The homocysteine pathway in human subfertility

Jolanda C. Boxmeer

The homocysteine pathway in human subfertility

The work presented in this thesis was performed at the Department of Obstetrics and Gynaecology, Division of Reproductive Medicine, Erasmus MC, University Medical Centre, Rotterdam, The Netherlands.

ISBN 978-90-8559-477-2

Coverdesign: hanontwerper.nl
Layout: Optima Grafische Communicatie, Rotterdam
Printing: Optima Grafische Communicatie, Rotterdam

Parts of this thesis are based upon manuscripts that have been published previously. Published manuscripts have been reproduced with explicit permission from the publishers: Boxmeer JC, et al. Seminal plasma cobalamin significantly correlates with sperm concentration in males undergoing IVF or ICSI procedures. *J Androl. 2007;28(4):521-7*. Boxmeer JC, et al. Preconception folic acid treatment affects the micro-environment of the maturing oocyte in human. *Fertil Steril. 2008;89(6):1766-70*. Boxmeer JC, et al. Low folate in seminal plasma is associated with increased sperm DNA damage. *Fertil Steril. 2008 Aug 21*. [Epub ahead of print]. Boxmeer JC, et al. Homocysteine metabolism in the pre-ovulatory follicle during ovarian stimulation. *Hum Reprod. 2008 Jul 29*. [Epub ahead of print].

Printing of this thesis was financially supported by: Erasmus Universiteit Rotterdam, Schering-Plough, Merck Serono, Ferring BV, Wyeth Pharmaceuticals BV, Bayer Schering Pharma, Solgar BV, Memidis Pharma BV and Medical Dynamics.

The homocysteine pathway in human subfertility

Proefschrift

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

Prof.dr. S.W.J. Lamberts

en volgens het besluit van het College voor Promoties.

De openbare verdediging zal plaats vinden op
woensdag 14 januari om 11:45 uur

door

Jolanda Clasina Boxmeer
geboren te Delft

PROMOTIECOMMISSIE

Promotoren:	Prof.dr. E.A.P. Steegers
	Prof.dr. N.S. Macklon
Overige leden:	Prof.dr. C.H. Bangma
	Prof.dr. B.C.J.M. Fauser
	Prof.dr. F.H. de Jong
Co-promotor:	Dr. R.P.M. Steegers-Theunissen

Voor mijn vader

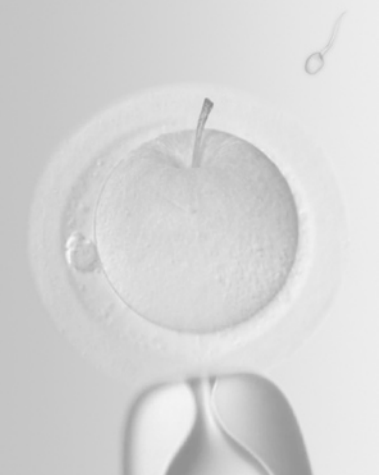
Contents

LIST OF ABBREVIATIONS

ART	Artificial reproduction technique
BMI	Body mass index
CCMO	Dutch Central Committee for Human Research
CD	Cycle day
CI	Confidence interval
DAG	Directed acyclic graph
DFI	DNA fragmentation index
DNA	Deoxyribonucleic acid
E2	Estradiol
ET	Embryo transfer
et al.	And others
FSH	Follicle stimulating hormone
hCG	human chorionic gonadotrophin
ICSI	Intracytoplasmic sperm injection
IU	International units
IVF	In vitro fertilization
L	Litre
MESA	Microsurgical epididymal sperm aspiration
mg	Milligram
µmol	Micromol
n	Number
nmol	Nanomol
NTD	Neural tube defects
OHSS	Ovarian hyperstimulation syndrome
OR	Odds Ratio
PESA	Percutaneous epididymal sperm aspiration
PGS	Preimplantation genetic screening
PLP	Pyridoxal'5-phosphate
pmol	Picomol
RBC	Red blood cell
rFSH	Recombinant follicle stimulating hormone
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SCSA	Sperm Chromatin Structure Assay
SD	Standard deviation
SHBG	Sex hormone binding globulin
tHcy	Total homocysteine
WHO	World Health Organisation

Chapter 1

General Introduction



Subfertility, defined as at least one year of unprotected intercourse with the same partner without conception, affects ten to fifteen percent of couples in the western world. Depending on the duration of the subfertility, a female factor is the dominant cause of subfertility in around 50%, a male factor in 20-26% and the cause is unexplained in 25-30% (1). In most couples, subfertility is of multifactorial origin and should therefore be considered as a complex disease (2). Whereas genetic causes are difficult to modulate, environmental and lifestyle factors implicated in reproduction are potentially amendable to curative or preventive measures. Worldwide, an increasing number of couples are treated by assisted reproductive technology (ART) to achieve pregnancy (3, 4). Currently, the chance of achieving a clinical pregnancy is around 28-31% per started in vitro fertilization (IVF) cycle with or without intracytoplasmic sperm injection (ICSI) in the Netherlands (5). The advantage of the introduction of IVF and ICSI treatment is that it gives access to and information about the direct environment of the gametes and early developing embryo. On the other hand, IVF and ICSI treatment is introducing an artifactual endocrinologic milieu which may affect the quality of the embryo. Whether concentrations of nutrients and enzymes change due to ovarian hyperstimulation is unclear, but it has already been demonstrated that different ovarian stimulation regimens increase the rates of embryo aneuploidy (6).

Follicular fluid represents the direct environment of maturing oocytes. Likewise, spermatozoa in the ejaculate are surrounded by seminal plasma, which contains a broad spectrum of components, including nutrients and enzymes, which protect the sperm and maintain its motility. Conventional determinants of sperm quality consist of the semen parameters volume, sperm concentration, motility and morphology. The DNA fragmentation index (DFI) as assessed by the Sperm Chromatin Structure Assay (SCSA), is also considered as an independent measure of sperm quality, with even a better diagnostic and prognostic capability than the conventional semen parameters (7, 8).

Evidence for an important role of the homocysteine pathway in fertility is increasing (9, 10). In this pathway the B-vitamins folate, cobalamin and pyridoxine serve as cofactors (Figure 1). Several intermediates of the homocysteine pathway are directly involved in the synthesis of proteins, the synthesis and repair of DNA, and balance the degree of oxidative stress, which are critical intermediates in gametogenesis. Therefore, derangements in this pathway in both women and men resulting in hyperhomocysteinaemia, are suggested to be detrimental for reproduction (9). Maternal hyperhomocysteinaemia is associated with adverse pregnancy outcome, such as recurrent miscarriages, pregnancy induced hypertension, abruptio placentae and several congenital abnormalities (11-16). The importance of the maternal folate status in the development of neural tube defects (NTD) and other congenital abnormalities, such as orofacial clefts and congenital heart disease is well known (17-19). In addition, a population-based, matched, case-control study demonstrated an association between low maternal plasma folate and an increased risk of early spontaneous abortion (20). Similarly, maternal low cobalamin (vitamin B12) status

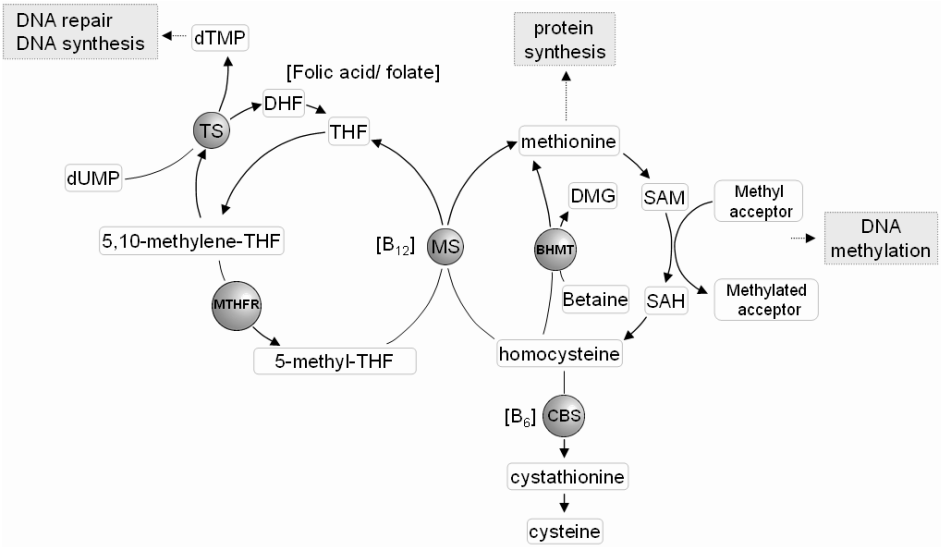


Figure 1. The homocysteine pathway
5-methyl-THF = 5-methyltetrafolate; MS = methionine synthase; B12 = cobalamin; B6 = pyridoxal '5-phosphate; dTMP = deoxythymidine '5-monophosphate; dUMP = deoxyuridine '5-monophosphate; TS = thymidylate synthase; DHF = dihydrofolate; THF = tetrahydrofolate; MTHFR = 5,10-methylene-THF reductase; DMG = dimethylglycine; BHMT = betaine homocysteine methyltransferase; SAM = S-adenosylmethionine; SAH = S-adenosylhomocysteine; CBS = cystathione synthase.

has been associated with a higher risk of neural tube defects and spontaneous abortions (10, 21-23). Recent case-control studies showed a reduction in the risk of orofacial clefts (24) and some decrease in congenital heart disease by pyridoxine (vitamin B6) (25). Pyridoxine may also have a role in the prevention of pre-eclampsia (26) and preterm birth (27). However, in a recent Cochrane review it was concluded that there is not enough evidence to detect clinical benefits of pyridoxine supplementation in pregnancy (28).

Recent publications suggest that the homocysteine pathway is not only important for reproductive outcome, but for fertility as well (10, 29, 30). This subject is rather new and requires further investigation. After an overview of the literature on the role of the homocysteine pathway in human subfertility, the objectives and outline of this thesis are described.

THE HOMOCYSTEINE PATHWAY IN HUMAN SUBFERTILITY

Homocysteine

In the homocysteine metabolism 5-methyltetrafolate (5-methyl-THF), the reduced form of folate, is the primary methyl donor for remethylation of total homocysteine (tHcy) into methionine (Figure 1). Methionine synthase, using cobalamin as co-factor, is essential

in this conversion. Methionine provides the methyl groups necessary for the formation of S-adenosylmethionine, which is involved in numerous cellular reactions, including methylation of phospholipids, proteins, DNA, RNA, amino acids and neurotransmitters. Hyperhomocysteinaemia can be induced by genetic factors, for example cystathione synthase (CBS) deficiencies or the MTHFR 677 C→T polymorphism, but also by a deficient intake of folate, cobalamin or pyridoxine (31). Around 50% of tHcy is transformed into cysteine for which pyridoxine is required as a cofactor.

The reported detrimental effects of high tHcy are direct cellular toxicity, endothelial damage, excessive generation of reactive oxygen species (ROS), inhibition of transmethylation reactions, and aberrant gene expressions. Physiological levels of ROS in follicular fluid are necessary for oocyte maturation (32), ovulation (33) and fertilization (9). However, high tHcy in follicular fluid may also increase the release of ROS, thereby inducing excessive oxidative stress.

A significant correlation between the concentration of tHcy in blood and follicular fluid has been established (34). A Polish group demonstrated significantly lower tHcy concentrations in follicular fluid and blood in folic acid supplemented women (35). Total homocysteine has also been determined in seminal plasma (29). Recently, an association has been demonstrated between high tHcy concentrations both in follicular fluid and the ejaculate and a higher risk of moderate to low embryo quality in IVF treatment (29).

Folate

Folate is a B-vitamin present in its natural form in fruits and vegetables. Folic acid is the synthetic form of folate and used in supplements and fortified foods, because of its high bioavailability and chemical stability. Folate is required for the synthesis of thymine and purines, precursors for DNA and RNA synthesis, and for DNA methylation and repair. A deficient intake of folate induces a mild hyperhomocysteinaemia. Since the early nineties periconception folic acid use is recommended to women planning pregnancy in order to prevent NTDs (36, 37). Whether the use of folic acid leads to an increased rate of twinning is still a subject of debate (38-41).

A significant correlation between the concentration of folate in blood and follicular fluid has been established (34). Similarly, folate concentrations in seminal plasma and blood are significantly correlated (42). Moreover, in seminal plasma other folate metabolites than methyl-tetrahydrofolate positively correlated with sperm density and total sperm count (42). Further evidence for the significant role of folate in spermatogenesis is derived from a randomized controlled trial demonstrating a 74% increase in sperm count in subfertile men after folic acid and zinc sulphate intervention for six months (30). Independent of the fertility state, sperm concentration significantly increased in wild types C677T MTHFR polymorphism, suggesting a role of folate genes in spermatogenesis (43).

Cobalamin

Cobalamin, or vitamin B12, is a B-vitamin present in animal products, such as beef, fish, eggs and milk. Several small studies and case reports in the sixties and seventies described cobalamin deficient subfertile women who became pregnant after cobalamin suppletion, but the underlying mechanisms are not clear (21, 44-47). Cobalamin concentrations in blood and follicular fluid are significantly correlated (34).

Likewise the effect of cobalamin on sperm quality is not clarified, although there is some evidence that this B-vitamin affects sperm parameters (48, 49). During the sixties and seventies several studies described the successful treatment of subfertile men following treatment of a cobalamin deficiency (50, 51).

Pyridoxine

Pyridoxine, or vitamin B6, is present in a variety of foods with a high content in soybeans, bran rice, walnuts and chicken breast. Vitamin B6-dependent coenzymes participate in numerous reactions involved in the metabolism of amino acids, heme, and myelin synthesis (28). Vitamin B6 deficiency has also been associated with impairment of enzymes involved in the structural integrity of arterial walls (52), which could affect implantation and early placental development.

In a prospective observational study in Chinese women, deficient pyridoxine concentrations in blood were associated with lower conception rates and a higher rate of early pregnancy loss (53). Pyridoxine has previously been determined in follicular fluid (34).

There is no information about the effects of pyridoxine on male reproduction. Pyridoxine has not been determined in human seminal plasma before. From animal studies it is known that high doses of pyridoxine impair sperm motility and sperm count and cause histopathological changes including degeneration of germinal epithelial cells (54, 55).

In conclusion, derangements of the homocysteine pathway are associated with parameters that may affect fertility. For the interpretation of the associations between biomarker concentrations and fertility outcome parameters it is important to know whether clinical factors, such as ovarian hyperstimulation, are affecting concentrations of the biomarkers of the homocysteine pathway. Moreover, most studies used multifollicular fluids instead of the more precise monofollicular fluids. Furthermore, biomarker concentrations have not been studied in association with sperm DNA-damage and the achievement of pregnancy. Therefore, the aim of this thesis is to further study the homocysteine pathway by improved methodologies en techniques in association with parameters of human fertility.

OBJECTIVES OF THIS THESIS

The main objective was to investigate the role of the homocysteine pathway in human fertility.

The detailed objectives of Part I (Women):

- To identify factors that affect folate and tHcy in follicular fluid.
- To investigate whether ovarian hyperstimulation changes B-vitamins and tHcy in blood.
- To determine associations between B-vitamins and tHcy in blood and follicular fluid and parameters of oocyte quality.
- To investigate associations between B-vitamins and tHcy in blood and follicular fluid and the outcome of IVF.

The detailed objectives of Part II (Men):

- To investigate associations between B-vitamins and tHcy in blood and seminal plasma.
- To investigate associations between B-vitamins and tHcy in blood and seminal plasma and conventional semen parameters and DFI.

OUTLINE OF THE THESIS

In Part I biomarkers of the homocysteine pathway are studied in women undergoing an IVF or ICSI treatment. Chapter 2 describes a pilot study in which factors affecting concentrations of biomarkers from the homocysteine pathway in follicular fluid are investigated. In chapter 3 associations between these biomarkers in multifollicular fluid and IVF outcome are investigated in the same study. Subsequently, a prospective observational study is performed in which blood, monofollicular fluid and information about the IVF outcome is obtained from women undergoing IVF or ICSI treatment. The effects of ovarian hyperstimulation on tHcy and B-vitamins determined in blood are studied in chapter 4. In chapter 5 associations between these biomarkers in blood and monofollicular fluid and IVF outcome are investigated.

In Part II biomarkers of the homocysteine pathway are studied in men of couples undergoing an IVF or ICSI treatment. In chapter 6 and 7, associations between concentrations of tHcy, folate, cobalamin, and pyridoxine in blood and seminal plasma are determined. Chapter 6 describes associations between biomarker concentrations and semen parameters, including volume, sperm concentration, total sperm count, progressive motility and

normal morphology. In addition to the conventional semen analysis, the DFI is studied in chapter 7. In this chapter associations between the biomarkers of the homocysteine pathway in blood and seminal plasma and conventional semen parameters as well as DFI are studied.

Finally, chapter 8 provides a general discussion of the main findings and reflects on implications for clinical practice and future research.

REFERENCES

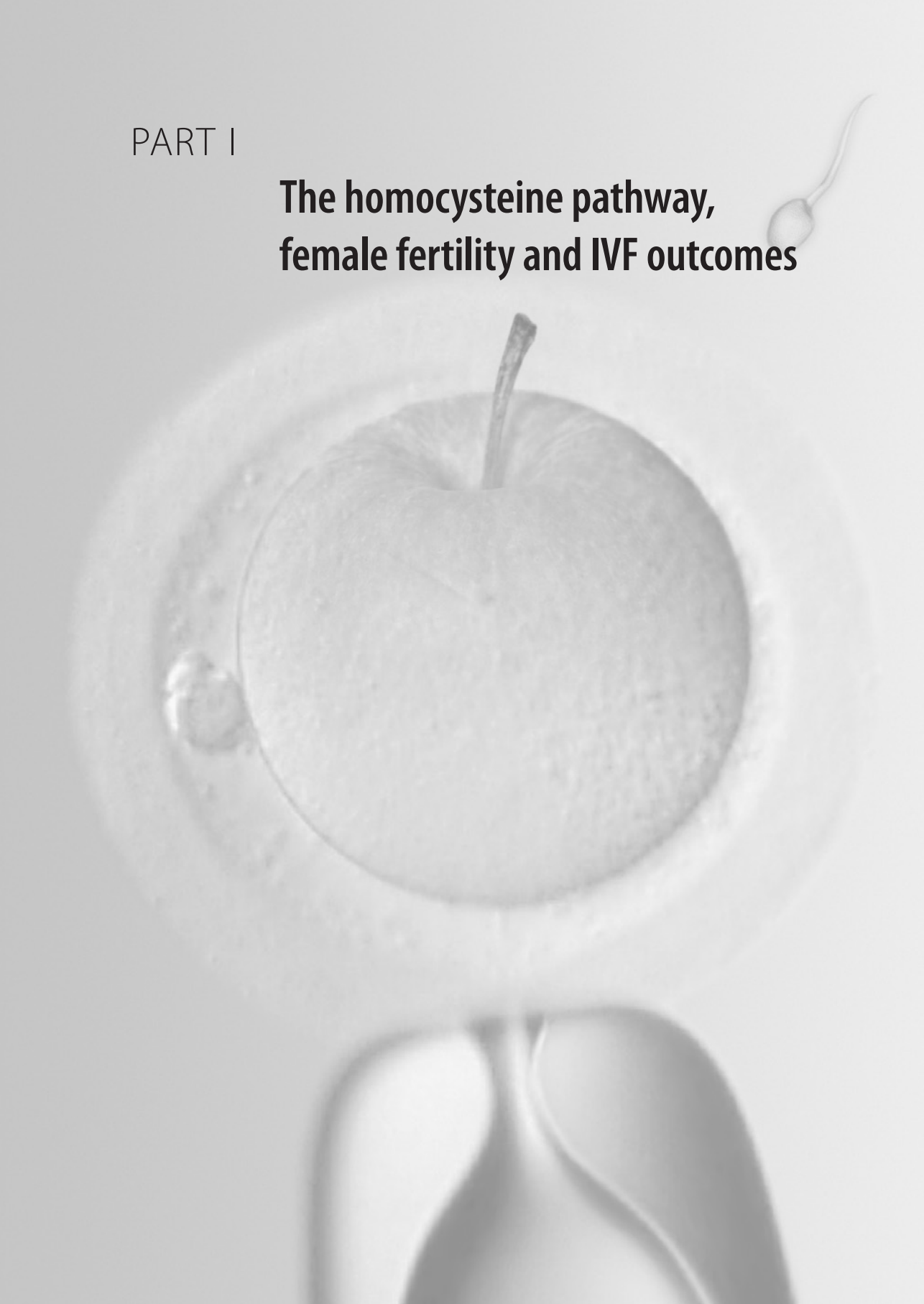
1. Evers JL. Female subfertility. Lancet 2002;360:151-9.
2. Steegers-Theunissen RP, Steegers EA. Nutrient-gene interactions in early pregnancy: a vascular hypothesis. Eur J Obstet Gynecol Reprod Biol 2003;106:115-7.
3. Jain T, Gupta RS. Trends in the use of intracytoplasmic sperm injection in the United States. N Engl J Med 2007;357:251-7.
4. Andersen AN, Goossens V, Gianaroli L, Felberbaum R, de Mouzon J, Nygren KG. Assisted reproductive technology in Europe, 2003. Results generated from European registers by ESHRE. Hum Reprod 2007; 22:1513-25.
5. Kremer J. Landelijke IVF-cijfers 1996-2006. In: LIR S, ed.: NVOG, 2007.
6. Baart EB, Martini E, Eijkemans MJ, Van Opstal D, Beckers NG, Verhoeff A *et al.* Milder ovarian stimulation for in-vitro fertilization reduces aneuploidy in the human preimplantation embryo: a randomized controlled trial. Hum Reprod 2007;22:980-8.
7. Agarwal A, Said TM. Role of sperm chromatin abnormalities and DNA damage in male infertility. Hum Reprod Update 2003;9:331-45.
8. Giwercman A, Richthoff J, Hjollund H, Bonde JP, Jepson K, Frohm B *et al.* Correlation between sperm motility and sperm chromatin structure assay parameters. Fertil Steril 2003;80:1404-12.
9. Ebisch IM, Thomas CM, Peters WH, Braat DD, Steegers-Theunissen RP. The importance of folate, zinc and antioxidants in the pathogenesis and prevention of subfertility. Hum Reprod Update 2007;13: 163-74.
10. Forges T, Monnier-Barbarino P, Alberto JM, Gueant-Rodriguez RM, Daval JL, Gueant JL. Impact of folate and homocysteine metabolism on human reproductive health. Hum Reprod Update 2007;13: 225-38.
11. Hague WM. Homocysteine and pregnancy. Best Pract Res Clin Obstet Gynaecol 2003;17:459-69.
12. Steegers-Theunissen RP, Boers GH, Blom HJ, Trijbels FJ, Eskes TK. Hyperhomocysteinaemia and recurrent spontaneous abortion or abruptio placentae. Lancet 1992;339:1122-3.
13. Wong WY, Eskes TK, Kuijpers-Jagtman AM, Spauwen PH, Steegers EA, Thomas CM *et al.* Nonsyndromic orofacial clefts: association with maternal hyperhomocysteinemia. Teratology 1999;60:253-7.
14. Verkleij-Hagoort AC, Verlinde M, Ursem NT, Lindemans J, Helbing WA, Ottenkamp J *et al.* Maternal hyperhomocysteinaemia is a risk factor for congenital heart disease. BJOG 2006;113:1412-8.
15. Steegers-Theunissen RP, Boers GH, Trijbels FJ, Eskes TK. Neural-tube defects and derangement of homocysteine metabolism. N Engl J Med 1991;324:199-200.
16. Nelen WL, Blom HJ, Steegers EA, den Heijer M, Thomas CM, Eskes TK. Homocysteine and folate levels as risk factors for recurrent early pregnancy loss. Obstet Gynecol 2000;95:519-24.
17. Tamura T, Picciano MF. Folate and human reproduction. Am J Clin Nutr 2006;83:993-1016.
18. Botto LD, Olney RS, Erickson JD. Vitamin supplements and the risk for congenital anomalies other than neural tube defects. Am J Med Genet C Semin Med Genet 2004;125:12-21.
19. van Rooij IA, Ocke MC, Straatman H, Zielhuis GA, Merkus HM, Steegers-Theunissen RP. Periconceptional folate intake by supplement and food reduces the risk of nonsyndromic cleft lip with or without cleft palate. Prev Med 2004;39:689-94.
20. George L, Mills JL, Johansson AL, Nordmark A, Olander B, Granath F *et al.* Plasma folate levels and risk of spontaneous abortion. JAMA 2002;288:1867-73.
21. Bennett M. Vitamin B-12 deficiency, infertility and recurrent fetal loss. J Reprod Med 2001;46:209-12.
22. Ray JG, Wyatt PR, Thompson MD, Vermeulen MJ, Meier C, Wong PY *et al.* Vitamin B12 and the risk of neural tube defects in a folic-acid-fortified population. Epidemiology 2007;18:362-6.
23. Groenen PM, van Rooij IA, Peer PG, Gooskens RH, Zielhuis GA, Steegers-Theunissen RP. Marginal maternal vitamin B12 status increases the risk of offspring with spina bifida. Am J Obstet Gynecol 2004;191:11-7.

24. Krapels IP, van Rooij IA, Ocke MC, van Cleef BA, Kuijpers-Jagtman AM, Steegers-Theunissen RP. Maternal dietary B vitamin intake, other than folate, and the association with orofacial cleft in the offspring. Eur J Nutr 2004;43:7-14.
25. Czeizel AE, Puho E, Banhidy F, Acs N. Oral pyridoxine during pregnancy: potential protective effect for cardiovascular malformations. Drugs R D 2004;5:259-69.
26. de la Calle M, Usandizaga R, Sancha M, Magdaleno F, Herranz A, Cabrillo E. Homocysteine, folic acid and B-group vitamins in obstetrics and gynaecology. Eur J Obstet Gynecol Reprod Biol 2003;107: 125-34.
27. Ronnenberg AG, Goldman MB, Chen D, Aitken IW, Willett WC, Selhub J *et al.* Preconception homocysteine and B vitamin status and birth outcomes in Chinese women. Am J Clin Nutr 2002;76:1385-91.
28. Thaver D, Saeed MA, Bhutta ZA. Pyridoxine (vitamin B6) supplementation in pregnancy. Cochrane database of systematic reviews (Online) 2006:CD000179.
29. Ebisch IM, Peters WH, Thomas CM, Wetzels AM, Peer PG, Steegers-Theunissen RP. Homocysteine, glutathione and related thiols affect fertility parameters in the (sub)fertile couple. Hum Reprod 2006; 21:1725-33.
30. Wong WY, Merkus HM, Thomas CM, Menkveld R, Zielhuis GA, Steegers-Theunissen RP. Effects of folic acid and zinc sulfate on male factor subfertility: a double-blind, randomized, placebo-controlled trial. Fertil Steril 2002;77:491-8.
31. Refsum H, Smith AD, Ueland PM, Nexo E, Clarke R, McPartlin J *et al.* Facts and recommendations about total homocysteine determinations: An expert opinion. Clin Chem 2004;50:3-32.
32. Riley JC, Behrman HR. Oxygen radicals and reactive oxygen species in reproduction. Proc Soc Exp Biol Med 1991;198:781-91.
33. Miyazaki T, Sueoka K, Dharmarajan AM, Atlas SJ, Bulkley GB, Wallach EE. Effect of inhibition of oxygen free radical on ovulation and progesterone production by the in-vitro perfused rabbit ovary. J Reprod Fertil 1991;91:207-12.
34. Steegers-Theunissen RP, Steegers EA, Thomas CM, Hollanders HM, Peereboom-Stegeman JH, Trijbels FJ *et al.* Study on the presence of homocysteine in ovarian follicular fluid. Fertil Steril 1993;60:1006-10.
35. Szymanski W, Kazdepka-Zieminska A. [Effect of homocysteine concentration in follicular fluid on a degree of oocyte maturity]. Ginekol Pol 2003;74:1392-6.
36. Botto LD, Lisi A, Robert-Gnansia E, Erickson JD, Vollset SE, Mastroiacovo P *et al.* International retrospective cohort study of neural tube defects in relation to folic acid recommendations: are the recommendations working? BMJ 2005;330:571.
37. Gezondheidsraad V. Vervolgadvies inzake foliumzuurvoorziening in relatie tot neuraalbuisdefecten. In: Gezondheidsraad DH, ed., 1993.
38. Boxmeer JC, Fauser BC, Macklon NS. Effect of B vitamins and genetics on success of in-vitro fertilisation. Lancet 2006;368:200.
39. Muggli EE, Halliday JL. Folic acid and risk of twinning: a systematic review of the recent literature, July 1994 to July 2006. Med J Aust 2007;186:243-8.
40. Berry RJ, Kihlberg R, Devine O. Impact of misclassification of in vitro fertilisation in studies of folic acid and twinning: modelling using population based Swedish vital records. BMJ 2005;330:815.
41. Haggarty P, McCallum H, McBain H, Andrews K, Duthie S, McNeill G *et al.* Effect of B vitamins and genetics on success of in-vitro fertilisation: prospective cohort study. Lancet 2006;367:1513-9.
42. Wallock LM, Tamura T, Mayr CA, Johnston KE, Ames BN, Jacob RA. Low seminal plasma folate concentrations are associated with low sperm density and count in male smokers and nonsmokers. Fertil Steril 2001;75:252-9.
43. Ebisch IM, van Heerde WL, Thomas CM, van der PN, Wong WY, Steegers-Theunissen RP. C677T methylenetetrahydrofolate reductase polymorphism interferes with the effects of folic acid and zinc sulfate on sperm concentration. Fertil Steril 2003;80:1190-4.

44. Jackson I, Doig WB, McDonald G. Pernicious anaemia as a cause of infertility. Lancet 1967;290:1159-60.
45. Girdwood RH, Eastwood MA, Finlayson ND, Graham GS. Pernicious anaemia as a cause of infertility in twins. Lancet 1971;1:528-30.
46. Varadi S. Pernicious anaemia and infertility. Lancet 1967;290:1350.
47. Mahmood A. Pernicious anaemia as a cause of infertility. Lancet 1971;297:753.
48. Watson AA. Seminal vitamin B12 and sterility. Lancet 1962;280:644.
49. Tomaszewski L, Zmudzka B, Nadworny J. Seminal vitamin B12 and sterility. Lancet 1963;281:170.
50. Blair JH, Stearns HE, Simpson GM. Vitamin B 12 and fertility. Lancet 1968;1:49-50.
51. Sharp AA, Witts LJ. Seminal vitamin B 12 and sterility Lancet 1962;280:779.
52. Levene CI, Murray JC. The aetiological role of maternal vitamin-B6 deficiency in the development of atherosclerosis. Lancet 1977;1:628-30.
53. Ronnenberg AG, Venners SA, Xu X, Chen C, Wang L, Guang W *et al.* Preconception B-vitamin and homocysteine status, conception, and early pregnancy loss. Am J Epidemiol 2007;166:304-12.
54. Plassmann S, Urwyler H. Improved risk assessment by screening sperm parameters. Toxicol Lett 2001; 119:157-71.
55. Tsutsumi S, Tanaka T, Gotoh K, Akaike M. Effects of pyridoxine on male fertility. J Toxicol Sci 1995;20: 351-65.

