI00296099	129	-0.2	0.72
Ribonuclease 4	IPI00029699	17	0	0.98
Metalloproteinase inhibitor 2	IPI00027166	24	0.2	0.49
Lysosome-associated membrane glycoprotein 1	IPI00884105	45	0	0.98
Plasminogen activator inhibitor 1	IPI00007118	45	-0.3	0.94
Isoform XB of Tenascin-X	IPI00025276	464	0.1	0.87
Galectin-3-binding protein	IPI00023673	65	0.2	0.83
Isoform 2 of Apolipoprotein L1	IPI00186903	46	0.3	0.41
L-lactate dehydrogenase B chain	IPI00219217	37	0.1	0.92

Supplementary Table. Continued from previous page.

Name	Accession No	MW (kDa)	Folic acid supplement use (Log2 fold change)	p
Isoform Beta of Poliovirus receptor	IPI00219425	40	0.3	0.46
Apolipoprotein M	IPI00030739	21	0.6	0.44
Apolipoprotein C-III	IPI00021857	11	0.7	0.62
Transforming growth factor beta receptor type 3	IPI00304865	94	0.1	0.94
FLJ00385 protein (Fragment)	IPI00168728	56	0	0.93
Spondin-1	IPI00171473	91	-0.1	0.91
72 kDa type IV collagenase	IPI00027780	74	0.1	0.22
Endoplasmin	IPI00027230	92	0.1	0.93
Collagen alpha-1(I) chain	IPI00297646	139	0	1
Xaa-Pro dipeptidase	IPI00257882	55	-0.1	0.91
Fibrinogen beta chain	IPI00298497	56	0	0.99
Platelet glycoprotein Ib alpha chain	IPI00748955	69	0.1	0.96
Low affinity immunoglobulin gamma Fc region receptor III-A	IPI00218834	29	-0.5	0.83
Neutrophil defensin 1	IPI00005721	10	0	0.99
Keratin, type I cytoskeletal 10	IPI00009865	59	-2.9	0.023*
Apolipoprotein C-II	IPI00021856	11	1.3	0.11
Protein S100-A9	IPI00027462	13	0.1	0.98
Isoform 1 of Probable serine protease HTRA3	IPI00027862	49	0.3	0.076
Insulin-like growth factor-binding protein 6	IPI00029235	25	0	0.93
Profilin-1	IPI00216691	15	0.1	0.83
Collagen alpha-1(VI) chain	IPI00291136	109	0.1	0.95
Nucleobindin-1	IPI00295542	54	0.1	0.81
Peroxiredoxin-2	IPI00027350	22	-0.1	0.87
ADP-ribosyl cyclase 2	IPI00026240	36	0	0.96
Isoform 1 of Noelin	IPI00017841	55	0	0.61
Exostosin-like 2	IPI00002732	37	0.1	0.8

Supplementary Table. Continued from previous page.

Name	Accession No	MW (kDa)	Folic acid supplement use (Log2 fold change)	p
Isoform LAMP-2A of Lysosome-associated membrane glycoprotein 2	IPI00009030	45	0.2	0.64
Isoform A of Decorin	IPI00012119	40	0.3	0.73
Isoform 1 of Cell surface glycoprotein MUC18	IPI00016334	72	0.1	0.94
Isoform Gamma-B of Fibrinogen gamma chain	IPI00021891	52	0	0.98
Cartilage oligomeric matrix protein	IPI00028030	83	0.1	0.94
Cathepsin B	IPI00295741	38	-0.2	0.88
Neutrophil gelatinase-associated lipocalin	IPI00299547	23	-0.3	0.92
Leukocyte immunoglobulin-like receptor subfamily A member 3	IPI00329104	47	-0.3	0.8
Farnesyl pyrophosphate synthetase like-4 protein (Fragment)	IPI00382869	40	0.6	0.87
Cathepsin D	IPI00011229	45	-0.2	0.53
Cornifin-A	IPI00017987	10	-0.5	0.92
Isoform 1 of Collectin-11	IPI00031490	29	0.1	0.96
Insulin-like growth factor-binding protein 1	IPI00031086	28	-0.1	0.94

V

Preconception folic acid use modulates estradiol and follicular responses to ovarian stimulation

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ABSTRACT

Background

Folate is a methyl donor. Availability of folate affects DNA methylation profiles, and thereby gene expression profiles. Here, we investigate the effects of low dose folic acid use (0.4 mg/day) on the ovarian response to mild and conventional ovarian stimulation in women.

Methods

In a randomized trial among subfertile women, 24 and 26 women received conventional- and mild ovarian stimulation, respectively. Blood samples were taken during the early follicular phase of the cycle preceding the treatment cycle and on the day of hCG administration for determination of serum homocysteine, -AMH, -estradiol and -folate. Folic acid supplement use is validated by questionnaire and serum folate levels. Preovulatory follicles were visualized, counted and diameters recorded using transvaginal ultrasound. The relation between folic acid supplement use and ovarian response is assessed using linear regression analysis.

Results

Folic acid supplement use modified the ovarian response to ovarian stimulation. The estradiol response was higher in nonfolic acid users receiving conventional treatment ($\beta^{interaction}$=0.52, [0.07-0.97]; p=0.03), this effect was independent of serum AMH levels and preovulatory follicle count. In the conventional treatment the mean follicle number was also greater in non-users compared to the users group (14 vs. 9; p=0.03)

Conclusion

Low dose folic acid supplement use attenuates follicular and endocrine responses to conventional stimulation, independent of AMH and follicle count. The nature of the observation suggests that the effects of folic acid are most prominent during early follicle development, affecting immature follicles. Deleterious effects of folate deficiency, like hypomethylation of DNA and oxidative stress can help explain the described observations.

INTRODUCTION

In women undergoing controlled ovarian hyperstimulation (COH) during assisted reproductive treatment (ART) there is unpredictable inter- and intraindividual variability in the ovarian response to COH.[7] Ovarian response is defined as 'the endocrine and follicular reaction of the ovaries to a stimulus'.[7] There is an optimum quantitative ovarian response to COH, where both a low and high ovarian response to COH is associated with an unfavourable treatment outcome.[4] Knowledge of which determinants influence the ovarian response to gonadotropins would improve predictions of the response to COH. This would reduce the number of treatment cycles required to achieve pregnancy, reduce the incidence of treatment complications or offer the possibility to tailor the treatment protocol according to patient characteristics. Accurately predicting the ovarian response to gonadotropins is currently not possible.

Folate deficient women undergoing COH have a lower oocyte quality, lower pregnancy rates and impaired ovarian function.[40, 41, 44, 46, 160] The underlying mechanism, however, is not known. The natural B-vitamin folate is involved in numerous metabolic pathways including cell cycle regulation, amino acid biosynthesis and protein processing.[130] Folate primarily serves as a methylgroup donor for these reactions. In a recent study in ewes fed a methyl deficient diet, low methylgroup availability increased mRNA expression of genes involved in mediating the ovarian response to gonadotropins.[144]

Epigenetic mechanisms aim to maintain the gene expression profile of cells after mitotic division. Epigenetic mechanisms are essentially post-replication modifications superimposed on the genome, regulating gene expression without causing changes in DNA sequence. In contrast to the genetic background of the individual, the epigenetic composition of the genome is sensitive to environmental influences, including nutrition, which effectively modify gene expression.[161, 162] DNA methylation is an epigenetic mechanism, dependent on the availability of methyl groups. In experimental animal and retrospective human studies, in utero exposure to low levels of methyl group donors leads to an altered DNA methylation profile and phenotype in offspring.[62, 163]

Due to the nature of complex pathways in which folate is involved, especially highly proliferating cells, including those in developing ovarian follicles, are affected by folate deficiencies. Therefore, we hypothesized that the ovarian response to gonadotropins is affected by the availability of methyldonors, like folate. In a randomized clinical trial, comparing a mild- and conventional ovarian stimulation protocol, we sought to study the effect of low dose folic acid supplement use on specific biomarkers of the folate dependent methionine pathway and estradiol concentrations following conventional- and mild ovarian stimulation treatment.

MATERIALS AND METHODS

Study design

The FOod Lifestyle and Fertility Outcome (FOLFO) study was designed to investigate the influence of periconception nutrition and lifestyle factors on biochemical, clinical fertility and pregnancy outcome parameters following IVF/ICSI treatment. The FOLFO study comprises FOLFO I (an observational study) and FOLFO II (a randomized controlled trial). The FOLFO II study is designed to compare mild ovarian stimulation treatment with conventional ovarian stimulation treatment with regard to maternal biochemical, endocrine and clinical parameters.

Eligible couples visiting the Erasmus MC, University Medical Centre in Rotterdam, The Netherlands, with an indication for IVF treatment were invited to participate in the FOLFO II study. Exclusion criteria for the FOLFO II study were: oocyte donation, endometriosis, hydrosalpinx, a priori indication for ICSI treatment, age >37 years old, BMI <18 or >29 kg/m^2, irregular menstrual cycle, previous IVF treatment without embryo transfer, recurrent abortion, abnormal karyotype of man/woman and/or uterus anomalies. These criteria served to select only those patients with unexplained subfertility in order to be able to assess the role of food and lifestyle factors. A higher cancellation rate before oocyte retrieval and fewer embryos were expected following mild ovarian stimulation.[164] Therefore, randomization to one of two treatment groups was performed according to a computer generated randomization schedule in a ratio of 2:3 (conventional group : mild group), assigned via numbered sealed envelopes. After the patient agreed to participate, the treating physician opened the next available numbered envelope on entry into the study during the preparatory IVF consultation. Of all eligible couples (n=161), 54 participated in the FOLFO II study and 49 participated in other clinical studies. Fifty-eight couples did not participate in any of the studies because the extra effort for participation relative to normal treatment was considered too great or they did not see a clear benefit in participating. At the end of the inclusion period, 24 couples were randomized to the conventional protocol and 30 couples to the mild protocol. After randomization 4 women with exclusion criteria appeared to be included into the study, they were therefore excluded from the final analysis.

When the allocated treatments commenced all couples completed a questionnaire regarding nutrition, lifestyle, medication and disease history. Blood samples were collected for all couples on cycle day (CD) 2, before treatment commenced. On the day of hCG administration serum was collected from women only.

The study protocol was approved by the Central Committee for Human Research (CCMO) in The Hague, The Netherlands and the Medical Ethical Committee (MEC) and Institutional Review Board of the Erasmus MC, University Medical Centre in Rotterdam, The Netherlands. All participants gave their written informed consent and all materials and questionnaires were anonymously processed.

IVF procedure

After randomization, patients assigned to conventional ovarian stimulation treatment were treated with the GnRH agonist Triptorelin (Decapeptyl®, Ferring BV, Hoofddorp, The Netherlands) at 0.1 mg/day s.c., starting on CD21 of the menstrual cycle preceding the actual stimulation cycle. After two weeks of the GnRH-agonist regimen, co-treatment with rFSH 225 IU/day s.c. (Puregon®, Schering-Plough, Oss, The Netherlands) was initiated. Patients assigned to mild ovarian stimulation treatment were treated with a fixed dose of 150 IU/day rFSH s.c. (Puregon®, Schering-Plough, Oss, The Netherlands) from CD5 onwards. As soon as the leading follicle reached a diameter of 14mm, a GnRH-antagonist (Orgalutran®, Schering-Plough, Oss, The Netherlands) was administered at 0.25 mg/day s.c.. To induce final oocyte maturation a single s.c. dose of 10,000 IE hCG (Pregnyl®, Schering-Plough, Oss, The Netherlands) was administered in both regimens as soon as the leading follicle reached a diameter of at least 18 mm and at least one additional follicle reached a diameter of 15 mm or more. Oocytes were retrieved 36 hours after hCG injection by transvaginal ultrasound-guided aspiration of follicles.

Sample collection and analysis

Isolated oocytes were washed and transferred to a separate droplet of medium in order to monitor their quality. The monofollicular fluid samples were centrifuged for 10 min at 1,700 rpm to separate red blood cells (RBC), leucocytes and granulosa cells. The samples were frozen without preservatives and stored at -20°C until assayed.

Venous blood samples were drawn from each woman on CD2, i.e. the early follicular phase of the menstrual cycle preceding the treatment cycle and on the day of hCG administration. For the determination of folate, cobalamin, pyridoxine and hormones, venous blood samples were drawn into dry vacutainer tubes and allowed to clot. After centrifugation at 2,000 x g, serum was collected for assay. Anti-Müllerian Hormone (AMH) levels were measured using an enzyme-linked immunosorbent assay (ELISA) (Immunotech-Coulter, Marseille, France). Folate and cobalamin were analysed using an immunoelectrochemoluminescence

assay (Roche Modular E170, Roche Diagnostics GmbH, Mannheim, Germany). Serum concentrations of FSH were measured by luminescence-based immunometric assay (Immulite 2000, Siemens Diagnostics, Los Angeles, CA, USA). Estradiol was determined using a coated tube radioimmunoassay obtained from the same supplier. For the determination of plasma total homocysteine (tHcy) and pyridoxine in whole blood, venous blood samples were drawn into ethylenediamine tetra-acetate (EDTA) containing vacutainer tubes. The EDTA-blood samples were placed on ice and within 1 hour, plasma was separated by centrifugation. Total homocysteine in EDTA plasma and pyridoxine as pyridoxal-5-phosphate in whole blood was determined using high-performance liquid chromatography with reversed phase separation and fluorescence detection.

Inter-assay coefficients of variation for folate were 4.5% at 13 nmol/L and 5.7% at 23 nmol/L; for cobalamin 3.6% at 258 pmol/L and 2.2% at 832 pmol/L; for tHcy 4.8% at 14.6 mmol/L and 3.3% at 34.2 mmol/L; for AMH this coefficient was <10%; for FSH < 5.8%; and for estradiol, <8.8%. The detection limit for folate was 1.36 nmol/L, for cobalamin 22 pmol/L, for pyridoxine 5 nmol/L; for tHcy 4 mmol/L, for AMH 0.1 mg/L, for FSH 0.1 U/L and for estradiol 10 pmol/L.

Statistical analysis

Prior to statistical analyses, all continuous variables were log-transformed, obtaining a near normal distribution of data suitable for parametric statistical testing. Normality was assessed using histograms and Q-Q plots. Measures of location and spread are depicted as Geometric Mean and Interquartile Range (IQR), respectively. When a suitable distribution was not attained after transformation, the variable is presented as median (range).

Comparison of the two treatment groups and subsequent sub-groups was done using an unpaired t-test or Mann-Whitney-U test, were appropriate. Proportions were compared using a chi-square test. The influence of COH on the biochemical levels after COH was determined using paired t-tests.

To allow for adjustment, linear regression methods were used to investigate the relation between baseline biomarkers and endocrine responses after COH. Regression parameters are reported with their 95% confidence intervals. Collinearity was assessed using the VIF statistic, a VIF of ≥4 was considered to indicate collinearity. In the final model the highest observed VIF was 3.4. The presented coefficients from the log-log linear regression model should be interpreted as follows: β is % change in Y for a 1% change in X.