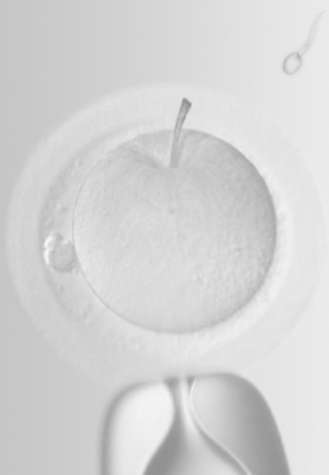
PART I

The homocysteine pathway, female fertility and IVF outcomes



Chapter 2

Preconception folic acid treatment affects the micro-environment of the maturing oocyte in human



Jolanda C. Boxmeer, R. Montserrate Brouns, Jan Lindemans, Eric A.P. Steegers, Elena Martini, Nick S. Macklon, Régine P.M. Steegers-Theunissen

Fertil Steril. 2008;89(6):1766-70

ABSTRACT

Study objective: To investigate the influence of folic acid supplementation on the follicular fluid concentrations of folate and total homocysteine and their relationship to the diameter of the follicle.

Design: Observational study.

Setting: Tertiary referral fertility clinic at the Erasmus MC, University Medical Center, Rotterdam, The Netherlands.

Patients: Thirty-seven women undergoing IVF or ICSI treatment.

Interventions: No interventions other than routine stimulation treatment and the recommendation of folic acid supplementation.

Main outcome measures: Concentrations of folate and total homocysteine in monofollicular and pooled follicular fluid, and the diameter of the follicle.

Results: Folic acid supplementation significantly increased folate and decreased total homocysteine concentrations in pooled follicular fluid. In monofollicular fluid folate concentrations only were significantly increased in supplemented women. The total homocysteine concentration appeared to be significantly correlated with the diameter of the follicle ($r = 0.27$). Samples from single follicles were less prone to artifacts in the measurements of the folate and total homocysteine concentration.

Conclusions: Preconception folic acid supplementation significantly alters both folate and total homocysteine concentrations in follicular fluid. The correlation between the diameter of the follicle and total homocysteine concentration in follicular fluid warrants further investigation.

INTRODUCTION

A primary deficiency of natural folate resulting in an increase of the total homocysteine (tHcy) concentration may be detrimental to the quality of the oocyte, subsequent fertilization, implantation, embryogenesis and fetal outcome (1-3). The presence of folate and tHcy in ovarian follicular fluid has previously been demonstrated (4). Interestingly, a low follicular fluid homocysteine concentration has been reported to be associated with a higher degree of maturation of the oocyte (5). More recently, a significant inverse association between the tHcy concentration in follicular fluid and embryo quality was demonstrated in women undergoing in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) treatment (6). Considerable evidence supports the preventive effects of periconception synthetic folic acid supplementation against the birth prevalence rate of several complex congenital malformations (7). Other beneficial reproductive effects of periconception folic acid use has been suggested as well (8). Although it has been suggested that folic acid supplementation stimulates the twinning rate, this is not yet clear (9, 10).

The B-vitamin folate is an important substrate in the synthesis of pyrimidines and purines. Moreover, folate is an important supplier of methyl groups to remethylate homocysteine into methionine. The methionine derivative S-adenosylmethionine is the most important methyl donor in the body for the methylation of lipids, proteins and DNA. Therefore, the cellular folate status is important for protein and DNA synthesis as well as the regulation of DNA expression by methylation. This is substantiated by molecular biologic studies that indicate that both folate deprivation and folic acid supplementation affect multiple metabolic pathways by controlling the expression of genes through methylation (11, 12).

During the preconception period gametogenesis and folliculogenesis take place, in which the requirement for folate is enhanced (13). After fertilization the blastocyst is demethylated, that is followed by the selective methylation of genes (12). A well-known marker of folate deficiency is an increased mean cell volume of red blood cells, which is defined as megaloblastic anemia. The underlying mechanism of this feature, however, is so far unknown. Whether other rapidly dividing cells show similar morphological features is not clear.

To further improve the methodology of future investigations into the influence of the microenvironment of the oocyte on its quality and the chance of successful fertilization, it is important to study some methodological issues as well. Therefore, the aims of this study are to examine: [1] the effects of preconception folic acid supplementation on follicular fluid concentrations of folate and tHcy in women undergoing IVF or ICSI treatment, [2] the differences between folate and tHcy concentrations in monofollicular and pooled follicular fluid, and [3] the correlations between folate, tHcy, and the diameter of the follicle.

MATERIALS AND METHODS

Population

Thirty-seven women with a median age of 34.4 years (range 27.2 - 40.7 years) underwent an IVF or ICSI treatment at the Department of Obstetrics and Gynecology, Division of Reproductive Medicine, Erasmus MC, University Medical Center, and were included in the study. The women were asked on the day of ovum pick up to report whether or not they were using supplements containing folic acid. The study protocol was performed, and the remnant follicular fluid samples were collected according to the guidelines of the Medical Ethical and Institutional Board of the Erasmus MC, University Medical Center in Rotterdam, The Netherlands.

Sample Collection

Follicular fluid was collected from each woman during ovum pick up from the largest follicle in the first ovary and the smallest follicle 10 mm diameter or more in the other ovary; the mean diameters (range) were 22 (15 - 28) and 16.1 (10 - 20) mm, respectively. The fluids collected from all other aspirated follicles were pooled per woman. After oocyte retrieval for the IVF or ICSI procedure, the follicular fluid samples were centrifuged for 10 minutes at 1,700 x g to separate red blood cells, leucocytes, and granulosa cells.

Laboratory Analysis

Follicular fluid folate concentrations were measured using electrochemiluminescence immunoassay (Elecsys 2010, Roche GmbH, Mannheim, Germany), and tHcy was determined using high-performance liquid chromatography with fluorescence detection (14, 15). The lowest detection limit for folate was 1.36 nmol/L and for tHcy 4 μmol/L. To adjust folate and tHcy concentrations for dilution by the medium used to flush the aspiration needle, total protein was measured colorimetrically using biuret reagent (Hitachi 917, Roche GmbH).

Statistical Methods

Statistical analyses were performed by the paired and two sample Wilcoxon rank tests. $P \leq .05$ was considered statistically significant. In the comparisons of the folate and tHcy concentrations between monofollicular and pooled follicular fluid women were only included when the concentrations could be determined in two monofollicular fluid samples. All data were analysed in a mixed-model analysis to determine the correlation between the folate and tHcy concentrations and the diameter of the follicles.

RESULTS

The median concentrations of folate and tHcy in monofollicular and pooled follicular fluids stratified for preconception folic acid supplementation are presented in Table 1. The concentrations of folate and tHcy are given as protein ratios to adjust for dilution by the medium used to flush the aspiration needle. The median folate concentrations were higher and the median tHcy were lower in the follicular fluid samples of folic acid supplement users compared with nonsupplement users. The differences were all significant in the pooled follicular samples. In the monofollicular samples, the folate concentrations only were statistically significantly higher in supplement users compared with nonsupplement users.

Overall the median folate concentrations were significantly higher in the pooled samples compared to the monofollicular samples (median, 0.55 and 0.48 nmol/g, respectively; $P \leq 0.001$), whereas tHcy concentrations were comparable in both samples (median, 0.12 µmol/g).

The mixed-model analysis revealed a significant positive correlation between the monofollicular tHcy concentration and the diameter of the follicle ($r = 0.268$; $P \leq 0.001$. Every increase of the tHcy concentration by 0.0025 µmol/g increased the follicle diameter by 1 mm ($P \leq 0.001$). An inverse correlation, albeit small and not significant, between the concentration of folate and the diameter of the follicle was established ($r = -0.052$; $P = 0.11$). The total protein concentration was not significantly correlated with the follicular diameter (Figure 1).

Sometimes the pooled samples were contaminated with blood. Therefore, we investigated whether blood contamination may cause an artifactual increase in the follicular fluid folate concentration. In the pooled follicular fluid samples of six women we determined the folate concentration in two clear and in two visibly blood-contaminated samples of which one sample was centrifuged within 1 hour and one after 24 hours. The median folate concentration within 1 hour was 0.37 nmol/g in the clear samples and 0.47 nmol/g

Table 1. Median folate and tHcy concentrations determined in monofollicular and pooled follicular fluids in IVF patients supplemented and nonsupplemented with folic acid.

	Folic acid supplemented	Folic acid not supplemented	*P*
Monofollicular samples[a]:			
Folate/ total protein (range), nmol/g	0.50 (0.18-0.61) (n=16)	0.32 (0.08-0.38) (n=5)	≤ 0.001
tHcy/ total protein (range), µmol/g	0.12 (0.06-0.18) (n=18)	0.12 (0.09-0.22) (n=8)	NS
Pooled follicular samples:			
Folate/ total protein (range), nmol/g	0.57 (0.16-1.03) (n=21)	0.18 (0.10-0.79) (n=8)	≤ 0.01
tHcy/ total protein (range), µmol/g	0.12 (0.08-0.22) (n=21)	0.15 (0.11-0.36) (n=8)	≤ 0.05

[a] Two single follicular fluid samples per patient.

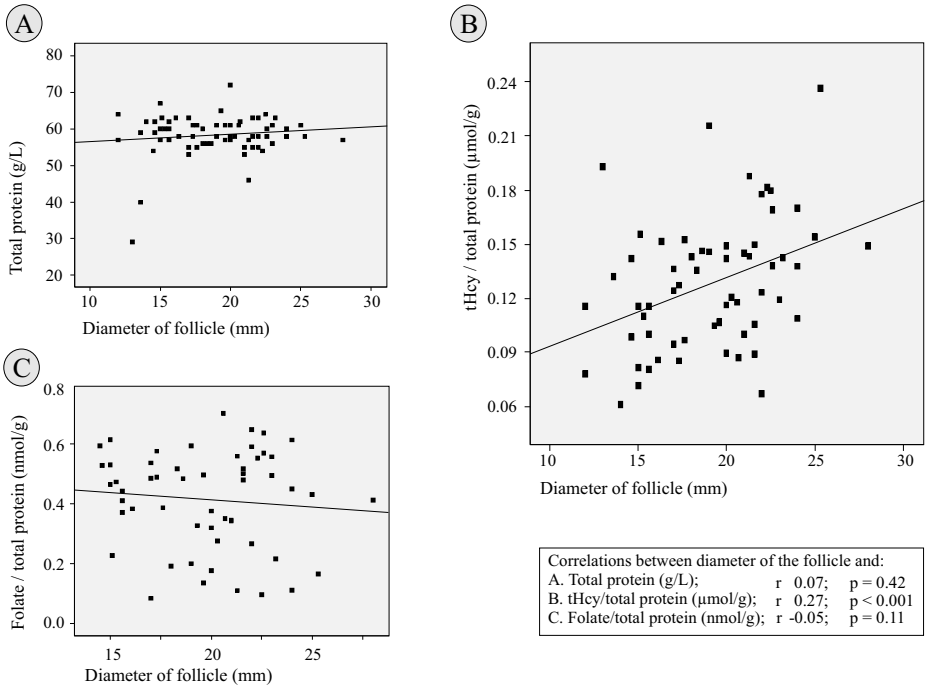


Figure 1. Correlations between the total protein, the folate/protein, and tHcy/protein ratios in follicular fluid and follicular diameter. The follicular fluid total protein concentration (graph A) and folate/protein ratio (graph B) are not significantly correlated with the diameter of the follicle. Graph C shows that the diameter of the follicle and the tHcy/protein ratio are significantly correlated. Every increase of the tHcy concentration by 0.0025 µmol/g, 95% confidence interval 0.0012 - 0.0036, was associated with an increase of the follicle diameter by 1 mm; $P < 0.001$.

in the blood-contaminated samples. After 24 hours, the median folate concentrations were, respectively, 0.39 and 0.48 nmol/g.

DISCUSSION

This study shows that folic acid supplementation significantly increases the folate concentration and decreases the tHcy concentrations, albeit not significantly in mono-follicular fluid, in the microenvironment of the maturing oocyte. The effect of folic acid supplementation on the folate-dependent homocysteine metabolism in follicular fluid was comparable to that in blood (4, 16).

The finding of a significant positive correlation between the diameter of the follicle and the follicular fluid concentration of tHcy was unexpected. Follicle fluid is a mixture of serum exudate and locally produced substances that are related to the metabolic activity of ovarian cells (17). It is not clear what determines the concentration of tHcy and folate

in follicular fluid. The statistically significant positive correlation between diameter and follicular fluid tHcy concentration may indicate that there is an increased production or leakage of tHcy via the granulosa cells or the oocyte and/or an enhanced requirement for folate in larger follicles. This might be caused by the evolution of the cells and/or the reaction to the hCG administration during the IVF treatment. Another explanation may be that there is an alteration of the exchange between the follicle and the surrounding cell layers during the maturation of the follicle, which causes a decrease of folate and an accumulation of tHcy. These findings, however, are in line with our previous study and that of others (5, 6).

Serum folate concentrations are inversely correlated with de size of red blood cells. Whether the same mechanisms are responsible for the growth or extension of the follicles and red blood cells is unknown. It is possible that osmolarity plays a role. However, our data may suggest that tHcy is involved as well. Whether an enhanced oxidative stress due to the high concentrations of tHcy affects the diameter of cells involved should be further elucidated. The inverse association between the follicular diameter and follicular folate concentration was statistically not significant. This may, however, be due to the small number of samples.

The consequences of a high concentration of tHcy in follicular fluid are not clear. Homocysteine is a thiol known to induce the release of reactive oxygen species (ROS). Although ROS is important for oocyte maturation and fertilisation, high levels of ROS in the culture medium of embryos have been shown to result in low cleavage rates, high embryonic fragmentation and low rates of blastocyst formation (18). This is consistent with the reported inverse correlation between the tHcy concentration in follicular fluid, seminal plasma and embryo quality (5, 6). The observation that the prolongation of the follicular phase during IVF treatment decreases the chance of achieving an ongoing pregnancy may be explained by the extended detrimental exposure to high follicular tHcy concentrations that decreases oocyte quality (19, 20). More studies on this field, however, are required to determine the role of folate and tHcy in follicular fluid as an indicator of oocyte quality. Such studies are now underway.

We have shown that blood contamination is a problem in the collection of pooled follicular samples. The results also demonstrated that the difference in folate concentration in follicular fluid as a result of oocyte aspiration could not be prevented by centrifugation within 1 hour after collection.

However, the absence of statistically significant differences in those concentrations with and without centrifugation within one hour after collection may be due to the small number of samples. It was shown that blood contamination could be avoided in most cases by taking monofollicular samples from the first follicle aspirated during oocyte pickup. Therefore, we recommend that monofollicular fluid samples or clear follicular fluid

samples should be collected in the future investigation of these nutrients in follicular fluid in association with several outcome parameters.

We calculated protein ratios of the folate and tHcy concentrations with the aim to of adjusting for dilution by the medium used to flush the aspiration needle. Total protein, however, appeared a constant determinant in follicular fluid, independent of the diameter of the follicle.

In conclusion, this study demonstrates a significant effect of folic acid supplementation on the follicular fluid folate and tHcy concentrations that thereby influence the microenvironment of the maturing oocyte. Of interest also is the finding that the diameter of the follicle is inversely correlated with the follicular fluid tHcy concentration. This may suggest that there is a particular range of the follicle diameter that reflects an optimal microenvironment of the maturing oocyte. Finally, blood contamination of the follicular fluid overestimates the folate concentration in follicular fluid. Therefore, in future studies, the collection of follicular fluid samples from monofollicles in women with and without folic acid supplementation is recommended.

Acknowledgments:

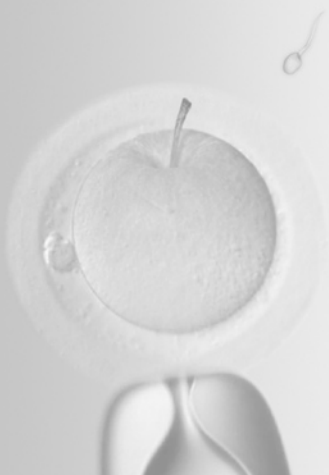
The authors gratefully acknowledge the contribution of the IVF team of the department of Obstetrics and Gynecology / Division of Reproductive Medicine, for the sample collection and the laboratory technicians and research assistants of the Laboratory of Clinical Chemistry of the Erasmus MC, University Medical Center in Rotterdam, The Netherlands for the laboratory determinations.

REFERENCES

1. Hague WM. Homocysteine and pregnancy. Best PractRes Clin Obstet Gynaecol 2003;17(3):459-69.
2. Steegers-Theunissen RP, Steegers EA. Nutrient-gene interactions in early pregnancy: a vascular hypothesis. Eur J Obstet Gynecol Reprod Biol 2003;106(2):115-7.
3. Ebisch IM, Thomas CM, Peters WH, Braat DD, Steegers-Theunissen RP. The importance of folate, zinc and antioxidants in the pathogenesis and prevention of subfertility. Hum Reprod Update 2007;13(2): 163-74.
4. Steegers-Theunissen RP, Steegers EA, Thomas CM, Hollanders HM, Peereboom-Stegeman JH, Trijbels FJ, et al. Study on the presence of homocysteine in ovarian follicular fluid. Fertil Steril 1993;60(6): 1006-10.
5. Szymanski W, Kazdepka-Zieminska A. [Effect of homocysteine concentration in follicular fluid on a degree of oocyte maturity]. Ginekol Pol 2003;74(10):1392-6.
6. Ebisch IM, Peters WH, Thomas CM, Wetzels AM, Peer PG, Steegers-Theunissen RP. Homocysteine, glutathione and related thiols affect fertility parameters in the (sub)fertile couple. Hum Reprod 2006; 21(7):1725-33.
7. Botto LD, Olney RS, Erickson JD. Vitamin supplements and the risk for congenital anomalies other than neural tube defects. Am J Med Genet C Semin Med Genet 2004;125(1):12-21.
8. Bailey LB, Berry RJ. Folic acid supplementation and the occurrence of congenital heart defects, orofacial clefts, multiple births, and miscarriage. Am J Clin Nutr 2005;81(5):1213S-7S.
9. Haggarty P, McCallum H, McBain H, Andrews K, Duthie S, McNeill G, *et al.* Effect of B vitamins and genetics on success of in-vitro fertilisation: prospective cohort study. Lancet 2006;367(9521):1513-9.
10. Muggli EE, Halliday JL. Folic acid and risk of twinning: a systematic review of the recent literature, July 1994 to July 2006. Med J Aust 2007;186(5):243-8.
11. Bliek JB, de Klein A, Luider TM, Lindemans J, Hulsman L, Guzel C, et al. New approach for the identification of folate-related pathways in human embryogenesis. Cell Mol Biol (Noisy-le-grand) 2004;50(8): 939-44.
12. Finnell RH, Spiegelstein O, Wlodarczyk B, Triplett A, Pogribny IP, Melnyk S, et al. DNA methylation in Folbp1 knockout mice supplemented with folic acid during gestation. J Nutr 2002;132(8 Suppl): 2457S-61S.
13. Kelly TL, Neaga OR, Schwahn BC, Rozen R, Trasler JM. Infertility in 5,10-methylenetetrahydrofolate reductase (MTHFR)-deficient male mice is partially alleviated by lifetime dietary betaine supplementation. Biol Reprod 2005;72(3):667-77.
14. Pfeiffer CM, Huff DL, Gunter EW. Rapid and accurate HPLC assay for plasma total homocysteine and cysteine in a clinical laboratory setting. Clin Chem 1999;45(2):290-2.
15. de Jonge R, Griffioen PH, van Zelst B, Brouns RM, Visser W, Lindemans J. Evaluation of a shorter methionine loading test. Clin Chem Lab Med 2004;42(9):1027-31.
16. Refsum H, Smith AD, Ueland PM, Nexo E, Clarke R, McPartlin J, et al. Facts and recommendations about total homocysteine determinations: An expert opinion. Clin Chem 2004;50(1):3-32.
17. Gerard N, Loiseau S, Duchamp G, Seguin F. Analysis of the variations of follicular fluid composition during follicular growth and maturation in the mare using proton nuclear magnetic resonance (1H NMR). Reproduction 2002;124(2):241-8.
18. Bedaiwy MA, Falcone T, Mohamed MS, Aleem AA, Sharma RK, Worley SE, et al. Differential growth of human embryos in vitro: role of reactive oxygen species. Fertil Steril 2004;82(3):593-600.
19. Kolibianakis EM, Albano C, Camus M, Tournaye H, Van Steirteghem AC, Devroey P. Prolongation of the follicular phase in in vitro fertilization results in a lower ongoing pregnancy rate in cycles stimulated with recombinant follicle-stimulating hormone and gonadotropin-releasing hormone antagonists. Fertil Steril 2004;82(1):102-7.
20. Clark L, Stanger J, Brinsmead M. Prolonged follicle stimulation decreases pregnancy rates after in vitro fertilization. Fertil Steril 1991;55(6):1192-4.

Chapter 3

Relationships between homocysteine follicular fluid concentrations in women undergoing IVF/ICSI treatment and the number of oocytes and pregnancy



Jolanda C. Boxmeer, R. Montserrate Brouns, Jan Lindemans, Eric A.P. Steegers, Elena Martini, Nick S. Macklon, Marinus J.C. Eijkemans, Régine P.M. Steegers-Theunissen

Submitted for publication

ABSTRACT

Derangements in the B-vitamin dependent homocysteine pathway are implicated in the pathophysiology of reproductive failures, but the role in fertility has scarcely been studied. Therefore, we determined the concentrations of total homocysteine (tHcy), folate, cobalamin and pyridoxine in follicular fluid of 143 women undergoing IVF or ICSI treatment. Relationships were determined between these biomarkers in follicular fluid and the number of oocytes, percentage of fertilization and the occurrence of biochemical pregnancy. Significant correlations were demonstrated between tHcy, folate, cobalamin, and pyridoxine concentrations in follicular fluid. Not any correlation was established between the biomarkers and fertilization. An inverse correlation was determined between tHcy and the number of oocytes after adjustment for age and treatment (r = -0.16; P < 0.05). Moreover, the higher the tHcy concentration per follicle, the lower the chance of biochemical pregnancy (odds ratio = 0.56, 95% CI 0.34 - 0.95). These data suggest that a high follicular fluid tHcy concentration is detrimental for the number of maturated oocytes and the chance of achieving a biochemical pregnancy.

INTRODUCTION

Subfertility occurs in around 10% of the couples during reproductive life. In about 30% of these couples no cause can be found (1). Evidence is increasing that nutritional factors play a significant role in reproduction. Of interest is the homocysteine pathway in which several nutrients are involved. This pathway has widely been studied in association with birth defects, miscarriages and hypertension-complicated pregnancies but only scarcely with regard to fertility parameters (2, 3).

The homocysteine pathway is important for delivering methyl groups by the intermediate S-adenosylmethionine, which is essential for the methylation of DNA and therefore genome regulation. Folate is an essential substrate in the remethylation of homocysteine into methionine by methionine synthase and for the synthesis of DNA- and RNA-precursors. Cobalamin (vitamin B12) serves as a cofactor for methionine synthase. Vitamin B6, or pyridoxine, acts as a cofactor for cystathionine-beta-synthase enabling the transsulphuration of homocysteine into cystathionine and cysteine. A deficiency of one of these vitamins causes mild hyperhomocysteinemia, which is associated with cardiovascular and cerebrovascular diseases (4). Associations between maternal hyperhomocysteinemia and congenital malformations, recurrent pregnancy loss and abruptio placentae have been previously demonstrated (5-7). Folic acid treatment significantly reduces elevated total homocysteine (tHcy) concentrations (4). This may partially explain the beneficial effect of periconception folic acid supplementation against several complex congenital malformations (8). Whether folic acid supplementation may also increase the occurrence of twins is still a matter of debate (9, 10).

In the early nineties the presence of tHcy, folate, cobalamin and pyridoxine in follicular fluid was demonstrated (11). Moreover, women who received folic acid supplementation showed a significantly lower tHcy concentration in follicular fluid (12). Ebisch et al. showed that a high tHcy concentration was inversely associated with embryo quality (13). From this background we hypothesized that a derangement in the homocysteine pathway is detrimental to the quality of the oocyte, fertilization, implantation and embryogenesis. Therefore we aimed to investigate the relationships between the tHcy, folate, cobalamin and pyridoxine concentrations in follicular fluid of women undergoing in-vitro fertilization (IVF) with or without intracytoplasmic sperm injection (ICSI) treatment and the number of oocytes, fertilization and the occurrence of biochemical pregnancy.

MATERIALS AND METHODS

Subjects

One hundred forty-three women, median age (range); 33.7 (23.3 – 43.2) years, undergoing IVF with or without ICSI treatment at the Department of Obstetrics and Gynecology, Division of Reproductive Medicine of the Erasmus MC, University Medical Center were included. On the day of ovum pick up the use of folic acid containing supplements was reported.

Follicular fluid

From each subject follicular fluid from all aspirated follicles was collected and pooled for analysis. Specimens were collected in accordance with the guidelines of the Medical Ethical and Institutional board of the Erasmus MC, University Medical Center in Rotterdam, The Netherlands. After oocyte retrieval for the IVF or ICSI procedure, the samples were centrifuged for 10 minutes at 1,700 g to separate red blood cells, leucocytes and granulosa cells from the follicular fluid.

The follicular fluid samples were analyzed for folate and cobalamin during routine laboratory procedures using an immunoelectrochemoluminescence assay (Roche Modular E170, Roche Diagnostics GmbH, Mannheim, Germany). Pyridoxine and tHcy were determined during routine laboratory procedures using high performance liquid chromatography with reversed phase separation and fluorescence detection (14, 15). Pyridoxine was determined as pyridoxal'5-phosphate (PLP).

In order to adjust the concentrations for dilution by the medium used to flush the IVF aspiration needle, the total protein concentration was measured colorimetrically using biuret reagent (Hitachi 917, Roche GmbH, Mannheim, Germany). The detection limit for tHcy was 4 µmol/L, folate 1.36 nmol/L, cobalamin 22 pmol/L and PLP 5 nmol/L.

Fertility outcome parameters

The proportion of fertilized oocytes was calculated by dividing the number of oocytes with two pronuclei the day after oocyte retrieval by the total number of oocytes retrieved. Biochemical pregnancy was defined as a positive urinary hCG pregnancy test performed on day 15 after ovum pick up.

Statistical analysis

The results are expressed in median (range), number and percentage and analyzed for statistical significance using nonparametric tests, because of the skewed distributions of the B-vitamins and tHcy. Spearman rank correlation coefficients were calculated to determine associations between tHcy, folate, cobalamin and PLP concentrations in follicular fluid.

To adjust for possible confounding variables, multivariable linear regression analysis was performed with the number of oocytes and the percentage of fertilized oocytes as dependent outcome variable. Logistic regression was performed for the occurrence of pregnancy as dependent outcome variable. Because age, the type of treatment (IVF or ICSI) and the presence of female factor subfertility are strong determinants of fertility outcome, these factors were considered as potential confounders. A *P*-value of < 0.05 was considered statistically significant. Statistical analyses were performed using SPSS 11.5 for Windows software (SPSS Inc, Chicago, Il, USA)

RESULTS

In Table 1 the characteristics of the study population and the B-vitamin and tHcy concentrations in follicular fluid are presented. Due to a limited sample volume the follicular fluid concentrations of tHcy, folate, cobalamin and PLP could not be determined in 1, 25, 3 and 16 samples, respectively. The unadjusted median concentrations were for tHcy 6.6 µmol/L (range 3.1-33.4), folate 21.9 nmol/L (range 4.4 – 43.6), cobalamin 187.1 pmol/L (range 59.9-561.4) and PLP 46 nmol/L (range 13-334).

Table 1. Characteristics of the 143 women undergoing IVF/ICSI

Age (years) median (range)	33.7 (23.3-43.2)
Folic acid supplement use % (n)	63 (78)
Oocytes per patient, median (range)	8 (0-30)
ICSI % (n)	30 (43)
Proportion fertilized oocytes % (range)	59.8 (0-100)
Embryo transfer rate % (n)	85.3 (122)
Biochemical pregnancy rate % (n)	40.6 (58)
Follicular fluid concentrations, median (range)*:	Per gram of protein
tHcy (µmol/g)	0.13 (0.07-0.70)
Folate (nmol/g)	0.41 (0.09-1.03)
Cobalamin (pmol/g)	3.88 (1.22-16.04)
Pyridoxine (nmol/g)	0.89 (0.28-9.21)

* Concentrations are determined in the individually pooled follicle fluid. The ratio of these concentrations per gram of total protein is calculated to adjust for dilution by the flushing medium.

In Table 2 the correlations between tHcy and the B-vitamins are shown. Table 3 depicts the correlations between the concentrations of tHcy and B-vitamins and the outcome of the IVF-treatment. A weak, but statistically significant inverse correlation between follicular fluid tHcy and the number of retrieved oocytes could be established ($r = –0.16$; $P < 0.05$) (Figure 1). Multivariable regression analysis revealed age and the type of treatment as significant confounders for the association between tHcy, B-vitamins and the number

Table 2. Spearman rank correlation coefficients (r) between tHcy and B-vitamins per gram of protein in follicle fluid

	tHcy (µmol/g) r*P*	Folate (nmol/g) r*P*	Cobalamin (pmol/g) r*P*
Folate (nmol/g)	-0.29 (0.002)		
Cobalamin (pmol/g)	-0.16 (0.066)	0.25 (0.007)	
Pyridoxine (nmol/g)	-0.20 (0.020)	0.26 (0.002)	0.13 (0.154)

of oocytes. Female factor subfertility was a significant confounder for the correlations between tHcy, B-vitamins and the percentage of fertilized oocytes. No significant confounders were found for the correlations between tHcy, B-vitamins and the occurrence of pregnancy. The correlations in Table 3 are adjusted for significant confounders.

In addition, for tHcy, folate, cobalamin and PLP the concentration per oocyte was calculated and Spearman correlation coefficients were calculated between the concentration of tHcy and B-vitamins per oocyte and IVF-outcome. There was a weak, but statistically significant inverse correlation between the concentration of tHcy per oocyte and the occurrence of biochemical pregnancy (odds ratio = 0.56, 95% CI 0.34-0.95, $P < 0.05$).

Table 3. Adjusted correlations coefficients (95% confidence interval (CI)) and odds ratios (95% CI) between tHcy and B-vitamins in follicle fluid and IVF/ICSI outcome in 143 women

	Number of oocytes correlation coefficients (95% CI) (SE)	Fertilization rate correlation coefficients (95% CI) (SE)	Pregnancy rate Odds Ratios (95% CI) (SE)
tHcy (µmol/g)	-0.16* (-0.31 - -0.01) (0.08)	-0.02 (-0.17 – 0.14) (0.08)	0.49 (0.17-1.38) (0.53)
Folate (nmol/g)	0.05 (-0.12 - 0.23) (0.09)	-0.04 (-0.22 – 0.15) (0.09)	0.67 (0.14 – 3.17) (0.80)
Pyridoxine (nmol/g)	0.01 (-0.15 - 0.18) (0.08)	-0.11 (-0.27 – 0.06) (0.08)	1.06 (0.61 – 1.84) (0.23)
Cobalamin (pmol/g)	0.03 (-0.14 - 0.20) (0.09)	-0.00 (-0.17 – 0.17) (0.08)	1.58 (0.67 – 3.71) (0.44)

SE = standard error. The correlations between tHcy, B-vitamins and number of oocytes are adjusted for age and the type of treatment (IVF or ICSI). The correlations between tHcy, B-vitamins and proportion of fertilized oocytes are adjusted for female factor subfertility.

* $P < 0.05$

DISCUSSION

This study demonstrates for the first time an inverse correlation between tHcy concentrations in follicular fluid, the number of retrieved oocytes and the occurrence of biochemical pregnancy in women undergoing IVF or ICSI-treatment. Homocysteine is a thiol known to induce the release of reactive oxygen species (ROS) that are scavenged by endogenous glutathione. ROS is important for oocyte maturation and fertilization. However, high levels of ROS may induce apoptosis of oocytes resulting in a lower number. In addition, high ROS in the culture medium of embryos have been shown to result in low cleavage rates, high embryonic fragmentation and low rates of blastocyst formation (16). Our findings are also

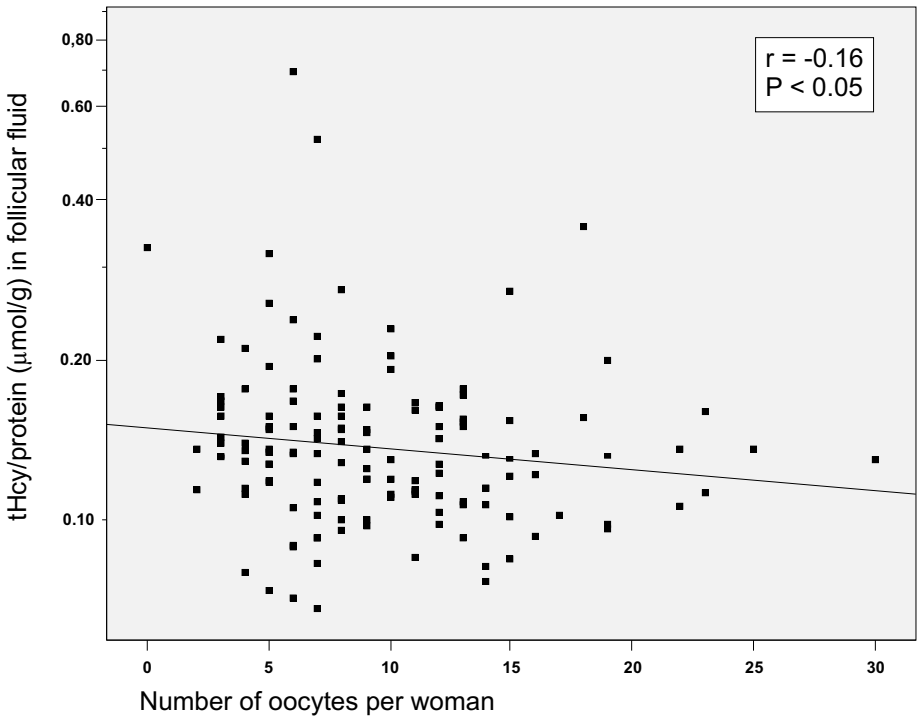


Figure 1. Correlation between logtransformed tHcy concentrations in follicular fluid and the number of oocytes retrieved per woman.

in line with the recently reported inverse correlation between the tHcy concentration in follicular fluid, seminal plasma and embryo quality (13). Moreover, the inverse correlation between follicular fluid tHcy concentration and oocyte maturity reported by Szymanski and Kazdepka-Zieminska supports our data (12). Therefore, the homocysteine pathway and tHcy in particular may play a role in gametogenesis and follicle development, which confirms our hypothesis that hyperhomocysteinaemia is detrimental to the quality of the oocyte and subsequent fertilization and pregnancy.

The unadjusted tHcy and B-vitamin concentrations are slightly higher compared with the concentrations measured in our previous study (11). Besides the larger study group of the current study, this may also be explained by changes in dietary intake and lifestyle during the last fifteen years. Another explanation may be a difference in the amount of flushing medium that was used in each study. However, the adjusted follicular fluid concentrations of tHcy in the present study are comparable with the adjusted concentrations recently reported by Ebisch *et al.* (13).

During the sixties and seventies, several small studies and case reports already reported an association between female subfertility and cobalamin deficiency. The described women became pregnant within a year after vitamin B12 administration (17-21). In the

present study cobalamin concentrations in follicular fluid were not correlated with any of the fertility outcome parameters. This may be explained by the absence of severe cobalamin deficient participants.

Some strengths and weaknesses of the study have to be addressed. The study group was homogenous with respect to the inclusion of subfertile couples only. However, the group is heterogeneous with regard to the causes of subfertility. We did not determine the vitamins and tHcy concentrations in blood, because the aim was to study the closest environment of the oocyte. Moreover, significant correlations between serum tHcy, folate, cobalamin and follicular fluid concentrations have been reported before (11). A strong finding is that the correlations between tHcy and the B-vitamins in follicular fluid are comparable with the correlations in blood.

In this study, we pooled the follicular fluid samples from all aspirated follicles and therefore we can only present general correlation coefficients between tHcy and the B-vitamin concentrations and fertility outcome parameters. Studies in animals reported a change of follicular fluid composition during follicular growth and maturation and it is feasible that B-vitamins and tHcy concentrations differ between follicles (22, 23). In future studies it would be preferable to measure concentrations in the separate follicles and to correlate them with the development and outcome of that specific oocyte.

Our study indicates that the homocysteine pathway, in which B-vitamins and methionine as nutrients are involved, plays a significant role in fertilization and the occurrence of early pregnancy. Because nutrition is part of lifestyle it is amenable for intervention. Therefore, more studies should focus on possible beneficial effects of nutritional intervention in the treatment of subfertility.

Acknowledgements

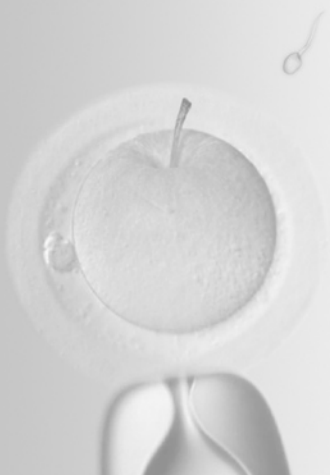
The authors gratefully acknowledge the contribution of the IVF team of the department of Obstetrics and Gynecology / Division of Reproductive Medicine for the sample collection, and the laboratory technicians and research assistants of the Laboratory of Clinical Chemistry of the Erasmus MC, University Medical Center in Rotterdam, The Netherlands for the laboratory determinations.

REFERENCES

1. Snick HK, Snick TS, Evers JL, Collins JA. The spontaneous pregnancy prognosis in untreated subfertile couples: the Walcheren primary care study. Hum Reprod 1997;12:1582-8.
2. Steegers-Theunissen RP, Steegers EA. Nutrient-gene interactions in early pregnancy: a vascular hypothesis. Eur J Obstet Gynecol Reprod Biol 2003;106:115-7.
3. Ebisch IM, Thomas CM, Peters WH, Braat DD, Steegers-Theunissen RP. The importance of folate, zinc and antioxidants in the pathogenesis and prevention of subfertility. Hum Reprod Update 2007;13: 163-74.
4. Refsum H, Smith AD, Ueland PM, Nexo E, Clarke R, McPartlin J *et al.* Facts and recommendations about total homocysteine determinations: An expert opinion. Clin Chem 2004;50:3-32.
5. Steegers-Theunissen RP, Boers GH, Trijbels FJ, Eskes TK. Neural-tube defects and derangement of homocysteine metabolism. N Engl J Med 1991;324:199-200.
6. Steegers-Theunissen RP, Boers GH, Blom HJ, Trijbels FJ, Eskes TK. Hyperhomocysteinaemia and recurrent spontaneous abortion or abruptio placentae. Lancet 1992;339:1122-3.
7. Hague WM. Homocysteine and pregnancy. Best Pract Res Clin Obstet Gynaecol 2003;17:459-69.
8. Botto LD, Olney RS, Erickson JD. Vitamin supplements and the risk for congenital anomalies other than neural tube defects. Am J Med Genet C Semin Med Genet 2004;125:12-21.
9. Haggarty P, McCallum H, McBain H, Andrews K, Duthie S, McNeill G *et al.* Effect of B vitamins and genetics on success of in-vitro fertilisation: prospective cohort study. Lancet 2006;367:1513-9.
10. Muggli EE, Halliday JL. Folic acid and risk of twinning: a systematic review of the recent literature, July 1994 to July 2006. Med J Aust 2007;186:243-8.
11. Steegers-Theunissen RP, Steegers EA, Thomas CM, Hollanders HM, Peereboom-Stegeman JH, Trijbels FJ *et al.* Study on the presence of homocysteine in ovarian follicular fluid. Fertil Steril 1993;60:1006-10.
12. Szymanski W, Kazdepka-Zieminska A. [Effect of homocysteine concentration in follicular fluid on a degree of oocyte maturity]. Ginekol Pol 2003;74:1392-6.
13. Ebisch IM, Peters WH, Thomas CM, Wetzels AM, Peer PG, Steegers-Theunissen RP. Homocysteine, glutathione and related thiols affect fertility parameters in the (sub)fertile couple. Hum Reprod 2006; 21:1725-33.
14. Schrijver J, Speek AJ, Schreurs WH. Semi-automated fluorometric determination of pyridoxal-5'-phosphate (vitamin B6) in whole blood by high-performance liquid chromatography (HPLC). Int J Vitam Nutr Res 1981;51:216-22.
15. Pfeiffer CM, Huff DL, Gunter EW. Rapid and accurate HPLC assay for plasma total homocysteine and cysteine in a clinical laboratory setting. Clin Chem 1999;45:290-2.
16. Bedaiwy MA, Falcone T, Mohamed MS, Aleem AA, Sharma RK, Worley SE *et al.* Differential growth of human embryos in vitro: role of reactive oxygen species. Fertil Steril 2004;82:593-600.
17. Jackson I, Doig WB, McDonald G. Pernicious anaemia as a cause of infertility. Lancet 1967;290:1159-60.
18. Varadi S. Pernicious anaemia and infertility. Lancet 1967;290:1350.
19. Hall M, Davidson RJL. Prophylactic folic acid in women with pernicious anaemia pregnant after periods of infertility. J Clin Path 1968;21:599-602.
20. Mahmood A. Pernicious anaemia as a cause of infertility. Lancet 1971;297:753.
21. Girdwood RH, Eastwood MA, Finlayson ND, Graham GS. Pernicious anaemia as a cause of infertility in twins. Lancet 1971;1:528-30.
22. Gerard N, Loiseau S, Duchamp G, Seguin F. Analysis of the variations of follicular fluid composition during follicular growth and maturation in the mare using proton nuclear magnetic resonance (1H NMR). Reproduction 2002;124:241-8.
23. Nandi S, Kumar VG, Manjunatha BM, Gupta PS. Biochemical composition of ovine follicular fluid in relation to follicle size. Dev Growth Differ 2007;49:61-6.

Chapter 4

Homocysteine metabolism in the pre-ovulatory follicle during ovarian stimulation



Jolanda C. Boxmeer, Régine P.M. Steegers-Theunissen, Jan Lindemans, Mark F. Wildhagen, Elena Martini, Eric A.P. Steegers, Nick S. Macklon

Hum Reprod. 2008 Jul 29. [Epub ahead of print]

ABSTRACT

Background: Ovarian stimulation gives rise to supraphysiological estradiol levels, which may affect oocyte quality. This study aims to investigate whether ovarian stimulation deranges the homocysteine pathway thereby affecting the pre-ovulatory follicle.

Methods: Blood samples were collected on cycle day 2 and the day of hCG administration in 181 women undergoing ovarian stimulation for IVF. In each subject the diameter of the two leading follicles was measured and the corresponding follicular fluids were collected. In blood and follicular fluid samples total homocysteine (tHcy), folate, cobalamin and pyridoxal'5-phosphate (PLP) were determined. According to the blood folate levels, women were classified as either folic acid supplemented (n = 113) or non-supplemented (n = 32).

Results: Ovarian hyperstimulation resulted in a significant decrease in blood tHcy and cobalamin levels (both $P \leq 0.001$). The blood concentrations of tHcy, folate, cobalamin and PLP were significantly correlated with the corresponding follicular fluid concentrations (all $P \leq 0.001$). Follicular fluid tHcy concentrations were inversely correlated with follicular diameter ($P \leq 0.05$). In folic acid supplemented women, follicular fluid folate was inversely correlated with follicular diameter ($P \leq 0.05$).

Conclusions: Ovarian hyperstimulation deranges blood and follicular fluid biomarkers of the homocysteine pathway. High ovarian follicular fluid tHcy and folate levels may have detrimental effects on follicular development.

INTRODUCTION

Involuntary childlessness imposes a heavy burden on most subfertile couples (1, 2). Many couples therefore seek help by means of artificial reproduction techniques such as *in vitro* fertilization (IVF) to achieve pregnancy. While these techniques have improved the treatment of subfertile couples, the chance of achieving a clinical pregnancy remains around 25% per started cycle (3). In order to obtain multiple embryos from which to select for transfer to the uterus, exogenous gonadotrophins are administered to stimulate multifollicular development in the ovaries. This results in supraphysiological serum levels of gonadotrophins and estradiol which themselves may have a detrimental effect on oocyte and embryo quality (4) Although the mechanisms of this effect remain to be elucidated, derangement of intrafollicular homocysteine pathway may be involved. Mild to moderate hyperhomocysteinemia is associated with detrimental effects on reproductive outcome, ranging from congenital malformations and miscarriages to pregnancy induced hypertension and low birth weight (5-7). The main causes of hyperhomocysteinemia include a dysbalance between the intake of folate, cobalamin, pyridoxine and methionine, metabolic derangements and related genetic variations (8).

Exogeneous estrogens have been shown to affect several endocrine and metabolic pathways in women, including homocysteine metabolism. For instance, artificial estrogen treatment of postmenopausal women significantly lowers the total homocysteine (tHcy) levels (9). Further evidence for sex steroid modulation of tHcy levels comes from studies showing levels to be significantly lower in the luteal phase than in the follicular phase of the normovulatory cycle (10). Moreover, blood tHcy levels decrease during pregnancy from preconception onwards (11-13).

Whether estrogens affect the homocysteine pathway directly and/or via intermediates and cofactors is not clear. In some studies, a significant decrease of pyridoxine (14) or cobalamin (15) was observed in postmenopausal women taking estradiol supplements, but this could not be confirmed by others (14, 16).

Given the previously described detrimental effects of hyperhomocysteinaemia on embryo quality (17), suppression of tHcy concentrations by increasing estradiol levels might be expected to have a beneficial effect. However, recent data has indicated that supraphysiological estradiol levels are correlated to higher levels of embryo aneuploidy (18) suggesting a negative effect on oocyte quality. At present, little is known about the influence of ovarian hyperstimulation treatment on the homocysteine pathway in the direct environment of the maturing follicle and oocyte in human, or the relationship between follicular tHcy concentrations and follicular growth. Moreover, the extent to which folate supplementation modifies these relationships is not known. Therefore, the principal aims of the present study were to investigate the influence of ovarian stimulation with exogenous gonadotropins on the levels of biomarkers of the homocysteine pathway

in blood and the follicular fluid of the maturing oocyte, and to study the correlations between the levels of these biomarkers and the follicular diameter. Finally, the impact of periconception folate supplementation on these correlations was determined.

MATERIALS AND METHODS

Population

The FOod, Lifestyle and Fertility Outcome-study (FOLFO-study) is a periconception cohort study focused on the influence of the preconception health of the couple on fertility parameters and pregnancy outcome. Between September 2004 and October 2006 subfertile couples undergoing an IVF with or without intracytoplasmic sperm injection (ICSI) procedure at the Erasmus MC, University Medical Centre, Rotterdam, The Netherlands, were invited to participate. Exclusion criteria were oocyte donation cycles, the diagnosis of endometriosis or hydrosalpinx and age above 43 years. Seventy-four percent of eligible couples consented to participate in the study. Data from these women have not been previously published. All obtained materials and questionnaires were processed anonymously. The study protocol was approved by the Dutch Central Committee for Human Research (CCMO) and the Medical Ethical and Institutional Review Board of the Erasmus MC, University Medical Centre in the Netherlands.

In vitro fertilization procedure

All women started daily subcutaneous injections of 150 IU recombinant FSH, on the second day of a spontaneous cycle (Puregon®, NV Organon, Oss, The Netherlands or Gonal-F®, Serono Benelux BV, The Hague, The Netherlands). Daily subcutaneous administration of 0.25 mg GnRH antagonist (Orgalutran®, NV Organon, or Cetrotide®, Serono Benelux BV) was started when at least one follicle measured ≥14 mm. To induce final oocyte maturation, a single dose of 5,000 or 10,000 IU human chorionic gonadotrophin sc (hCG, Pregnyl®, NV Organon) was administered as soon as the largest follicle reached at least 18 mm in diameter and at least 1 additional follicle of > 15 mm was present. Oocyte retrieval was carried out 35 hours after hCG injection by transvaginal ultrasound-guided puncture of the follicles. Immediately prior to this procedure, the diameter of the leading follicle in both the right and left ovary was measured in three dimensions. Fluid from each of these two follicles was aspirated and stored separately before continuing with the oocyte retrieval procedure. After oocyte retrieval the follicular fluid samples were centrifuged for 10 minutes at 1,700 x *g* to separate red blood cells, 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 cycle day 2 and the day of hCG administration. At both time points tHcy, folate, cobalamin, pyridoxine, total protein and estradiol

were determined. In addition, on cycle day 2, FSH was determined. Blood was collected in dry vacutainer tubes, ethylenediamine tetra-acetate (EDTA) containing vacutainer tubes and lithium heparin containing vacutainers.

After clotting, the blood collected in dry vacutainer tubes was centrifuged at 2,000 x g and the sera were stored at 4°C before being assayed. The venous blood samples collected in the EDTA containing vacutainer tubes were kept on ice for a maximum of one hour after which the plasma was separated after centrifugation and stored at 4°C before being assayed. Blood samples drawn into lithium heparin containing vacutainers were stored at 4°C before being assayed.

tHcy in EDTA plasma and follicular fluid and pyridoxine as pyridoxal'5-phosphate (PLP) in whole blood and follicular fluid were determined using high-performance liquid chromatography with reversed phase separation and fluorescence detection (19, 20). For the determination of folate and cobalamin an immunoelectrochemoluminescence immunoassay was used (Roche Modular E170, Roche Diagnostics GmbH, Mannheim, Germany). For the determination of red blood cell (RBC) folate, 100 µL blood out of one EDTA tube was hemolyzed with 2 mL freshly prepared ascorbic acid (0.05g 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 (Groffin Meyvis, Etten-Leur, The Netherlands). The hemolysate was centrifuged for 10 minutes at 2,000 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 * 21) - (nmol/L serum folate * (1- hematocrit)) / hematocrit = nmol/L RBC folate. Total protein concentrations were determined photometrically on a Hitachi 917 (Roche Diagnostics GmbH, Mannheim, Germany). Levels of FSH were measured by luminescence-based immunometric assay (Immulite 2000, Diagnostic Products Corporation, Los Angeles, USA). Estradiol was measured using coated tube radioimmunoassay obtained from the same supplier.

Inter-assay coefficients of variation for tHcy were 4.8% at 14.6 µmol/L and 3.3% at 34.2 µmol/L, for folate 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 PLP 1.8% at 40 nmol/L and 1.3 % at 115 nmol/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 tHcy was 4 µmol/L, for folate 1.36 nmol/L, for cobalamin 22 pmol/L, for pyridoxine 5 nmol/L, for total protein 0.1 g/L, for FSH 0.1 U/L, and for estradiol 10 pmol/L.

Questionnaires

After oocyte retrieval, women filled out a general questionnaire from which the following data were extracted: medical history, body length and weight, ethnicity, and lifestyle factors, such as smoking and the use of vitamin supplements. Ethnicity was classified according to the definitions of Statistics Netherlands (21).

Women were considered to be taking folic acid supplements when folate levels in serum were > 22.4 nmol/L on both cycle day 2 and the day of hCG administration (22).

Statistical Methods

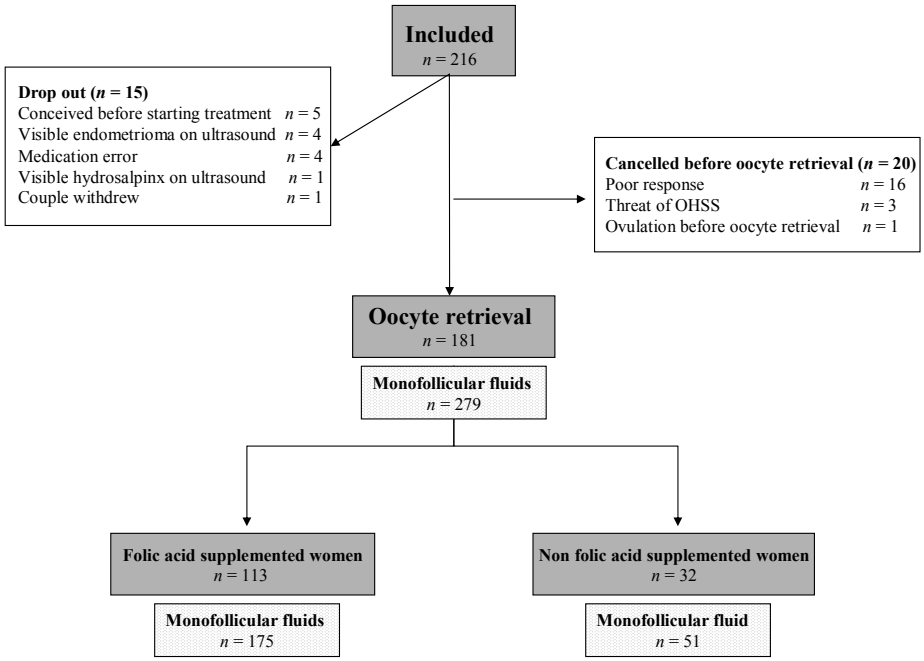
Follicular fluid total protein concentrations vary with follicular maturity (23). Concentrations of tHcy and B-vitamins in follicular fluid are therefore also expressed in concentration per gram of protein. The results were expressed in median (range), because of the skewed distributions of tHcy, B-vitamins, FSH and estradiol. Consequently these variables were log transformed before statistical testing. Because the distribution was normal after log transformation, paired t-tests were performed to determine differences between biomarker concentrations on cycle day 2 and the day of hCG administration and to determine differences between biomarker concentrations in blood and follicular fluid. Pearson's correlation coefficients were calculated to determine correlations between the deltas of the estradiol, tHcy, and B-vitamin concentrations.

In most subjects, but not all, two monofollicular fluid samples were obtained. In order to avoid a possible statistical bias arising from subjects producing just one fluid sample, a mixed model analysis was performed to determine correlations between the biomarker concentrations in blood and follicular fluid, and the diameter of the follicles. An independent t-test was performed with one follicle per women to determine differences between biomarkers in follicular fluid of supplemented and non-supplemented women. A P-value of ≤ 0.05 was considered statistically significant. All statistical analyses were performed using SPSS 14.0 for Windows software (SPSS Inc., Chicago, Il, USA).

RESULTS

The flow chart of the study is presented in Figure 1 and the baseline characteristics of the participating women are given in Table 1. Of the 216 included couples, 10 dropped out before commencing the study. Five women conceived spontaneously before the start of IVF treatment, and 5 were diagnosed with a visible endometrioma or hydrosalpinx at the start of the IVF treatment. Four couples were excluded from analysis due to incorrect use of the IVF-medication, and one couple withdrew. In the remaining 201 couples, treatments were cancelled before oocyte retrieval due to a poor response ($n = 16$), the threat of ovarian hyperstimulation syndrome (OHSS) ($n = 3$) and the occurrence of ovulation before oocyte retrieval ($n = 1$). The mean number of follicles > 10 mm measure on the day of hCG administration was 8 (SD 5) and the mean number of retrieved oocytes was 7 (SD 5). In 4 cases, blood samples were not taken at the correct day. Consequently, 177 cycle day 2 blood samples and 177 samples taken on the day of hCG administration were available for analysis.

Figure 1. Flowchart of the study



Women were considered to be taking folic acid supplements when folate levels in serum were greater than 22.4 nmol/L on both cycle day 2 and the day of hCG administration (22). In 24 women of the included 181 women, serum concentrations indicated an irregular folic acid intake.

Table 1. Characteristics of the study subjects (*n* = 181)

Age (years) median (range)	36	(23.7 – 43.7)
Body Mass Index, kg/m2, median (range)	23.1	(16.1 – 36.3)
Ethnicity:		
Dutch % (n)	72	(113)
Non-Dutch European % (n)	8.9	(14)
Non-European % (n)	19.1	(30)
Folic acid containing supplement, yes % (n)	87.2	(143)
Smokers % (n)	9	(15)
Duration of subfertility, median (range)	36	(4-121)
Cause of subfertility:		
Female factor % (n)	18.2	(33)
Male factor % (n)	44.2	(80)
Male and female factor % (n)	8.3	(15)
Idiopathic % (n)	29.3	(53)
Baseline FSH (U/L), cycle day 2, median (range)	8.3	(0.4 – 30.3)
Baseline estradiol (pmol/L), cycle day 2, median (range)	137	(41 – 307)

Ethnicity was classified according to the definitions of Statistics Netherlands (http://www.cbs.nl/en-GB/default.htm 2007)

Of the 299 collected monofollicular fluid samples, 20 samples were excluded from analysis due to contamination with the culture medium, which contains very high concentrations of cobalamin (> 14000 pmol/L) and folate (> 950 nmol/L). The mean diameter of the follicles from which fluid was collected was 19.2 mm (SD 2.8 mm). This mean diameter was similar in the subgroups of folic acid supplemented and non-supplemented. Moreover, no significant differences were observed between young and old participants (19.0 vs 19.4 in the subgroups of ≤ and > 36 years, respectively).

Blood and follicular fluid concentrations of estradiol, total protein and biomarkers of the homocysteine pathway are presented in Table 2. One hundred and thirteen women (67%) were classified as folic acid supplement users, with median folate concentrations of 36.4 nmol/L (range 22.9 – 908.0) on cycle day 2 and 38.8 nmol/L (range 22.8 – 174.3) on the day of hCG administration. Thirty two women (19%) were classified as non-supplemented, with median folate concentration of 14.8 nmol/L (range 6.4 – 20.7) on cycle day 2, and 15.3 nmol/L (range 6.6 – 22.3) on the day of hCG administration. The median age of non-supplemented and supplemented women was not significantly different. In folic acid non-supplemented women, subfertility was more often caused by both a female and a

Table 2. Biomarkers in blood and follicular fluids, and the correlations between the biomarkers in blood and follicular fluids

	Blood					Follicular fluid				
	Cycle day 2 (*n* = 177)		hCG day (*n* = 177)		Diff.[a]	concentration (*n* = 279)		protein ratio[b] (*n* = 279)		estimate[c]
Estradiol (pmol/L)	136.5	(41 – 307)	2137	(233 – 11978)	*					
tHcy (µmol/L)	9.1	(5.0 – 75.3)	8.4	(4.3 – 71.6)	*	6.6	(0.9 – 73.6)	0.12	(0.03 – 1.27)	0.98*
Folate (nmol/L)	30.5	(6.4 – 908.0)	32.6	(6.6 – 174.3)		29.8	(5.9 – 234.2)	0.55	(0.11 – 4.04)	0.73*
Folate RBC (nmol/L)	1407	(459 – 3611)	1347	(427 – 4413)						
Cobalamin (pmol/L)	319	(74 – 1856)	303	(75 – 1046)	*	208	(59 – 1136)	3.81	(1.02 – 34.42)	0.87*
Pyridoxine (nmol/L)	80	(32 – 310)	76	(35 – 310)		72	(2 - 310)	1.37	(0.03 – 5.85)	1.18*
Total protein (g/L)	72	(58 – 84)	71	(59 – 83)	*	57	(21 – 70)			

Note: values are given as median (range).

[a] Diff. = difference between serum concentrations at cycle day 2 and the day of hCG administration;

[b] follicular fluid concentrations of B-vitamins and tHcy expressed per gram total protein;

[c] Mixed model analysis with 2log transformed biomarker concentrations; correlations between concentration in blood on the day of hCG administration and the corresponding concentration per gram total protein in follicular fluid.

Folate RBC = red blood cell folate

* $P \leq 0.001$

male factor compared to the supplemented women (28% versus 3%) ($P \leq 0.01$). Other causes for subfertility were not significantly different between these two subgroups. In the remaining 24 women, serum concentrations reflected sporadic folate intake, and the associated data was considered non-informative in this case.

Samples taken following ovarian stimulation (on the day of hCG administration) revealed tHcy concentrations to be significantly lower in follicular fluid than in blood. Women taking folic acid supplementation had significantly lower follicular fluid levels of tHcy, median 6.4 µmol/L (range 3.5 - 73.6 µmol/L) than non-supplemented women, median 7.1 µmol/L, (range 4.0 - 47.0) ($P \leq 0.005$). Folate concentrations were also observed to be consistently higher in blood than in follicular fluid. Folate concentrations in follicular fluid were significantly higher in supplemented women, median 35.8 nmol/L (range 13.0 - 234.2) compared with non-supplemented women, median 15.1 nmol/L (range 6.3 - 39.8) ($P \leq 0.001$). Cobalamin and pyridoxine concentrations were also significantly lower in follicular fluid than in blood ($P \leq 0.001$). All biomarker concentrations in blood were significantly correlated with follicular fluid levels (all $P \leq 0.001$) (Table 2).

During ovarian stimulation, serum estradiol concentrations significantly increased and blood tHcy, cobalamin and total protein levels all decreased significantly (Table 2). However, no significant correlations were found between the increase of the estradiol concentrations and the change in those biomarker concentrations in the blood. Likewise, the increase of the estradiol concentrations were not correlated with the biomarker concentrations in follicular fluid.

In the group as a whole, a significant inverse correlation between follicular fluid tHcy protein ratio and the follicular diameter was demonstrated (Table 3). A two-fold increase of the tHcy concentration was associated with a 0.06 mm decrease of the follicular diameter ($P \leq 0.05$). In non-supplemented women this inverse correlation was much stronger (1.64 mm; $P \leq 0.01$). In supplemented women a two-fold increase of the follicular folate concentration was associated with a 0.74 mm decrease of the follicular diameter ($P \leq 0.05$).

Table 3. Correlations between follicular fluid biomarker concentrations and follicular diameter stratified for folic acid supplement use

	Total group ($n = 279$ samples)	Supplement use ($n = 175$ samples)	Non-supplement use ($n = 51$ samples)
tHcy (µmol/g)	-0.06 s.*	-0.15 n.s.	-1.64 s.**
Folate (nmol/g)	-0.17 n.s.	-0.74 s.*	0.44 n.s.
Cobalamin (pmol/g)	0.49 n.s.	0.49 n.s.	0.25 n.s.
Pyridoxine (nmol/g)	0.29 n.s.	0.17 n.s.	0.73 n.s.

Correlations of Mixed Model Analysis are given as estimate and *P*-value; n.s. = not significant; s. = significant; * $P \leq 0.05$; ** $P \leq 0.01$

DISCUSSION

This study demonstrates that ovarian hyperstimulation in women undergoing IVF treatment is associated with a decrease of tHcy and cobalamin levels both in blood and in follicular fluid. Furthermore, inverse correlations are shown between follicular fluid tHcy and folate concentrations, and follicular diameter.

The observed decline in blood levels of tHcy and cobalamin levels during ovarian hyperstimulation treatment may be due to estradiol mediated induction of general enzyme activity through which the metabolization and clearance of both biomarkers might be increased. Two smaller studies reported no significant effect of ovarian hyperstimulation on biomarkers of the homocysteine pathway (24, 25). However, the inverse effect of rising estradiol levels on tHcy concentration is consistent with the results of studies comparing pre- and post-menopausal women (26, 27) and trials in which the administration of estrogen was compared with placebo in post-menopausal women (9, 14, 28). Similarly, a decrease in the cobalamin levels in estrogen supplemented post-menopausal women has been recently demonstrated (15).