Serum folate levels were used to confirm folic acid supplement use. Patients were considered folic acid supplement users when serum folate level was ≥22.5 umol/L.[137]

At the moment of study initiation there was no literature on a possible effect size, therefore a sample size estimate was not done. A p-value <0.05 was considered statistically significant. All statistical analyses were done using SPSS 15.0 for Windows software (SPSS Inc., Chicago, IL, USA).

RESULTS

Study population

At baseline, there were no significant differences with regard to patient characteristics between treatment groups (**Table I**). In the conventional group, self-reported folic acid use was 66.7%, in the mild group this was 80.8%. Self reported use is confirmed in CD2 serum levels in 62.5% and 76.9%, respectively.

Table I. Baseline characteristics of women undergoing ovarian stimulation treatment (n=50).

	Conventional (n=24)	Mild (n=26)
Age (years) mean (IQR)	32.7 (31.0-35.7)	34.0 (33.0-36.0)
Body Mass Index (kg/m^2) mean (IQR)	21.8 (19.5-23.5)	22.7 (21.0-24.0)
Ethnicity, % (n)		
Dutch	57.1 (12)	66.7 (16)
Non-Dutch European	9.5 (2)	16.7 (4)
Non-European	33.3 (7)	16.7 (4)
Education, % (n)		
Low	22.7 (5)	11.5 (3)
Intermediate	31.8 (7)	38.5 (10)
High	45.5 (10)	50.0 (13)
Fertilization procedure, % (n)		
IVF	91.7 (22)	87.5 (21)
ICSI	8.3 (2)	12.5 (3)
Folic acid containing supplement (yes), % (n)	66.7 (14)	80.8 (21)
Smoking (yes), % (n)	13.4 (3)	3.8 (1)
Duration of subfertility (months), median (range)	41 (16-101)	42 (3-135)

Biochemical parameters

At baseline, there were no significant differences with regard to biochemical parameters between treatment groups (**Table II**).

Baseline AMH levels predict the ovarian response to COH (β^{AMH} = 0.43 [0.14-0.72]; p<0.01). On the day of hCG administration treatment groups showed considerable differences with respect to estradiol concentrations, with the response in the conventional protocol being the highest (**Table II**). Also, within each treatment group we observed a decline of tHcy levels relative to basal concentrations, where the median decline was more profound in the conventional stimulation treatment arm than that observed in the mild stimulation treatment arm (-1.20 umol/L vs. -0.7 umol/L; p<0.01 and p=0.01, respectively). This observation was further analysed using linear regression analysis, which showed a differential tHcy decline between the stimulation protocols ($\beta^{protocol}$ = 0.15 [0.06-0.24]; p<0.01). Furthermore, there was a differential decline in AMH levels after stimulation treatment, which was higher after conventional stimulation treatment ($\beta^{protocol}$ = 0.57 [0.34-0.79]; p<0.001).

After stratification for folic acid supplement use and stimulation treatment, women in the conventional stimulation protocol, who did not use folic acid supplements, had an increased ovarian response to COH (**Table III**). To confirm effect modification, this finding was further investigated in a linear regression model, which showed an interaction between baseline serum folate levels and stimulation protocol with regard to the estradiol response to COH ($\beta^{interaction}$=0.52 [0.07-0.97]; p=0.03). The effect of folate was independent of preovulatory follicle count and AMH levels and baseline concentrations of pyridoxine and cobalamin did not alter the effects of folate on the outcomes.

Clinical outcome parameters after IVF/ICSI treatment

As presented in **Tables III** and **IV**, the mean number of preovulatory follicles and the median number of retrieved oocytes differed between treatment groups. The number of follicles was positively correlated with the estradiol response (r=0.78; p<0.001). Furthermore, in the nonusers stratum the conventional group had a higher number of preovulatory large antral follicles, an effect that was not observed in the low dose users stratum (**Table III**). Fertilization rates were comparable, and the number of transferred embryos did not differ (**Table IV**). The number of ongoing pregnancies was not different (**Table IV**).

DISCUSSION

The results of our study suggest that the ovarian response to gonadotropins is subject to the availability of the methyl donor folate. After conventional

Table II. Biochemical markers of women undergoing ovarian stimulation treatment (n=50).

	Conventional (n=24)	Mild (n=26)	p
Follicles	11 (8-16)	9 (7-9)	<0.01
Baseline serum			
FSH (U/L)	7.7 (6.5-8.8)	8.0 (6.8-9.7)	ns
Estradiol (pmol/L)	141.6 (103.5-203.6)	160.1 (119.2-199.0)	ns
AMH (ug/L)	4.4 (2.9-6.1)	5.6 (3.3-10.1)	ns
Cobalamin (pmol/L)	351.0 (259.5-480.0)	309.3 (256.8-387.0)	ns
Pyridoxine (nmol/L)	81.5 (66.0-91.0)	83.2 (61.0-108.0)	ns
Folate (nmol/L)	27.3 (17.9-37.4)	37.1 (19.1-72.5)	ns
Homocysteine (umol/L)	9.5 (8.1-11.2)	9.5 (7.7-10.7)	ns
hCG-day serum			
Estradiol (pmol/L)	4,293 (2,889-6,646)	2,706 (1,716-3,558)	0.03
AMH (ug/L)	1.8 (1.4-3.0)	3.5 (2.4-5.3)	0.01
Cobalamin (pmol/L)	333.4 (262.7-481.5)	300.0 (221.7-381.8)	ns
Pyridoxine (nmol/L)	76.9 (65.0-84.0)	82.1 (56.0-106.0)	ns
Folate (nmol/L)	31.5 (18.2-59.4)	35.7 (19.1-65.4)	ns
Homocysteine (umol/L)	7.9 (6.7-9.8)	8.9 (7.0-10.7)	ns

Variables are depicted: Geometric Mean (Interquartile Range)

stimulation treatment, women who did not use a low dose folic acid supplement had a higher ovarian response to stimulation treatment than those who did use folic acid supplements. This effect is independent of follicle count and AMH levels. Kanakkaparambil et al.[144] have previously observed the interplay between COH and low methyl group availability in ewes fed a methyl deficient (MD) diet. They reported a higher ovarian response after rFSH administration in MD ewes. Further in vitro analysis of granulosa cells revealed higher FSH receptor (*FSHR*) mRNA expression as homocysteine levels increased, reflecting low methyl group availability.

The ovarian response to mild stimulation treatment seems not affected by folic acid use. Incidentally, ovarian dynamics differ considerably between conventional- and mild ovarian stimulation treatment. After pituitary desensitizing using a GnRH-agonist, as is done in the conventional stimulation

Table III. Biochemical markers of women undergoing ovarian stimulation treatment by folic acid use and protocol (n=50).

	Folic acid use		**p**	**Nonfolic acid use**		**p**
	Conventional (n=15)	**Mild (n=20)**		**Conventional (n=9)**	**Mild (n=6)**	
Follicles (n)	9 (6-11)	8 (6-10)	ns	14 (9-20)	7 (5-10)	0.004
Baseline serum						
FSH (U/L)	7.1 (6.2-8.9)	8.0 (6.7-9.9)	ns	8.8 (7.0-8.8)	8.1 (7.2-9.0)	ns
Estradiol (pmol/L)	147.7 (103.5-272.4)	154.1 (122.7-201.0)	ns	131.4 (92.2-181.3)	181.7 (106.0-266.0)	ns
AMH (ug/L)	3.9 (2.6-5.5)	5.6 (2.9-10.1)	ns	5.4 (3.1-10.3)	5.4 (3.3-7.7)	ns
Cobalamin (pmol/L)	382.5 (289.0-515.0)	336.4 (280.7-373.0)	ns	304.0 (238.3-381.4)	233.7 (144.0-397.2)	ns
Pyridoxine (nmol/L)	89.7 (65.7-110.2)	88.8 (63.0-114.8)	ns	70.2 (58.5-80.5)	68.3 (51.5-81.6)	ns
Folate (nmol/L)	38.5 (31.1-43.5)	51.0 (33.6-76.7)	0.08	15.4 (12.8-18.8)	13.5 (12.1-15.0)	ns
Homocysteine (umol/L)	8.6 (6.9-10.4)	8.5 (7.5-9.5)	ns	11.1 (8.5-14.3)	13.7 (9.9-19.0)	0.08
hCG-day serum						
Estradiol (pmol/L)	3,482 (2,347-5,148)	2,978 (2,008-5,115)	ns	6,190 (4,110-10,409)	1,996 (1,613-2,552)	<0.001
AMH (ug/L)	1.5 (1.0-2.5)	3.5 (2.0-5.7)	0.001	2.4 (1.5-3.5)	3.7 (2.9-5.0)	ns
Cobalamin (pmol/L)	364.6 (274.0-527.0)	318.5 (262.0-369.0)	ns	287.2 (216.8-357.9)	247.2 (155.0-403.0)	ns
Pyridoxine (nmol/L)	82.1 (68.0-88.8)	87.9 (70.0-106.5)	ns	69.4 (58.4-80.5)	68.3 (51.5-81.6)	ns
Folate (nmol/L)	45.0 (27.2-70.6)	47.5 (33.3-69.1)	ns	17.4 (14.5-20.8)	13.5 (12.1-15.0)	ns
Homocysteine (umol/L)	7.1 (5.7-8.4)	7.9 (6.3-9.1)	ns	9.3 (7.8-11.2)	13.7 (9.9-19.0)	ns

Variables are depicted: Geometric Mean (Interquartile Range)

Table IV. Clinical outcome parameters after ovarian stimulation treatment (n=50).

	Conventional (n=24)	Mild (n=26)	p
Retrieved oocytes (n) median (range)	12 (1-28)	7 (0-17)	0.001
Fertilization rate	56.2	49.0	ns
Positive pregnancy test, % (n)	37.5 (9)	19.2 (5)	ns
Ongoing pregnancy, % (n)	30.4 (7)	16.0 (4)	ns
Embryo transfer, % (n)			ns
None	8.3 (2)	24.0 (6)	
Single	83.3 (20)	60.0 (15)	
Double	8.3 (2)	16.0 (4)	

treatment arm, the proportion of *immature*, FSH-responsive follicles has increased in size.[165] In addition to the higher doses of rFSH, this can partially underlie the overall higher ovarian response after conventional stimulation treatment. The mild stimulation protocol does not interfere with the initial follicle recruitment by the natural menstrual cycle, and the lower dose of rFSH only stimulates more mature follicles for which the FSH-threshold is higher.[166] Nevertheless, despite a higher oocyte yield, clinical outcome was comparable after conventional and mild stimulation treatment.[167] This suggests that the absolute number of competent oocytes is not different, but only the proportion of competent oocytes is higher after mild stimulation treatment. It seems that preconception folic acid use attenuates the ovarian response; by affecting only less mature follicles that are stimulated after GnRH-agonist treatment.

Clinical, animal and in vitro studies permit speculation on potential mechanisms underlying the observed effect. The intertwined folate-methionine cycle is the main route of utilization for folate. In the folate cycle, folate primarily serves as a substrate for DNA nucleotide synthesis, where deficiencies in folate can result in faulty DNA-repair and nucleotide synthesis.[27, 28] In the methionine cycle, folate serves as a substrate for the remethylation of homocysteine into methionine. Thereafter, methionine-adenosyltransferase metabolizes methionine into S-Adenosylmethionine (SAM), which is the substrate for virtually all methylation reactions. Deficiencies in folate can result in accumulation of homocysteine and S-Adenosylhomocysteine.[168] Deficiencies in cofactors for these reactions, like cobalamin and pyridoxine, can also aggravate derailment of the methionine-cycle. In our study, however, cobalamin and pyridoxine levels did not affect the results. Homocysteine is a reactive metabolite. Homocysteine metabolites can be wrongfully incorporated

in protein instead of methionine, affecting protein function and thereby possibly cellular function.[29] Also, homocysteine can generate reactive oxygen species (ROS).[169] Although ROS function as second messengers, an excess of ROS results in oxidative stress. Oxidative stress affects female fertility and ART success.[31] Also, folate availability affects the DNA methylation pattern and thereby the gene expression profile of a cell.[168] The reduction in SAM levels and accumulation of homocysteine due to folate deficiency inhibit the activity of DNA-methyltransferases.[170] Investigation of the mouse genome indicates that the *FSHR* gene contains CpG repeats, which when methylated inhibit *FSHR* gene transcription.[171] In addition, Kanakkaparambil et al.[144] showed that incubating granulosa cells with homocysteine increases *FSHR* mRNA levels. Similarly, the aromatase enzyme, which converts androgens into estrogens, expressed in bovine granulosa cells is regulated by DNA methylation.[172] Finally, steroidogenesis by the ovarian follicle is augmented by the Insulin-like Growth Factor (IGF) family, Insulin-like Growth Factors mainly elicit their effect through the type 1 IGF receptor, which is overexpressed in folate deficient cells.[173, 174] In proliferating tissues, shortages of methyl groups can inhibit the full methylation of hemi-methylated DNA strands by DNA methyltransferases, eventually leading to hypomethylation of DNA in progeny cells of originally methylated gene-loci.[161] Such a process of passive demethylation is also seen in the early zygote.[175] In addition, oxidative DNA products inhibit effective (re) methylation of DNA or induce loss of methylation.[176-178]

Folate status is associated with the quality of many parameters in human reproduction, from gamete- and embryo quality to the birth prevalence of congenital malformations.[160] In the current study, folate also attenuates the ovarian response to ovarian stimulation treatment. Studies in human show that there is an optimal ovarian response with regard to the number of retrieved oocytes and attained estradiol levels after stimulation treatment with respect to clinical outcome after ovarian stimulation treatment.[167, 179] High hormone levels affect oocyte competency and endometrial receptivity to the embryo.[5, 180] Possibly, folate affects ovarian stimulation treatment outcome by mediating the ovarian response to gonadotropins through interference with *FSHR*, aromatase availability and an increase in ROS.

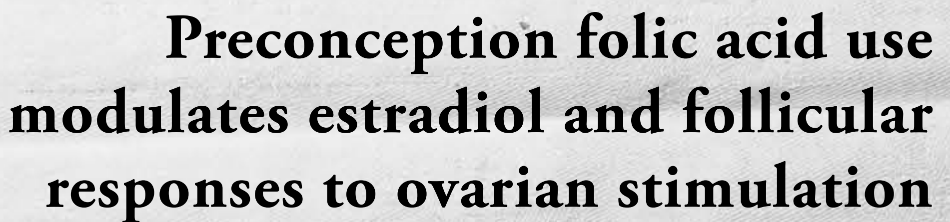
We also need to address strengths and limitations of our study. The sample size permits for less control for confounding and reduces precision. At the moment of study initiation, the effect estimates were unknown and therefore a sample size estimate was not possible. Nevertheless, the statistical analysis showed no complications common to small numbers and our outcome is supported by earlier observations.[144] Although, not primarily designed as a folic acid intervention study, folic acid supplement use across

treatment groups was similar. Nevertheless, we cannot exclude selective non-participation, however, because of the randomized design; confounding factors associated with folic acid supplement use will have an equal distribution over the two treatment groups.