In the present study, the concentration of tHcy in follicular fluid was inversely correlated with the follicular diameter. Since tHcy levels were adjusted for maturation by calculating the protein ratio (23), our data suggests that exposure of the follicles to high blood concentrations of tHcy may contribute to restricting follicular growth. The strong correlations between tHcy in blood and follicular fluid and the stronger correlation between tHcy and follicular diameter in non-supplemented women are consistent with this observation. Previously, our group demonstrated a positive correlation between tHcy in follicular fluid and follicular diameter (29). The levels of tHcy in follicular fluid in that study and the present study were similar. The discrepancy in results may reflect the small number of samples in our previous study.

On the basis of the present findings, it is not possible to determine whether high tHcy levels in follicular fluid are the cause or result of limited follicular growth. However, the present findings are consistent with our previously reported observation that a high tHcy in follicular fluid reduces embryo quality (17) and other reported associations between mild to moderate hyperhomocysteinemia and detrimental effects on reproductive outcome (5-7).

The toxic effects of tHcy may be due to the production of reactive oxygen species (ROS). A certain level of ROS in follicular fluid is necessary for oocyte maturation (30) and ovulation (31). Moreover, in a previous study, patients who became pregnant after IVF had significantly higher levels of ROS in their follicular fluid compared to non-pregnant women (32, 33). An excess of ROS results in a state of oxidative stress. High levels of ROS in culture medium of embryos are associated with low cleavage rates and high embryonic fragmentation rates (34). The results of our study seem to suggest that high levels of tHcy

have a detrimental effect on follicular maturation as reflected by the follicle diameter. This may be a consequence of oxidative stress. The decline of blood tHcy concentrations during ovarian hyperstimulation may suggest a beneficial effect of this hormonal treatment on follicle maturation. However, it is plausible that there is an optimal level of tHcy.

In the present study, a detrimental effect of both low and high folate levels on follicular growth is suggested. Folate supplementation appeared to protect follicular growth against the observed detrimental effects of high tHcy levels. Folic acid supplementation lowers tHcy levels by remethylation of tHcy into methionine. Surprisingly, we also observed that rising folate levels in follicular fluids of folic acid supplemented women, median 35.8 nmol/L, are associated with a significant smaller follicular diameter. There is an ongoing discussion about possible adverse effects of excessive folate levels with respect to increased twinning rates (35-37). Our results are consistent with the previously proposed concept of an 'optimal' level of supplementation above which reproductive outcomes may deteriorate. Such a concept would also reconcile two apparently contradictory observations: that supraphysiological estradiol concentrations are associated with suppression of tHcy concentrations in the follicle, yet may be detrimental to oocyte and embryo quality. It would appear that excessive suppression of tHcy levels by high estrogens may indeed be harmful to gamete and embryo quality.

Some limitations of this study have to be addressed. Our own study lacks a control group, because follicular fluid is not available from spontaneous cycles. There is little data about the spontaneous cycle, and only a decline of tHcy has been reported before (10). However, the extent of tHcy decrease as reported by Tallova et al., cannot be compared with the present results because of the different timing of sampling. The women recruited for the present study constitute a heterogeneous group in terms of race, age and indication for treatment. The effect of ovarian stimulation treatment on tHcy, folate, cobalamin and pyridoxine and the associations between the same biomarkers and follicular diameter may be more prominent in certain subgroups. However, the aim of our study was to investigate this in a representative population of women undergoing IVF or ICSI. The strength of our study is the standardised design, the large sample size and the biomarkers determined in blood and monofollicular fluids. Moreover, all participants were treated according to the same ovarian stimulation protocol. Future studies should address possible associations between nutritional biomarker concentrations in the follicle and serum, and clinical outcomes of IVF. Moreover, maternal gene polymorphisms involved in the homocysteine pathway have also been associated with an increased risk of offspring with Down syndrome possibly due to chromosomal damage (38-40). The findings of the present study indicate that further studies are required to address whether polymorphisms may also be associated with follicular growth.

In conclusion, ovarian stimulation resulted in a significant decline in tHcy and cobalamin blood concentrations but did not affect folate or pyridoxine levels. Follicular fluid tHcy

concentrations were inversely correlated with follicular diameter, and this association was much stronger in women who did not use a folic acid supplement. In folic acid supplemented women, follicular folate concentrations were inversely correlated with follicular diameter. This suggests that both high tHcy and high folate concentrations may be detrimental for follicular growth. This supports the need for finding the optimal folic acid dose not only to prevent neural tube defects but also to improve the quality of oogenesis.

Acknowledgements

The authors gratefully acknowledge the contribution of the IVF team of the division of Reproductive Medicine, and the laboratory technicians and research assistants of the Laboratory of Clinical Chemistry and the laboratory of Endocrinology of the Erasmus MC, University Medical Center in Rotterdam, The Netherlands for the laboratory determinations.

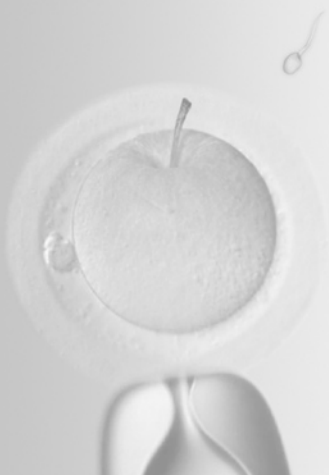
REFERENCES

1. Verhaak CM, Smeenk JM, Kremer JA, Braat DD, Kraaimaat FW. [The emotional burden of artificial insemination: increased anxiety and depression following an unsuccessful treatment]. Ned Tijdschr Geneeskd 2002;146:2363-6.
2. Lechner L, Bolman C, van Dalen A. Definite involuntary childlessness: associations between coping, social support and psychological distress. Hum Reprod 2007;22:288-94.
3. Andersen AN, Goossens V, Gianaroli L, Felberbaum R, de Mouzon J, Nygren KG. Assisted reproductive technology in Europe, 2003. Results generated from European registers by ESHRE. Hum Reprod 2007; 22:1513-25.
4. Macklon NS, Stouffer RL, Giudice LC, Fauser BC. The science behind 25 years of ovarian stimulation for in vitro fertilization. Endocr Rev 2006;27:170-207.
5. Steegers-Theunissen RP, Boers GH, Blom HJ, Trijbels FJ, Eskes TK. Hyperhomocysteinaemia and recurrent spontaneous abortion or abruptio placentae. Lancet 1992;339:1122-3.
6. Hague WM. Homocysteine and pregnancy. Best Pract Res Clin Obstet Gynaecol 2003;17:459-69.
7. Wouters MG, Boers GH, Blom HJ, Trijbels FJ, Thomas CM, Borm GF *et al.* Hyperhomocysteinemia: a risk factor in women with unexplained recurrent early pregnancy loss. Fertil Steril 1993;60:820-5.
8. Refsum H, Smith AD, Ueland PM, Nexo E, Clarke R, McPartlin J *et al.* Facts and recommendations about total homocysteine determinations: An expert opinion. Clin Chem 2004;50:3-32.
9. Madsen JS, Kristensen SR, Klitgaard NA, Bladbjerg EM, Abrahamsen B, Stilgren L *et al.* Effect of long-term hormone replacement therapy on plasma homocysteine in postmenopausal women: a randomized controlled study. Am J Obstet Gynecol 2002;187:33-9.
10. Tallova J, Tomandl J, Bicikova M, Hill M. Changes of plasma total homocysteine levels during the menstrual cycle. Eur J Clin Invest 1999;29:1041-4.
11. Velzing-Aarts FV, Holm PI, Fokkema MR, van der Dijs FP, Ueland PM, Muskiet FA. Plasma choline and betaine and their relation to plasma homocysteine in normal pregnancy. Am J Clin Nutr 2005;81: 1383-9.
12. Murphy MM, Scott JM, McPartlin JM, Fernandez-Ballart JD. The pregnancy-related decrease in fasting plasma homocysteine is not explained by folic acid supplementation, hemodilution, or a decrease in albumin in a longitudinal study. Am J Clin Nutr 2002;76:614-9.
13. Cikot RJ, Steegers-Theunissen RP, Thomas CM, de Boo TM, Merkus HM, Steegers EA. Longitudinal vitamin and homocysteine levels in normal pregnancy. Br J Nutr 2001;85:49-58.
14. Smolders RG, de Meer K, Kenemans P, Jakobs C, Kulik W, van der Mooren MJ. Oral estradiol decreases plasma homocysteine, vitamin B6, and albumin in postmenopausal women but does not change the whole-body homocysteine remethylation and transmethylation flux. J Clin Endocrinol Metab 2005; 90:2218-24.
15. Cagnacci A, Generali M, Pirillo D, Baldassari F, Volpe A. Effects of low- or high-dose hormone therapy on fasting and post-methionine homocysteine levels in postmenopausal women. Climacteric 2006;9: 388-95.
16. Berger PB, Herrmann RR, Dumesic DA. The effect of estrogen replacement therapy on total plasma homocysteine in healthy postmenopausal women. Mayo Clin Proc 2000;75:18-23.
17. Ebisch IM, Peters WH, Thomas CM, Wetzels AM, Peer PG, Steegers-Theunissen RP. Homocysteine, glutathione and related thiols affect fertility parameters in the (sub)fertile couple. Hum Reprod 2006; 21:1725-33.
18. Baart EB, Martini E, Eijkemans MJ, Van Opstal D, Beckers NG, Verhoeff A *et al.* Milder ovarian stimulation for in-vitro fertilization reduces aneuploidy in the human preimplantation embryo: a randomized controlled trial. Hum Reprod 2007;22:980-8.
19. Pfeiffer CM, Huff DL, Gunter EW. Rapid and accurate HPLC assay for plasma total homocysteine and cysteine in a clinical laboratory setting. Clin Chem 1999;45:290-2.

20. Schrijver J, Speek AJ, Schreurs WH. Semi-automated fluorometric determination of pyridoxal-5'-phosphate (vitamin B6) in whole blood by high-performance liquid chromatography (HPLC). Int J Vitam Nutr Res 1981;51:216-22.
21. http://www.cbs.nl/en-GB/default.htm Af. Statistics Netherlands [homepage on the Internet]. Voorburg/Heerlen: Classification of educational level and ethnicity Statistics Netherlands. [cited 2007 March 10]. In, 2007.
22. Brouwer IA, van Dusseldorp M, Thomas CM, Duran M, Hautvast JG, Eskes TK *et al.* Low-dose folic acid supplementation decreases plasma homocysteine concentrations: a randomized trial. Am J Clin Nutr 1999;69:99-104.
23. Spitzer D, Murach KF, Lottspeich F, Staudach A, Illmensee K. Different protein patterns derived from follicular fluid of mature and immature human follicles. Hum Reprod 1996;11:798-807.
24. Bettahar-Lebugle K, Feugeas O, Wittemer C, Ohl J, Rongieres C, Nisand I. [Evolution of homocysteine during ovarian stimulation for IVF or ICSI]. Gynecol Obstet Fertil 2002;30:121-8.
25. Roopnarinesingh R, Jackson B, Osman Z, Harrison R, Mayne P. Homocysteine in assisted reproduction: does oestradiol influence homocysteine levels? J Obstet Gynaecol 2006;26:59-62.
26. Morris MS, Jacques PF, Selhub J, Rosenberg IH. Total homocysteine and estrogen status indicators in the Third National Health and Nutrition Examination Survey. Am J Epidemiol 2000;152:140-8.
27. Hak AE, Polderman KH, Westendorp IC, Jakobs C, Hofman A, Witteman JC *et al.* Increased plasma homocysteine after menopause. Atherosclerosis 2000;149:163-8.
28. van Baal WM, Smolders RG, van der Mooren MJ, Teerlink T, Kenemans P. Hormone replacement therapy and plasma homocysteine levels. Obstet Gynecol 1999;94:485-91.
29. Boxmeer JC, Brouns RM, Lindemans J, Steegers EA, Martini E, Macklon NS *et al.* Preconception folic acid treatment affects the microenvironment of the maturing oocyte in humans. Fertil Steril. 2008; 89(6):1766-70.
30. Riley JC, Behrman HR. Oxygen radicals and reactive oxygen species in reproduction. Proc Soc Exp Biol Med 1991;198:781-91.
31. Miyazaki T, Sueoka K, Dharmarajan AM, Atlas SJ, Bulkley GB, Wallach EE. Effect of inhibition of oxygen free radical on ovulation and progesterone production by the in-vitro perfused rabbit ovary. J Reprod Fertil 1991;91:207-12.
32. Attaran M, Pasqualotto E, Falcone T, Goldberg JM, Miller KF, Agarwal A *et al.* The effect of follicular fluid reactive oxygen species on the outcome of in vitro fertilization. Int J Fertil Womens Med 2000; 45:314-20.
33. Bedaiwy MA, Miller K, Goldberg JM, Nelson DR, Agarwal A, Falcone T. Assessment of the predictive value of follicular fluid cytokines and reactive oxygen species in IVF cycles. Fertil Steril 2002;78:S5-S6.
34. Bedaiwy M, Agarwal A, Said TM, Goldberg JM, Sharma RK, Worley S *et al.* Role of total antioxidant capacity in the differential growth of human embryos in vitro. Fertil Steril 2006;86:304-9.
35. Haggarty P, McCallum H, McBain H, Andrews K, Duthie S, McNeill G *et al.* Effect of B vitamins and genetics on success of in-vitro fertilisation: prospective cohort study. Lancet 2006;367:1513-9.
36. Boxmeer JC, Fauser BC, Macklon NS. Effect of B vitamins and genetics on success of in-vitro fertilisation. Lancet 2006;368:200.
37. Muggli EE, Halliday JL. Folic acid and risk of twinning: a systematic review of the recent literature, July 1994 to July 2006. Med J Aust 2007;186:243-8.
38. Scala I, Granese B, Sellitto M, Salome S, Sammartino A, Pepe A *et al.* Analysis of seven maternal polymorphisms of genes involved in homocysteine/folate metabolism and risk of Down syndrome offspring. Genet Med 2006;8:409-16.
39. Coppede F, Colognato R, Bonelli A, Astrea G, Bargagna S, Siciliano G *et al.* Polymorphisms in folate and homocysteine metabolizing genes and chromosome damage in mothers of Down syndrome children. Am J Clin Genet 2007;143:2006-15.
40. Zintzaras E. Maternal gene polymorphisms involved in folate metabolism and risk of Down syndrome offspring: a meta-analysis. J Hum Genet 2007;52:943-53.

Chapter 5

IVF outcomes are associated with biomarkers of the homocysteine pathway in monofollicular fluid



Jolanda C. Boxmeer, Nick S. Macklon, Jan Lindemans, Nicole G.M. Beckers, Marinus J.C. Eijkemans, Joop S.E. Laven, Eric A.P. Steegers, Régine P.M. Steegers-Theunissen

Submitted for publication

ABSTRACT

Background: Maternal hyperhomocysteinemia is detrimental for reproduction, but the effects on embryo quality are unknown. The aim of this study was to investigate whether biomarkers of the homocysteine pathway are associated with IVF outcome.

Methods: In a prospective study we investigated associations between biomarkers of the homocysteine pathway and embryo quality and biochemical pregnancy in women undergoing IVF- or ICSI-treatment (*n* = 181). In the treatment cycle blood and monofollicular fluid samples were collected for determination of folate, cobalamin, and total homocysteine (tHcy) concentrations.

Results: Sixty-seven percent of the women used folic acid supplements. In blood, a significant correlation was established between high cobalamin and better embryo quality (standardized adjusted regression coefficient: – 0.17; 95% CI: -0.30, -0.01). In monofollicular fluid of non-supplemented women, high cobalamin correlated with better embryo quality (estimate: -0.87; 95% CI: -1.68, -0.06) whereas high tHcy resulted in poor embryo quality (estimate: 1.01; 95% CI: 0.08, 1.95). In monofollicular fluid of supplemented women high tHcy correlated with better embryo quality (estimate: -0.58; 95% CI: -1.12, -0.04). In the total group, a two-fold increase of monofollicular fluid folate corresponded with a 3.3 higher chance (95% CI: 1.09, 9.71) to achieve pregnancy.

Conclusions: An optimal homocysteine pathway in follicular fluid are associated with a better embryo quality and chance of pregnancy.

INTRODUCTION

In most couples who face difficulties in conceiving, the subfertility is considered to have a multifactorial origin. Whereas genetic causes are difficult to modulate, environmental and lifestyle factors also implicated in subfertility are potentially amenable to curative or preventive measures. Nutrition is an important remediable factor, but continues to be largely neglected when counselling and managing subfertile couples. In recent years, interest has increased in the role of folate and cobalamin as modulators of fertility outcome (1, 2). These B vitamins have a central role in the homocysteine (tHcy) pathway. Shortages of folate and vitamin B12 result in hyperhomocysteinemia, which is associated with a number of adverse pregnancy outcomes, such as recurrent miscarriages, pregnancy induced hypertension and abruptio placentae (3-5). Hyperhomocysteinemia is also associated with the occurrence of several congenital malformations, such as neural tube defects, orofacial clefts and congenital heart disease (6-10). This has led to the recommendation of periconception folic acid supplementation and, in some countries, even to folic acid fortification of flour. Recently, our group demonstrated also a detrimental effect of high tHcy in ejaculated sperm and follicular fluid on embryo quality (11).

A low cobalamin status has also been associated with a higher risk of neural tube defects, oral facial clefts, congenital heart disease and spontaneous abortions (12-16). Furthermore, several studies and case reports suggest an association between cobalamin deficiency and female subfertility (17, 18).

Assisted reproductive technologies provide the opportunity to study the effects of biomarkers of nutrients in follicular fluid on fertility treatment outcomes, such as embryo quality. Follicular fluid offers a window on the intimate environment of the developing cumulus-oocyte complex that can easily be obtained during in vitro fertilization (IVF) treatment. Our group previously demonstrated significant correlations between folate, cobalamin and total homocysteine (tHcy) in blood and follicular fluid (19). Moreover, follicular fluid folate concentrations have been demonstrated to be higher in folic acid supplemented women while tHcy concentrations are lower (20, 21).

From this background we aimed to investigate whether the concentrations of tHcy and the intermediates folate and cobalamin in blood and follicular fluid are associated with embryo quality and the chance of achieving biochemical pregnancy in couples undergoing IVF treatment with or without intracytoplasmic sperm injection (ICSI).

MATERIALS AND METHODS

Study population

The FOod, Lifestyle and Fertility Outcome-project (FOLFO-project) was set up to study the influence of preconception nutrition and lifestyle on fertility and pregnancy outcome. Between September 2004 and October 2006, 292 subfertile couples undergoing an IVF procedure with or without ICSI at the tertiary referral fertility clinic, the Erasmus MC, University Medical Centre, Rotterdam, The Netherlands, were invited to participate. Women diagnosed with endometriosis and women with a hydrosalpinx were excluded since these conditions detrimentally influence IVF outcome. In addition, couples undergoing oocyte donation could not participate. The study protocol was approved by the Dutch Central Committee for Human Research (CCMO) and the Medical Ethical and Institutional Review Board of the Erasmus Medical Centre in Rotterdam. Participants provided written informed consent and obtained materials and questionnaires were processed anonymously.

It has previously been demonstrated that in women of reproductive age using 250 µg folic acid per day for four weeks, serum folate concentrations increase to a mean value of 22.5 nmol/L (22). To minimize bias, we defined folic acid supplement users as women having a serum folate concentration on both cycle day two and the day of hCG administration above 22.5 nmol/L. Women with serum folate concentrations below this level at both time points were defined as non supplement users.

In order to investigate more precise whether concentrations of tHcy and B-vitamins in follicular fluid are correlated with the occurrence of biochemical pregnancy, additional analyses were performed in women with single embryo transfer of an embryo that developed out of a collected monofollicular fluid ('FOLFO-ET').

In vitro fertilization procedure

All women started the ovarian stimulation treatment with daily injections of 150 IU recombinant Follicle Stimulating Hormone (rFSH) sc on cycle day two (Puregon®, NV Organon, Oss, The Netherlands or Gonal-F®, Serono Benelux BV, The Hague, The Netherlands). Administration of daily sc Gonadotrophin Releasing Hormone (GnRH) antagonist (Orgalutran®, NV Organon, or Cetrotide®, Serono Benelux BV) was started when at least one follicle was ≥14 mm, as described previously (23). To induce final oocyte maturation, a single dose of 5,000 or 10,000 IU human Chorionic Gonadotropin (hCG) sc (Pregnyl®, NV Organon) was administered as soon as the largest follicle reached at least 18 mm in diameter and at least one additional follicle of more than 15 mm was observed. Oocyte retrieval was carried out 35 hours 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 three after oocyte pick up a maximum of two embryos were transferred.

Sample collection and analyses

During oocyte retrieval follicle diameters were remeasured and monofollicular fluid from the leading follicle was aspirated from each ovary and collected separately. Oocytes isolated from these monofollicular fluids were washed and transferred to a separate droplet of medium in order to monitor embryo quality. The monofollicular fluid samples were centrifuged for 10 minutes at 1,700x g 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 cycle day two before the first injection of rFSH and at the day of hCG administration. Folate, cobalamin, tHcy, FSH, estradiol (E2) and AMH concentrations were determined. For the 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. Blood serum and monofollicular fluid samples from each patient were analyzed during routine laboratory procedures for folate and cobalamin 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, Mannheim, Germany). Serum concentrations of FSH were measured by luminescence-based immunometric assay (Immulite 2000, Diagnostic Products Corporation (DPC), Los Angeles, CA, USA) and E2 was determined using coated tube radioimmunoassay obtained from the same supplier. AMH concentrations were assayed using an in-house double antibody enzyme-linked immunosorbent assay (ELISA), commercially available through Diagnostic Systems Laboratories (Webster, TX).

For the determination of RBC folate and plasma tHcy, venous blood samples were drawn into ethylenediamine tetra-acetate (EDTA) containing vacutainer tubes. The EDTA-blood samples were placed on ice and within one hour plasma was separated by centrifugation for determination of tHcy. Homocysteine in EDTA plasma and monofollicular fluid was determined during routine laboratory procedures using high performance liquid chromatography with reversed phase separation and fluorescence detection (24). For the determination of RBC folate, 100 µL blood from an EDTA tube was hemolyzed with 2 mL freshly prepared ascorbic acid (0.05g 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 (Groffin Meyvis, Etten-Leur, The Netherlands). The hemolysate was centrifuged for 10 minutes at 2,000 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 * 21) - (nmol/L serum folate * (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 µmol/L and 3.3 % at 34.2 µmol/L, for total protein 1.5 % at 55 g/L and 1.3 % at 84 g/L, for

FSH these coefficients were < 5.8 %, for E2 < 8.8 %, and for AMH <10%. The detection limit for folate was 1.36 nmol/L, for cobalamin 22 pmol/L, for tHcy 4 µmol/L, for total protein 0.1 g/L, for FSH 0.1 U/L, and for E2 10 pmol/L.

Questionnaires

In addition, on the day of oocyte pick up, participants were invited to fill out a general questionnaire from which the following data were extracted: medical history, height and weight, ethnicity, use of medication, and lifestyle factors, such as smoking and the use of vitamin supplements in the preceding month. Ethnicity was classified according to the definitions of Statistics Netherlands (25).

Major outcomes

The clinical endpoints of the study were embryo quality and the occurrence of biochemical pregnancy. On day three post oocyte retrieval embryo quality scores were assigned according to previously described criteria (26). These scores ranged from one (best quality) to five (poor quality and inadequate for embryo transfer). Biochemical pregnancy was determined by a urinary pregnancy test 15 days after oocyte retrieval.

Statistical analysis

Monofollicular fluid total protein concentrations vary with follicular maturity (27). Concentrations of tHcy and B-vitamins in monofollicular fluid are therefore expressed in concentration per gram of protein. Multiple linear and logistic regression analyses were performed forward to determine associations between tHcy, and B-vitamin concentrations in blood and the outcome parameters mean embryo score and biochemical pregnancy. In most subjects, two monofollicular fluid samples were obtained. In order to avoid a possible statistical bias arising from subjects producing just one fluid sample, a mixed model analysis was performed in which one or two samples per subjects could be analysed. With this analysis correlations between the concentrations of B-vitamins and tHcy in monofollicular fluid and embryo score and pregnancy were determined.

Age and smoking are major independent determinants of fertility outcome and therefore, were considered as potential confounders. In the analyses of biochemical pregnancy as outcome parameter the number of transferred embryos was included as additional potential confounder. Covariates were included as confounders in the model when the *P*-value was equal or less than 0.1.

In the subgroup of women with single embryo transfer of an embryo that developed from the oocyte derived of a collected monofollicular fluid ('FOLFO-ET'), logistic regression analyses were performed to determine correlations between the concentrations of B-vitamin and tHcy in monofollicular fluid and the occurrence of pregnancy.

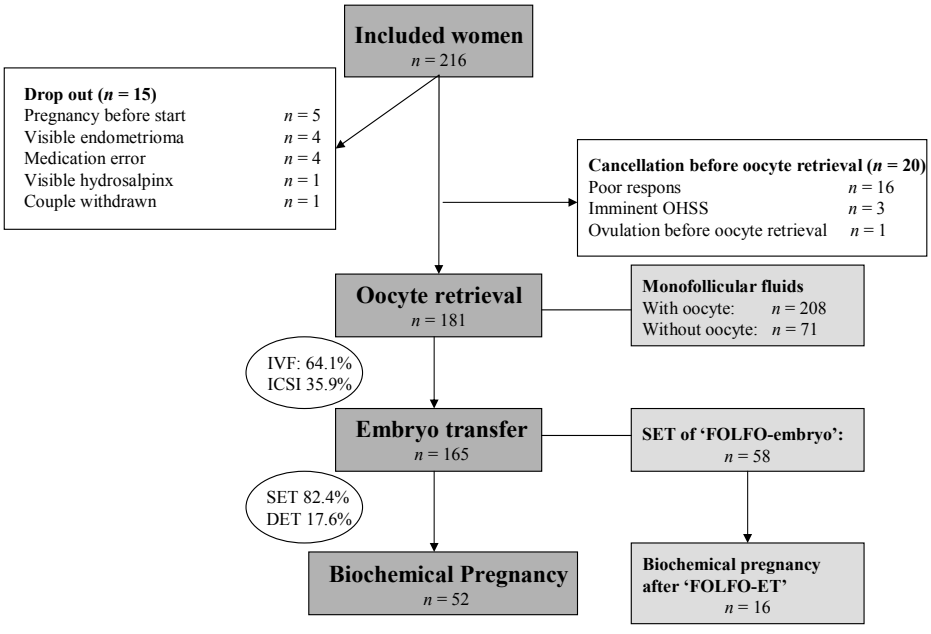
A *P*-value of < 0.05 was considered statistically significant. All statistical analyses were performed using SPSS 14.0 for Windows software (SPSS Inc, Chicago, Il, USA).

RESULTS

Study population

Seventy-four percent of the eligible couples participated in the study (*n* = 216). Reasons for not participating were: couples not being prepared to spend time visiting the hospital and/or completing questionnaires, having a language barrier, or a doctor forgetting to ask for participation. Of these couples, 15 dropped out and 20 women did not undergo oocyte retrieval for reasons specified in figure 1. The baseline characteristics of the 181 participating women are given in Table 1, along with overall treatment outcomes. Data on BMI, smoking and folic acid supplementation were missing in respectively 15, 14 and 17 women. Eighty-seven percent of the participants reported the use of folic acid containing supplements (*n* = 143). Of these women 24% (*n* = 34) reported to use more than one folic acid containing supplement. However, in 113 women serum folate concentrations were consistent with a regular folic acid intake. In 32 women, serum concentrations indicated

Figure 1. Flowchart of included couples



OHSS: Ovarian Hyper Stimulation Syndrome; SET: single embryo transfer; DET: double embryo transfer; FOLFO-ET: Single embryo transfer of an embryo that developed out of a collected monofollicular fluid

Table 1. Baseline characteristics of the study population and overall treatment outcome (n = 181).

Age (years), mean (SD)	35.5	(4.4)
BMI (kg/m^2), mean (SD)	23.9	(3.8)
Smoking, yes, % (no.)	9	(15)
Folic acid supplementation (questionnaire), yes, % (no.)	87.2	(143)
Folic acid supplementation (biochemistry), yes, % (no.)	66.9	(113)
Cause of subfertility: Female factor, % (no.)	18.2	(33)
Male factor, % (no.)	44.2	(80)
Male and female factor, % (no.)	8.3	(15)
Idiopathic, % (no.)	29.3	(53)
FSH, cycle day two (U/L), median (range)	8.4	(0.4 – 30.3)
Estradiol, cycle day two (pmol/L), median (range)	136.5	(41 – 307)
AMH, cycle day two (µg/L), median (range)	2.1	(0.1 - 18.6)
Embryo score of fertilized oocytes, mean (SD)	2.7	(0.6)
Biochemical pregnancy per oocyte retrieval, % (no.)	29	(52)

SD, standard deviation.

that folic acid supplements had not been taken. In the remaining 24 women serum concentrations indicated an irregular folic acid intake. One or both blood samples were missing from 12 women.

Serum parameters

Day two blood samples of the treatment cycle from 177 women were available for analyses (Table 2). The results were expressed in median (range) because of the skewed distributions of FSH, E2, B-vitamins and tHcy. Consequently these variables were log-transformed before statistical testing.

In the total group and the subgroup of non-supplemented women a significant correlation was established between a high cobalamin concentration in blood and a high mean embryo quality (low score) (standardized adjusted regression coefficient: – 0.17, 95% confidence interval (CI): -0.30, -0.01 and standardized adjusted regression coefficient: – 0.39, 95% CI: -0.81, -0.00 respectively). Folate and tHcy concentrations in blood were not correlated with mean embryo quality and none of the B-vitamins or tHcy concentrations in serum was significantly correlated with the occurrence of biochemical pregnancy.

Biomarkers in monofollicular fluid

Of the 299 collected monofollicular fluid samples from 181 women, 20 samples from 14 women were excluded from analysis due to contamination with culture medium, which itself contains very high concentrations of cobalamin (> 14000 pmol/L) and folate (> 950 nmol/L). Of the remaining 279 monofollicular fluid samples, 208 contained one oocyte. The results are expressed in median (range) because of the skewed distributions and the variables were consequently log-transformed before statistical testing (Table 3).

Table 2. Blood levels of B-vitamins and tHcy on cycle day two and associations with IVF/ICSI outcomes.

				Mean embryo score [c]		Biochemical pregnancy	
		Median (range)		Regression coefficient [d]	95% CI	Odds Ratio [e]	95% CI
Total group (n = 177)	Folate, serum, (nmol/L)	30.5	(6.4-908)	-0.05	-0.14, 0.07	1.07	0.72, 1.59 [1,3]
	Folate, RBC [d], (nmol/L)	1407	(459-3611)	-0.02	-0.20, 0.16	0.67	0.36, 1.25 [3]
	Cobalamin, serum, (pmol/L)	319	(74-1856)	-0.17*	-0.30, -0.01	1.19	0.68, 2.10 [1,3]
	tHcy, plasma, (µmol/L)	9.1	(5-75.3)	0.03	-0.18, 0.25	0.96	0.42, 2.20 [1,3]
Supplemented [a] (n = 113)	Folate, serum, (nmol/L)	36.4	(22.9-908)	-0.09	-0.22, 0.09	1.10	0.60, 2.02 [3]
	Folate, RBC, (nmol/L)	1561	(578-3611)	0.20	-0.04, 0.51	1.04	0.37, 2.92 [3]
	Cobalamin, serum, (pmol/L)	351	(74-1856)	-0.11	-0.28, 0.08 [2]	1.31	0.64, 2.70 [3]
	tHcy, plasma, (µmol/L)	8.8	(5-75.3)	0.08	-0.24, 0.57	1.72	0.99, 31.46 [3]
Non-supplemented [b] (n = 32)	Folate, serum, (nmol/L)	14.3	(6.4-20.7)	0.03	-0.54, 0.62	1.37	0.22, 8.72
	Folate, RBC, (nmol/L)	771.5	(459-2408)	-0.27	-0.66, 0.16	0.57	0.15, 2.14
	Cobalamin, serum, (pmol/L)	276.5	(130-589)	-0.39*	-0.81, -0.00 [1,2]	1.26	0.37, 4.25
	tHcy, plasma, (µmol/L)	10.7	(6.1-50.9)	-0.18	-0.54, 0.18	0.40	0.09, 1.88

RBC, red blood cells; CI, confidence interval.