CONCLUSION

Our results show that the ovarian response to ovarian stimulation treatment is amongst others subject to the availability of folate. The effect of folate is independent of AMH and preovulatory follicle count. The nature of the observation suggests that the effect of folate is most prominent during early follicle development, affecting less mature follicles. Our finding may offer a partial explanation for both the observed inter- and intraindividual variability in ovarian response to COH. Possibly, folate affects ovarian stimulation treatment outcome by mediating the ovarian response to gonadotropins through interference with *FSHR* and aromatase availability and an increase in ROS. However, folate availability might also influence the ovarian response through homocysteine metabolites, which might alter protein as well as cellular function.

Of interest for future studies is to investigate the proteome and DNA methylation patterns in human and animal granulosa cells and the deleterious effect of ROS on these entities.



Preconception folic acid use modulates estradiol and follicular responses to ovarian stimulation

VI

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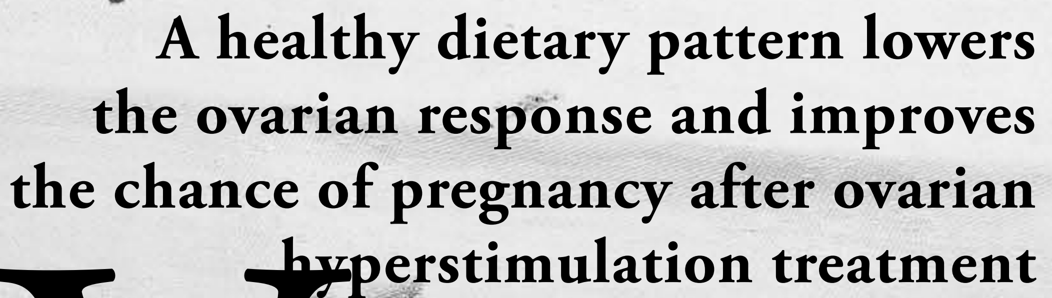

A healthy dietary pattern lowers the ovarian response and improves the chance of pregnancy after ovarian hyperstimulation treatment

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Submitted

ABSTRACT

Background

The ovarian response to controlled ovarian hyperstimulation treatment (COH) associates with assisted reproduction treatment outcome and studies show that nutrition is of influence on assisted reproduction treatment outcome. In this study we investigate the influence of nutrition on the ovarian response to COH and reproductive outcomes after IVF/ICSI treatment.

Materials and Methods

In a prospective periconception cohort of 203 women undergoing COH we assessed dietary intake using a Food Frequency Questionnaire and performed principal component analysis to identify dietary patterns. The pattern of interest was stratified into tertiles of adherence. Blood samples taken at cycle day 2 preceding the treatment cycle and on the day of ovulation induction are used for determination of serum biomarkers. The relation between dietary patterns and outcomes of interest was assessed using regression analysis.

Results

A Healthy dietary pattern, characterized by high intake of fruits, whole grains and margarine and low intake of refined grains and snacks associates with the estradiol response (-0.16; $p<0.05$). Adjusted for possible confounders, a one-unit increase in adherence to the Healthy dietary pattern lowered the estradiol response to COH by 14.4%. Similarly, strong adherence to the Healthy dietary pattern increased the chance of pregnancy (OR_{adj}: 1.61 [1.07-2.44]; $p=0.02$)

Conclusion

Strong adherence to the Healthy dietary pattern beneficially lowers the estradiol response to COH and increases the chance of ongoing pregnancy after IVF/ICSI treatment. This data supports the importance of using a preconception healthy diet and confirms the attenuation of the ovarian response by folic acid supplement use.

INTRODUCTION

In Vitro Fertilization/Intracytoplasmic Sperm Injection (IVF/ICSI) is a moderately successful technique to achieve pregnancy in subfertile couples, with a cumulative life birth rate after six IVF/ICSI treatment cycles just over 50%.[3] The propensity for IVF/ICSI treatment success is in part determined by the ovarian response to Controlled Ovarian Hyperstimulation (COH), i.e. the number of growing ovarian follicles and the quantity of steroid hormone produced. If one can identify modifiable factors, which affect the ovarian response to COH, one can use these factors to optimize IVF/ICSI treatment outcomes.

Demographic, ultrasound and endocrine markers predict the ovarian response to COH.[7] In an effort to decide on the optimal treatment strategy, age, BMI, antral follicle count, basal Follicle Stimulating Hormone (FSH) and Anti-Müllerian Hormone (AMH) concentrations are used to predict the ovarian response to COH.[7] Except for overweight status, these factors comprise non-modifiable factors with little variability between treatment cycles and are therefore not suitable to modify the ovarian response to COH.[6, 8] Modifiable factors, such as nutrition, can help control and modify the ovarian response to COH through improving nutrition.

Improved embryo quality and a more adequate nutritional environment for the developing embryo will obviously in part support the beneficial effect of nutrition on IVF/ICSI treatment outcomes. Previous studies show that B-vitamin status associates with embryo quality and the ovarian response to COH, and that the preconception diet associates with the chance of pregnancy after IVF/ICSI treatment.[44, 134, 181] Nevertheless the mechanism through which nutrition supports these IVF/ICSI treatment outcomes remains unqualified.

Both COH itself and supraphysiologic estradiol levels, such as those seen during COH cycles have a detrimental effect on endometrium development.[5, 182] Endometrial development to the 'receptive' stage is distorted when subjected to high estradiol levels and the composition of endometrial gland secretions, which facilitate early embryo development, is altered after a COH cycle.[5, 183-185] Thus, during traditional IVF/ICSI treatment cycles, the embryo is often transferred to a relatively unreceptive endometrium. Attaining an appropriate ovarian response can thus improve pregnancy chances after IVF/ICSI treatment through better synchronization of embryo and endometrium development.[5, 186]

MATERIALS AND METHODS

Study Design

The FOod, Lifestyle and Fertility Outcome study was designed to investigate the relationship between nutrition, lifestyle and IVF/ICSI treatment outcome.

This prospective periconception study has been described in detail before.[136] In short, between September 2004 and January 2007 subfertile couples undergoing IVF/ICSI treatment at the Erasmus MC, University Medical Centre, Rotterdam, The Netherlands, were invited to participate.

After inclusion and before treatment commenced participants received a self-administered questionnaire addressing age, educational level, medical history, BMI, ethnicity, medication use, smoking, and use of folic acid and other vitamin supplements. In addition, all participants completed a Food Frequency Questionnaire (FFQ) to estimate food intake covering the previous 4 weeks. This FFQ was developed by the division of Human Nutrition, Wageningen University, The Netherlands and validated for intake of energy, B-vitamins, and fatty acids.[187, 188] The FFQ was provided on the day of oocyte retrieval or semen sample collection and returned on the day of embryo transfer.

The Dutch Central Committee for Human Research and the medical ethical and institutional review board of the Erasmus MC University Medical Centre in Rotterdam approved the study protocol. Participants provided written informed consent and the obtained materials and questionnaires were processed anonymously.

IVF/ICSI Procedure

Patients were assigned one of two stimulation treatments, conventional and mild. Patients undergoing conventional ovarian stimulation treatment were treated with the GnRH agonist Triptorelin (Decapeptyl®, Ferring BV, Hoofddorp, The Netherlands) at 0.1 mg/day s.c., starting on Cycle Day (CD) 21 of the menstrual cycle preceding the actual stimulation cycle. After two weeks of the GnRH-agonist regimen, co-treatment with rFSH 225 IU/day s.c. (Puregon®, MSD Haarlem, Haarlem, The Netherlands) was initiated. Patients receiving mild ovarian stimulation treatment were treated with a fixed dose of 150 IU/day rFSH s.c. (Puregon®, MSD Haarlem, Haarlem, The Netherlands) from CD5 onwards. As soon as the leading follicle reached a diameter of 14mm, a GnRH-antagonist (Orgalutran®, MSD Haarlem, Haarlem, The Netherlands) was administered at 0.25 mg/day s.c. To induce final oocyte maturation a single s.c. dose of 10,000 IE hCG (Pregnyl®, MSD Haarlem, Haarlem, The Netherlands) was administered in both regimens as soon as the leading follicle reached a diameter of at least 18 mm and at least one additional follicle reached a diameter of 15 mm or more. Oocytes were retrieved 36 hours after hCG injection by transvaginal ultrasound-guided aspiration of follicles.

Sample collection and analysis

Isolated oocytes were washed and transferred to a separate droplet of medium in order to monitor their quality. The monofollicular fluid samples were centrifuged for 10 min at 1,700 rpm to separate red blood cells (RBC), leucocytes and granulosa cells. The samples were frozen without preservatives and stored at -20°C until assayed.

Venous blood samples were drawn from each woman on CD2, i.e. the early follicular phase of the menstrual cycle preceding the treatment cycle and on the day of hCG administration. For the determination of folate, cobalamin, pyridoxine and hormones, venous blood samples were drawn into dry vacutainer tubes and allowed to clot. After centrifugation at 2,000 x g, serum was collected for assay. AMH concentrations were measured using an enzyme-linked immunosorbent assay (Immunotech-Coulter, Marseille, France). Folate and cobalamin were analysed using an immunoelectrochemoluminescence assay (Roche Modular E170, Roche Diagnostics GmbH, Mannheim, Germany). Serum concentrations of FSH were measured by luminescence-based immunometric assay (Immulite 2000, Siemens Diagnostics, Los Angeles, CA, USA). Estradiol was determined using a coated tube radioimmunoassay obtained from the same supplier. For the determination of plasma total homocysteine (tHcy) and pyridoxine as pyridoxal-5-phosphate in whole blood, venous blood samples were drawn into ethylenediamine tetra-acetate (EDTA) containing vacutainer tubes. The EDTA-blood samples were placed on ice and within 1 hour, plasma was separated by centrifugation. Total homocysteine in EDTA plasma, pyridoxine in whole blood was determined using high-performance liquid chromatography with reversed phase separation and fluorescence detection. For the determination of RBC folate, 100ul blood from one EDTA tube was hemolyzed with 2 ml freshly prepared ascorbic acid (0.05 g ascorbic acid in 25 ml aqua dest) directly after blood sampling. Subsequently, the hematocrit of the EDTA blood was determined on a Sysmex XE-2100 (Goffin Meyvis, Etten-Leur, The Netherlands). The hemolysate was centrifuged for 10 min at 2000 x g shortly before the folate measurement. The folate concentration in the hemolysate was calculated in RBC folate using the following formula: (nmol hemolysate folate X 21) – (nmol/l serum folate X (1 – hematocrit))/ hematocrit = nmol/l RBC folate. Inter-assay coefficients of variation for folate were 4.5% at 13 nmol/L and 5.7% at 23 nmol/L; for cobalamin 3.6% at 258 pmol/L and 2.2% at 832 pmol/L; for tHcy 4.8% at 14.6 mmol/L and 3.3% at 34.2 mmol/L; for AMH this coefficient was <10%; for FSH < 5.8%; and for estradiol, <8.8%. The detection limit for folate was 1.36 nmol/L,

for cobalamin 22 pmol/L, for pyridoxine 5 nmol/L; for tHcy 4 mmol/L, for AMH 0.1 mg/L, for FSH 0.1 U/L and for estradiol 10 pmol/L.

Dietary pattern analysis

All 195 food items from the FFQ data of all participants were classified into 22 food groups and adjusted for total energy intake.[189] This was followed by Principal Components Analysis (PCA) applied on intake of the energy-adjusted food groups of women to construct overall dietary patterns by explaining the largest proportion of variation in the food group. Based on the scree plot and the proportion explained (eigen-value >1.25), the first three factors were considered for dietary pattern analysis (**Supplementary Table**). Each woman was assigned personal scores for the respective factors (i.e. dietary pattern), calculated as the product of the food group value and its factor loadings summed across foods. According to their personal score the group of women was divided into tertiles and classified as low, intermediate, or high adherence to the respective dietary pattern. The strength of adherence indicates the resemblance of the woman's diet compared with the respective dietary pattern identified by PCA.

Prior to analyses, all continuous variables were log-transformed, obtaining a near normal distribution of data suitable for parametric statistical testing. Normality was assessed using histograms and Q-Q plots. Measures of location and spread are depicted as Geometric Mean and Interquartile Range, respectively.

Comparison of the three tertiles of adherence was done using ANOVA. Proportions were compared using the Chi-square statistic, correlations using Pearson's correlation. To allow for adjustment for covariates, linear and logistic regression methods were used to investigate the relation between dietary patterns, patient characteristics and the ovarian response to COH and to address the influence hereof on the chance of pregnancy. For the effect of dietary patterns on the ovarian response to COH we considered baseline AMH, estradiol, FSH and serum and RBC folate concentrations, no. of follicles, stimulation protocol and age and BMI of the woman as covariates. For the effect of diet on ongoing pregnancy chance we considered number of transferred embryo's, ovarian response, age of the woman, BMI of the woman, treatment indication, fertilization rate, woman and partner smoking status, ethnicity and education. We applied a backward analysis, with the inclusion of higher order variables. During the backward process a variable was included into the analysis if its p-value <0.10 and inclusion changed the effect estimate of the variable of interest >10%.[190] For ease of interpretation, linear regression was done using log-transformed dependent variables and non-transformed independent variables, i.e. a log-level linear regression. The interpretation of the β is thus such that βx100 equals the % change in the

dependent variable for a 1 unit change in β. Relevant regression parameters are reported with their 95% confidence intervals. Collinearity was assessed using the VIF statistic, a VIF of ≥4 was considered to indicate collinearity. In the final model the highest observed VIF was 2.0. Statistical analysis was performed using SPSS 17.0 for Windows software (SPSS Inc, Chicago, IL).

RESULTS

Study population and dietary patterns

The three identified dietary patterns (**Supplementary Table**) were explored for associations with the ovarian response. The first two dietary patterns did not significantly associate with the outcomes of interest. The third dietary pattern, however, associated with the ovarian response and was therefore further analysed. This diet is characterized by high intake of fruits, whole grains, margarine, and low intake of refined grains and snacks, it explained 8.9% of variance and is named the Healthy dietary pattern. The Healthy dietary pattern associated with the estradiol response after stimulation treatment (r=-0.16; p=0.02). In addition, adherence to the Healthy dietary pattern associated with baseline RBC and serum folate concentrations (0.27; p=0.001 and 0.15; p=0.03), baseline estradiol (-0.15; p=0.03) and BMI of the woman (-0.15; p<0.05). To compare women over the range of scores of adherence to the Healthy dietary pattern (-2.5-4.6), we divided women into three equally sized groups of adherence, i.e. low (-2.5- -0.39), medium (-0.38-0.3) and high (0.31-4.58). **Table I** depicts baseline characteristics of women undergoing COH. In the high adherence group, a larger proportion of women reported use of a folic acid containing (multi)vitamin supplement, excluding women who did not use a vitamin supplement from the forthcoming analysis did not alter the results. Other baseline characteristics show a comparable distribution over the tertiles of adherence (**Table I**).