* $P = 0.04$

[a] Supplemented, women who had taken folic acid supplements according to serum folate concentrations on 2 time points.

[b] Non-supplemented, women who had not taken folic acid supplements according to serum folate concentrations on 2 time points.

[c] Embryo scores ranged from 1 (best quality) to 5 (poor quality and inadequate for embryo transfer).

[d] Regression coefficient, Standardized adjusted regression coefficient; the potential confounders [1]age and [2]smoking were only included in the model when $P \leq 0.1$.

[e] The potential confounders [1]age and the [3]number of transferred embryos were only included in the model when $P \leq 0.1$.

Table 3. Levels of the biomarkers of the homocysteine pathway in monofollicular fluid and associations with embryo quality.

				Embryoscore [d]		
		Median [c] (range)		Estimate [e]	95% CI	
Total group (*n* = 279 samples)	Folate (nmol/g)	0.55	(0.11-4.04)	-0.10	-0.33, 0.12	[1]
	Cobalamin (pmol/g)	3.81	(1.02-34.42)	-0.05	-0.32, 0.22	[1]
	tHcy (µmol/g)	0.12	(0.03-1.27)	0.07	-0.35, 0.48	[1]
Supplemented [a] (*n* = 175 samples)	Folate (nmol/g)	0.64	(0.36-4.04)	-0.04	-0.34, 0.26	
	Cobalamin (pmol/g)	3.91	(1.02-34.42)	0.04	-0.24, 0.33	
	tHcy (µmol/g)	0.12	(0.06-1.27)	-0.58	-1.12, -0.04	*
Non-supplemented [b] (*n* = 51 samples)	Folate (nmol/g)	0.27	(0.11-0.63)	-0.60	-1.50, 0.29	
	Cobalamin (pmol/g)	3.81	(1.65-7.00)	-0.87	-1.68, -0.06	[1] *
	tHcy (µmol/g)	0.14	(0.07-0.80)	1.01	0.08, 1.95	*

CI, confidence interval.

* $P = 0.04$

[a] Supplemented: defined as women who had taken folic acid supplements based on serum folate concentrations on 2 time points above 22.5 nmol/L

[b] Non-supplemented: defined as women who had not taken folic acid supplements based on serum folate concentrations on 2 time points below 22.5 nmol/L.

[c] Concentrations of biomarkers in follicular fluid are given in concentration per g total protein.

[d] Embryo scores ranged from 1 (best quality) to 5 (poor quality and inadequate for embryo transfer).

[e] Mixed model analysis with 2log transformed biomarker concentrations with the potential confounders [1]age included in the model ($P \leq 0.1$).

No correlations were found between B-vitamins and tHcy in follicular fluid and the quality of the corresponding embryo in the total group. However, higher tHcy concentrations in monofollicular fluid of supplemented women corresponded with better embryo quality (adjusted estimate: -0.58; 95% CI: -1.12, -0.04). In monofollicular fluid of non-supplemented women cobalamin and tHcy concentrations were significantly correlated with embryo quality of the corresponding follicle. Higher cobalamin concentrations in follicular fluid corresponded with a better embryo quality after adjustment for age (adjusted estimate: -0.87; 95% CI: -1.68, -0.06) while higher tHcy concentrations corresponded with a poorer embryo quality (adjusted estimate = 1.01, 95% CI: 0.08, 1.95).

In the subgroup of women with a single embryo transfer of an embryo derived from an individually aspirated and analyzed follicle (*n* = 58) cobalamin and tHcy were not correlated with the occurrence of biochemical pregnancy. However, after adjustment for age, a doubling in follicular folate concentrations increased the chance of biochemical pregnancy by a factor of 3.3 (95% CI: 1.09, 9.71) (Table 4). In this group, pregnant women were significantly younger compared to non-pregnant women (34 versus 37 years, $P = 0.02$). Pregnant and non-pregnant women were not different in BMI, smoking, and serum concentrations of FSH, E2, AMH, folate, cobalamin and tHcy.

Table 4. Associations between the levels of the biomarkers of the homocysteine pathway in monofollicular fluid and the chance of achieving pregnancy in women with single embryo transfer (n = 58).

	Median [a] (range)		Adjusted Odds Ratio	95% CI	*P*-value
Folate (nmol/g)	0.57	(0.12-4.04)	3.26	1.09, 9.71	0.03
Cobalamin (pmol/g)	3.84	(1.80-81.29)	1.21	0.69, 2.11	0.51
tHcy (µmol/g)	0.12	(0.07-0.80)	0.37	0.07, 1.95	0.24

Logistic regression analyses with 2logtransformed concentrations of the biomarkers. The Odds Ratio's are adjusted for the potential confounder age ($P \leq 0.1$). [a] Concentrations of biomarkers in monofollicular fluid are given in concentration per g total protein.
CI, confidence interval.

DISCUSSION

In this study, we have demonstrated that folate, cobalamin and tHcy levels in monofollicular fluid are related to embryo quality and the chance of achieving biochemical pregnancy in women undergoing IVF/ICSI.

These associations are of particular importance with regard to folic acid campaigns and food fortification programs. It is the Dutch policy to advise all women who want to become pregnant to take a folic acid supplement of 0.4 - 0.5 mg/day. Data from the questionnaire indicated that about 86 % of the subfertile patients took a folic acid containing supplement prior to and during IVF treatment. However, after verification of the intake by the biomarker concentration of folate in blood, thereby using the cut-of value of 22.5 nmol/L as defined by Brouwer et al (22), it revealed that 67% of the study population had consistently taken folic acid. Although the use of folic acid in our study population is much higher compared to that reported in Dutch pregnant women (28), it was surprisingly low for a highly motivated, informed group of subfertile patients participating in a study directed on nutrition and lifestyle. On the other hand, 24% of the supplemented women reported taking more than one folic acid containing supplement, resulting in very high folate concentrations in blood and follicular fluid. Therefore, even in highly motivated, subfertile patients more education about folic acid supplementation seems necessary.

Of most interest is the significant correlation between monofollicular fluid folate and the occurrence of biochemical pregnancy. The importance of folate as a determinant of embryo quality is illustrated by the correlation between monofollicular fluid cobalamin and embryo quality observed in non-supplemented women. The harmful effect of a relative shortage of cobalamin in monofollicular fluid could only be demonstrated when the folate concentrations were also relatively low. Folate is a methyl group donor in the homocysteine pathway but also for many other pathways involving protein synthesis. Therefore, a shortage of several folate dependent proteins is suggested to play a significant role in

the periconception period (29). Folate deficiency can also lead to mutations caused by the incorporation of uracil into the DNA instead of thymine (30, 31). Repair processes which remove the misincorporated uracil often fail, leading to double strand breaks and chromosome instability which promotes apoptosis (30, 31). Synthesis and repair of DNA as well as methylation of DNA is crucial in gametogenesis, fertilization and pregnancy (32, 33). The majority of the women had normal to high folate concentrations. However, the optimal folate concentration in monofollicular fluid to increase the chance of pregnancy still has to be determined.

In the total group of women and the subgroup of non-supplemented women the mean blood cobalamin concentration was positively correlated with mean embryo quality. In the subgroup of women who had not taken folic acid supplements, cobalamin in monofollicular fluid was also associated with a better quality of the corresponding embryo. Cobalamin is a cofactor for the folate-dependent methionine synthase, which is involved in homocysteine remethylation. A low cobalamin concentration reduces methionine synthase activity, lowering the concentration of methionine. Methionine is either incorporated in various peptides or transmethylated into S-adenosylmethionine (SAM). A deficiency of SAM reduces DNA methylation and consequently leads to hypomethylation of DNA which may lead to aberrant patterns of gene expression (34). Another consequence of low cobalamin concentrations is a decreased bioavailability of folate and a decreased activity of the mitochondrial methylmalonyl CoA mutase. Several small studies and case reports have reported an association between cobalamin deficiency and female subfertility (14, 17, 18) but the mechanisms behind this association are not clear. In the present study serum cobalamin concentrations were correlated with embryo quality but not with pregnancy. The latter observation may be explained by the power of the study. Thus, women without clinical symptoms of cobalamin deficiency, but with a marginal cobalamin state may benefit from additional cobalamin supplementation.

Interestingly, the association between monofollicular fluid tHcy concentrations and embryo quality differed between the subgroups of supplemented and non-supplemented women. In non-supplemented women the monofollicular tHcy concentrations were slightly but statistically significantly higher compared to supplemented women (0.14 versus 0.12 µmol/g protein). In these women tHcy was negatively correlated with embryo quality. This is in line with our previous findings (11). However, in supplemented women tHcy in monofollicular fluid was positively correlated with embryo quality. These data may suggest that there must be an optimal monofollicular tHcy concentration. The detrimental effect of high tHcy concentrations might be due to an excessive production of reactive oxygen species (ROS) thereby inducing excessive oxidative stress. However, physiological levels of ROS in follicular fluid are necessary for oocyte maturation (35), ovulation (36) and fertilization and therefore a very low tHcy concentration may be detrimental as well.

Correlations between tHcy, folate and embryo quality and pregnancy were only statistically significant in monofollicular fluid. Biomarker concentrations in blood and monofollicular fluid are significantly correlated but the levels are not similar (19). In order to demonstrate also significant correlations between biomarkers in blood and outcome parameters, it may be necessary to extend the sample size.

The limitations of this study have to be considered as well. The included patients are a sample of women undergoing IVF with or without ICSI in our clinic. They constitute a heterogeneous group in terms of race, age and indication of treatment. However, they are representative for this particular population. The participation in this study may have influenced lifestyle behaviors, which may explain the relatively high number of folic acid supplement users compared to the 43 % in the general Dutch population (28). To define folic acid users the cut-off value of women in reproductive age, i.e., 22.5 nmol/L, is used (22). The age range of our population was between 24 and 44 years and was only slightly older than those described by Brouwer et al. (aged 18-40 years). It is not very likely that these women react differently on folic acid supplementation. However, this cannot be excluded. Furthermore, there are no other cut off points available from populations more similar to ours. The strength of our study is the large sample size and the biomarkers determined in both blood and most importantly in monofollicular fluid. Although ongoing pregnancy and pregnancy outcome would have been interesting endpoints to study, for these associations the present study was underpowered.

In conclusion, we have shown that high concentrations of folate in monofollicular fluid are associated with an increased chance of achieving biochemical pregnancy. Cobalamin concentrations in early follicular phase serum and in monofollicular fluid are positively correlated with embryo quality, whereas both low and high concentrations of tHcy in monofollicular fluid are associated with reduced embryo quality, suggesting that there must be an optimal level. These findings support the importance of periconception folic acid supplementation and may suggest the additional intake of cobalamin. Clearly, further studies are now required to confirm these data and to find the best and safest B vitamin dosages.

Acknowledgements

The authors gratefully acknowledge the contribution of the IVF team, division of Reproductive Medicine for the sample collection and the laboratory technicians and research assistants of the Laboratories of Clinical Chemistry and Endocrinology of the Erasmus MC, University Medical Center in Rotterdam, The Netherlands for the laboratory determinations. We thank Professor F.H. de Jong for the facilitation of endocrinology determinations and Dr. M.F. Wildhagen for his excellent support of all aspects of data-base building, guidance and support.

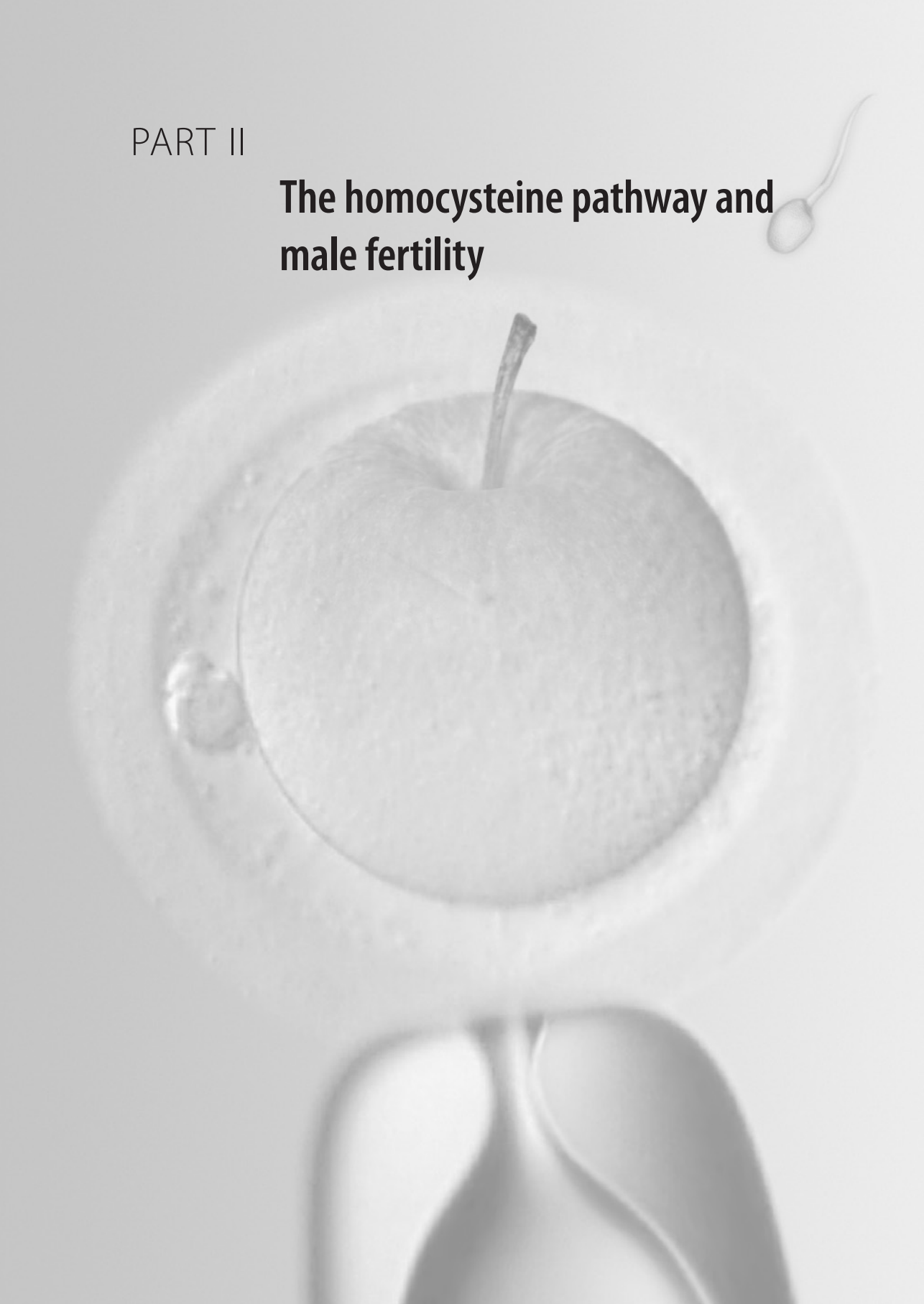
REFERENCES

1. Forges T, Monnier-Barbarino P, Alberto JM, Gueant-Rodriguez RM, Daval JL, Gueant JL. Impact of folate and homocysteine metabolism on human reproductive health. Hum Reprod Update 2007;13: 225-38.
2. Ebisch IM, Thomas CM, Peters WH, Braat DD, Steegers-Theunissen RP. The importance of folate, zinc and antioxidants in the pathogenesis and prevention of subfertility. Hum Reprod Update 2007;13: 163-74.
3. Steegers-Theunissen RP, Boers GH, Blom HJ, Trijbels FJ, Eskes TK. Hyperhomocysteinaemia and recurrent spontaneous abortion or abruptio placentae. Lancet 1992;339:1122-3.
4. Nelen WL, Blom HJ, Steegers EA, den Heijer M, Thomas CM, Eskes TK. Homocysteine and folate levels as risk factors for recurrent early pregnancy loss. Obstet Gynecol 2000;95:519-24.
5. Hague WM. Homocysteine and pregnancy. Best Pract Res Clin Obstet Gynaecol 2003;17:459-69.
6. Botto LD, Olney RS, Erickson JD. Vitamin supplements and the risk for congenital anomalies other than neural tube defects. Am J Med Genet C Semin Med Genet 2004;125:12-21.
7. Tamura T, Picciano MF. Folate and human reproduction. Am J Clin Nutr 2006;83:993-1016.
8. Wong WY, Eskes TK, Kuijpers-Jagtman AM, Spauwen PH, Steegers EA, Thomas CM *et al.* Nonsyndromic orofacial clefts: association with maternal hyperhomocysteinemia. Teratology 1999;60:253-7.
9. Steegers-Theunissen RP, Boers GH, Trijbels FJ, Eskes TK. Neural-tube defects and derangement of homocysteine metabolism. N Engl J Med 1991;324:199-200.
10. Verkleij-Hagoort AC, Verlinde M, Ursem NT, Lindemans J, Helbing WA, Ottenkamp J *et al.* Maternal hyperhomocysteinaemia is a risk factor for congenital heart disease. BJOG 2006;113:1412-8.
11. Ebisch IM, Peters WH, Thomas CM, Wetzels AM, Peer PG, Steegers-Theunissen RP. Homocysteine, glutathione and related thiols affect fertility parameters in the (sub)fertile couple. Hum Reprod 2006; 21:1725-33.
12. Verkleij-Hagoort AC, De Vries JH, Ursem NT, de Jonge R, Hop WC, Steegers-Theunissen RP. Dietary intake of B-vitamins in mothers born a child with a congenital heart defect. Eur J Nutr 2006;45:478-86.
13. Ray JG, Wyatt PR, Thompson MD, Vermeulen MJ, Meier C, Wong PY *et al.* Vitamin B12 and the risk of neural tube defects in a folic-acid-fortified population. Epidemiology 2007;18:362-6.
14. Bennett M. Vitamin B-12 deficiency, infertility and recurrent fetal loss. J Reprod Med 2001;46:209-12.
15. Groenen PM, van Rooij IA, Peer PG, Gooskens RH, Zielhuis GA, Steegers-Theunissen RP. Marginal maternal vitamin B12 status increases the risk of offspring with spina bifida. Am J Obstet Gynecol 2004;191:11-7.
16. van Rooij IA, Swinkels DW, Blom HJ, Merkus HM, Steegers-Theunissen RP. Vitamin and homocysteine status of mothers and infants and the risk of nonsyndromic orofacial clefts. Am J Obstet Gynecol 2003;189:1155-60.
17. Jackson I, Doig WB, McDonald G. Pernicious anaemia as a cause of infertility. Lancet 1967;290:1159-60.
18. Girdwood RH, Eastwood MA, Finlayson ND, Graham GS. Pernicious anaemia as a cause of infertility in twins. Lancet 1971;1:528-30.
19. Steegers-Theunissen RP, Steegers EA, Thomas CM, Hollanders HM, Peereboom-Stegeman JH, Trijbels FJ *et al.* Study on the presence of homocysteine in ovarian follicular fluid. Fertil Steril 1993;60:1006-10.
20. Szymanski W, Kazdepka-Zieminska A. [Effect of homocysteine concentration in follicular fluid on a degree of oocyte maturity]. Ginekol Pol 2003;74:1392-6.
21. Boxmeer JC, Brouns RM, Lindemans J, Steegers EA, Martini E, Macklon NS *et al.* Preconception folic acid treatment affects the microenvironment of the maturing oocyte in humans. Fertil Steril. 2008; 89(6):1766-70.

22. Brouwer IA, van Dusseldorp M, Thomas CM, Duran M, Hautvast JG, Eskes TK *et al.* Low-dose folic acid supplementation decreases plasma homocysteine concentrations: a randomized trial. Am J Clin Nutr 1999;69:99-104.
23. Hohmann FP, Macklon NS, Fauser BC. A randomized comparison of two ovarian stimulation protocols with gonadotropin-releasing hormone (GnRH) antagonist cotreatment for in vitro fertilization commencing recombinant follicle-stimulating hormone on cycle day 2 or 5 with the standard long GnRH agonist protocol. J Clin Endocrinol Metab 2003;88:166-73.
24. Pfeiffer CM, Huff DL, Gunter EW. Rapid and accurate HPLC assay for plasma total homocysteine and cysteine in a clinical laboratory setting. Clin Chem1999;45:290-2.
25. http://www.cbs.nl/en-GB/default.htm Af. Statistics Netherlands [homepage on the Internet]. Voorburg/Heerlen: Classification of educational level and ethnicity Statistics Netherlands. [cited 2007 March 10]. In, 2007.
26. Huisman GJ, Fauser BC, Eijkemans MJ, Pieters MH. Implantation rates after in vitro fertilization and transfer of a maximum of two embryos that have undergone three to five days of culture. Fertil Steril 2000;73:117-22.
27. Spitzer D, Murach KF, Lottspeich F, Staudach A, Illmensee K. Different protein patterns derived from follicular fluid of mature and immature human follicles. Hum Reprod 1996;11:798-807.
28. de Walle HE, de Jong-van den Berg LT. Growing gap in folic acid intake with respect to level of education in the Netherlands. Commun Genet 2007;10:93-6.
29. Bliek JB, de Klein A, Luider TM, Lindemans J, Hulsman L, Guzel C *et al.* New approach for the identification of folate-related pathways in human embryogenesis. Cell Mol Biol (Noisy-le-grand) 2004;50: 939-44.
30. Blount BC, Mack MM, Wehr CM, MacGregor JT, Hiatt RA, Wang G *et al.* Folate deficiency causes uracil misincorporation into human DNA and chromosome breakage: implications for cancer and neuronal damage. Proc Natl Acad Sci USA 1997;94:3290-5.
31. Koury MJ, Horne DW, Brown ZA, Pietenpol JA, Blount BC, Ames BN *et al.* Apoptosis of late-stage erythroblasts in megaloblastic anemia: association with DNA damage and macrocyte production. Blood 1997;89:4617-23.
32. Jaroudi S, SenGupta S. DNA repair in mammalian embryos. Mutat Res 2007;635:53-77.
33. Kiefer JC. Epigenetics in development. Dev Dyn 2007;236:1144-56.
34. Reik W. Stability and flexibility of epigenetic gene regulation in mammalian development. Nature 2007;447:425-32.
35. Riley JC, Behrman HR. Oxygen radicals and reactive oxygen species in reproduction. Proc Soc Exp Biol Med 1991;198:781-91.
36. Miyazaki T, Sueoka K, Dharmarajan AM, Atlas SJ, Bulkley GB, Wallach EE. Effect of inhibition of oxygen free radical on ovulation and progesterone production by the in-vitro perfused rabbit ovary. J Reprod Fertil 1991;91:207-12.

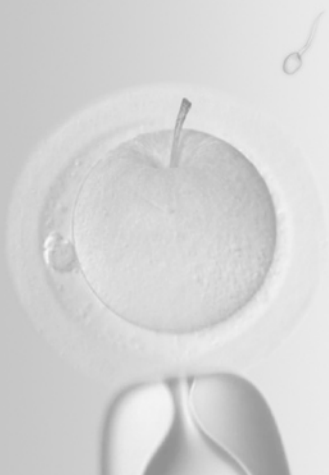
PART II

The homocysteine pathway and male fertility



Chapter 6

Seminal plasma cobalamin significantly correlates with sperm concentration in males undergoing IVF or ICSI procedures.



Jolanda C. Boxmeer, Marij Smit, Robertus F. Weber, Jan Lindemans, Johannes C. Romijn, Marinus J. Eijkemans, Nick S. Macklon, Régine P.M. Steegers-Theunissen

J Androl. 2007;28(4):521-7

ABSTRACT:

A mild hyperhomocysteinemia is caused by B-vitamin deficiencies. We hypothesize that these biochemical derangements detrimentally affect spermatogenesis. Therefore, the aim of this study was to investigate the folate, cobalamin, pyridoxine and homocysteine concentrations in blood and seminal plasma and the associations between these biomarkers and semen parameters in males participating in an in vitro fertilization or intracytoplasmic sperm injection program. From 73 men (median age [range]: 37 [28-53] years) a blood and semen sample were obtained for the determination of serum and red blood cell (RBC) folate, serum total cobalamin, whole-blood pyridoxal'5-phosphate, plasma total homocysteine (tHcy) and serum total testosterone. Semen analysis included sperm concentration, motility and morphology according to World Health Organization criteria. The B-vitamins and tHcy concentrations were significantly correlated in blood but not in seminal plasma. The serum and RBC folate concentrations were significantly correlated also with the total folate concentration in seminal plasma ($r = 0.44$; $P < 0.001$ and $r = 0.39$; $P < 0.001$, respectively). Likewise, the total cobalamin concentration in serum and seminal plasma was significantly correlated ($r = 0.55$; $P = 0.001$). Of interest is that the total cobalamin concentration in seminal plasma was significantly correlated with the sperm concentration ($r = 0.42$; $P < 0.001$). This is in contrast to the absence of significant associations between the other vitamins and tHcy in blood and seminal plasma and any of the semen parameters. These findings suggest that folate and cobalamin are transferred from the blood to the male reproductive organs and emphasize the role of cobalamin in spermatogenesis in human.

INTRODUCTION

Spermatogenesis is influenced by a combination of endocrine, genetic and environmental factors, including nutrition and lifestyle (1, 2). Evidence is increasing that nutritional factors are important in reproduction and thus in spermatogenesis as well. Of main interest are the B-vitamins folate, cobalamin and pyridoxine involved in homocysteine metabolism.

Folate is essential as a substrate in the synthesis of DNA and RNA precursors and in the remethylation of homocysteine into methionine. In folate-dependent homocysteine remethylation, vitamin B12 (cobalamin) is a cofactor for the methionine synthase enzyme. Vitamin B6 (pyridoxal'5-phosphate [PLP]), however, is as a cofactor for cystathionine-β-synthase, which is necessary for the transsulphuration of homocysteine into cystathionine and cysteine. A deficiency of these vitamins causes a mild hyperhomocysteinemia in blood plasma, which is associated with several health problems, including cardiovascular and cerebrovascular diseases (3). Furthermore, hyperhomocysteinemia has been associated with reproductive disorders, such as recurrent pregnancy loss, abruptio placentae and congenital malformations (4, 5). Folic acid treatment significantly reduces the plasma total homocysteine (tHcy) concentrations (3). The beneficial effects of folic acid supplementation in the reduction of congenital malformations can partly be explained by the correction of the hyperhomocysteinemia (3). We recently showed a significant inverse association between embryo quality following in vitro fertilization (IVF)/ intracytoplasmic sperm injection (ICSI) treatment and the tHcy concentration in seminal plasma and ovarian follicular fluid (6).

Total seminal plasma folate concentrations are significantly correlated with blood plasma folate concentrations (7). Moreover, folate derivatives other than 5-methyltetrahydrofolate in seminal plasma appear to be correlated with sperm count (7). Further evidence for the role of synthetic folic acid in spermatogenesis is derived from our randomized controlled trial demonstrating a 74% increase in sperm count after folic acid and zinc sulphate intervention for 26 weeks (8).

The effect of cobalamin in reproduction is less defined, although there is some evidence that this B-vitamin affects sperm parameters (9, 10). During the sixties and seventies several studies described the successful treatment of subfertility in men and women following treatment of a cobalamin deficiency (11-15). There is no information about the effects of pyridoxine on human reproduction. From animal studies it is known that high doses of pyridoxine impair sperm motility and sperm count and cause histopathological changes including degeneration of germinal epithelial cells (16).

From this background, we investigated the total folate, total cobalamin, pyridoxine as pyridoxal-5'-phosphate and tHcy concentrations in blood and seminal plasma in males, and the association with semen parameters.

METHODS

Study Subjects

As part of an ongoing prospective study focused on the role of nutrition, in particular folate, in fertilization, implantation and embryo quality (FOLFO-Study), we enrolled couples undergoing an IVF or ICSI procedure at the Erasmus MC, University Medical Center in the Netherlands. Fertile and subfertile males were eligible for enrolment unless frozen or surgically retrieved sperm was to be used for the assisted reproductive treatment. Fertility is defined by a sperm concentration of more than 20 x 10^6 cells/mL. Of the eligible IVF/ICSI population 66% of the males participated in the FOLFO-Study resulting in the analysis of the data of 73 males. The study protocol was approved by the Central Committee for Human Research (CCMO) in The Hague, The Netherlands and the Medical Ethical and Institutional Review Board of the Erasmus MC, University Medical Center in Rotterdam, The Netherlands. All participants gave their written informed consent.

Study protocol

At the intake visit the IVF treatment couples were asked to participate in the study. Between 2 weeks before and 2 weeks after oocyte retrieval men visited the andrology outpatient clinic for fertility evaluation comprising semen analysis, blood withdrawal, and scrotal ultrasound. One semen sample and blood sample were collected from each man. All laboratory analyses were performed without knowledge of the clinical diagnosis of the participants. The ultrasonic volume of each testis was calculated from 3 perpendicular measurements in the equation V (mL) = π x length x width x depth (all in cm)/6. The mean testicular volume was calculated for each participant.

Semen samples were produced via masturbation into polypropylene containers. After liquefaction, semen analysis according to the World Health Organization (WHO) guidelines were performed (17). Subsequently an aliquot of semen was centrifuged at 2,500 x *g* for 10 minutes. The supernatant seminal plasma was frozen without preservatives and stored at –20°C until assayed.

Venous blood samples were drawn into dry vacutainer tubes and allowed to clot. After centrifugation at 2,000 x *g*, the blood serum was collected before being assayed for the concentrations of total folate, total cobalamin and testosterone. For the determination of red blood cell (RBC) folate and plasma tHcy, venous blood samples were drawn into ethylenediamine tetra-acetate (EDTA)-containing vacutainer tubes. The EDTA-blood samples were kept on ice, and plasma was separated by centrifugation within 1 hour for determination of tHcy. For the determination of pyridoxine, blood was drawn into lithium-heparin-containing vacutainers.

Blood serum and seminal plasma samples from each patient were analyzed during routine laboratory procedures for total folate and total cobalamin using an

immunoelectrochemoluminescence assay (Roche Modular E170, Roche Diagnostics GmbH, Mannheim, Germany). Directly after blood sampling, 0.1 mL blood out of an EDTA tube was hemolyzed with 0.9 mL freshly prepared 1.0% ascorbic acid. Subsequently the hematocrit of the EDTA-blood was determined on an ADVIA 120 Hematology Analyzer (Bayer Diagnostics, Germany). The hemolysate was centrifuged for five minutes at 1,000 x *g* shortly before the folate measurement. The folate concentration in the hemolysate was recalculated in RBC folate using the following formula: (nM hemolysate folate x 10 / hematocrit) - (nM serum folate x [1- hematocrit] / hematocrit) = nM RBC folate. Pyridoxine in whole blood and seminal plasma and tHcy in EDTA plasma and seminal plasma were determined during routine laboratory procedures using high-performance liquid chromatography with reversed-phase separation and fluorescence detection (18, 19). We determined total pyridoxine with PLP being the most common form. Testosterone was measured using Coat-a-Count radioimmunoassay (Diagnostic Products Corp., Los Angeles, CA).