Biochemical parameters

Table II shows the biochemical markers in blood serum measured prior to COH on CD2 and after stimulation treatment on the day of hCG administration. First, although not significant, baseline estradiol and AMH concentrations are lower in the tertile of highest adherence (**Table II**). In addition, serum concentrations of pyridoxine and RBC folate concentrations are higher in the highest tertile (**Table II**). After COH, the differences are comparable with pyridoxine and RBC folate concentrations higher in the highest tertile of adherence. In addition, AMH and estradiol concentrations are lowest in the highest tertile (**Table II**). When data were adjusted for stimulation protocol,

Table I. Baseline characteristics of women undergoing controlled ovarian hyper-stimulation, stratified for adherence to the Healthy dietary pattern (n=203).

	Low (n=68)	Medium (n=68)	High (n=67)	p
Age (years)	34.3 (31.0-38.0)	33.9 (31.3-37.0)	35.1 (33.0-38.0)	ns
Body Mass Index (kg/m^2)	23.7 (21.0-26.3)	22.9 (20.0-25.0)	22.6 (20.0-25.0)	ns
Ethnicity, % (n)				ns
Dutch	65.6 (42)	72.7 (48)	72.1 (44)	
Non-Dutch European	9.4 (6)	10.6 (7)	11.5 (7)	
Non-European	25.0 (16)	16.7 (11)	16.4 (11)	
Education, % (n)				ns
Low	13.4 (9)	20.6 (14)	13.4 (9)	
Intermediate	38.8 (26)	41.2 (28)	41.8 (28)	
High	47.8 (32)	38.2 (26)	44.8 (30)	
Cause of subfertility, % (n)				ns
Male	30.9 (21)	35.3 (24)	46.3 (31)	
Female	29.4 (20)	22.1 (15)	14.9 (10)	
Combined	5.9 (4)	5.9 (4)	3.0 (2)	
Unknown	33.8 (23)	36.8 (25)	35.8 (24)	
Multivitamin supplement use (yes), % (n)	80.9 (55)	83.8 (57)	94.0 (63)	0.024
Stimulation protocol, % (n)				0.49
GnRH-agonist	7.4 (5)	13.2 (9)	9.0 (6)	
GnRH-antagonist	92.6 (63)	86.8 (59)	91.0 (61)	
Smoking (yes), % (n)	7.3 (5)	13.2 (9)	7.6 (5)	ns
Duration of subfertility (months)	33.1 (24.0-50.0)	36.3 (27.3-51.0)	35.2 (25.3-51.0)	ns

Variables are depicted: Geometric Mean (Interquartile Range)

age, BMI, number of visualized follicles and baseline AMH concentrations, a non-linear association between adherence to the Healthy dietary pattern and hCG-day estradiol concentrations (r-model=0.73; $\beta_{adherence}$=-0.092 [-0.16 - -0.023]; p<0.01 and $\beta_{adherence, squared}$ =-0.052 [-0.088 - -0.016]; p<0.01) could be established. In other words, a one-point increase in adherence to the Healthy dietary pattern lowers hCG-day estradiol concentrations by 14.4%.

Table II: Biochemical markers of women undergoing controlled ovarian hyperstimulation, stratified for adherence to the Healthy dietary pattern (n=203).

	Low (n=68)	Medium (n=68)	High (n=67)	p
Follicles (n)	7 (5-11)	8 (5-12)	7 (5-11)	ns
Baseline serum				
FSH (U/L)	8.5 (6.9-10.8)	8.1 (6.4-9.7)	8.3 (6.9-10.1)	ns
Estradiol (pmol/L)	143.6 (109.5-185.8)	146.1 (115.0-185.8)	134.0 (108.0-167.0)	ns
AMH (ug/L)	4.2 (2.7-7.4)	4.7 (2.5-7.9)	3.6 (2.2-7.6)	ns
Cobalamin (pmol/L)	329.0 (243.0-401.0)	326.9 (245.0-454.0)	323.6 (256.3-407.3)	ns
Pyridoxine (nmol/L)	80.9 (62.0-101.0)	71.9 (60.0-88.3)	86.8 (64.0-112.0)	0.014
RBC folate (nmol/L)	1,245 (830-1,681)	1,256 (922-1,860)	1,493 (1,070-2,033)	0.057
Folate (nmol/L)	30.6 (20.5-42.2)	27.9 (16.4-39.8)	33.9 (25.1-40.6)	ns
Homocysteine (umol/L)	9.0 (7.8-10.2)	10.0 (7.9-11.2)	9.5 (7.8-10.9)	ns
hCG-day serum				
Estradiol (pmol/L)	2,582 (1,574-3,924)	2,580 (1,695-4,292)	2,240 (1,409-3,419)	ns
AMH (ug/L)	2.5 (1.9-4.2)	2.6 (1.7-5.1)	1.8 (1.0-3.9)	0.06
Cobalamin (pmol/L)	314.3 (235.0-407.0)	320.1 (241.8-439.3)	305.7 (232.8-390.5)	ns
Pyridoxine (nmol/L)	77.7 (61.5-97.0)	73.2 (57.0-89.5)	84.2 (65.8-107.3)	ns
RBC folate (nmol/L)	1,322 (997-1,937)	1,242 (975-1,730)	1,503 (1,125-1,931)	0.045
Folate (nmol/L)	31.8 (20.2-48.7)	31.3 (20.7-41.8)	38.2 (25.9-64.4)	ns
Homocysteine (umol/L)	8.3 (7.0-9.6)	9.1 (7.4-10.4)	8.5 (6.9-9.7)	ns

Variables are depicted: Geometric Mean (Interquartile Range)

Clinical outcome parameters after IVF/ICSI treatment

Table III shows the clinical outcome parameters after IVF/ICSI treatment. There are no statistically significant differences between the groups. Nevertheless, despite a lower fertilization rate in the highest tertile (**Table III**) the proportion of women with a positive pregnancy test and ongoing pregnancy was considerably higher in the highest group of adherence (**Table III**). The number of embryos transferred was similar between groups. When adjusted for the number of transferred embryo's, age of the woman, BMI of the woman, treatment indication, fertilization rate and woman smoking status we show a significant association between adherence to the Healthy dietary pattern and the chance of ongoing pregnancy (OR_{adj}: 1.61 [1.07-2.44]; p=0.02).

DISCUSSION

The results of this study shows that strong adherence to a Healthy dietary pattern characterized by high intake of fruit, whole grains and margarine and low intake of refined grains and snacks is associated with a beneficial reduction in the ovarian response to COH and an increase in the chance of achieving an ongoing pregnancy after IVF/ICSI treatment.

The results from this study should be considered within the context of its strengths and limitations. The FOLFO study is a prospective periconception observational study specifically designed to address the influence of nutrition and lifestyle factors on IVF/ICSI treatment outcomes. Generating dietary patterns using PCA from FFQ data results in sensible food-item based

Table III. Clinical outcome parameters after controlled ovarian hyperstimulation, stratified for adherence to the Healthy dietary pattern (n=203).

	Low (n=68)	Medium (n=68)	High (n=67)	p
Retrieved oocytes (n), median (IQR)	7 (4-10)	7 (5-12)	6 (4-9)	ns
Fertilization rate	65	51	50	ns
Positive pregnancy test, % (n)	23.5 (16)	25.0 (17)	35.8 (24)	ns
Ongoing pregnancy, % (n)	17.9 (12)	19.1 (13)	26.9 (18)	ns
Embryo transfer, % (n)				ns
None	11.9 (8)	9.7 (6)	9.1 (6)	
Single	71.6 (48)	79.0 (49)	74.2 (49)	
Double	16.4 (11)	11.3 (7)	16.7 (11)	

outcomes that are easily translated into patient care. However, in interpreting these components, it is important to consider the tendency of human to over represent socially desirable food habits and generally underestimate their overall food intake. Also, food habits are culturally determined, sometimes requiring an adapted FFQ for different ethnicities.[191] In this study excluding non-western ethnicities did not alter the result. Even though PCA tends to emphasize sample specific factors; the high accordance between the various studies in the generated dietary patterns that are considered healthy and their associations with treatment outcome is a testament to the external validity of this method.[181, 192]

Controlling the ovarian response to COH using modifiable factors such as nutrition can help improve the chance of pregnancy after COH by improving endometrial receptivity and reducing the risk of complications. Various epidemiological studies investigated the association between post stimulation estradiol concentrations and did indeed not always show adverse effects of high estradiol on implantation rates or pregnancy after IVF/ICSI treatment.[179, 193-195] Nevertheless, the effect of a high average number of transferred embryos (≥3) in these studies is likely to dilute or distort any effects of the ovarian response on these clinical outcomes. In vitro studies and studies focussing on endometrial gene expression patterns show that high estradiol concentrations are associated with endometrial gene expression profiles which are considered not beneficial for implantation.[5, 183] Furthermore, a more prolonged follow-up suggests that a high ovarian response increases the risk for trophoblast related pregnancy complications, which suggests an effect of high estradiol concentrations on the endometrium.[196] Finally, cryopreserving successfully fertilized oocytes and delaying embryo transfer until the next menstrual cycle doubled the chance of ongoing pregnancy after IVF/ICSI treatment.[186]

In our study, the chance of pregnancy was highest in women with strong adherence to the Healthy dietary pattern. Previous studies by Vujkovic et al. shows that nutrition beneficially affects the chance of pregnancy after IVF/ICSI treatment, the underlying mechanism through which these endpoints were reached are unclear.[181] The beneficial effects of reduced oxidative stress and folic acid supplement use on embryo and placental quality could lead to such outcomes, possibly without affecting the ovarian response.[44, 65, 75, 197] This suggestion is supported by the finding that nutrition also affects the chance of pregnancy in couples not undergoing IVF/ICSI treatment.[192] Improved pregnancy rates and a lower ovarian response can pose two separate effects of nutrition. Further studies need to address whether the association between nutrition, the ovarian response and improved pregnancy rates have a common causal pathway and if so whether there is a cumulative effect of

nutrition on IVF/ICSI treatment outcomes by affecting both embryo quality and endometrial receptivity through the ovarian response.

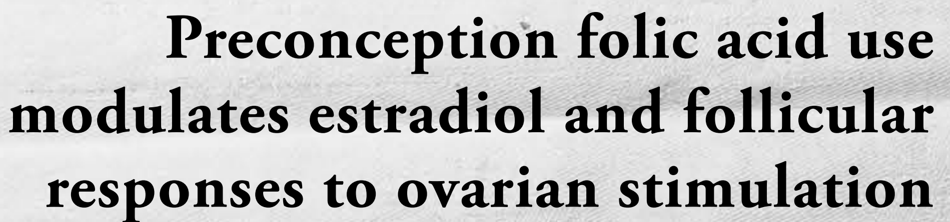
Earlier studies allow for speculation on possible mechanisms through which nutrition affects the ovarian response. Previously we showed that folic acid supplement use lowered the ovarian response to COH as did a diet high in fruit and whole grains in the current study.[134] Additionally, Kanakkaparambil et al. similarly showed an increased ovarian response in methyl-deficient ewes.[144] Possibly, the effects of the Healthy dietary pattern are even mediated by elevated folate concentrations. The anti-oxidant capability of folate and the high anti-oxidant content of fruits propose a mechanism through which both nutrition and folate can independently affect the ovarian response through a similar mechanism, reducing oxidative stress. In the study of Kanakkaparambil et al., increasing homocysteine concentrations, acting as an oxidant and used as an indicator of a low methyl group status, associated with increasing FSH Receptor (*FSHR*) expression levels.[144] Increased *FSHR* expression will facilitate an increased follicular steroid production and overall ovarian response to gonadotropin stimulation. An in vitro study showed that anti-oxidant treatment of follicular cells had similar anti-apoptotic properties as did *FSHR* stimulation.[49] Indeed, *FSHR* stimulation mediated a cellular response to oxidative stress by stimulating synthesis of the endogenous anti-oxidant glutathione.[47] Possibly, up regulation of *FSHR* is an attempt to re-equilibrate the redox balance through production of glutathione. An inherent (side-)effect is the increased perceived stimulus for growth and steroid production upon FSH stimulation.

CONCLUSION

We show that adherence to the Healthy dietary pattern lowers the ovarian response to COH, while increasing the chance of ongoing pregnancy after IVF/ICSI treatment. These findings support the rational and necessity for the use of a healthy preconception diet. It is unclear whether the lower ovarian response, improved embryo quality through improved nutrition or both is causal for the higher pregnancy rates or poses two separate effects of nutrition. The anti-oxidant effects of high fruit consumption may explain the effects on both the ovarian response, improved pregnancy rates and is in accordance with our previous studies.

Supplementary Table. Food group loadings for eligible dietary patterns.

	First dietary pattern (Variance explained: 10.3%)	Second dietary pattern (Variance explained: 9.5%)	Third (Healthy) dietary pattern (Variance explained: 8.9%)
Alcohol	.039	.009	-.009
Cereals	.198	.559	.026
Butter	.013	.666	.130
Dairy	-.033	-.136	.098
Eggs	-.081	.453	.139
Fish	.633	-.012	.125
Fruits	.357	.044	.622
Legumes	.526	.090	.161
Margarine	-.089	-.684	.293
Mayonnaise	-.021	-.186	-.130
Meat	-.517	-.211	-.008
Non-alcohol	-.056	-.029	.019
Nuts	.078	.079	.148
Refined grains	.393	-.092	-.579
Potato	-.020	.060	-.111
Sauces	.079	-.038	-.075
Snacks	-.096	-.231	-.448
Soup	.057	-.095	.031
Sugars	-.190	.259	-.096
Vegetable oils	.138	.208	-.189
Vegetables	.683	.073	.096
Whole grains	.042	-.235	.705



Preconception folic acid use modulates estradiol and follicular responses to ovarian stimulation

VII

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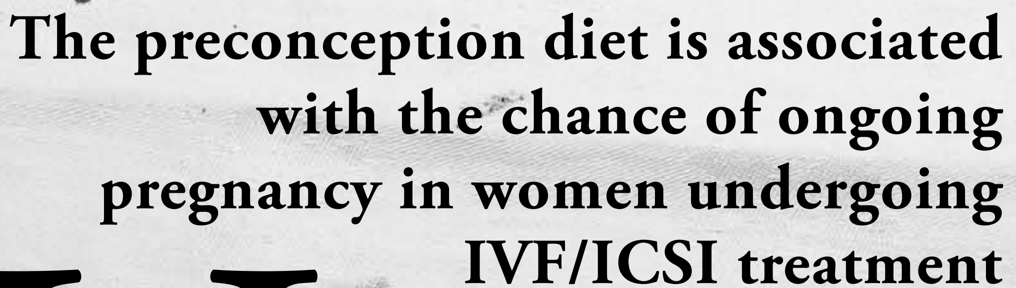

V

The preconception diet is associated with the chance of ongoing pregnancy in women undergoing IVF/ICSI treatment

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ABSTRACT

Background

Subfertility and poor nutrition are increasing problems in Western countries. Moreover, nutrition affects fertility in both women and men. In this study, we investigate the association between adherence to general dietary recommendations in couples undergoing IVF/ICSI treatment and the chance of ongoing pregnancy.