The between-run coefficient of variation for serum total cobalamin was 5.1% at 125 pmol/L and 2.9% at 753 pmol/L; for serum total folate these coefficients of variation were 9.5% at 8.3 nmol/L and 3.2% at 20.2 nmol/L, for tHcy 3.3% at 14.55 µmol/L and 2.3% at 34.23 µmol/L, for pyridoxine these coefficients of variation were 1.8% at 40 nmol/L and 1.3% at 115 nmol/L, for testosterone these coefficients of variation were less than 7.5%. The detection limit for total folate was 1.36 nmol/L, for total cobalamin 22 pmol/L, for pyridoxine 5 nmol/L and for tHcy 4 µmol/L, for testosterone 0.1 nmol/L. In addition, males filled out a general questionnaire from which the following data were extracted: medical history, education, use of medication, and lifestyle factors, such as smoking and the use of vitamin supplements.

Statistical Analysis

The results were expressed as median (range) or as a percentage and analyzed for statistical significance using nonparametric tests, because of a skewed distribution of the semen parameters and B-vitamins and tHcy in blood and seminal plasma. Spearman rank correlation coefficients were calculated to determine associations between semen parameters and total folate, total cobalamin, PLP and tHcy concentrations in blood and seminal plasma. In the figures we present the log transformed variables.

To adjust for possible confounding variables, a multiple linear regression analysis was performed with the logarithm of semen concentration as dependent variable. In this analysis, age, body mass index (BMI), smoking, alcohol, medication, intake of multivitamin supplements, intake of folic acid supplements, serum total testosterone, mean testicular volume measured by ultrasound, a history of urologic surgery, and the presence of a varicocèle were considered as potential confounding factors.

RESULTS

The population characteristics and semen parameters are presented in Table 1. Due to a low semen volume, concentrations of total folate and total cobalamin could not be determined in 2 samples, and PLP and tHcy concentrations were missing in 7 and 19 semen samples, respectively. The median serum testosterone was 14.5 nmol/L (range: 5.8 – 31.9). The median of the mean testicular volume was 10.9 mL (range: 5.5 – 22.0). In the study population a varicocele was diagnosed in 17 participants (26%) and 5 men (7%) had a history of urologic surgery. Twelve men used medication for the following categories of diseases [n]: cardiovascular [3], enterologic [3], psychologic [2], respiratory [2], metabolic [1] and dermatologic [1]. None of these drugs are known to affect sperm parameters or the concentrations of B-vitamins and tHcy. According to the sperm concentration in the semen sample for this study 34% of the participants were subfertile (≤ 20 x 10^6 cells/mL)

Table 1. Characteristics and semen parameters of 73 males in an IVF/ICSI program

Characteristics	
Age median, years (range)	37 (28-53)
Body mass index median (range)	22.3 (17.7 – 28.9)
Smoking – yes, % (n)	25 (16)
Medication – yes, % (n)	19 (12)
Folic acid supplement - yes, % (n)	8 (5)
Multivitamin supplement - yes, % (n)	37 (23)
Educational level Low, % (n)	14 (9)
Intermediate, % (n)	41 (26)
High, % (n)	44 (28)
Cause of subfertility: Male facto,r % (n)	26 (19)
Female factor, % (n)	23 (17)
Male and female factor, % (n)	1 (1)
Idiopathic, % (n)	49 (36)
Sperm concentration (x 10^6 cells/mL),* median (range)	30 (1.6-278)
Sperm progressive motility (%),* median (range)	35 (8-65)
Normal sperm morphology (%),* median (range)	5 (0-15)

*Determination according to World Health Organization criteria (1999). Normal reference values are > 20 x 10^6 cells/mL sperm concentration, >50% motility, and ≥ 30% normal morphology. The cause of subfertility is based on the medical history and previous examinations of the couples.

Table 2. Concentrations of B vitamins and tHcy in blood and seminal plasma

Vitamins	Blood median (range)	Seminal plasma median (range)
Total Folate serum (nmol/L)	14.7 (8.9-131.1)	25.7 (13.8-78.6)
Folate RBC (nmol/L)	973.5 (558-2195)	
Total Cobalamin (pmol/L)	317 (141-696)	509 (94-1260)
Pyridoxal'5-phosphate (nmol/L)	76 (40-300)	24 (0-300)
tHcy (µmol/L)	11.6 (7-28.7)	6.8 (1.7-35.5)

The total folate and total cobalamin concentrations in serum were significantly lower than in seminal plasma ($P < 0.001$ and $P < 0.001$), and RBC folate and pyridoxine concentrations were significantly higher ($P < 0.001$ and $P < 0.001$) in blood than in seminal plasma. The tHcy concentrations in serum and seminal plasma were comparable ($P = 0.137$) (Table 2). In blood, the total folate, total cobalamin, PLP and tHcy concentrations were significantly correlated with each other, but in seminal plasma no significant correlations between these B-vitamins and tHcy could be determined. In addition, significant correlations were determined between serum, RBC and seminal plasma total folate as well as between serum and seminal plasma total cobalamin (Figure 1). No significant correlations were observed between the tHcy and PLP concentrations in blood and seminal plasma.

A significant correlation was determined between cobalamin in seminal plasma and sperm concentration ($r = 0.42$; $P \leq 0.001$) (Figure 2). The other B-vitamins and tHcy in blood

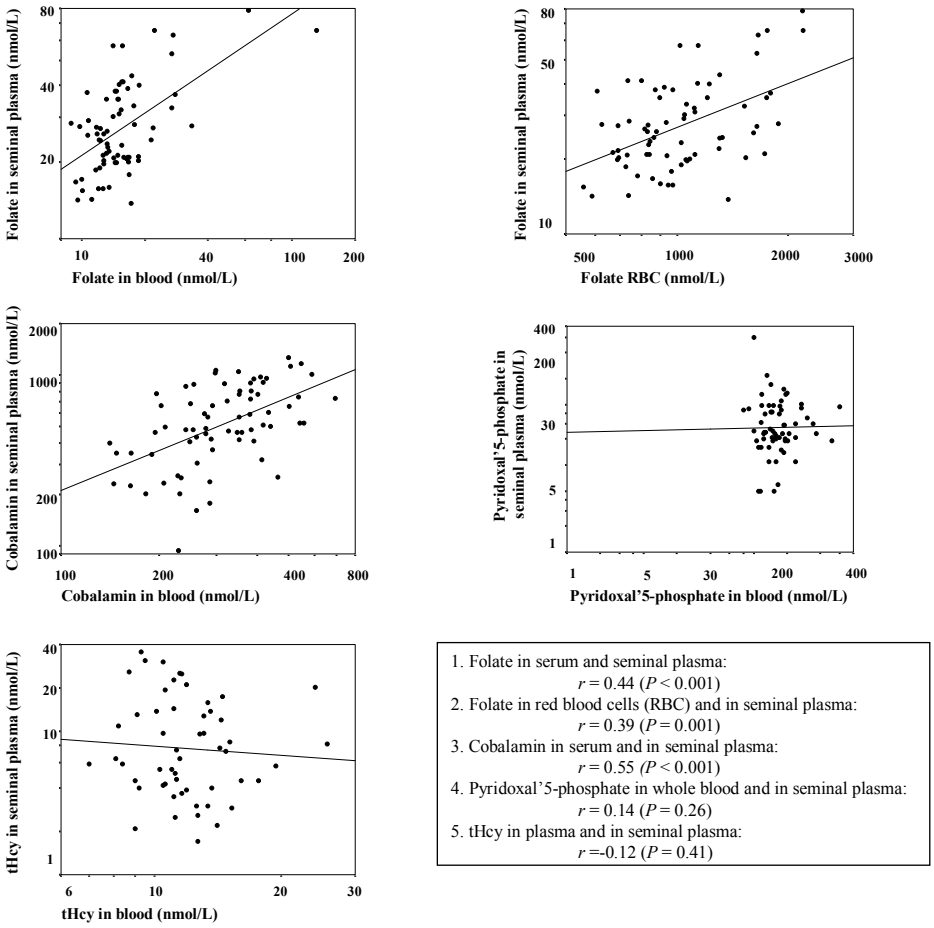
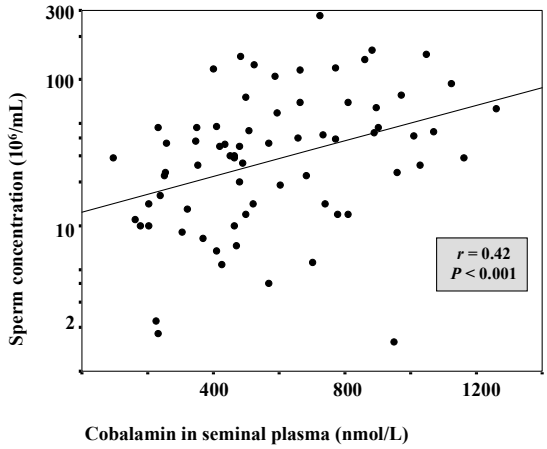


Figure 1. Logtransformed B-vitamin and tHcy concentrations in blood and seminal plasma.

Figure 2. Correlation between logtransformed cobalamin concentrations in seminal plasma and logtransformed sperm concentration.



and seminal plasma were not significantly correlated with any of the semen parameters. We adjusted this significant correlation for possible confounders by multiple linear regression analysis with the logarithm of the semen concentration as dependent variable and the concentration of cobalamin in seminal plasma as independent variable. None of the factors considered as potential confounders (age, BMI, smoking, alcohol, medication, intake of multivitamin supplements, intake of folic acid supplements, serum total testosterone, mean testicular volume measured by ultrasound, a history of urologic surgery, and the presence of a varicocèle) reached statistical significance. In this linear regression model the corresponding standardized regression coefficient was 0.35 ($P = 0.012$). After correction for the previously described variables, the regression coefficient was only marginally different compared to the crude estimate ($r = 0.34$; $P = 0.013$).

DISCUSSION

In this study, we clearly demonstrate a significant positive correlation between the total cobalamin concentration in seminal plasma and the sperm concentration in men participating in an IVF or ICSI procedure. Moreover, the total cobalamin concentration in serum was significantly correlated with the seminal plasma concentration.

Tomaszewski et al. (1963) found lower cobalamin concentrations in males with oligospermia than in males with a normal semen concentration, which is in line with our finding (10). It was previously suggested that cobalamin influences the maturation of human spermatozoa (9). Our data, however, are not showing a significant correlation between sperm morphology and cobalamin concentrations. Differences in the distributions of the

sperm morphology in both studies and the method of determining sperm morphology may explain these differences.

According to the criteria of the WHO, all males participating in the present study should be classified as subfertile, because of their low sperm morphology scores (17). The threshold for sperm morphology, however, is widely debated and several authors have advocated a lower threshold (20, 21). For that reason, we defined male subfertility in our study by the sperm concentration of less than 20 x 10^6 cells/mL.

Of interest is that in the 1960s and 1970s subfertile, cobalamin-deficient men were described who became fertile after treatment with cobalamin (11, 14). In our study, however, only 1 man showed a borderline cobalamin concentration (141 pmol/L; normal: > 145 pmol/L) with a normal semen analysis (118 x 10^6 cells/mL, 43% progressive motility, 7% normal sperm morphology). Although the sperm morphology was below the WHO criteria, there is still a lot of discussion about the definition of 'normal' sperm morphology (17, 21).

Another interesting finding is the significant correlation between the total folate concentration in blood and seminal plasma. The total folate concentrations in serum and seminal plasma were slightly lower and in RBC slightly higher than the pre-intervention concentrations in fertile and subfertile males reported by us previously (8). This might be due to laboratory differences, such as the use of different assays, population differences and changes in dietary patterns and life styles in the last decade in the Netherlands. This is also in line with the slightly higher tHcy concentrations in seminal plasma compared to the concentrations reported by Ebisch et al (6).

A significant correlation between the total folate concentration in seminal plasma and sperm parameters could not be determined. This is in contrast to the results of Wallock et al, which demonstrated a significant correlation between the nonmethylated folate derivatives, in particular, and sperm count (7). This difference may be due to the fact that they determined a subgroup of nonmethylated folate derivatives while we measured mainly methyltetrahydrofolate.

The absence of a correlation between tHcy and sperm parameters is consistent with the results from Ebisch et al (6). However, in that study we assessed a significant association between the embryo quality and the tHcy concentration in both seminal plasma and follicular fluid. This emphasizes the importance of considering both male and female parameters in the investigation of (sub) fertility.

In general, the correlations between total cobalamin, total folate and PLP in blood are rather small, which is very likely due to the many factors that determine these biomarkers, such as diet, medication, supplement use, metabolism, and genetic variations. The significant correlations between tHcy and total folate and total cobalamin are well known (3). The absence of significant correlations between total folate, total cobalamin and PLP in seminal plasma, however, are new.

The rationale for the determination of seminal plasma concentrations of the B-vitamins and tHcy was to gain more insight into tissue-specific concentrations and the associations with sperm parameters. The correlations between blood and seminal plasma for total folate and total cobalamin suggest a transfer of these nutrients between the 2 compartments. On the other hand, seminal plasma is not only derived from the testis but also from the epididymis and prostate. Therefore, the best but not very popular manner to get more information into the role of these nutrients in human spermatogenesis is to determine these concentrations in local samples obtained by a testicular biopsy.

Some limitations of the present study have to be addressed. The study has been performed in a population of subfertile couples and in 27% of the couples a male factor was present. It would be interesting to repeat our study in extended groups of both fertile and subfertile men. For the group of fertile males, couples with female factor fertility can be considered but males with healthy children after spontaneous pregnancies would be even better. Moreover, considering normal physiology we expect that the associations are independent of the fertility status of the males.

Furthermore, the finding of a correlation between total cobalamin in seminal plasma and sperm concentration does not equate to causality. More research on this subject should be performed to clarify the role of cobalamin in spermatogenesis.

In summary, the strong correlation between seminal plasma total cobalamin and sperm concentration supports the role of this vitamin, besides that of folate, in spermatogenesis in human. Because male factor subfertility due to cobalamin deficiency is amendable to curative and/or preventive action by supplementation, studies should focus on the determination of cut off points of cobalamin concentration in blood and seminal plasma and the efficacy and safety of supplementation on semen parameters and fertility as outcome measures.

Acknowledgements

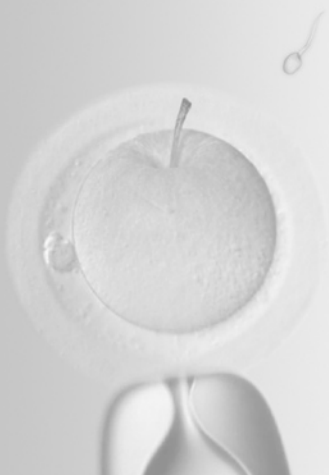
The authors gratefully acknowledge the contribution of the IVF team of the Division of Reproductive Medicine, and the laboratory technicians and research assistants of the Department of Andrology and Laboratory of Clinical Chemistry of the Erasmus MC, University Medical Center in Rotterdam, The Netherlands for the collection and laboratory determinations.

REFERENCES

1. Kuroki Y, Iwamoto T, Lee JW, Yoshiike M, Nozawa S, Nishida T, et al. Spermatogenic ability is different among males in different Y chromosome lineage. J Hum Genet 1999;44(5):289-92.
2. Wong WY, Thomas CM, Merkus JM, Zielhuis GA, Steegers-Theunissen RP. Male factor subfertility: possible causes and the impact of nutritional factors. Fertil Steril 2000;73(3):435-42.
3. Refsum H, Smith AD, Ueland PM, Nexo E, Clarke R, McPartlin J, et al. Facts and recommendations about total homocysteine determinations: An expert opinion. Clin Chem 2004;50(1):3-32.
4. Steegers-Theunissen RP, Boers GH, Trijbels FJ, Eskes TK. Neural-tube defects and derangement of homocysteine metabolism. N Engl J Med 1991;324(3):199-200.
5. Steegers-Theunissen RP, Boers GH, Blom HJ, Trijbels FJ, Eskes TK. Hyperhomocysteinaemia and recurrent spontaneous abortion or abruptio placentae. Lancet 1992;339(8801):1122-3.
6. Ebisch IM, Peters WH, Thomas CM, Wetzels AM, Peer PG, Steegers-Theunissen RP. Homocysteine, glutathione and related thiols affect fertility parameters in the (sub)fertile couple. Hum Reprod 2006; 21(7):1725-33.
7. Wallock LM, Tamura T, Mayr CA, Johnston KE, Ames BN, Jacob RA. Low seminal plasma folate concentrations are associated with low sperm density and count in male smokers and nonsmokers. Fertil Steril 2001;75(2):252-9.
8. Wong WY, Merkus HM, Thomas CM, Menkveld R, Zielhuis GA, Steegers-Theunissen RP. Effects of folic acid and zinc sulfate on male factor subfertility: a double-blind, randomized, placebo-controlled trial. Fertil Steril 2002;77(3):491-8.
9. Watson AA. Seminal vitamin B12 and sterility. Lancet 1962;280(7257):644-.
10. Tomaszewski L, Zmudzka B, Nadworny J. Seminal vitamin B12 and sterility. Lancet 1963;281(7273): 170.
11. Sharp AA, Witts LJ. Seminal vitamin B 12 and sterility. Lancet 1962;280(7259):779.
12. Jackson I, Doig WB, McDonald G. Pernicious anaemia as a cause of infertility. Lancet 1967;290(7527): 1159-60.
13. Varadi S. Pernicious anaemia and infertility. Lancet 1967;290(7529):1350.
14. Blair JH, Stearns HE, Simpson GM. Vitamin B 12 and fertility. Lancet 1968;1(7532):49-50.
15. Mahmood A. Pernicious anaemia as a cause of infertility. Lancet 1971;297(7702):753.
16. Plassmann S, Urwyler H. Improved risk assessment by screening sperm parameters. Toxicology Letters 2001;119(2):157-71.
17. World Health Organization. (1999) Laboratory Manual for the examination of human semen and semen-cervical mucus interaction. 4th ed, Cambridge University Press, Cambridge.
18. Schrijver J, Speek AJ, Schreurs WH. Semi-automated fluorometric determination of pyridoxal-5'-phosphate (vitamin B6) in whole blood by high-performance liquid chromatography (HPLC). Int J Vitam Nutr Res 1981;51(3):216-22.
19. Pfeiffer CM, Huff DL, Gunter EW. Rapid and accurate HPLC assay for plasma total homocysteine and cysteine in a clinical laboratory setting. Clin Chem 1999;45(2):290-2.
20. Ombelet W, Bosmans E, Janssen M, Cox A, Vlasselaer J, Gyselaers W, et al. Semen parameters in a fertile versus subfertile population: A need for change in the interpretation of semen testing. Hum Reprod 1997;12(5):987-93.
21. van der Merwe FH, Kruger TF, Oehninger SC, Lombard CJ. The use of semen parameters to identify the subfertile male in the general population. Gynecol Obstet Invest 2005;59(2):86-91.

Chapter 7

Low folate in seminal plasma is associated with increased sperm DNA damage



Jolanda C. Boxmeer, Marij Smit, Elaine Utomo, Johannes C. Romijn, Marinus J.C. Eijkemans, Jan Lindemans, Joop S.E. Laven, Nick S. Macklon, , Eric A.P. Steegers, Régine P.M. Steegers-Theunissen.

Fertil Steril. 2008 Aug 21 [Epub ahead of print]

ABSTRACT

Objective: To determine associations between B vitamin status, homocysteine (tHcy), semen parameters and sperm DNA damage.

Design: Observational study.

Setting: A tertiary referral fertility clinic.

Patients: Two hundred fifty-one men of couples undergoing in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) treatment. Subgroups of fertile (n = 70) and subfertile men (n = 63) were defined according to semen concentration and proven fertility.

Interventions: None.

Main outcome measure(s): The DNA fragmentation index (DFI) as marker of sperm DNA damage was determined using the Sperm Chromatin Structure Assay (SCSA), and semen parameters were assessed according to World Health Organization criteria. In seminal plasma and blood, tHcy, folate, cobalamin and pyridoxine concentrations were determined.

Results: In the total group of fertile and subfertile men, all biomarkers in blood were significantly correlated with those in seminal plasma. No correlation was found between the biomarkers in blood and the semen parameters. In seminal plasma, both tHcy and cobalamin positively correlated with sperm count. Folate, cobalamin, and pyridoxine were inversely correlated with ejaculate volume. In fertile men, seminal plasma folate showed an inverse correlation with the DNA fragmentation index.

Conclusions: Low concentrations of folate in seminal plasma may be detrimental for sperm DNA stability.

INTRODUCTION

In general, male subfertility is of multifactorial origin. Whereas genetic causes of subfertility are difficult to modulate, other conditions such as accessory gland infections and vas deferens obstruction are potentially treatable. Environmental and lifestyle factors are also potentially amenable and thereby may cure or prevent the subfertility. Nutrition is an important modifier, but one that is largely ignored in the counseling and treatment of these patients. In recent years, interest has increased in the role of B-vitamins as modulators of fertility outcome (1, 2). Deficient B vitamin concentrations cause elevated homocysteine concentrations and impair the remethylation cycle. This metabolism is involved in the methylation of phospholipids, proteins, DNA, and RNA, and in the synthesis and repair of DNA. These processes are essential in spermatogenesis; therefore, derangements in this pathway may be detrimental for reproduction. Recently, our group demonstrated an adverse effect of a high total homocysteine (tHcy) concentration in ejaculated sperm and follicular fluid on embryo quality (3). Moreover, we showed in a randomized, placebo-controlled trial that during 6 months of intervention with folic acid and zinc phosphate a 74% increase of total normal sperm count in subfertile men could be achieved (4). The effect of cobalamin in reproduction is less defined, although there is some evidence that this B vitamin affects sperm parameters as well (5-8).

Conventional semen analysis consists of measuring a variety of semen parameters including volume, sperm concentration, motility, and morphology. Nowadays, the DNA fragmentation index (DFI) can be assessed by the Sperm Chromatin Structure Assay (SCSA), an independent measure of sperm quality with a better diagnostic and prognostic capability than the conventional semen analysis alone (9, 10). During spermatogenesis, most of the cytoplasm of the spermatozoa is discarded, through which the availability of nutrients and defensive cytosolic enzymes is limited. This may result in a higher sensitivity for DNA damage. During ejaculation, the microenvironment of the spermatozoa is formed by the seminal plasma. It contains a broad spectrum of nutrients, enzymes, and hormones necessary to maintain normal metabolism and function of the spermatozoa. Deficiencies or excessive concentrations may be detrimental to the spermatozoa. So far, the effects of seminal plasma tHcy and B vitamins on semen parameters have scarcely been studied. Our study investigated the associations between 1) B vitamin and homocysteine concentrations in blood and seminal plasma, 2) the biomarkers and conventional semen parameters, and 3) the biomarkers and sperm DNA damage.

MATERIALS AND METHODS

Study Population

Between September 2004 and January 2007, subfertile couples undergoing in vitro fertilization (IVF) with or without intracytoplasmic sperm injection (ICSI) treatment at the Erasmus MC, University Medical Center in Rotterdam, the Netherlands were included in the prospective FOod, Lifestyle and Fertility Outcome-study (FOLFO-study). The FOLFO project was set up to study the influence of preconception nutrition and lifestyle on fertility and pregnancy outcome. In the present analysis, the male participants are evaluated. Fertile and subfertile men were eligible for enrollment unless semen was cryopreserved or obtained by microsurgical or percutaneous epididymal sperm aspiration (MESA or PESA). Couples were invited to participate in the study at the intake visit. The study protocol was approved by the Dutch Central Committee for Human Research in and the Medical Ethical and Institutional Review Board of Erasmus MC, University Medical Center in Rotterdam, the Netherlands. All participants gave their written informed consent.

For the standard fertility evaluation, men visited the andrology outpatient clinic. The fertility evaluation comprised semen analysis, blood sampling, a physical examination including a scrotal ultrasound, and a general questionnaire. All obtained materials were processed anonymously. During physical examination scrotal ultrasonography was performed using a Toshiba Nemio 20 with a 12-Hz transducer (Toshiba, Tokyo, Japan). A varicocele was diagnosed when at least two venous vessels with a diameter of at least 3 mm were present, in addition to reflux or diameter increase during Valsalva's maneuver.

Furthermore, all participants filled out a general questionnaire from which the following data are extracted: medical history, body height and weight, ethnicity, and lifestyle factors such as the use of alcohol, cigarettes, and vitamin supplements.

To stratify the participants in subgroups of fertile and subfertile men, fertile men were defined by a sperm concentration of $\geq 20 \times 10^6$ cells/mL and a prior conception with the current or previous partner. Subfertile men were defined by a sperm concentration of $< 20 \times 10^6$ cells/mL and no prior conception.

Semen Collection and Analysis

Semen specimens were produced via masturbation after a required abstinence period of 3 to 5 days. After liquefaction, the semen parameters of volume, sperm concentration, sperm count, percentage progressive motility, and percentage normal morphology were assessed according to the WHO guidelines (11). An aliquot of unprocessed semen was stored at -80°C to determine the DFI at a later stage. Subsequently, the remainder of semen was centrifuged at 2500 x *g* for 10 minutes. The supernatant seminal plasma was frozen without preservatives and stored at -20°C until assayed.

SCSA

The principles and procedures of measuring sperm DNA damage by a FACScan flow cytometry SCSA have been described in detail previously (12). In short, semen samples were diluted with TNE buffer to a concentration of 1-2 x 10^6 sperm cells/mL in a volume of 0.20 mL. This cell suspension was mixed with 0.40 mL of acid detergent solution and then stained with 1.2 mL Acridine Orange (AO) staining solution. A reference sample treated in the same way was run prior to the actual measurements and used to adjust the voltage gains of the flow cytometer FL3 and FL1 photomultipliers that detected red and green fluorescence respectively. An aliquot of reference sample was stained and run again after every 5 to 10 samples. Data collection of the fluorescent pattern in 5000 cells was performed at 3 minutes after acid treatment. Each sperm sample was analyzed twice.

The extent of DNA damage was expressed as the DNA fragmentation index (DFI), reflecting the ratio for red fluorescence to total fluorescence. Cell Quest Pro and WinList software (Becton Dickinson, San Jose, CA, USA) were used to calculate the DFI of each sample.

Determination of Biomarkers in Blood and Seminal Plasma

Concentrations of tHcy, folate, cobalamin and pyridoxine were determined in venous blood and seminal plasma samples. In blood samples red blood cell (RBC) folate, testosterone, sex hormone-binding globulin (SHBG), and inhibin B were determined as well. Venous blood was collected in dry Vacutainer tubes (Becton Dickinson), ethylenediamine tetra-acetate (EDTA) containing Vacutainer tubes and lithium heparin containing Vacutainers. After clotting, the blood collected in dry Vacutainer tubes was centrifuged at 2,000 x *g* and sera were stored at 4°C before being assayed. The venous blood samples collected in the EDTA containing Vacutainer tubes were kept on ice for a maximum of one hour after which the plasma was separated after centrifugation and stored at 4°C before being assayed. The venous blood that was drawn into lithium heparin containing Vacutainer was stored at 4°C before being assayed.

Concentrations of tHcy in EDTA plasma and seminal plasma and pyridoxine as pyridoxal '5-phosphate (PLP) in whole blood and seminal plasma were determined during routine laboratory procedures using high performance liquid chromatography with reversed phase separation and fluorescence detection (13, 14). For the determination of folate and cobalamin in blood and seminal plasma an immunoelectrochemoluminescence immuno-assay was used (Roche Modular E170, Roche Diagnostics GmbH, Mannheim, Germany).

For the determination of RBC folate, 100 µL of blood out of one EDTA tube was hemolyzed with 2 mL freshly prepared ascorbic acid (0.05g 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 (Groffin Meyvis, Etten-Leur, The Netherlands). The hemolysate was centrifuged for 10 minutes at 2,000 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 * 21) - (nmol/L serum folate * (1- hematocrit)) / hematocrit = nmol/L RBC folate.

Testosterone concentrations were determined using a non-extraction coated tube radioimmunoassay (Coat-a-Count, Diagnostic Products Corporation, Los Angeles, USA). We used an immunometric technique on an Immulite Analyzer (Diagnostic Products) to determine SHBG, and serum inhibin B was measured by immunoenzymometric assay (Oxford Bio-Innovation, Kidlington, Oxford, United Kingdom).

Interassay coefficients of variation for tHcy were 4.8% at 14.6 µmol/L and 3.3% at 34.2 µmol/L; folate 4.5% at 13 nmol/L and 5.7% at 23 nmol/L; PLP 1.8% at 40 nmol/L and 1.3 % at 115 nmol/L; cobalamin 3.6% at 258 pmol/L and 2.2% at 832 pmol/L; and for SHBG 6.1% at 11.6 nmol/L and 6.9% at 93 nmol/L. For testosterone, these coefficients of variation were less than 7.5%, and for inhibin B these coefficients of variation were less than 15%. The detection limit for tHcy was 4 µmol/L, folate 1.36 nmol/L, pyridoxine 5 nmol/L, cobalamin 22 pmol/L, testosterone 0.1 nmol/L, SHBG 5 nmol/L and inhibin B 10 ng/L. In seminal plasma the lower detection limit of tHcy was defined as 3*SD = 2 µmol/L.

Statistical Analysis

Biomarker concentrations were expressed in median (range), because of the skewed distributions. Consequently these variables were log transformed prior to statistical analysis. Pearson correlation coefficients were calculated to determine associations between the biomarkers in serum and seminal plasma. Differences between fertile and subfertile men were tested with independent *t*-test or chi-square tests.

To determine associations between DFI and the semen parameters, between the biomarkers and DFI, and semen parameters, we made a causal diagram known as directed acyclic graph (DAG) (15). To adjust for potential confounders, multiple linear regression analyses were performed. The DAG consisted of 3 blocks: one block with the biomarkers, one block with semen parameters (including DFI), and one block with potential confounders. Age, body mass index (BMI), smoking, alcohol use, and the presence of a varicocele were considered as potential confounders.

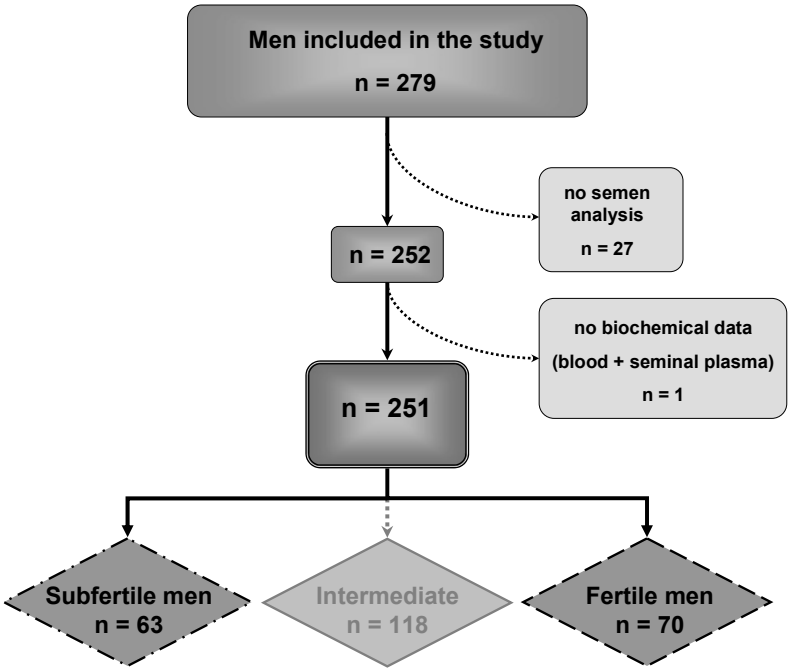
The first step was to determine correlations between the variables within the block of biomarkers and the block of semen parameters. Secondly, the correlations between the two blocks were adjusted for the potential confounders and the covariates of the tested variables within each block. The covariates were included in the model at a significance level of < 0.1 in a forward stepwise regression model. The analyses were performed in the total group and in the two subgroups. For statistical analysis SPSS 12.0.1 was used (SPSS Inc., Chicago, IL,). *P* values ≤ 0.05 were considered statistical significant.