Methods

Between October 2007 and October 2010, couples planning pregnancy visiting the outpatient clinic of the department of Obstetrics and Gynaecology of the Erasmus Medical Centre in Rotterdam, the Netherlands, were offered preconception counselling. Self-administered questionnaires on general characteristics and diet were completed and checked during the visit. Six questions, based on dietary recommendations of the Netherlands Nutrition Centre, covered the intake of six main food groups (fruits, vegetables, meat, fish, whole-wheat products and fats). Using the questionnaire results, we calculated the Preconception Dietary Risk score (PDR-score), providing an estimate of nutritional habits. Dietary quality increases with an increasing PDR-score. We define ongoing pregnancy as an intrauterine pregnancy with positive heart action confirmed by ultrasound. For this analysis we selected all couples (n=199) who underwent their first IVF/ICSI treatment within six months after preconception counselling. We applied adjusted logistic regression analysis on the outcomes of interest using SPSS.

Results

After adjustment for age of the woman, PDR-score of the partner, BMI and smoking of the couple, we show an association between the PDR-score of the woman and the chance of ongoing pregnancy after IVF/ICSI treatment (OR_{adj}: 1.65 [1.08-2.52]; p=0.02). Thus, a one-point increase in the PDR-score associates with a 65% increased chance of ongoing pregnancy.

Conclusions

Our results show that increasing adherence to Dutch dietary recommendations in women undergoing IVF/ICSI treatment increases the chance of ongoing pregnancy. This data warrants further confirmation in couples achieving a spontaneous pregnancy and in randomized controlled trials.

INTRODUCTION

The preconception diet is often inadequate in women planning pregnancy.[198] Nutrition and lifestyle factors, comprising diet, exercise, stress, alcohol- and drug use, smoking, and obesity affect reproductive performance, also after assisted reproduction.[199] Despite the available knowledge on the relation between poor nutrition and lifestyles and the risk of fertility problems, congenital malformations and maternal pregnancy complications, there are few initiatives that aim to structurally offer preconception counselling on these topics in a clinical setting to couples planning pregnancy.[84, 181, 200-203] Screening is the first step in counselling and screening on lifestyle factors, such as smoking and obesity, compared to nutritional quality is relatively straightforward.[191, 204] There are many different questionnaire tools to address nutrition, designed to measure diet and nutrient intake as accurate as possible. These tools differ in their mode of administration, elaborateness and validity.[191, 204] However, most of these tools are not suitable for clinical practice, because of time and financial constraints. If we can screen nutritional behaviours using a simple clinically applicable questionnaire, however, this may be a first step in changing poor nutritional behaviours in order to ultimately contribute to the improvement of reproductive capacity and performance.

Studies performed on the relation between (micro)nutrients and fertility provide evidence that nutrition affects fertility in both men and women.[55, 200] Indeed, the aim of nutrition and nutritional advice is to ensure an adequate intake and status of (micro)nutrients. Ensuring that an adequate nutrient status is attained through counselling is difficult and troubled by various factors such as nutrient interactions, ethnic variation in nutritional habits, day-to-day variation in intake, misreporting and the method of evaluation.[191, 204] In addition, there is still much debate on what is the recommended daily intake for specific nutrients.[204] At present, the Food Frequency Questionnaire is the most applied and appropriate tool to quantify and qualify nutritional habits.

Time and costs are major issues also in the development of preconception care. As a result, screening and counselling on nutrition in a clinical setting is a trade-off between validity and applicability. The obtained information and offered advices should be simple, understandable and applicable for the patient-couple, regardless of differences in demographics.

The Netherlands Nutrition Centre is the official government funded institution that undertakes public health initiatives to improve nutrition in the Dutch population.[205] Based on the general recommendations of the Netherlands Nutrition Centre for a healthy diet, which are developed to ensure a sufficient nutrient intake, we devised a questionnaire to estimate nutritional intake in subfertile patient-couples using six simple questions. In the current

clinical study, using this simple questionnaire we addressed the influence of a healthy diet on the chance of ongoing pregnancy after IVF/ICSI treatment.

MATERIALS AND METHODS

Study design

Between October 2007 and October 2010, patient-couples planning pregnancy, visiting the outpatient clinic of the department of Obstetrics and Gynaecology of the Erasmus MC, University Medical Centre Rotterdam, the Netherlands, were offered preconception counselling at the clinic 'Achieving a Healthy Pregnancy' (AHP). At the first gynaecological visit, couples were referred for preconception counselling on nutrition and lifestyle after having received a flyer with information and a self-administered questionnaire. The questionnaires were filled out at home and included six questions on nutrition. We extracted the following additional data from the questionnaires: age, ethnicity, educational level, indication for referral, lifestyle factors (smoking, alcohol, exercise levels (type of exercise, frequency and duration), stress (yes/no and cause of stress), and drug use), use of medication (over the counter and prescription) and vitamin supplement use. During the preconception counselling, the questionnaires were checked by the counsellor and discussed in detail with the patient-couple. Height and weight were measured to calculate the Body Mass Index (BMI = weight in kilograms divided by squared height in meters). Within 3 weeks after counselling, the participating couples and the gynaecologist received a letter in which in detail the identified (un)healthy lifestyle and nutritional factors and recommendations were reported. Every patient-couple was invited for a follow-up visit to evaluate their compliance to the recommendations. In our population, 46.2% made use of this opportunity.

The six nutritional questions covered the intakes of the main food groups: whole wheat (including cereal consumption), fats, vegetables, fruit, meat and fish. This was based on the dietary recommendations of the Netherlands Nutrition Centre. These questions provide an overall estimate of the nutrition of a person. The current guidelines are at least four slices of whole wheat bread daily (or comparable servings of cereals), the use of monounsaturated or polyunsaturated oils, ≥200 grams of vegetables daily, ≥2 pieces of fruit daily, ≥3 servings of meat or meat replacers weekly and ≥1 servings of fish weekly. Based on these questions we calculated the Preconception Dietary Risk score (PDR-score). When the intake of each food group met the recommendations of the Netherlands Nutrition Centre, a score of one point was assigned. Thus, the maximum PDR-score was six and represented highly adequate nutrition according to recommendations of the Netherlands Nutrition Centre.

Patient-couples who received their first IVF/ICSI treatment with embryo transfer within six months after their first AHP consultation were selected for our analysis. The information on the IVF/ICSI treatment was extracted from the database of the Erasmus MC fertility clinic and included treatment protocol, no. of retrieved oocytes and transferred embryos and treatment outcome. The primary outcome was ongoing pregnancy, defined as a pregnancy with positive foetal heart action at around 10 weeks after embryo transfer, confirmed by ultrasonography.

Statistical analysis

Data analysis was carried out using SPSS 17.0 for Windows. We applied univariate analysis and statistical comparison between groups is done using the non-parametric Mann-Whitney U-test. Variables are reported as Median (Interquartile Range). Proportions are compared using the Chi-Square statistic. To address the influence of nutrition on the primary outcome, we applied multivariable logistic regression with expression of the effect estimate in Odds Ratio´s (OR). We considered several confounders: age, BMI, treatment indication, smoking status, alcohol use, exercise levels, stress, ethnicity, education, no. of transferred embryos, PDR-score and smoking of the man and ovarian stimulation protocol. Because couples are offered a second visit to the AHP consult, we also considered the change in the PDR-score, with those visiting just once receiving a conservative score of '0', reflecting no change. We applied a backward analysis, with the inclusion of higher order variables. During the backward process a variable was included into the analysis if its p-value <0.10 and inclusion changed the effect estimate of the variable of interest >10%.[190]

RESULTS

Of the 1,270 patient-couples participating in this study up to the date of analysis, 199 couples received their first IVF/ICSI treatment with embryo transfer within 6 months after AHP consultation. Women undergoing IVF/ICSI treatment were more often of Dutch origin and less often of non-European origin (60.8% vs. 53.1%; p=0.04). In addition, treated women were more likely to consume alcoholic beverages (62.8% vs. 48.6%; p<0.001), which resulted from the different ethnicity distribution between the groups. Women undergoing IVF/ICSI treatment were older and the duration of subfertility longer than those who did not receive this treatment (33.6 vs. 32.1 years; p<0.001 and 28 vs. 22 months; p<0.001, respectively) and had a slightly lower BMI (23.8 vs. 24.8 kg/m^2; p<0.01).

Of the 199 couples undergoing their first IVF/ICSI treatment, 35% became pregnant, of which 26% was ongoing whereas in 62% no pregnancy

could be achieved. For 3%, the outcome of treatment is unknown. Of the 199 couples, 92 made use of the opportunity to visit the AHP consult a second time. In this group, those fallen pregnant were slightly overrepresented (56.9% vs. 43.0%; p=0.09).

Table I shows the characteristics of couples undergoing IVF/ICSI treatment stratified for pregnancy status. Women with an ongoing pregnancy tended to be younger. In addition, women and men with an ongoing pregnancy were more often non-smokers (**Table I**). The proportion of adherence to the recommendations for the main food groups comprised in the PDR-score, i.e. what proportion of women scored a point for the PDR-score for each food group was comparable between those fallen pregnant and those that did not. Without adjusting for any confounders there are no differences in the PDR-score between women with and without an ongoing pregnancy (**Table I**).

However, the logistic regression analysis shows a significant association between the PDR-score of the woman and the probability of ongoing pregnancy after IVF/ICSI treatment (**Table II**). A beneficial increase in the PDR-score with one point associates with an increase of 65% in the chance of ongoing pregnancy after the first IVF/ICSI treatment within 6 months after counselling. The OR is adjusted for the confounders: treatment indication, age of the woman (squared), BMI and smoking status of the woman and the PDR-score and BMI of the partner. Interestingly, an increasing PDR-score of the men seemed to reduce the chance of pregnancy after IVF/ICSI treatment. After including the interaction between treatment indication and PDR-score of the man, the association between the PDR-score of the man and the chance of pregnancy was conditional on female factor infertility. Thus, the PDR-score of the man did not affect the chance of pregnancy after IVF/ICSI treatment. Factors such as alcohol use, exercise and stress levels, ethnicity, education level, ovarian stimulation protocol, smoking of the man, the change in the PDR-score at the second visit, duration of subfertility and indication for treatment did not show effects according to the pre-specified criteria.[190]

DISCUSSION

In this study, we show that the quality of the preconception diet of patient-couples undergoing a first IVF/ICSI treatment associates with the chance of ongoing pregnancy after IVF/ICSI treatment within 6 months after preconception counselling tailored on nutrition and lifestyle. After adjusting for several covariates a beneficial one-point increase in the PDR-score reflects a 65% increased chance for ongoing pregnancy.

There are many different tools to measure nutrition.[191, 204] The goal of these tools is to estimate qualitative and quantitative nutrient intake through the

Table I. Baseline characteristics for couples undergoing IVF/ICSI treatment (n=193).

	Ongoing pregnancy (n=51)	No pregnancy (n=142)	p
Women			
Age (years), median (IQR)	32.5 (30.2-35.3)	33.6 (29.7-38.7)	0.06
Ethnicity, % (n)			ns
Dutch	66.7 (34)	58.5 (83)	
European	13.7 (7)	10.6 (15)	
Non-European	19.6 (10)	31.0 (44)	
Education level, % (n)			0.08
High	50.0 (24)	37.6 (50)	
Intermediate	39.6 (19)	42.9 (57)	
Low	10.4 (5)	19.5 (26)	
Subfertility, % (n)			ns
Primary	60.8 (31)	59.2 (84)	
Secondary	39.2 (20)	40.8 (58)	
Reason of subfertility, % (n)			ns
E.c.i.	22.5 (32)	27.5 (14)	
Male	39.4 (56)	43.1 (22)	
Female	27.5 (39)	15.7 (8)	
Combined	10.6 (15)	13.7 (7)	
Duration of subfertility (months), median (IQR)	34 (17-44)	28 (18-45)	ns
Smoking (yes), % (n)	9.8 (5)	24.6 (34)	0.03
Alcohol use (yes), % (n)	62.7 (32)	61.3 (87)	ns
Body Mass Index (kg/m^2), median (IQR)	24.0 (21.8-29.2)	23.8 (21.8-27.2)	ns
PDR-score, median (IQR)	3 (2-4)	3 (2-4)	ns
Whole wheat (yes), % (n)	43.1 (22)	38.7 (55)	ns
Fats (yes), % (n)	64.7 (33)	62.7 (89)	ns
Vegetables (yes), % (n)	23.5 (12)	22.5 (32)	ns
Fruits (yes), % (n)	23.5 (12)	15.5 (22)	ns
Meats (yes), % (n)	92.2 (47)	90.1 (128)	ns
Fish (yes), % (n)	56.9 (29)	47.9 (68)	ns

Table I. Continued from previous page.

	Ongoing pregnancy (n=51)	No pregnancy (n=142)	p
Men			
Smoking (yes), % (n)	20.4 (10)	36.5 (50)	0.04
Body Mass Index (kg/m^2), median (IQR)	26.1 (24.4-27.8)	26.2 (24.0-28.9)	ns
PDR-score, median (IQR)	3 (2-4)	3 (2-4)	ns
Whole wheat (yes), % (n)	58.8 (30)	74.6 (106)	0.03
Fats (yes), % (n)	66.7 (34)	64.8 (92)	ns
Vegetables (yes), % (n)	19.6 (10)	19.7 (28)	ns
Fruits (yes), % (n)	15.7 (8)	15.5 (22)	ns
Meats (yes), % (n)	94.1 (48)	93.7 (133)	ns
Fish (yes), % (n)	54.9 (28)	47.3 (70)	ns

PDR-score (Preconception Dietary Risk score)

diet mainly in a research setting.[204] Our simple clinically applicable tool is based on the recommendations of the Netherlands Nutrition Centre, which are developed to ensure sufficient nutrient intake.[205] This tool does not estimate quantitative or qualitative nutrient intake but in a binary fashion it addresses whether daily nutrient intake of six main food groups (fruits, vegetables, meat, fish, whole-wheat products and fats) is sufficient. Although we were able to account for several potential confounders, it is important to realize the considerable variability in the reporting of day-to-day nutritional habits conditional on obesity, education level, perceived health and ethnicity.[191] Nevertheless, because we discuss the questionnaire in detail with the patient-couple during the consultation, we were able to address any ambiguity in the reporting of nutritional habits and ask for additional information if necessary.