RESULTS

Baseline Characteristics

The flowchart of the study is presented in Figure 1. Seventy-four percent of the eligible couples participated in the study. Semen samples could not be obtained from 27 men, and the biochemical data were missing for one individual. Thus, we evaluated the semen analyses and biochemical data of 251 men. According to our definition 70 men were classified as fertile and 63 as subfertile.

Figure 1. Flowchart of included men



Fertile men: sperm concentration of equal or more than $20x10^6$ cells/mL and a prior conception with the current or previous partner. Subfertile men: a sperm concentration of less than $20x10^6$ cells/mL and no prior conception.

The baseline characteristics of the participating men and the subgroups are given in Table 1. Fertile men were significantly older and showed significantly higher inhibin B concentrations than subfertile men. Forty-one men (16 %) used one or more medicine for the following categories of diseases: respiratory system (10 men), gastroenterology (7 men), endocrinology (7 men), cardiovascular (6 men), psychology (5 men), metabolic (4 men), skin (1 man) and miscellaneous (3 men). Only few of these drugs are known to affect

Table 1. General characteristics

	Total group of men (*n* = 251)		N	Subfertile men (*n* = 63)		N	Fertile men (*n* = 70)		N	*P* value[b]
Age (years)[a]	37.0	(23.1 – 59.1)	251	34.0	(23.1 – 46.2)	63	39.1	(30.5 – 54.0)	70	≤0.001
BMI (kg/m^2)[a]	25.6	(18.4 – 38.4)	226	24.0	(19.6 – 37.9)	60	25.8	(18.8 – 38.4)	60	n.s.
Etnicity			227			60			60	n.s.
European Dutch Natives	181	(79.7)		51	(85)		41	(68.3)		
European others	16	(7.0)		3	(5)		5	(8.3)		
Non-European	30	(13.2)		6	(10)		14	(23.3)		
Smoking			227			60			60	n.s.
Yes	57	(25.1)		14	(23.3)		15	(25)		
No	170	(74.9)		46	(76.7)		45	(75)		
Alcohol use (units/week)[a]	4.3	(0.0 – 50.3)		3.5	(0 – 17.5)		2.1	(0 – 27.6)		n.s.
Varicocele			220			59			59	n.s.
Yes	37	(16.8)		12	(20.3)		6	(10.2)		
No	183	(83.2)		47	(79.7)		53	(89.8)		
Vitamin use			217			57			58	n.s.
Yes, with folic acid	44	(20.3)		15	(26.3)		8	(13.8)		
Yes, without folic acid	13	(6.0)		5	(8.8)		2	(3.4)		
No	160	(73.7)		37	(64.9)		48	(82.8)		
Endocrinology[a]										
Testosterone (nmol/L)	15.1	(5.8 – 36.0)	248	15.3	(8.8 – 34.5)	61	14.7	(7.6 – 25.2)	70	n.s.
SHBG (nmol/L)	26.1	(8.1 – 70.7)	248	27.8	(11.4 – 52.1)	61	25.7	(8.1 – 70.7)	70	n.s.
Inhibin B (ng/L)	160.0	(2.0 – 411.0)	248	126.0	(2.0 – 301.0)	61	186.5	(61.0 – 411.0)	70	≤0.001

Note: Results are presented as number (%), unless otherwise indicated

[a] median (range); [b] difference between subfertile and fertile men; independent T-test or Chi-square (two-tailed) if appropriate; n.s. = not significant

Table 2. Concentrations of biomarkers in blood and seminal plasma and semen parameters

	Total group of men (n = 251)		N	Subfertile men (n = 63)		N	Fertile men (n = 70)		N	P value[a]
Blood										
tHcy (µmol/L)	11.7	(6.8 – 56.4)	250	11.9	(7.0 – 34.3)	63	11.9	(7.7 – 56.4)	70	n.s.
Folate (nmol/L)	15.7	(7.1 – 131.1)	251	15.6	(8.6 – 45.0)	63	15.5	(7.5 – 55.9)	70	n.s.
Folate RBC (nmol/L)	1025	(340 – 2329)	247	948	(340 – 2329)	62	1044	(532 – 2045)	70	n.s.
Cobalamin (pmol/L)	316	(108 – 989)	250	323	(131 – 802)	63	337	(138 – 989)	69	n.s.
Pyridoxine (nmol/L)	80	(39 – 310)	250	80	(51 – 310)	62	79	(40 – 310)	70	n.s.
Seminal plasma										
tHcy (µmol/L)	4.3	(1.2 – 35.5)	172	3.7	(1.2 – 35.5)	51	4.7	(1.7 – 30.4)	50	n.s.
Folate (nmol/L)	25.3	(11.7 – 78.6)	225	23.9	(11.7 – 59.2)	61	24.5	(13.8 – 58.6)	62	n.s.
Cobalamin (pmol/L)	558	(94 – 5704)	225	474	(111 – 4360)	61	674	(94 – 5516)	62	n.s.
Pyridoxine (nmol/L)	28	(0 – 310)	194	26	(0 – 211)	55	29	(0 – 310)	54	n.s.
Semen parameters										
DFI (%)	22.8	(1.3 – 74.8)	226	28.3	(1.3 – 65.9)	59	19.6	(3.7 – 74.8)	64	n.s.
Ejaculate volume (mL)	2.7	(0.2 – 8.1)	250	3.2	(0.2 – 6.0)	63	2.5	(0.2 – 6.5)	70	≤0.01
Sperm concentration (x10^6 cells/mL)	26	(0 – 278)	250	6	(0 – 19)	62	51	(20 – 215)	70	≤0.001
Sperm count (x10^6 cells)	65	(0 – 1557)	249	14.8	(0 – 95)	62	127	(10 – 690)	70	≤0.001
Sperm progressive motility (%)	34	(0 – 74)	244	26	(0 – 58)	59	41	(5 – 73)	70	≤0.001
Sperm normal morphology (%)	4	(0 – 15)	244	3	(0 – 12)	59	6	(1 – 14)	69	≤0.001

Note: Results are presented as median (range) [a] difference between fertile and subfertile men; independent T-test; n.s. = not significant; DFI = DNA fragmentation index

sperm parameters or the concentrations of B-vitamins and tHcy. Due to the small numbers and heterogeneity it was not possible to perform subanalysis.

Correlations between Blood and Seminal Plasma

Table 2 shows the biomarkers in blood and seminal plasma. Twelve men (5%) had hyperhomocysteinemia (22.6 - 56.4 umol/L; reference value >22 umol/L) while only 3 men (1%) had a mild folate deficiency (7.1, 7.5 and 7.6 nmol/L; reference value <8 nmol/L). There were no significant differences between the biomarker concentrations in either blood or seminal plasma in fertile and subfertile men.

Table 3 depicts a matrix with the correlations between biomarkers in blood and seminal plasma and semen parameters in the total group. Total homocysteine concentrations in blood and seminal plasma were significantly correlated (r = 0.16, $P \leq 0.05$). Similarly, folate, cobalamin and pyridoxine in serum and seminal plasma were significantly correlated (respectively r = 0.47, $P \leq 0.001$, and r = 0.36, $P \leq 0.001$ and r = 0.23, $P \leq 0.01$). The tHcy and pyridoxine concentrations were significantly lower in seminal plasma compared to serum (respectively $P \leq 0.05$ and $P \leq 0.001$), and the folate and cobalamin concentrations in seminal plasma were significantly higher compared to serum (both $P \leq 0.001$). In serum,

Table 3. Correlation matrix of B-vitamin and homocysteine concentrations in blood, seminal plasma and semen parameters of 251 participants

	Blood					Seminal plasma				Semen parameters					
	tHcy	serF	RBCF	B12	B6	tHcy	Fol	B12	B6	DFI	Volume	Concen	Count	Motility	Morphol
Blood															
tHcy															
serF	-0.56 [c]														
RBCF	-0.39 [c]	0.62 [c]													
B12	-0.39 [c]	0.23 [c]	0.22 [c]												
B6	-0.31 [c]	0.49 [c]	0.35 [c]	0.32 [c]											
SemP															
tHcy	0.16 [a]	-0.11	-0.01	-0.03	-0.07										
Fol	-0.15 [a]	0.47 [c]	0.34 [c]	0.08	0.30 [c]	-0.01									
B12	-0.22 [c]	0.10	0.17 [b]	0.36 [c]	0.18 [b]	0.26 [c]	0.26 [c]								
B6	0.01	0.12	0.02	0.03	0.23 [b]	-0.01	0.06	0.04							
Semen															
DFI	0.04	0.01	0.03	0.08	-0.05	-0.02	-0.12	0.02	-0.04						
Volume	-0.02	0.03	-0.02	0.01	-0.08	-0.09	-0.18 [b]	-0.21 [b]	-0.17 [a]	0.21 [b]					
Concen	-0.01	-0.05	-0.04	0.05	-0.06	0.30 [c]	0.07	0.19 [b]	-0.01	-0.24 [c]	-0.04				
Count	0.00	-0.04	-0.05	0.05	-0.07	0.25 [c]	0.03	0.13	-0.04	-0.17 [a]	0.25 [c]	0.95 [c]			
Motility	0.03	0.02	-0.02	0.01	0.03	0.07	-0.03	0.01	0.03	-0.49 [c]	0.06	0.49 [c]	0.49 [c]		
Morphol	0.03	-0.08	-0.07	0.05	-0.08	0.19 [a]	0.02	0.00	-0.10	-0.25 [c]	0.04	0.63 [c]	0.62 [c]	0.45 [c]	

Note: Pearson correlation coefficients were calculated after log transformation of the parameters

SemP = Seminal plasma; Semen = Semen parameters; serF = serum Folate; RBCF = Folate in red blood cells; B12 = Cobalamin; B6 = Pyridoxine; DFI = DNA fragmentation index; Volume = Ejaculate volume; Concen = Sperm concentration; Count = Sperm count; Motility = Sperm progressive motility; Morphol = Sperm normal morphology; [a] $P \leq 0.05$; [b] $P \leq 0.01$; [c] $P \leq 0.001$

the B-vitamins were inversely correlated with tHcy and positively correlated with each other (all $P \leq 0.001$). In seminal plasma, cobalamin was positively correlated with folate and tHcy (both $r = 0.26$, $P \leq 0.001$). The directed acyclic graph presents the independent correlations between the B-vitamins and tHcy in seminal plasma (Figure 2). Cobalamin also correlated with tHcy after adjustment for the other B-vitamins (standardized adjusted regression coefficient 0.25, $P \leq 0.01$).

Semen Parameters

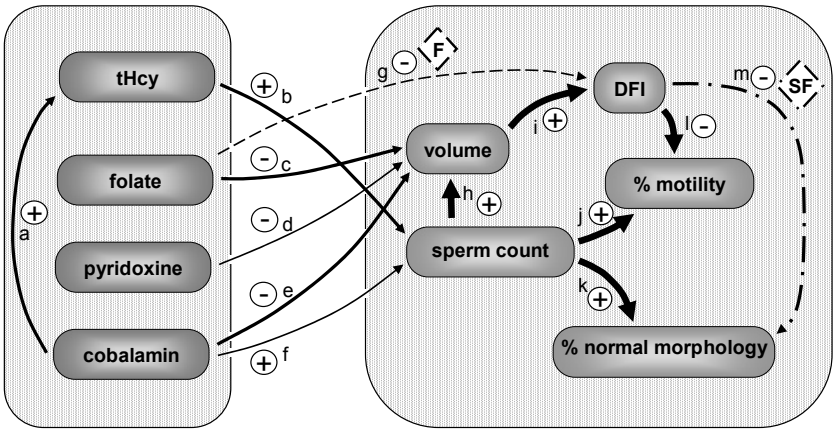
The semen parameters and DFI are presented in Table 2. Ejaculate volume was significantly higher and sperm concentration, sperm count, percentage progressive motility and the percentage normal morphology were significantly lower in subfertile men compared to fertile men (all $P \leq 0.001$). In subfertile men the DFI was higher compared to fertile men, albeit not significantly.

Figure 2 shows the DAG. As can be expected, sperm count was significantly correlated with volume (standardized adjusted regression coefficient 0.25, $P \leq 0.001$), the percentage progressive motility (standardized adjusted regression coefficient 0.39, $P \leq 0.001$), and the percentage normal morphology (standardized adjusted regression coefficient 0.39, $P \leq 0.001$). We found a positive correlation between ejaculate volume and DFI (standardized adjusted regression coefficient 0.23, $P \leq 0.001$); and DFI inversely correlated with the percentage progressive motility (standardized adjusted regression coefficient -0.41, $P \leq 0.001$). In the subgroup of subfertile men, DFI was also inversely correlated with the percentage sperm cells with normal morphology (standardized adjusted regression coefficient -0.33, $P \leq 0.01$).

Correlations between the Biomarkers and Semen Parameters

The biomarkers in blood were not correlated with any of the semen parameters. In seminal plasma both tHcy and cobalamin correlated with sperm count (standardized adjusted regression coefficient 0.21, $P \leq 0.01$ and 0.15, $P \leq 0.05$, respectively). Folate, cobalamin, and pyridoxine concentrations in seminal plasma inversely correlated with ejaculate volume (standardized adjusted regression coefficient -0.20, $P \leq 0.01$, -0.19, $P \leq 0.01$ and -0.16, $P \leq 0.05$). In the total group of men biomarkers were not correlated with DFI, motility, or morphology. However, in the subgroup of fertile men, folate concentrations in seminal plasma were inversely correlated with DFI (standardized adjusted regression coefficient -0.36, $P \leq 0.05$).

Figure 2. Correlations between B-vitamins and homocysteine in seminal plasma and semen parameters



Directed Acyclic Graph (n = 251); in the left block biomarkers in seminal plasma, in the right block semen parameters. Age, body mass index (BMI), smoking, alcohol use and the presence of a varicocele were considered as potential confounders. To adjust for potential confounders, multiple linear regression analyses were performed. Only statistically significant correlations are depicted. **(+)** = positive correlations; **(–)** = inverse correlations.

The strength of P values is depicted by arrow thickness: → = $P \leq 0.05$; ➡ = $P \leq 0.01$; ➧ = $P \leq 0.001$

----- <F> = Fertile men: sperm concentration of equal or more than $20x10^6$ cells/mL and a prior conception with the current or previous partner (n = 70).

-.-.-.- <SF> = Subfertile men: a sperm concentration of less than $20x10^6$ cells/mL and no prior conception (n = 63).

a: standardized coefficient 0.25, $P \leq 0.01$, b: standardized coefficient 0.21, $P \leq 0.01$,
c: standardized coefficient -0.20, $P \leq 0.01$, d: standardized coefficient -0.16, $P \leq 0.05$,
e: standardized coefficient -0.19, $P \leq 0.01$, f: standardized coefficient 0.15, $P \leq 0.05$,
g: standardized coefficient -0.36, $P \leq 0.05$ in fertile men,
h: standardized coefficient 0.25, $P \leq 0.001$,
i: standardized coefficient 0.23, $P \leq 0.001$, j: standardized coefficient 0.39, $P \leq 0.001$,
k: standardized coefficient 0.39, $P \leq 0.001$, l: standardized coefficient -0.41, $P \leq 0.001$,
m: standardized coefficient -0.33, $P \leq 0.01$ in subfertile men.

DISCUSSION

Our study found statistically significant correlations between B-vitamin and homocysteine concentrations in blood and seminal plasma. Furthermore, we have demonstrated for the first time that a low folate concentration in seminal plasma is associated with more sperm DNA damage in fertile men. This novel finding is in line with the role of folate in DNA synthesis and DNA and protein methylation processes. It has been shown before that folate shortage increases DNA fragility due to the misincorporation of uracil instead of thymine (16, 17). During normal repair processes, when the removal of the misincorporated uracil fails, double strand breaks resulting in chromosome instability may occur (16-18). Folate

shortage also decreases the supply of methyl groups, which are important substances for the protection of DNA against harmful exposures (16).

In contrast with fertile men, we suggest that in subfertile men other much stronger causes for subfertility than folate shortage are responsible for the sperm DNA damage (19). Covariates such as testosterone, SHBG concentration, cigarette smoking, and the presence of a varicocele were not significantly different between the subgroups. As expected, inhibin B was statistically significantly lower in the subfertile men. Inhibin B is strongly correlated with spermatogenesis (20, 21), but no correlation was found between inhibin B and DFI in a recent study (22). After adjustment for inhibin B the association between seminal plasma folate and DFI did not become significant in the total group and in subfertile men but the association remained significant in the fertile men. Age is significantly correlated with sperm DNA damage (23, 24). Because subfertile men were significantly younger than fertile men in our study population, all calculations were adjusted for age.

We determined an inverse correlation between DFI and the percentage of progressive motile sperm. In the subgroup of subfertile men, DFI was also inversely correlated with the percentage sperm cells with normal morphology. These findings are in line with several studies (10, 22, 25), although other groups could not demonstrate associations between DNA damage and motility and morphology (26, 27). The number and selection of men in the study may explain the different results.

In our study, cobalamin in seminal plasma correlated with sperm count. This is in line with our previous report (28) and an older study by Tomaszewski et al. (7). Of interest are the reports of 1960 and 1970s in which subfertile cobalamin-deficient males became fertile after treatment with cobalamin (5, 6). Both folate and cobalamin concentrations in seminal plasma were on average 1.6 and 1.8 times higher compared to the blood concentrations in the total group. This is in line with the report by Wallock et al. (29). The tHcy and pyridoxine concentrations were lower in seminal plasma than in blood. Similar differences in concentrations have been shown for zinc (4). This may suggest a passive or active transfer from blood to seminal plasma of folate and cobalamin. In future studies it may be interesting to determine folate and vitamin B12-binding proteins in seminal plasma. It is possible that tHcy and pyridoxine either cannot pass the blood-testis barrier or are actively resorbed.

Some limitations of the present study must be addressed. Some information is missing due to questionnaires that were not returned. Furthermore, we had some difficulties with the determination of the biomarkers due to the fact that there was sometimes not enough seminal plasma available. Furthermore, due to the high protein content and viscosity of seminal plasma we encountered some technical problems with the tHcy determination. Nevertheless, the missing data are equally distributed among the subgroups, so it is not very likely that our results are significantly affected by selection bias. In addition, from our previous study we were aware about the difficulties of measuring biomarkers, in particularly tHcy, in seminal plasma (3). Thus, we performed dilution experiments that revealed

linear dose-response curves. Furthermore, the concentrations in seminal plasma were calculated from recoveries from each individually spiked sample. We realize that these validation procedures are not the final proof but are rather suggestive for the absence of matrix effects. Furthermore, we used the HPLC-method to determine tHcy, which is a generally accepted reference procedure. Although the quality of the tHcy assay has much improved, the data should be carefully interpreted.

The use of one semen sample for distinguishing fertile from subfertile men may have introduced some misclassification. For this reason, we added the criterium of a prior conception, thereby proving the fertility of the individual. A group of healthy men without a history of a fertility problem whose partners conceived spontaneously within one year of regular unprotected intercourse would have been the ideal fertile group.

In the current study population, 5% of the subjects had a mild to severe hyperhomocysteinemia. This may be due to a polymorphism in the B-vitamin metabolism and/or a deficient B-vitamin intake. Previously, we have studied the associations between the MTHFR C677T polymorphism and male fertility in another study group (30), but it was not the aim of the current periconceptional observational study to investigate the effect of polymorphisms on semen parameters.

Seminal plasma is a mixture of the secretion of several glands of which the Cowper and Littre glands (5%), prostate (15 - 30%) and seminal vesicles (60 – 70%) contribute the majority (31). Thus about 90% of the seminal plasma is derived from glands outside testicular tissue and may have a composition different from the liquid in the seminiferous tubules and epididymis where spermatogenesis and maturation takes place. Therefore, the composition of the seminal plasma may not be representative for conditions during spermatogenesis. Comparison of the composition of seminal plasma with material obtained from MESA/PESA procedures would be interesting. However, due to the low volume of material derived from this technique, the invasiveness of these procedures, and medical ethical issues involved, there is limited use of this material for research.

In the present study the biomarkers in seminal plasma correlated with semen parameters, but not with the biomarkers in blood. This might be due to the fact that active mechanisms regulate biomarker concentrations in seminal plasma in a normal range. Therefore, this study may be repeated in a population with very low and very high biomarker concentrations through which also correlations between biomarkers in blood and semen parameters may be found as well. Folic acid and cobalamin supplementation may be a useful therapy to improve male fertility, but more research should be performed to study both the efficacy and safety of dose and duration. In addition it may be useful to establish the dietary intake of these vitamins.

In conclusion, this study emphasizes the importance of B-vitamin status in spermatogenesis in humans. We found that low folate concentrations in the seminal plasma of a subgroup of fertile men were associated with increased levels of sperm DNA damage.

This may suggest that low folate concentrations in the microenvironment of spermatozoa may be detrimental for sperm DNA stability. High concentrations of sperm DNA damage are associated with poor sperm cell motility and morphology. Future research is needed to determine the importance of sufficient B-vitamin status in men on fertilization and subsequent pregnancy outcome.

Acknowledgements

The authors thank the IVF team of the division of Reproductive Medicine, Department of Obstetrics and Gynecology and the laboratory technicians and research assistants of the Department of Andrology and Laboratory of Clinical Chemistry of the Erasmus MC, University Medical Center in Rotterdam, The Netherlands for the collection and laboratory determinations.

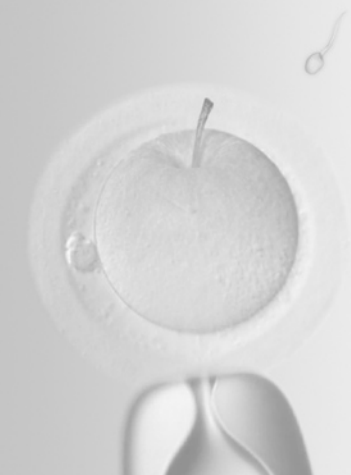
REFERENCES

1. Ebisch IM, Thomas CM, Peters WH, Braat DD, Steegers-Theunissen RP. The importance of folate, zinc and antioxidants in the pathogenesis and prevention of subfertility. Hum Reprod Update 2007;13: 163-74.
2. Forges T, Monnier-Barbarino P, Alberto JM, Gueant-Rodriguez RM, Daval JL, Gueant JL. Impact of folate and homocysteine metabolism on human reproductive health. Hum Reprod Update 2007;13: 225-38.
3. Ebisch IM, Peters WH, Thomas CM, Wetzels AM, Peer PG, Steegers-Theunissen RP. Homocysteine, glutathione and related thiols affect fertility parameters in the (sub)fertile couple. Hum Reprod 2006; 21:1725-33.
4. Wong WY, Merkus HM, Thomas CM, Menkveld R, Zielhuis GA, Steegers-Theunissen RP. Effects of folic acid and zinc sulfate on male factor subfertility: a double-blind, randomized, placebo-controlled trial. Fertil Steril 2002;77:491-8.
5. Blair JH, Stearns HE, Simpson GM. Vitamin B 12 and fertility. Lancet 1968;1:49-50.
6. Sharp AA, Witts LJ. Seminal vitamin B 12 and sterility. Lancet 1962;280:779.
7. Tomaszewski L, Zmudzka B, Nadworny J. Seminal vitamin B12 and sterility. Lancet 1963;281:170.
8. Grasbeck R, Salonen EM. Vitamin B12. Prog Food Nutr Sci 1976;2(5):193-231.
9. Agarwal A, Said TM. Role of sperm chromatin abnormalities and DNA damage in male infertility. Hum Reprod Update 2003;9:331-45.
10. Giwercman A, Richthoff J, Hjollund H, Bonde JP, Jepson K, Frohm B *et al.* Correlation between sperm motility and sperm chromatin structure assay parameters. Fertil Steril 2003;80:1404-12.
11. World Health Organization. Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction. 4th ed. Cambridge: Cambridge University Press, 1999.
12. Smit M, Dohle GR, Hop WC, Wildhagen MF, Weber RF, Romijn JC. Clinical correlates of the biological variation of sperm DNA fragmentation in infertile men attending an andrology outpatient clinic. Int J Androl 2007;30:48-55.
13. Pfeiffer CM, Huff DL, Gunter EW. Rapid and accurate HPLC assay for plasma total homocysteine and cysteine in a clinical laboratory setting. Clin Chem 1999;45:290-2.
14. Schrijver J, Speek AJ, Schreurs WH. Semi-automated fluorometric determination of pyridoxal-5'-phosphate (vitamin B6) in whole blood by high-performance liquid chromatography (HPLC). Int J Vitam Nutr Res 1981;51:216-22.
15. Hernan MA, Hernandez-Diaz S, Werler MM, Mitchell AA. Causal knowledge as a prerequisite for confounding evaluation: an application to birth defects epidemiology. Am J Epidemiol 2002;155: 176-84.
16. Blount BC, Mack MM, Wehr CM, MacGregor JT, Hiatt RA, Wang G *et al.* Folate deficiency causes uracil misincorporation into human DNA and chromosome breakage: implications for cancer and neuronal damage. Proc Natl Acad Sci USA 1997;94:3290-5.
17. Koury MJ, Horne DW, Brown ZA, Pietenpol JA, Blount BC, Ames BN *et al.* Apoptosis of late-stage erythroblasts in megaloblastic anemia: association with DNA damage and macrocyte production. Blood 1997;89:4617-23.
18. Steegers-Theunissen RP, Smith SC, Steegers EA, Guilbert LJ, Baker PN. Folate affects apoptosis in human trophoblastic cells. BJOG 2000;107:1513-5.
19. Zini A, Libman J. Sperm DNA damage: clinical significance in the era of assisted reproduction. CMAJ 2006;175:495-500.
20. Jensen TK, Andersson AM, Hjollund NH, Scheike T, Kolstad H, Giwercman A *et al.* Inhibin B as a serum marker of spermatogenesis: correlation to differences in sperm concentration and follicle-stimulating hormone levels. A study of 349 Danish men. J Clin Endocrinol Metab 1997;82:4059-63.
21. Pierik FH, Vreeburg JT, Stijnen T, De Jong FH, Weber RF. Serum inhibin B as a marker of spermatogenesis. J Clin Endocrinol Metab 1998;83:3110-4.

22. Appasamy M, Muttukrishna S, Pizzey AR, Ozturk O, Groome NP, Serhal P *et al.* Relationship between male reproductive hormones, sperm DNA damage and markers of oxidative stress in infertility. Reprod Biomed Online 2007;14:159-65.
23. Siddighi S, Chan CA, Patton WC, Jacobson JD, Chan PJ. Male age and sperm necrosis in assisted reproductive technologies. Urol Int 2007;79:231-4.
24. Plastira K, Msaouel P, Angelopoulou R, Zanioti K, Plastiras A, Pothos A *et al.* The effects of age on DNA fragmentation, chromatin packaging and conventional semen parameters in spermatozoa of oligoasthenoteratozoospermic patients. J Assist Reprod Genet 2007;24:437-43.
25. Spano M, Kolstad AH, Larsen SB, Cordelli E, Leter G, Giwercman A *et al.* The applicability of the flow cytometric sperm chromatin structure assay in epidemiological studies. Asclepios. Hum Reprod 1998; 13:2495-505.
26. Bakos HW, Thompson JG, Feil D, Lane M. Sperm DNA damage is associated with assisted reproductive technology pregnancy. Int J Androl 2007 [Epub ahead of print].
27. Khalili MA, Aghaie-Maybodi F, Anvari M, Talebi AR. Sperm nuclear DNA in ejaculates of fertile and infertile men: correlation with semen parameters. Urol J 2006;3:154-9.
28. Boxmeer JC, Smit M, Weber RF, Lindemans J, Romijn JC, Eijkemans MJ *et al.* Seminal plasma cobalamin significantly correlates with sperm concentration in males undergoing IVF or ICSI procedures. J Androl 2007;28(4):521-7.
29. Wallock LM, Tamura T, Mayr CA, Johnston KE, Ames BN, Jacob RA. Low seminal plasma folate concentrations are associated with low sperm density and count in male smokers and nonsmokers. Fertil Steril 2001;75:252-9.
30. Ebisch IM, van Heerde WL, Thomas CM, van der PN, Wong WY, Steegers-Theunissen RP. C677T methylenetetrahydrofolate reductase polymorphism interferes with the effects of folic acid and zinc sulfate on sperm concentration. Fertil Steril 2003;80:1190-4.
31. Owen DH, Katz DF. A review of the physical and chemical properties of human semen and the formulation of a semen simulant. J Androl 2005;26:459-69.

Chapter 8

General Discussion

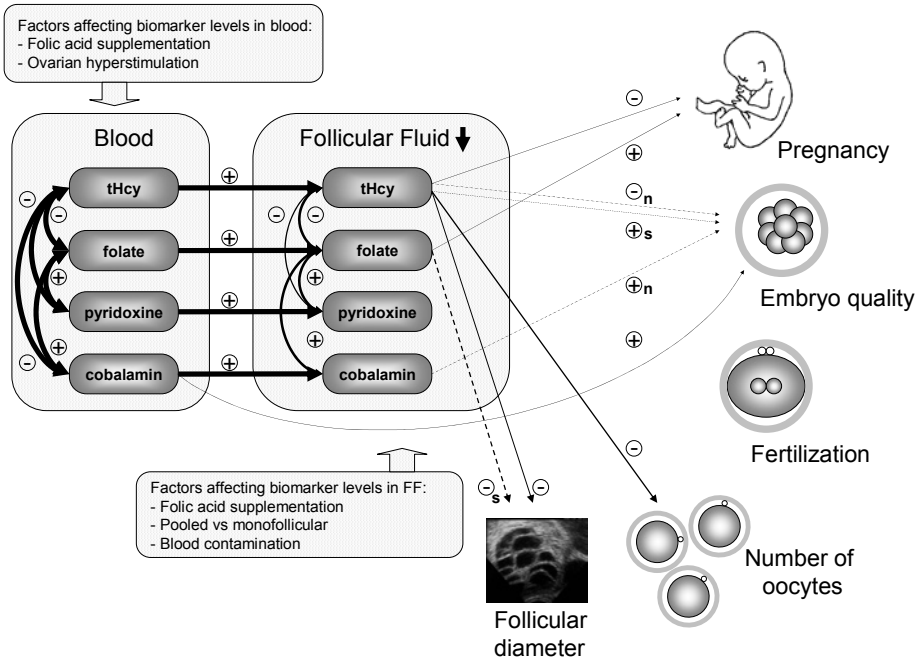


The main objective of this thesis was to study associations between the biomarkers of the homocysteine pathway in blood, follicular fluid and seminal plasma, and semen parameters and in vitro fertilization (IVF) outcomes in women (part I) and men (Part II).