Previously, Vujkovic et al. have investigated the association between adherence to the Mediterranean diet using the FFQ, comprising 195 questions, and the chance of biochemical pregnancy after IVF/ICSI treatment.[181] This diet comprised high intakes of vegetable oils, vegetables, fish, and legumes and low intakes of snacks. High adherence to the Mediterranean diet associates with a 40% increased chance of biochemical pregnancy on day 15 after embryo transfer. Here we show a comparable effect size for the adherence to

Table II. Predictors for ongoing pregnancy after IVF/ICSI treatment (n=193).

	β	**Odds Ratio (95% CI)**	**p**
Age^2 (woman)	-0.022	0.98 (0.96-0.99)	<0.01
Body Mass Index (woman)	0.078	1.1 (0.99-1.19)	ns
Body Mass Index (man)	-0.064	0.94 (0.85-1.04)	ns
Smoking (woman)	-0.99	0.37 (0.12-1.11)	0.08
PDR-score (woman)	0.50	1.65 (1.08-2.52)	0.02
PDR-score (man)	-0.054	0.95 (0.48-1.86)	ns
Treatment indication			ns
E.c.i	REF	-	
Male	-0.49	0.61 (0.23-1.62)	ns
Female	-1.21	0.30 (0.09-1.01)	ns
Combined	-0.19	0.83 (0.48-1.86)	ns
Treatment indication by PDR-score (man)			ns
E.c.i	REF	-	
Male	-0.31	0.74 (0.32-1.70)	ns
Female	-1.14	0.32 (0.11-0.92)	ns
Combined	-0.42	0.66 (0.22-1.97)	ns

PDR-score (Preconception Dietary Risk score)

the recommendations of the Netherlands Nutrition Centre and the chance of ongoing pregnancy after IVF/ICSI treatment only using a lower resolution and clinically applicable questionnaire.

Studies mostly focus on the relation of (micro)nutrient status, or markers thereof, and reproductive outcomes.[206] Given that (micro)nutrients are primarily derived from the diet, it should be interesting to investigate through which specific (micro)nutrients the effect of nutrition is mediated. Here, the effects of B-vitamins, such as folate, on reproduction can help provide a mechanism through which nutrition mediates its effects on reproduction. Indeed, various studies by Vujkovic et al. show that certain dietary patterns are beneficial with regard to reproductive outcomes and associate with B-vitamin concentrations.[84, 181, 202, 203] Nevertheless, despite this knowledge, there are few initiatives in reproductive medicine to translate these findings into clinical practice. Combined with a recent study from our group by Hammiche et al., we show that preconception counselling on nutrition seems effective, and now also results in an improved chance of ongoing pregnancy after IVF/ICSI treatment.[207]

During the AHP consult we counsel patient-couples to adopt, if necessary, a healthier diet, stop smoking, alcohol and drug use, to increase their exercise levels and subsequently lose weight. To review their compliance, patient-couples are invited for a follow-up visit. Possibly, not only the baseline difference in nutrition affects the chance of ongoing pregnancy after IVF/ICSI treatment but also the improvement in nutrition. Of the couples that returned for a second AHP consult, there was a significant improvement of the PDR-score, but no significant difference in the change of the PDR-score between women getting pregnant and those who did not (data not shown). Including this change of the PDR-score in the final model did not result in any changes to the effect estimate of the PDR-score, suggesting that the effect of nutrition is mediated through baseline differences. The study by Hammiche et al. in the ongoing AHP cohort in patient-couples, who consulted the AHP twice, shows a beneficial increase in the PDR-score.[207] This was most notably driven by an improvement in fruit (+15%) and fish intake (+13%). Additionally, also self-reported exercise levels (+44%) and folic acid containing supplement use (+17%) improved, which are not included in the PDR-score. Although not significant in the study of Hammiche et al., self-reported use of vegetables, whole wheat products, vegetable oil and meat, comprised in the PDR-score, and the distribution of the BMI groups improved, with fewer obese people (-3%).[207] These findings suggest a significant uptake of the counselling. Therefore, it would be interesting to address further in depth whether an increase in the PDR-score at a second visit associates with an increased chance of ongoing pregnancy after IVF/ICSI treatment.

Paternal dietary and lifestyle factors affect semen quality and thereby could influence assisted reproduction treatment success.[202, 208] Here, we are not able to identify a significant influence of the PDR-score of the man on IVF/ICSI treatment outcome. Only in the case of female factor infertility, the PDR-score of the man appeared to influence the chance of pregnancy. Nevertheless, the unintuitive direction of this association likely reflects the more adequate nutritional habits of men of couples that did not fall pregnant and experiencing female factor infertility as opposed to those that did fall pregnant (mean +1 PDR-score point, data not shown). Apparently, and not surprising, the influence of female factor infertility is not influenced by nutrition of the man. In addition, because our study consisted of IVF/ICSI treatment cycles that resulted in a good quality embryo suitable for transfer, this suggests that the effect of maternal nutrition on IVF/ICSI treatment outcome is primarily mediated through provision of adequate nutrients to the early transferred embryo.

In addition to various nutritional factors that affect the chance of pregnancy after IVF/ICSI treatment, lifestyle factors such as smoking and overweight status, but also exercise levels and stress could affect the chance of pregnancy

after IVF/ICSI treatment.[209-212] To our knowledge the only study that specifically addresses the influence of physical exercise on IVF/ICSI treatment outcomes is by Morris et al.[210] This study with a large sample size failed to find an association between current exercise levels and IVF/ICSI treatment outcomes, which is how this topic is addressed during the AHP consult. Including this measurement of physical exercise in the analysis did not affect the influence of the PDR-score on the chance of ongoing pregnancy. During the consult we also obtain general information on stress, giving a crude estimate of stress. Here we fail to show an effect of stress on the chance of pregnancy. Given the evidence in the literature for the relation between IVF/ICSI treatment success and physical exercise and stress, implementing more detailed measures for both features could help to further detail the influence of nutrition on IVF/ICSI treatment. Although marginal and not significant, the BMI appears beneficially associated with the chance of ongoing pregnancy. This is in contrast with firmly established evidence, which shows a decline in IVF/ICSI treatment success with an increasing BMI.[209] As noted, we counsel couples to adopt a healthier lifestyle. Although it is not possible to show such an association in the sample for this analysis, possibly the largest improvement in dietary and lifestyle factors is noted in overweight/obese people who require and perceive the greatest necessity for change. Studies focussing on weight loss, suggest that it is not necessary to obtain a healthy weight but weight reduction of 5-10% is already sufficient to improve fertility and endocrine parameters.[213, 214] Thus, considering the time-lag between treatment and counselling, the discussed results from Hammiche et al.,[207] the fact that a slight weight reduction is often sufficient to improve the chance of pregnancy and the notion that overweight people could perceive the greatest necessity to take up counselling; it might me possible that BMI measured prior to treatment is a correlate of dietary and lifestyle improvements and thereby explain the positive association between BMI and pregnancy chance in this study.

CONCLUSION

We show here that adherence to recommendations of the Netherlands Nutrition Centre associates with an increased chance of ongoing pregnancy after the first IVF/ICSI treatment. We addressed this topic using a low-resolution and clinically applicable questionnaire. In line with studies on weight reduction, this study provides evidence that programs aimed at beneficially changing preconception nutrition and lifestyle factors should be considered a first-choice treatment for unexplained subfertility.[213, 215] A next step for these investigations would be to address whether a beneficial change in nutrition associates with an increase in the chance of ongoing pregnancy after IVF/ICSI treatment.

Introduction & Aim

VIII

General Discussion

I

This thesis reports on the influence of preconception nutrition of a woman on her ovarian response to exogenous administered gonadotropins (i.e. Controlled Ovarian Hyperstimulation) and the success of achieving pregnancy after IVF/ICSI treatment. In the following discussion we elaborate on the main findings from this thesis and discuss the implications they might have for preconception care and future research.

PRECONCEPTION NUTRITION, FOLIC ACID SUPPLEMENT USE AND THE OVARIAN RESPONSE

The ovarian response is a crude and clinically relevant measure of the numerous extrinsic- and intrinsic ovarian factors that are implicated in follicle growth. Gaining insight into determinants of the ovarian response to COH improves the understanding of mechanisms that regulate ovarian follicle growth and those involved in oocyte quality. Additionally, such determinants could be used to tailor the COH treatment protocol to a woman's specific needs and requirements; or to optimize a woman's condition before undergoing COH. In this thesis we show that healthy nutrition as well as folic acid supplement use are determinants that comparably influence the ovarian response to COH (**Chapter V and VI**). The ensuing recommendations to optimize the maternal preconception nutritional status to improve the ovarian response to COH are in line with and conducive to current recommendations with regard to an uneventful development of, as well as an improved outcome of pregnancy. The question remains, however, how these data can be implemented, in particular for couples undergoing IVF/ICSI treatment.

When subjected to COH, the typical ovarian follicle undergoes an 1800-fold increase in cell count.[216] Therefore, based on the results presented in this thesis, we propose that an adequate nutritional environment enables the ovarian follicle to adequately respond to the gonadotropin stimulus, in contrast to a possible negative or inhibitory influence of the nutritional status on the ovarian response. Since follicle and oocyte maturation are interdependent, the magnitude of the ovarian response could have consequences for oocyte quality, subsequent embryo viability and the chance of pregnancy as well. Indeed, similarly, the use of an nutritionally insufficient diet to loose weight lowers the chance of pregnancy after IVF/ICSI treatment.[217] Moreover, under the aggravated growth circumstances posed by COH (i.e. stimulating follicles to grow that otherwise would have not), it is not unlikely that the presence and influence of (genetic, lifestyle, or metabolic) factors on the ovarian response, are in part mediated or ameliorated by adequate nutrition.[213, 215, 217]

In more detail, previous studies show an optimum in the relation between the ovarian response to COH and the chance of pregnancy after IVF/ICSI treatment.[4] This raises the question: could the reduction in the ovarian response to COH due to healthy nutrition and folic acid supplement use also be harmful for IVF/ICSI treatment outcomes? Achieving an optimal ovarian response to COH is the result of the appropriate balance between determinants of the ovarian response and the applied stimulation protocol. Modified by external (environmental) factors, a given combination of constitutive factors that influence the ovarian response dictate an optimal ovarian response, to which a stimulation protocol is adapted. Therefore, a reduction of the ovarian response through optimization of extrinsic factors, such as nutrition and overweight status, does not necessarily constitute a negative influence on the ovarian response but aligns the ovarian response to the anticipated optimal ovarian response.

Adherence to a Healthy dietary pattern characterized by high intake of fruit and whole grain lowers the ovarian response to COH (**Chapter VI**), comparable to the effect of folic acid supplement use (**Chapter V**); nevertheless, how do these independent findings from the same cohort compare and agree? To compare these findings, the results from chapter V are transformed to a log-level model, similar to the type in chapter VI. Second, serum folate concentrations from chapter V are binned into seven categories to obtain a similar resolution as that of the component of the Healthy dietary pattern in chapter VI. Now, a one-unit change of serum folate concentrations lowers the ovarian response by 4.9% (-18.2% - 8.4%) compared to 14.4% (-24.8% - -0.7%) for a one-unit change of the component for the Healthy dietary pattern. Indeed, the larger influence of nutrition on the ovarian response suggests that more pathways influencing ovarian follicle metabolism are affected through nutrition than by a single nutrient, i.e. folate.

The food items comprised in the Healthy dietary pattern provide antioxidants and various cofactors and substrates for the one-carbon pathway but are not the principal sources of folate. Nevertheless, adherence to the Healthy dietary pattern associated with serum and RBC folate concentrations, and folic acid supplement use had a similar, albeit smaller, influence on the ovarian response (**Chapter V**). Other studies also consistently show associations between healthy dietary patterns and biomarkers of the folate dependent one-carbon pathway.[84, 181, 202, 203] Therefore, we hypothesize that the effect of nutrition is in part mediated through the one-carbon pathway. Furthermore, this allows for an explanation that a Healthy dietary pattern rich in anti-oxidants similarly influences the ovarian response; a deficiency of the one-carbon pathway results in oxidative stress.[29] The one-carbon pathway is the underlying pathway for the synthesis of DNA nucleotides,

several amino acids and phospholipids. Moreover, this pathway provides the methyl groups that are used for epigenetic processes such as DNA and histone methylation and detoxification of various substrates, which are implicated in programming of the oocyte and granulosa cell epigenome.[11]

It is surprising that ovarian follicles developing in a presumably poor nutritional environment show increased metabolic activity as reflected by increased steroid synthesis. Especially so since previous studies show that folate deficient women are at increased risk of anovulatory infertility, which is in turn supported by studies in chimpanzees that have an increased proportion of atretic and cystic follicles after folate depletion.[37, 218] Possibly, this is the phenotype acquired after prolonged folate depletion. Indeed, in vitro studies on human, ovine, bovine and rodent granulosa cells show that a compromised one-carbon pathway or oxidative stress can indeed establish an increased ovarian response to COH. The studies in human described in this thesis, but also by Kanakkaparambil et al. in ewes, show that these mechanisms also act in vivo.[144] First, expression of the FSH-Receptor (*FSHR*) and aromatase is regulated through one-carbon pathway dependent DNA methylation.[172, 219] Cells with a high mitotic index subjected to a compromised one-carbon pathway could lose epigenetic marks, which would result in reduced repression of these two proteins that promote the ovarian response.[25] Also, a compromised one-carbon pathway results in oxidative stress, which can explain the larger effect of a diet high in anti-oxidants than folic acid supplement use alone.[29] Indeed elevated homocysteine, as the best-studied marker of a compromised one-carbon pathway, and oxidative stress result in an up regulation of *FSHR*, apparently in order to stimulate production of the endogenous anti-oxidant glutathione.[47, 49, 144] Concomitantly, increased *FSHR* expression will result in an increased perceived stimulus for estradiol production through up regulation of aromatase activity in granulosa cells. Possibly, increased estradiol production is even the purpose. In vivo, estradiol stimulates up regulation of anti-oxidant genes and enzymes.[220] Thus, the increased ovarian response might be an expression of the cellular response to negate the increased oxidative stress due to a compromised one-carbon pathway or poor anti-oxidant intake through the diet, through up regulation of estradiol dependent anti-oxidant enzymes. A physiologic response to oxidative stress that is inadequate in the presence of a supraphysiologic FSH stimulus while undergoing COH and a poor nutritional environment.

FOLIC ACID SUPPLEMENT USE AND THE MICROENVIRONMENT OF THE OOCYTE

The functions of the one-carbon pathway are ubiquitous and it seems highly unlikely that changes in the one-carbon pathway result in pathology mediated through a single mechanism.[130, 135, 221] Therefore, in **chapter III and IV** we aimed to qualify and quantify the effects of folic acid supplement use on ovarian follicle metabolism by interrogating the follicle fluid proteome.

The use of follicle fluid and proteomics tools to investigate the effects of folate on ovarian follicle metabolism is one of many methodological possibilities. Follicle fluid is readily available after COH and its investigation is non-invasive with regard to the oocyte and embryo. Follicle fluid is the product of follicular cell excretions and an exudate of blood serum; therefore, follicle fluid provides insight into the intra-follicular environment resulting from local and systemic influences, which are in turn influenced by genetic, metabolic and lifestyle factors.[108] The contents of follicle fluid are directly linked with oocyte quality.[44, 112-114, 136] More specific studies into gene expression patterns of follicular cells can help disentangle the independent systemic as well as local contributions to the follicle fluid. This knowledge can help the direction of specific interventions or development of biomarkers to assess oocyte quality but also allows more in-depth evaluation through which mechanisms folic acid supplement use improves reproductive outcomes. Unfortunately, isolating follicular cells from monofollicular fluid and validating the obtained sample as follicular cells is at present not feasible without culturing. This precludes studies into alternative 'omes' because in vivo effects on the epigenome, transcriptome and proteome are possibly erased in vitro.

The proteomic studies of human follicle fluid described in this thesis confirm the previously reported complexity of the follicle fluid proteome.[109-111] An important distinction compared to previous studies is that we used monofollicular fluids for proteomic analysis instead of multi-follicular fluids. Since the ultimate goal is to relate the follicle fluid proteome to oocyte and embryo quality, the use of monofollicular fluid is a prerequisite. Of interest is that we show an increased abundance of lipoproteins and a lower inflammatory state of follicle fluid in folic acid supplement users as compared to non-supplement users. This observation is very similar to findings in mice where the one-carbon pathway was compromised.[222] Also, follicles of users appeared more mature as suggested by lower cytokeratin abundance. With regard to oocyte development and embryo competence, both high lipoprotein concentrations and low CRP concentrations are beneficial for embryo quality.[153, 223] Although these findings do not provide conclusive evidence as to what mechanism would underlie the effects of a healthy diet and folic

acid supplement use on the ovarian response, they support the proposal that follicle maturation is disrupted due to folate or nutritional deficiencies, as is also reflected by cytokeratin concentrations in follicle fluid.[155, 224]

In conclusion, the results of the proteomics studies highlight the involvement of possible pathways through which folic acid supplement use can improve oocyte and embryo quality after IVF/ICSI treatment.

NUTRITION AND PREGNANCY AFTER IVF/ICSI TREATMENT

In the studies described in **chapter VI and VII** we study the influence of nutrition on the chance of achieving pregnancy after IVF/ICSI treatment. For scientists, nutrition as consumed by humans is a complex exposure. Nutrition comprises a wide range of food items with large inter- and intra-item nutrient differences. Furthermore, regardless of nutrient content, eventual bioavailability of nutrients is affected by amongst others nutrient-nutrient interactions, constitutive traits of the consumer, the mode of preparation, storage conditions and the age of the food item.[225] The problem would only be mathematical in case the magnitudes of all involved factors are known. Unfortunately this is not the case. Nevertheless, the relevance of nutrition studies ultimately resort from the same reason that underlies its difficulties, human consumes a constellation of food items, which comprise the diet and which provides all substrates for maintenance of homeostasis.

Nutrition and folic acid supplement use influence the chance of pregnancy after IVF/ICSI treatment, independent of the ovarian response.[181, 226] The question is how could the ovarian response contribute to an improved chance of pregnancy after IVF/ICSI treatment (**Chapter VI**)? In addition to the previously discussed follicular maturation mechanism, there is also the influence of the ovarian response on the endometrium. The endometrium in which the embryo implants is very sensitive to steroid hormone concentrations and indeed, high estradiol concentrations have a detrimental effect on endometrial receptivity.[5] The endometrial gene expression pattern and endometrial gland secretion composition after COH, is shifted to one less favourable for embryo implantation.[5, 184, 227] Indeed, a Healthy diet and folic acid supplement use may also improve the chance of pregnancy after IVF/ICSI treatment, through improved embryo quality regardless of endometrial receptivity; which is supported by the findings that folic acid supplement use and healthy nutrition associate with the birth prevalence of congenital malformations.[11] Nevertheless, under the hypothesis that the ovarian response to COH is a surrogate for healthy follicle maturation, a

lower ovarian response can associate with improved embryo quality. In part mediated by the one-carbon pathway, the effects of nutrition on ovarian follicle metabolism, embryo quality and endometrial receptivity through the ovarian response after COH, could additively cooperate to improve the chance of pregnancy after IVF/ICSI treatment as described in chapter VI.

The goal of the studies described in **chapters VI and VII** is to provide points of applications to improve preconception counselling of couples undergoing IVF/ICSI treatment. Preconception counselling is best done using simple and understandable recommendations. Because of time and financial constraints, the tools commonly used to meticulously map nutrition in a controlled study environment are often not feasible or available in most clinical settings. Indeed, with regard to tailored nutritional counselling, in a clinical setting there is clearly a trade-off between applicability and validity. To prevent undesirable outcomes, as is the purpose of counselling, it is necessary that the screening tool is sufficiently accurate and therefore able to identify outcomes of interest. In **chapter VII** we use a 6-item questionnaire based on recommendations of the Netherlands Nutrition Centre to screen nutrition in couples.[205] The results from the study in **chapter VII**, and the study by Hammiche et al., show that it is possible to screen nutritional habits in a clinical setting.[207] Also, we observe that an increase in adherence to the recommendations of the Netherlands Nutrition Centre associates with an improved chance of ongoing pregnancy after IVF/ICSI treatment, with an effect-size similar to that described in **chapter VI** and by Vujkovic et al.[181] Based on these results, it is clearly possible to screen nutritional habits and relate these to clinically relevant outcomes.

METHODOLOGICAL CONSIDERATIONS

The studies described in this thesis are embedded in two observational studies, the FOod Lifestyle and Fertility Outcome- and Rotterdam Predict study. Indeed, such observational studies are subject to common issues that reflect on the validity and generalizability of observational studies. All participants in the studies described in this thesis are part of a highly selected population, i.e. subfertile couples requiring assisted reproduction to achieve pregnancy. In addition, the question remains, does an improvement in nutritional and lifestyle behaviour indeed improve the chance of a spontaneous pregnancy? It is important to realize the considerable variability in the reporting of day-to-day nutritional habits conditional on nutrition and lifestyle factors which are perceived to be (un)healthy, but also the possibility of residual confounding.[191] Nevertheless, despite the day-to-day variation in nutritional

habits, the observed effects of nutrition on health are consistent, arguing for the validity of dietary pattern based exposure measures.

In **chapters III and IV** we applied mass spectrometry based proteomics to elucidate the monofollicular fluid proteome. Such high-throughput technologies give rise to a large number of protein identifications, with possible false positive identifications. Various methods have been developed to tackle this issue, which are also enacted on our datasets, resulting in a prudent and sensible estimate of the follicle fluid proteome. Unfortunately, given the nature of these data and methods it is not possibly to adjust for confounding factors in order to specify the influence of folic acid supplement use in more detail.

Despite such possible shortcomings, the FOLFOstudy is specifically designed to address the influence of nutrition and lifestyle factors on IVF/ICSI treatment outcome, excluding women with known risk factors for adverse IVF/ICSI treatment outcome. In addition, the agreement between the various studies that have addressed the influence of nutrition on the ovarian response and chance of pregnancy is a testament to the validity of these findings. Also, the Rotterdam Predict/Achieving a Healthy Pregnancy study described in **chapter VII** is part of the ongoing preconception counselling clinic tailored on nutrition and lifestyle in which patient-care and research are combined, which allows for a clinically relevant evaluation of the feasibility of preconception counselling initiatives and the to be expected change in nutritional and lifestyle behaviours.

IMPLICATIONS FOR PRECONCEPTION CARE AND RESEARCH

A next step in evaluating the influence of nutrition and addressing the potential for preconception counselling on IVF/ICSI treatment is to investigate whether improvements in nutrition also result in improved treatment outcomes. Moreover, would improving a woman's nutritional status also increase the chance of a spontaneous conception? Finally, should preconception counselling be restricted to the woman or should it also address men?

Nutritional and lifestyle behaviours have great influence on offspring and reproductive health; therefore preconception counselling should be available to all couples trying to conceive, indeed with great barriers to be overcome in case one is trying to address the reproductive population which does not require fertility treatment. Given the consistencies in the reported effects and the long-term health benefits of a healthier lifestyle, along with the possibility that poor nutritional and lifestyle factors are the attributable factor for subfertility, preconception counselling should be regarded as a first step in the

evaluation of a subfertile couple. Even if poor nutrition is not the attributable factor for subfertility, healthy nutrition, as the results in this thesis emphasize, improves the chance of pregnancy after IVF/ICSI treatment. This is enforced by the notion that the influence of nutrition in both **chapter VI and VII** was independent of the cause of subfertility and that nutritional quality also coincides with the chance of unassisted conception.[192]

Even though subfertile couples have a strong incentive to change lifestyle and nutritional behaviours, it is still difficult to achieve weight-loss, quit smoking and improve the nutritional status in a given couple in this population. With the notable exception, not only the lack of initiatives to support couples in improving nutrition and lifestyle habits but also the perceived solely medical origin of the failure to conceive hamper preconception counselling initiatives. Successfully implementing preconception counselling is therefore dependent on a paradigm shift in medicine itself, more aimed towards primary prevention and the perception that nutrition is an important determinant of (reproductive) health and longevity. Equally important is the realisation of society that health and disease is in large parts the result of ones own behaviour, and that nutrition is a factor of considerable importance herein.

The onset and ongoing attention for the influence of nutrition on fertility and the ensuing preconception counselling initiatives will likely subject IVF/ICSI treatment cohorts to selection bias. Indeed, couples with a healthy diet were less likely to visit their physician for difficulty trying to conceive.[192] Based on previous studies on the influence of weight loss and the risk of needing IVF/ICSI treatment, healthy nutrition can be considered a competing risk with regard to requiring ART, selecting those in who nutrition was not the attributable factor for subfertility or those in whom unhealthy lifestyle habits are persistent.[213] Therefore, with the rise of preconception counselling initiatives, failure to prove any influence of nutrition on reproductive success after assisted reproduction does not necessarily mean the lack of influence. We could advocate that in the reproductive population healthy nutrition is a prerequisite for receiving assisted reproduction treatment and should be the primary goal to achieve before treatment is initiated. Nonetheless, meticulous follow-up of all participants in prospective studies, such as the Rotterdam Predict study, that include subfertile couples before treatment and preconception counselling or comparable Randomized Controlled Trials will show how much benefit is to be gained by improving nutritional habits with regard to the need of assisted reproduction. In any case, the beneficial influence of healthy nutrition on foetal development has been well established.[11]

Furthermore, the fashion in which we address IVF/ICSI treatment outcomes need to be readdressed. It remains unclear if there is much room left

for improvement of the per treatment cycle success rates after single embryo transfer, and thus if odds ratios such as reported in this thesis adequately reflect the influence of nutrition on IVF/ICSI treatment outcome. If treatment is still required, clinical studies should consider cumulative outcomes for the complete assisted reproduction treatment course, starting with preconception counselling. Human reproduction seems inefficient, since also in normal fertile women or women with tubal factor infertility implantation rates are low.[228, 229] Moreover, there are unknown intra-patient prognostic factors for treatment success.[9] Studying cumulative outcomes for the entire treatment cycle could prove more effective in identifying such prognostic factors and account for the apparent inefficiencies in human reproduction. It might well be possibly that interventions not necessarily improve the chance of pregnancy after a single treatment cycle, but do ameliorate the generally observed decline of success in subsequent cycles and thus increase the cumulative chance of success.

CONCLUSION

Healthy nutrition lowers the ovarian response to COH and improves the chance of pregnancy after IVF/ICSI treatment. The folate mediated one-carbon pathway is a likely intermediate through which the effect of nutrition on ovarian follicle metabolism and the chance of pregnancy after IVF/ICSI treatment are mediated. In-depth studies of follicular cells will provide suggestions for underlying pathways that mediate the ovarian response to COH conditional on one-carbon metabolism and what the role of the ovarian response to COH is in determining the chance of pregnancy after IVF/ICSI treatment.

Finally, the results from this thesis emphasize the necessity to optimize the preconception nutritional status of women undergoing COH and IVF/ICSI treatment and to change the perceived relation between nutrition, general health and reproductive health. Not only to improve the ovarian response to COH, but also to improve the chance of pregnancy after IVF/ICSI treatment and consequently the chance of an uncomplicated healthy pregnancy.

Introduction & Aim

IX

Summary / Samenvatting

I

ENGLISH SUMMARY

Controlled Ovarian Hyperstimulation treatment (COH) and In Vitro Fertilization/Intracytoplasmic Sperm Injection (IVF/ICSI) are assisted reproduction techniques used to achieve pregnancy in subfertile couples. Although COH and IVF/ICSI treatment are moderately successful in achieving pregnancy, there is room for improvement. There are various approaches to improve IVF/ICSI treatment, ranging from the improvement of embryo culture conditions to optimizing modifiable patient characteristics. However, also the intensity of the ovarian response to COH is thought to influence the chance of IVF/ICSI treatment success. In the studies described in this thesis we aimed to identify modifiable nutritional factors that associated with the one-carbon pathway and influence the ovarian response to COH and the chance of ongoing pregnancy after IVF/ICSI treatment. First we elaborate on mechanisms through which the effect of nutrition on ovarian follicle metabolism can be established and the magnitude of influence of nutrition on the ovarian response to COH and IVF/ICSI treatment outcome. Moreover, we addressed whether a simple clinically applicable questionnaire to screen nutritional habits associated with IVF/ICSI treatment outcome.

To specify mechanisms through which nutrition and the folate dependent one-carbon pathway influence ovarian follicle metabolism we initiated proteomics studies of the ovarian follicle fluid. In **chapter III** we show that IEF assisted proteomics is a sensitive method to investigate the follicle fluid proteome. Additionally we studied the influence of folic acid supplement use on the follicle fluid proteome in **chapter IV**. In this study we elaborate on difficulties associated with the use of follicle fluid as a marker of intra-follicular processes. Folic acid supplement use associated with higher abundance of apolipoproteins that predominantly associate with High Density Lipoproteins. These findings show how folic acid supplement use can beneficially influence embryo quality after IVF/ICSI treatment. Also follicles of folic acid supplement users appeared more mature, as reflected by cytokeratin abundance, which can be a reflection of the degree of oocyte maturation. Finally, in follicle fluid of supplement users, there was a lower abundance of C-reactive protein, suggesting a lower inflammatory status of the follicle fluid in folic acid supplement users.

In **chapter VI** we studied the diet of 203 women undergoing COH for IVF/ICSI treatment using a validated Food Frequency Questionnaire. With the use of Principal Component Analysis we identified a dietary pattern that associated with the ovarian response to COH. Each woman was assigned a personal score, which reflected adherence to this dietary pattern. The Healthy dietary pattern consisted of high intake of fruit, whole grain products and

margarine and low intake of snacks and refined grain products. In adjusted analyses we consequently show a 14.4% reduction in the ovarian response for each gained point that reflects adherence to the Healthy dietary pattern. Similarly adherence to the Healthy dietary pattern increased the chance of ongoing pregnancy after IVF/ICSI by 60%. In **chapter V** we investigate whether the vitamin folate could explain the influence of nutrition on the ovarian response. Here we show that folic acid supplement use indeed similarly influenced the ovarian response to COH, as did the Healthy dietary pattern. However, because the groups were very small, we did not study the influence of folic acid supplement use on the chance of pregnancy.

In **chapter VII** we describe the clinical implementation of the findings of nutritional studies. Using a clinically applicable questionnaire comprised of six questions that addresses nutritional adequacy based on guidelines of the Netherlands Nutrition Centre, we show an association between the measure of nutritional adequacy (the Preconception Dietary Risk score) and the chance of pregnancy.

Finally, in **chapter VIII** we integrate and discuss the findings from **chapters III-VII** and provide tentative future directions for COH and IVF/ICSI research. We emphasize the necessity for both further experimental and observational studies into the influence of nutrition and folic acid supplement use on ovarian follicle metabolism but also the required changes in approach to clinical IVF/ICSI treatment study design.

NEDERLANDS SAMENVATTING

Ovariële hyperstimulatie behandeling (OH) en In Vitro Fertilisatie /Intracytoplasmatische Semen Injectie (IVF/ICSI), zijn vruchtbaarheidsbehandelingen voor paren met een verminderde vruchtbaarheid. Hoewel OH en IVF/ICSI behandeling redelijk succesvol zijn in het bewerkstelligen van een zwangerschap, is er nog veel ruimte voor verbetering. Er zijn verschillende benaderingen voor verbetering mogelijk, variërend van verbetering in de kweekomstandigheden van het embryo tot het optimaliseren van eigenschappen van patiënten. Echter, ook de ovariële response op OH heeft invloed op de kans van slagen van de IVF/ICSI behandeling. De studies beschreven in dit proefschrift hadden tot doel om, beïnvloedbare voeding- en leefstijl gewoonten te identificeren die een rol spelen bij de ovariële response en de kans op zwangerschap na IVF/ICSI behandeling. Hierbij is ook gekeken naar de invloed van dergelijke factoren, onder welke foliumzuur, op het micromilieu van de ontwikkelende eicel. Verder hebben we bestudeerd of het mogelijk is om tijdens een preconceptioneel consult met korte en simpele vragenlijsten de voeding- en leefstijl gewoonten van paren met verminderde vruchtbaarheid te screenen en deze te relateren aan het succes van de IVF/ICSI behandeling.

In **hoofdstuk III** worden twee methoden om de eiwitsamenstelling van follikelvocht te bestuderen beschreven. Hieruit blijkt dat de IEF methode een gevoelige methode is om eiwitten in follikelvocht te identificeren. Om de invloed van voeding en foliumzuur beter in kaart te brengen hebben wij in **hoofdstuk IV** onderzocht wat de invloed van het gebruik van een foliumzuur tablet op de eiwitsamenstelling van follikelvocht is. Uit deze studie blijkt dat foliumzuur vele verschillende processen beïnvloed, waaronder die van de cholesterol stofwisseling. Er werden hogere gehaltes van apolipoproteinen gemeten in het follikelvocht van foliumzuur supplement gebruikers. Opmerkelijk was dat foliumzuur gebruikers een lager C-reactief eiwit gehalte in het follikelvocht hadden, wat een maat is voor actieve ontstekingsprocessen. Ook lijkt het erop dat de follikels van foliumzuur gebruikers beter ontwikkeld zijn, blijkend uit de lagere concentraties van cytokeratinen.

Het doel van de studie beschreven in **hoofdstuk VI** was om de invloed van het dieet van de vrouw op de ovariële response te kwalificeren en kwantificeren. Met behulp van Principal Component Analyse identificeren wij een dieet dat werd gekenmerkt door veel fruit, volkoren producten en margarine. In volgende analyses stellen wij vast dat naarmate dit dieet sterker wordt aangehouden, de ovariële response op OH lager wordt. Op dezelfde wijze is er een verband tussen het gebruik van dit dieet en een hogere kans op een doorgaande zwanger na de IVF/ICSI behandeling. In **hoofdstuk V**, komt

naar voren dat de foliumzuur concentraties in het bloed een vergelijkbaar verband vertonen met de ovariële response op OH. Gezien de relatief kleine groepen was het niet mogelijk om de invloed van foliumzuur op de kans op een doorgaande zwangerschap te kwantificeren.

In **hoofdstuk VII** wordt ingegaan op de klinische implementatie van de bevindingen uit dit proefschrift aangaande de invloed van voeding op de kans op een doorgaande zwangerschap. Hierin laten wij zien dat het mogelijk is om voeding- en leefstijl gewoonten te screenen met behulp van een simpele klinisch toepasbare vragenlijst en dat de berekende preconceptionele voedingsscore samenhangt met de kans op een doorgaande zwangerschap na IVF/ICSI behandeling. Hiermee worden de bevindingen in **hoofdstuk VI** bevestigd.

In **hoofdstuk VIII** wordt ingegaan op de implicaties van de bevindingen uit dit proefschrift voor toekomstig onderzoek en preconceptiezorg. Hierbij ligt de nadruk op het verder kwantificeren en kwalificeren van de invloed van voeding en foliumzuur op de stofwisseling van de ovariële follikel. Ook wordt de studie opzet besproken die nodig is om de invloed van voeding- en leefstijlfactoren op IVF/ICSI behandeling in kaart te brengen.

Introduction & Aim

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REFERENCES
AUTHORS AND AFFILIATIONS
LIST OF ABBREVIATIONS
PHD PORTFOLIO
ABOUT THE AUTHOR
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LIST OF ABBREVIATIONS

5-mTHF	5-methyltetrahydrofolate
5,10-mTHF	5,10-methylenetetrahydrofolate
AHCY	S-Adenosylhomocysteine hydrolase
AMH	Anti-Müllerian Hormone
AHP	Achieving a Healthy Pregnancy
ART	Assisted Reproduction Technique
BHMT	Betaine-Homocysteine Methyltransferase
BMI	Body Mass Index
CBS	Cystathione-β-Synthase
CD	Cycle Day
CHD	Congenital Heart Defects
COH	Controlled Ovarian Hyperstimulation
CRP	C-Reactive Protein
CTH	Cystathione-y-lyase
dTMP	Deoxythymidine Monophosphate.
dUMP	Deoxyuridine Monophosphate.
DS	Down Syndrome
DHF	Dihydrofolate
FFQ	Food Frequency Questionnaire
FOLFO	Food Lifestyle and Fertility Outcome
FSH	Follicle Stimulating Hormone
FSHR	Follicle Stimulating Hormone Receptor
FR	Folate Receptor
GnRH	Gonadotropin Releasing Hormone
GM	Geometric Mean
GNMT	Glycine-N-Methyltransferase
hCG	human Chorionic Gonadotropin
HDL	High Density Lipoprotein
ICSI	Intracytoplasmic Sperm Injection
IEF	Isoelectric Focusing
IGF	Insulin-like Growth Factor
IVF	In Vitro Fertilization
IQR	Interquartile Range
LC-MS/MS	Liquid Chromatography Tandem Mass Spectrometry
MAT	Methionine Adenosyltransferase
MD	Methyl Deficient
MTHFR	5,10-methylenetetrahydrofolate Reductase
MTR	Methionine synthase
MTRR	Methionine synthase reductase

NTD	Neural Tube Defects
OFC	Orofacial Clefts
PCA	Principal Components Analysis
PDR	Preconception Dietary Risk Score
RBC	Red Blood Cell
ROS	Reactive Oxygen Species
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SAH	S-Adenosylhomocysteine
SAM	S-Adenosylmethionine
SHMT	Serine Hydroxymethyltransferase
tHcy	total Homocysteine
THF	Tetrahydrofolate
TYMS	Thymidylate synthase

PHD PORTFOLIO

Name PhD student:	J.M. Twigt
Erasmus MC Dept.:	Obstetrics & Gynaecology
Supervisors:	Prof. Dr. R.P.M. Steegers-Theunissen Prof. Dr. J.S.E. Laven

General courses	**Year**
- Principles of Research in Medicine and Epidemiology	2007
- Methods of Clinical Research	2007
- Clinical Trials	2007
- Pharmaco-epidemiology	2007
- Case-control studies	2007
- Introduction to Decision-making in Medicine	2007
- Study Design	2007
- Introduction to Data-analysis	2008
- Regression Analysis	2008
- Topics in Meta-analysis	2008
- Survival Analysis	2008
- Modern Statistical Methods	2008
- Introduction to Clinical Research	2008
- Advanced Topics in Decision-making in Medicine	2008
- Intervention Research and Clinical Trials	2008
- Diagnostic Research	2008
- Prognosis Research	2008
- Biomedical English Writing and Communication	2009
- Management in Health Care Organizations	2009
- Ethical Basis: Health Care Delivery	2009
- Pharmaco-epidemiology and Drug Safety	2010
- Advanced Analysis of Prognosis Studies	2010

Seminars and workshops	
- Reprotox meeting, RIVM Rijswijk	2009
- Reprotox meeting, TNO Driebergen	2010
- RCOG onderzoeksdag / Wladimiroff symposium, Rotterdam	2009/2010/2012
- VFS Meeting, Leuven	2010
- Wetenschapslunch Cluster 12	2010/2011/2012

Presentations

- Preconception folic acid use modulates the endocrine and follicular response to ovarian stimulation treatment (poster)[1]	2009
- Proteomic analysis of human follicular fluid (poster)[1]	2009
- Does folate modulate the inhibitory effect of AMH on aromatase? (poster)[2]	2010
- Foliumzuur supplement gebruik verlaagd de ovariele response op ovariele stimulatiebehandeling (poster)[3]	2011
- Dieet aanbevelingen en de kans op doorgaande zwangerschap na IVF/ICSI (oral)[5]	2011
- The preconception diet is associated with ongoing pregnancies in women undergoing IVF/ICSI treatment (oral)[4]	2011
- Preconception folic acid use modulates estradiol response and follicle count after ovarian stimulation in women (poster)[6]	2011
- Preconception nutritional and lifestyle counselling applied in a tertiary clinical setting, uptake of advice and the association with IVF/ICSI treatment outcomes (oral)[6]	2011
- De preconceptionele voedingsinname heeft invloed op de kans van slagen van de IVF/ICSI behandeling (poster)[8]	2012
- The preconception diet influences the chance of pregnancy after IVF/ICSI treatment (poster)[9]	2012
- The ovarian response to stimulation treatment is beneficially modified by a diet high in fruit and whole grains (poster)[9]	2012

(Inter)national conferences

- Society of Gynaecological Investigation, Glasgow, UK[1]	2009
- Society of Gynaecological Investigation, Orlando, US[2]	2010
- Gynaecongres, Breda, NL[3]	2010
- Society of Gynaecological Investigation, Miami, US[4]	2011
- Nationaal Preconceptiezorg congres, Nieuwegein, NL[5]	2011
- Periconceptional Develop. Program, Jerusalem, IL[6]	2011
- EPMA World Congress, Bonn, DE[7]	2011
- Gynaecongres, Arnhem, NL[8]	2011
- Society of Gynaecological Investigation, San Diego, US[9]	2012

Supervising practicals and excursions, tutoring

- Principles of research in medicine and epidemiology, teaching assistant, NIHES	2010
- Supervising practical, Basic introduction course to SPSS, Molmed	2011

Supervising Master's theses

- The preconception diet is associated with ongoing pregnancies in women undergoing IVF/ICSI treatment, Mette Bolhuis	2011
- The effect of folic acid on DNA methylation of zebra fish embryos, Josja Graafland	2011

Other

- Preconception counselling of subfertile couples	2009-2012

ABOUT THE AUTHOR

John Twigt is geboren op 17 januari 1986 en woont de eerste twee jaar van zijn leven in Berkel en Rodenrijs. In 1988 verhuist hij naar Helmond, waarna Zwolle (1990), Genemuiden (1992) en Woerden (1999) als woonplaatsen volgen. In 2007 kiest John voor het eerst zijn eigen woonplaats uit: Rotterdam. Al deze periodes dragen bij aan zijn persoonlijkheid in zijn jonge leven: het bourgondische van Brabant, de nuchterheid van het Noorden en de bravoure uit het Westen zijn terug te vinden in wie hij nu is.

In Genemuiden brengt hij het grootste deel van zijn jeugd door. Op school komt John goed mee, maar het liefst is hij toch aan het spelen met vrienden. In Zwolle begint hij op het Carolus Clusius College aan zijn middelbare schooltijd, die hij in Woerden op het Kalsbeek college met het behalen van zijn HAVO en VWO diploma in 2005 afrond.

Sportief gezien is John zijn eerste jaren als voetballer actief bij SC Genemuiden. Zijn middelbare schooltijd staat op dat vlak echter in het teken van het squashen. All-in in Utrecht is zijn thuisbasis en iedere week is hij daar of elders in het land te vinden voor trainingen en wedstrijden. Die periode levert hem plezier en vriendschappen op en leren hem gedisciplineerd en geconcentreerd aan doelen te werken.

Wat John ‘later wil worden' is voor iedereen lang onduidelijk. Rond zijn zestiende denk hij eraan om piloot te worden en in het laatste jaar van het VWO komt daar de interesse voor geneeskunde bij. De aanmelding voor die opleidingen zijn dan ook zijn keuzes na behalen van zijn VWO diploma. Vanaf het moment dat hij aan zijn studie begint, is het duidelijk dat hij zijn plek heeft gevonden. In juni 2010 studeert John af en in augustus van dat jaar behaalt hij zijn Master. De mogelijkheid om te promoveren op de afdeling Gynaecologie pakt John met beide handen aan. Hij bezoekt daarbij verschillende congressen in de VS, Engeland en Israël waar hij zelf ook presentaties geeft over de resultaten van zijn onderzoek.

Inmiddels is John bezig met zijn co-schappen en staat hij opnieuw voor belangrijke keuzes. In de eerste zeventwintig jaar van zijn leven heeft hij zich ontwikkeld tot een evenwichtig man met vele talenten en interesses. Hij is intelligent, onafhankelijk, zelf bewust met zo af en toe een vleugje arrogantie. Tegelijkertijd is hij warm en zorgzaam en zit zijn hart op de goede plek.

Fred en Marieke Twigt

DANKWOORD

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Now this is not the end.
It is not even the beginning of the end.
But it is, perhaps, the end of the beginning.
Sir Winston Churchill, November 1942