MAIN FINDINGS

Biomarkers in follicular fluid and seminal plasma, which represent the immediate gamete environment, were more significantly associated with fertility outcome than biomarkers in blood. In women, homocysteine (tHcy), folate and cobalamin in follicular fluid as well as cobalamin in blood were correlated with IVF outcomes. Significant associations were demonstrated between the biomarkers and follicular diameter, number of retrieved

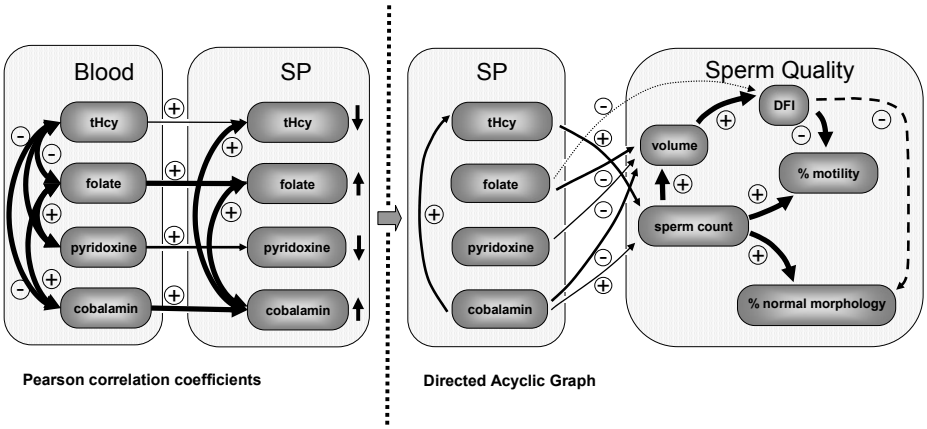
Figure 1. Biomarkers of the homocysteine pathway in women



Correlations between biomarkers of the homocysteine pathway in blood and follicular fluid and IVF outcome parameters. Only statistically significant correlations are depicted.
The strength of *P* values is depicted by arrow thickness: thin arrows = $P \leq 0.05$; medium arrows = $P \leq 0.01$; thick arrows = $P \leq 0.001$.
(+) = positive correlation; (-) = inverse correlation
-----s = only statistically significant in the subgroup folic acid supplemented women
_._._.n = only statistically significant in the subgroup non-supplemented women

oocytes, embryo quality and the occurrence of biochemical pregnancy (Figure 1). In men significant associations were demonstrated between these biomarker concentrations in seminal plasma and sperm quality (Figure 2). Ovarian stimulation was observed to result in a significant decrease in blood concentrations of homocysteine and cobalamin, whereas homocysteine and folate in both blood and follicular fluid were shown to be affected by folic acid supplementation. Although folic acid supplementation is recommended in fertile women, we have observed that even in a highly motivated group of subfertile patients participating in a study directed on nutrition and lifestyle, the rate of proper folic acid supplement intake is surprisingly low.

Figure 2. Biomarkers of the homocysteine pathway in men



Correlations between biomarkers of the homocysteine pathway in blood and follicular fluid and semen parameters. Only statistically significant correlations are depicted. The strength of *P* values is depicted by arrow thickness: thin arrows = $P \leq 0.05$; medium arrows = $P \leq 0.01$; thick arrows = $P \leq 0.001$ (+) = positive correlation; (-) = inverse correlation.
… <F> = Fertile men: sperm concentration of equal or more than $20x10^6$ cells/mL and a prior conception with the current or previous partner; --- <SF> = Subfertile men: a sperm concentration of less than $20x10^6$ cells/mL and no prior conception;
SP = seminal plasma; DFI = DNA fragmentation index.
Directed Acyclic Graph: To adjust for potential confounders, multiple linear regression analyses were performed. Age, body mass index (BMI), smoking, alcohol use and the presence of a varicocele were considered as potential confounders.

BIOMARKERS OF THE HOMOCYSTEINE PATHWAY IN WOMEN

Homocysteine, folate, cobalamin and pyridoxine levels in blood are known to correlate with each other (1) and are affected by dietary and vitamin supplement intake as well as genetic factors. In Chapter 4 we observed a significant decrease in both tHcy and cobalamin blood levels during ovarian hyperstimulation. While this may be due to estradiol

mediated induction of liver enzyme activity and protein synthesis, a direct correlation between estradiol and those biomarkers could not be demonstrated. Although two other studies reported no significant effect of ovarian hyperstimulation on biomarkers of the homocysteine pathway, this may reflect the small sample sizes (2, 3). The decrease of tHcy we observed after an increase of estradiol is consistent with studies in pre- and postmenopausal women (4, 5) and trials with estrogens in postmenopausal women (6-8). The reported decrease in cobalamin in estrogen supplemented postmenopausal women is also consistent with our findings (9). Given the previously described detrimental effects of hyperhomocysteinaemia in follicular fluid on embryo quality (10), and the currently observed inverse associations between tHcy and the number of oocytes retrieved, follicular growth, embryo quality, and the occurrence of pregnancy, suppression of elevated tHcy by estradiol levels would appear to offer beneficial effects. On the other hand, excessive suppression of both tHcy and cobalamin by the supraphysiological estradiol levels which arise during ovarian stimulation may be detrimental for these IVF outcomes.

Recent data has indicated that supraphysiological estradiol levels are associated with a higher frequency of embryo aneuploidy (11). It may be hypothesized that disruption of the homocysteine pathway by ovarian stimulation is an underlying mechanism, because several intermediates of this pathway are directly involved in the synthesis and repair of DNA. The optimal levels still have to be determined. To investigate this further, studies of milder ovarian hyperstimulation protocols, in which embryo aneuploidy determined by pre-implantation genetic screening (PGS) is correlated to monofollicular fluid biomarkers are required.

Biomarkers in follicular fluid are strongly correlated with those in blood (Chapter 3 and 4) and both are affected by folic acid supplementation (Chapter 2). Homocysteine and B-vitamin concentrations are slightly lower in follicular fluid than in blood. These findings are consistent with those of a previous study (12). Follicular fluid is a product of both the transfer of blood plasma across the blood-follicular barrier and locally produced substances by granulosa cells (13). Therefore, passive diffusion of these biomarkers into follicular fluid is suggested to determine concentrations.

We observed that folate in follicular fluid was higher in individually pooled follicular fluid compared to monofollicular samples of the same woman, while tHcy concentrations were comparable. This finding is likely to be due to blood contamination of the pooled follicular fluid as a consequence of the multiple punctures from each woman (Chapter 2). This can be addressed in future studies by restricting sampling to the first follicle aspirated during oocyte pick up.

BIOMARKERS OF THE HOMOCYSTEINE PATHWAY IN MEN

Biomarkers of the homocysteine pathway in blood were shown to be correlated with those in seminal plasma (Chapter 7). However, compared to blood levels, tHcy and pyridoxine levels were significantly lower in seminal plasma, whereas folate and cobalamin were significantly higher (Chapter 7). In addition, the correlations between biomarkers in seminal plasma appeared to be different from the correlations in blood. This may suggest an active transfer from blood to seminal plasma of folate and cobalamin. It is also possible that tHcy and pyridoxine either cannot pass the seminal-blood barrier or are actively resorbed. The inverse correlation between ejaculate volume and the B-vitamins in seminal plasma may indicate dilution (Chapter 7), which may indicate secretion by glands that contribute to seminal plasma, such as the prostate (14). In contrast, the lack of the correlation between tHcy and the ejaculate volume might be explained by the production of tHcy by sperm cells, because tHcy and sperm count were strongly positively correlated.

BIOMARKERS OF THE HOMOCYSTEINE PATHWAY AND HUMAN FERTILITY

These data now enable us to describe relationships between biomarkers of the homocysteine pathway, and a number of outcome parameters related to female and male subfertility. However, we also demonstrated that the mechanisms which determine the concentrations of biomarkers of the homocysteine pathway differ between follicular fluid and seminal plasma. This has to be kept in mind during the interpretation of the associations between these biomarkers and IVF outcome parameters, because cause and effect may easily be confused.

Homocysteine

Whether the significant decrease of tHcy in blood observed during ovarian hyperstimulation affects the follicular fluid composition and/or IVF outcome is not clear, because it is not feasible in humans to puncture follicular fluid during the first days of an ovarian hyperstimulation treatment.

In the presented studies, we were unable to shown any correlation between blood homocysteine levels in women and men with any of the reported fertility outcome parameters. However, follicular fluid tHcy was positively associated with follicular diameter (Chapter 2). Interestingly, this correlation could not be substantiated in the much larger study described in chapter 4. The difference between chapter 2 and 4 may be explained by the difference in sample size.

The observed inverse correlation between follicular fluid tHcy and follicular diameter in Chapter 4 is in line with the inverse correlations observed between tHcy and the number

of retrieved oocytes (Chapter 3), embryo quality (Chapter 5) and the occurrence of biochemical pregnancy (Chapter 3). An adverse effect of tHcy on embryo quality has also recently been reported by Ebisch et al (10). Therefore, we suggest that higher levels of homocysteine are detrimental for follicular growth and may also be detrimental for oocyte and subsequent embryo quality.

Interestingly, the inverse correlation between tHcy in follicular fluid and embryo quality was only present in women who did not take folic acid supplements, while low tHcy in supplemented women corresponded with poor embryo quality (Chapter 5). We therefore conclude that there may be an optimal level of tHcy in follicular fluid. Homocysteine induces the production of reactive oxygen species (ROS) and physiologic levels of ROS in follicular fluid are necessary for oocyte maturation and subsequent ovulation (15). Previous studies demonstrated that patients who became pregnant after IVF had significantly higher levels of ROS in follicular fluid compared to those who did not achieve pregnancy (16, 17). An excess of ROS results in increased oxidative stress. High levels of ROS in culture medium of embryos are associated with low cleavage rates and high embryonic fragmentation (18). The concept of an 'optimal level' of tHcy is not only consistent with the physiological principle of homeostasis, but also reconciles two apparently contradictory observations: that supraphysiological estradiol concentrations are associated with suppression of tHcy concentrations in blood, yet may be detrimental to oocyte and embryo quality. It would appear that excessive suppression of tHcy levels by high estrogens may indeed be harmful to gamete and embryo quality. However, the effect of the decrease of tHcy in blood during ovarian hyperstimulation may be minor compared to other effects of supraphysiological estradiol concentrations.

In Chapter 7 we determined a significant correlation between seminal plasma tHcy concentrations and sperm count. It seems quite likely that tHcy is produced by sperm cells. This is supported by previous studies showing that human spermatozoa generate ROS in physiologic amounts, which play a role during sperm capacitation, acrosome reaction and oocyte fusion (19). Excessive seminal oxidative stress is caused by an imbalance between ROS and anti-oxidant scavenging activities (20). This may explain why we did not find associations between seminal plasma tHcy concentrations and sperm DNA-damage or other sperm parameters.

Folate

Folate has an important role in DNA synthesis and repair which are crucial processes in gametogenesis, fertilization and pregnancy (21, 22). Although folate in blood was not associated with any of the fertility outcome parameters, significant associations were determined between folate in both follicular fluid and seminal plasma and some of these outcomes. The inverse correlation between follicular folate and follicular diameter in women who have been taken folic acid supplements is consistent with the associations

between tHcy and follicular diameter (Chapter 4). The inverse correlation between follicular tHcy concentrations and follicular diameter was stronger in women who did not take folic acid supplements. These associations may indicate that, as with tHcy, both very low and very high levels of folate are detrimental for follicular growth. Very high folate concentrations may be harmful due to an excessive decrease of tHcy, but detrimental effects may also be caused by the presence of unmetabolized synthetic folic acid in blood and/or follicular fluid. The effect of exposure to this form of folate is not clear (23, 24). It would be interesting to differentiate between unmetabolized synthetic folic acid and natural folates in future studies.

In a subgroup of 58 women with single embryo transfer we were able to study associations between biomarkers in follicular fluid of the oocyte from which the transferred embryo was derived and the chance of successful implantation. After adjustment for age, a doubling in follicular folate was associated with a 3.3 fold increase in the chance of clinical pregnancy. This finding is in line with our observations in men, which showed an inverse correlation between seminal plasma folate and DNA-damage as assessed by Sperm Chromatin Structure Assay (SCSA) (Chapter 7). The absence of this correlation in subfertile men may be due to much stronger causes for subfertility responsible for the sperm DNA damage in this subgroup (25). In a randomized, placebo controlled trial a significantly increased sperm count was demonstrated in subfertile men after supplementation with folic acid and zinc (26). However, the percentage abnormal sperm morphology also significantly increased. In that trial participants took daily 5 mg folic acid, which is about ten times the dosage that has been taken by a small part of the participants in the present studies. In line with our observations in women, a very high folate concentration may also have detrimental effects on sperm quality.

Cobalamin

Cobalamin in blood was positively correlated with embryo quality (Chapter 5). Therefore the decrease of cobalamin in blood during ovarian hyperstimulation may be harmful. Similarly, cobalamin in monofollicular fluid was positively correlated with the quality of the corresponding embryo (Chapter 5). The latter association was only statistically significant in women who did not take folic acid supplements, suggesting that sufficient levels of folate can compensate a marginal deficient cobalamin status. However, the intake of folic acid supplements may also indicate a better cobalamin status since about 40% of the folic acid supplemented women also took a cobalamin containing supplement. This may suggest a threshold of cobalamin below which additional cobalamin supplementation may improve embryo quality.

The positive associations between cobalamin and embryo quality are in line with several case reports of subfertility and recurrent miscarriages in women with cobalamin deficiency (27-31). Similarly, subfertile, cobalamin deficient men showed improved semen

parameters after cobalamin supplementation (32, 33). In the present studies seminal plasma cobalamin was positively correlated with sperm concentration and sperm count (chapter 6 and 7). In contrast with seminal plasma tHcy, cobalamin is likely to have a causal effect on sperm count. Cobalamin in seminal plasma inversely correlated with ejaculate volume and this effect of dilution indicates excretion by one of the contributing glands instead of sperm cells. In contrast to a study by Watson et al, cobalamin in seminal plasma was not associated with sperm cell morphology in the present studies (34). This may be due to the small number of participants with a cobalamin deficiency (6 out of 251) or a different assessment of sperm cell morphology.

In spite of an absence of participants with a severe cobalamin deficiency, positive associations between cobalamin and sperm count and embryo quality were observed. Thus, women and men without clinical symptoms of cobalamin deficiency, but with a marginal cobalamin state may benefit from additional cobalamin supplementation.

Pyridoxine

In women and men no associations between pyridoxine and fertility outcome parameters could be demonstrated. Recently, pyridoxine deficiency in Chinese women was associated with lower conception rates and a higher rate of early pregnancy loss (35). Possibly only severe pyridoxine deficiencies affect fertility and in the populations we studied only 2 - 4.5% of the participating women and men had a mild pyridoxine deficiency (minimum 32 nmol/L; reference 46 nmol/L). The absence of severe pyridoxine deficiencies in our study population may explain the absence of associations between pyridoxine and fertility outcome parameters.

STRENGTHS AND LIMITATIONS, AND HOW MIGHT THESE BE ADDRESSED IN FUTURE STUDIES.

One of the strengths of this thesis is that associations between biomarkers of the homocysteine pathway and fertility outcomes are studied in both women and men. At the outset, the intention was to analyze the data of women and men together, but this approach appeared to be too complex. Therefore, we decided to determine associations in women and men separately. All women were treated with a similar ovarian hyperstimulation protocol which prevented the introduction of a bias due to differences in treatment. In a representative population of subfertile couples samples and phenotypic data were standardised collected. Other strengths of our study are the prospective design and the relative large sample size. Moreover, biomarkers were determined in both blood, seminal plasma and most importantly in monofollicular fluid. However, most of the significant associations observed with fertility outcome parameters were restricted to biomarker

levels in follicular fluid and seminal plasma. These fluids represent the closest environment of the gametes. It is likely that a larger study population would be required to demonstrate associations between biomarkers of the homocysteine pathway in blood and fertility outcome parameters.

The present results suggest that active transport and/or resorption of biomarkers takes place in seminal plasma, and that biomarkers may originate from different sources. Although biomarkers in seminal plasma were correlated with the corresponding biomarkers in blood, these transport mechanisms may make it difficult to predict the effect of supplementation and require further research. Comparison of the composition of seminal plasma with material obtained from MESA/PESA procedures would be informative. However, due to the low volume of material derived from this technique, the invasiveness of these procedures, and medical ethical issues there is limited use of this material for research. In addition, it would be interesting to study several consecutive semen samples of individuals. Semen parameters are known to be subject to individual biological variation (36), and variation of biomarkers of the homocysteine pathway has not previously been studied.

Technically it is easier to collect individually pooled follicular fluid samples than monofollicular fluid samples. However, monofollicular fluid samples are recommended in future studies to minimize blood contamination and to provide a more precise reflection of the closest environment of a single oocyte. According to the present results it is not clear whether biomarker levels are affecting follicular growth or are themselves affected by the maturation of the cumulus-oocyte complex. The associations were determined after adjustment for total protein concentrations which have been demonstrated to vary with follicular maturation (37). Also the strong correlation between biomarkers in serum and follicular fluid supports the theory that biomarker levels are affecting follicular growth.

In the present studies only couples undergoing IVF/ICSI treatment were included. In order to investigate the effect of ovarian hyperstimulation on the biomarkers of the homocysteine pathway, it would be interesting to determine the changes of biomarkers in blood during a spontaneous cycle and different ovarian hyperstimulation protocols. Furthermore, in our male study, fertile men were defined by a sperm concentration of equal or more than $20x10^6$ cells/mL and a prior conception with the current or previous partner. Ideally, the subgroup of fertile men would have comprised a group of healthy men without a history of a fertility problem whose partners conceived spontaneously within one year of regular unprotected intercourse.

Ongoing pregnancy and ultimately live birth of a healthy child are the most meaningful outcome parameters of assisted reproductive technology (ART). The present studies were underpowered for these outcome parameters. There are indications that both folic acid and cobalamin supplementation may increase fertility. However, before wide-scale implementation of the recommendation to periconception supply both women and men

with folic acid and cobalamin, it is necessary to perform additional research. A prospective dose-finding study in which the effect on women and men is investigated with a healthy child as endpoint would be the best study design, but this will be hardly feasible.

IMPLICATIONS FOR CLINICAL PRACTICE AND FUTURE RESEARCH

In many couples who face difficulties in conceiving, their subfertility is of multifactorial origin. Environmental and lifestyle factors also implicated in subfertility are potentially amenable to curative or preventive measures. These factors are likely important for both human reproduction and postnatal health. This thesis supports the role of some periconception nutritients in human subfertility.

Several studies have shown that periconception maternal intake of folic acid reduces the risk of neural tube defects (38) and possibly other birth defects (39). Therefore periconception folic acid supplementation has been recommended to women since the early 1990's. Despite these recommendations, the reported intake of this vitamin is still low in most countries (24, 40-42). In a recent survey, 51% of the Dutch women reported that they used folic acid in the entire advised period, which is 4 weeks before conception till 8 weeks after (42). The main reason given for not taking supplements properly was 'becoming pregnant earlier than expected' or 'hearing about it too late in the pregnancy' (43). However, the present study shows that even women undergoing ART are not all using folic acid whereas these women are in a position to prepare their pregnancy, and might be expected to receive extra information during their visits at the fertility clinic. Moreover, a substantial number of women reported taking folic acid, but their blood samples showed inadequate intake. Blood samples taken on two time points revealed that only 67% of the participating women took folic acid supplements regularly. There is a clear need to investigate why these women are not taking the recommended folic acid in order to improve compliance. Providing additional information may help, but stimulation and repeated reminders may be even more effective. In several countries food fortification with folic acid has been implemented in order to increase the average serum folate concentration. In the Netherlands no fortified foods are yet available due to the Dutch law (44). However, also with such policy it is difficult to reach the daily intake of 400 µg folate in every woman of childbearing age (45).

On the other hand, 24% of the women using folic acid reported taking more than one supplement and these women were showed to have very high folate concentrations in both serum and follicular fluid (Chapter 5). Until now, most attention has been paid to the beneficial effects of folate and the detrimental effects of tHcy. However, very high folate levels and very low tHcy levels may be detrimental for human reproduction as well. In line with this, there is an ongoing discussion about possible adverse effects of excessive folate

levels with respect to increased twinning rates (46-48). It can therefore be concluded that the current provision of information regarding folic acid supplementation is inadequate.

Although at present the use of folic acid supplements is not recommended for men, 20% of men participating in the present study reported to take folic acid containing supplements. Interestingly, this percentage was higher in subfertile men (26%) compared to fertile men (14%) (Chapter 7). The reason for the use of folic acid containing supplements by men undergoing IVF/ICSI treatment has not been investigated in this thesis. Participants may have heard of the previously reported positive effect of folic acid on sperm count (26). The present results also indicate that some men may benefit from folic acid supplementation.

In addition, cobalamin supplementation may be advantageous in some couples, because we observed a positive association between cobalamin concentrations in seminal plasma and sperm count in men (Chapter 7) and positive associations between cobalamin concentrations in female blood and follicular fluid and embryo quality (Chapter 5).

The present studies clearly demonstrated that the homocysteine pathway is involved in human subfertility. The most interesting new findings are the positive association between folate and the achievement of pregnancy and the inverse association between folate and sperm DNA damage. Previously, most studies focused on the detrimental effects of low folate concentrations and high tHcy concentrations. The results of this thesis indicate that very high levels of folate and deficient levels of tHcy may be harmful as well. Therefore, the concept of an 'optimal' level is proposed. Additional prospective dose-finding studies are now required to find the optimal dosages.

REFERENCES

1. Refsum H, Smith AD, Ueland PM, Nexo E, Clarke R, McPartlin J *et al.* Facts and recommendations about total homocysteine determinations: An expert opinion. Clin Chem 2004;50:3-32.
2. Bettahar-Lebugle K, Feugeas O, Wittemer C, Ohl J, Rongieres C, Nisand I. [Evolution of homocysteine during ovarian stimulation for IVF or ICSI]. Gynecol Obstet Fertil 2002;30:121-8.
3. Roopnarinesingh R, Jackson B, Osman Z, Harrison R, Mayne P. Homocysteine in assisted reproduction: does oestradiol influence homocysteine levels? J Obstet Gynaecol 2006;26:59-62.
4. Morris MS, Jacques PF, Selhub J, Rosenberg IH. Total homocysteine and estrogen status indicators in the Third National Health and Nutrition Examination Survey. Am J Epidemiol 2000;152:140-8.
5. Hak AE, Polderman KH, Westendorp IC, Jakobs C, Hofman A, Witteman JC *et al.* Increased plasma homocysteine after menopause. Atherosclerosis 2000;149:163-8.
6. Smolders RG, de Meer K, Kenemans P, Jakobs C, Kulik W, van der Mooren MJ. Oral estradiol decreases plasma homocysteine, vitamin B6, and albumin in postmenopausal women but does not change the whole-body homocysteine remethylation and transmethylation flux. J Clin Endocrinol Metab 2005; 90:2218-24.
7. van Baal WM, Smolders RG, van der Mooren MJ, Teerlink T, Kenemans P. Hormone replacement therapy and plasma homocysteine levels. Obstet Gynecol 1999;94:485-91.
8. Madsen JS, Kristensen SR, Klitgaard NA, Bladbjerg EM, Abrahamsen B, Stilgren L *et al.* Effect of long-term hormone replacement therapy on plasma homocysteine in postmenopausal women: a randomized controlled study. Am J Obstet Gynecol 2002;187:33-9.
9. Cagnacci A, Generali M, Pirillo D, Baldassari F, Volpe A. Effects of low- or high-dose hormone therapy on fasting and post-methionine homocysteine levels in postmenopausal women. Climacteric 2006;9: 388-95.
10. Ebisch IM, Peters WH, Thomas CM, Wetzels AM, Peer PG, Steegers-Theunissen RP. Homocysteine, glutathione and related thiols affect fertility parameters in the (sub)fertile couple. Hum Reprod 2006; 21:1725-33.
11. Baart EB, Martini E, Eijkemans MJ, Van Opstal D, Beckers NG, Verhoeff A *et al.* Milder ovarian stimulation for in-vitro fertilization reduces aneuploidy in the human preimplantation embryo: a randomized controlled trial. Hum Reprod 2007;22:980-8.
12. Steegers-Theunissen RP, Steegers EA, Thomas CM, Hollanders HM, Peereboom-Stegeman JH, Trijbels FJ *et al.* Study on the presence of homocysteine in ovarian follicular fluid. Fertil Steril 1993;60:1006-10.
13. Gerard N, Loiseau S, Duchamp G, Seguin F. Analysis of the variations of follicular fluid composition during follicular growth and maturation in the mare using proton nuclear magnetic resonance (1H NMR). Reproduction 2002;124:241-8.
14. Owen DH, Katz DF. A review of the physical and chemical properties of human semen and the formulation of a semen simulant. J Androl 2005;26:459-69.
15. Ebisch IM, Thomas CM, Peters WH, Braat DD, Steegers-Theunissen RP. The importance of folate, zinc and antioxidants in the pathogenesis and prevention of subfertility. Hum Reprod Update 2007;13: 163-74.
16. Attaran M, Pasqualotto E, Falcone T, Goldberg JM, Miller KF, Agarwal A *et al.* The effect of follicular fluid reactive oxygen species on the outcome of in vitro fertilization. Int J Fertil Womens Med 2000; 45:314-20.
17. Bedaiwy MA, Miller K, Goldberg JM, Nelson DR, Agarwal A, Falcone T. Assessment of the predictive value of follicular fluid cytokines and reactive oxygen species in IVF cycles. Fertil Steril 2002;78:S5-S6.
18. Bedaiwy MA, Falcone T, Mohamed MS, Aleem AA, Sharma RK, Worley SE *et al.* Differential growth of human embryos in vitro: role of reactive oxygen species. Fertil Steril 2004;82:593-600.

19. de Lamirande E, Jiang H, Zini A, Kodama H, Gagnon C. Reactive oxygen species and sperm physiology. Reviews of reproduction 1997;2:48-54.
20. Sharma RK, Pasqualotto FF, Nelson DR, Thomas AJ, Jr., Agarwal A. The reactive oxygen species-total antioxidant capacity score is a new measure of oxidative stress to predict male infertility. Hum Reprod 1999;14:2801-7.
21. Jaroudi S, SenGupta S. DNA repair in mammalian embryos. Mutat Res 2007;635:53-77.
22. Kiefer JC. Epigenetics in development. Dev Dyn 2007;236:1144-56.
23. Sweeney MR, McPartlin J, Scott J. Folic acid fortification and public health: report on threshold doses above which unmetabolised folic acid appear in serum. BMC public health 2007;7:41.
24. Smith AD, Kim YI, Refsum H. Is folic acid good for everyone? Am J Clin Nutr 2008;87:517-33.
25. Zini A, Libman J. Sperm DNA damage: clinical significance in the era of assisted reproduction. CMAJ 2006;175:495-500.
26. Wong WY, Merkus HM, Thomas CM, Menkveld R, Zielhuis GA, Steegers-Theunissen RP. Effects of folic acid and zinc sulfate on male factor subfertility: a double-blind, randomized, placebo-controlled trial. Fertil Steril 2002;77:491-8.
27. Hall M, Davidson RJL. Prophylactic folic acid in women with pernicious anaemia pregnant after periods of infertility. J Clin Path 1968;21 599-602.
28. Girdwood RH, Eastwood MA, Finlayson ND, Graham GS. Pernicious anaemia as a cause of infertility in twins. Lancet 1971;1:528-30.
29. Bennett M. Vitamin B-12 deficiency, infertility and recurrent fetal loss. J Reprod Med 2001;46:209-12.
30. Jackson I, Doig WB, McDonald G. Pernicious anaemia as a cause of infertility. Lancet 1967;290:1159-60.
31. Mahmood A. Pernicious anaemia as a cause of infertility. Lancet 1971;297:753.
32. Sharp AA, Witts LJ. Seminal vitamin B 12 and sterility Lancet 1962;280:779.
33. Blair JH, Stearns HE, Simpson GM. Vitamin B 12 and fertility. Lancet 1968;1:49-50.
34. Watson AA. Seminal vitamin B12 and sterility. Lancet 1962;280:644.
35. Ronnenberg AG, Venners SA, Xu X, Chen C, Wang L, Guang W *et al.* Preconception B-vitamin and homocysteine status, conception, and early pregnancy loss. Am J Epidemiol 2007;166:304-12.
36. Alvarez C, Castilla JA, Martinez L, Ramirez JP, Vergara F, Gaforio JJ. Biological variation of seminal parameters in healthy subjects. Hum Reprod 2003;18:2082-8.
37. Spitzer D, Murach KF, Lottspeich F, Staudach A, Illmensee K. Different protein patterns derived from follicular fluid of mature and immature human follicles. Hum Reprod 1996;11:798-807.
38. Lumley J, Watson L, Watson M, Bower C. Periconceptional supplementation with folate and/or multivitamins for preventing neural tube defects. Cochrane database of systematic reviews (Online) 2001:CD001056.
39. Botto LD, Olney RS, Erickson JD. Vitamin supplements and the risk for congenital anomalies other than neural tube defects. Am J Med Genet C Semin Med Genet 2004;125:12-21.
40. Nilsen RM, Vollset SE, Gjessing HK, Magnus P, Meltzer HM, Haugen M *et al.* Patterns and predictors of folic acid supplement use among pregnant women: the Norwegian Mother and Child Cohort Study. Am J Clin Nutr 2006;84:1134-41.
41. de Jong-van den Berg LT, Hernandez-Diaz S, Werler MM, Louik C, Mitchell AA. Trends and predictors of folic acid awareness and periconceptional use in pregnant women. Am J Obstet Gynecol 2005;192: 121-8.
42. de Walle HE, de Jong-van den Berg LT. Ten years after the Dutch public health campaign on folic acid: the continuing challenge. Eur J Clin Pharmacol 2008;64:539-43.
43. de Walle HE, de Jong-van den Berg LT. Growing gap in folic acid intake with respect to level of education in the Netherlands. Community genetics 2007;10:93-6.
44. Health Council of the N. Health Council of the Netherlands: Risks of folic acid fortification. In: Hague T, ed., 2000:publication no. 2000/21.

45. Yang QH, Carter HK, Mulinare J, Berry RJ, Friedman JM, Erickson JD. Race-ethnicity differences in folic acid intake in women of childbearing age in the United States after folic acid fortification: findings from the National Health and Nutrition Examination Survey, 2001-2002. Am J Clin Nutr 2007;85:1409-16.
46. Haggarty P, McCallum H, McBain H, Andrews K, Duthie S, McNeill G *et al.* Effect of B vitamins and genetics on success of in-vitro fertilisation: prospective cohort study. Lancet 2006;367:1513-9.
47. Boxmeer JC, Fauser BC, Macklon NS. Effect of B vitamins and genetics on success of in-vitro fertilisation. Lancet 2006;368:200.
48. Muggli EE, Halliday JL. Folic acid and risk of twinning: a systematic review of the recent literature, July 1994 to July 2006. Med J Aust 2007;186:243-8.

Summary

Approximately ten to fifteen percent of all couples are affected by subfertility, defined as at least one year of unprotected intercourse with the same partner without conception. In most couples, subfertility is of multifactorial origin. The use of assisted reproductive technology (ART) can increase the chance of conception, but also provides access to the direct environment of gametes and the early developing embryo. Applying these techniques has greatly increased our knowledge of the factors involved in periconceptional development. Furthermore, it is becoming clear that also environmental, nutritional and genetic factors contribute to the outcome of treatment and subsequent birth outcome.

Whereas genetic factors are difficult to modulate, environmental and lifestyle factors are potentially amenable to curative or preventive measures. Nutrition is an important remediable factor, but continues to be largely neglected when counselling and managing subfertile couples. The homocysteine pathway may play an important role in fertility because several nutrients are intermediates in this pathway and as such directly involved in the synthesis of proteins and DNA, which are critical in gametogenesis.

After a general introduction concerning the current knowledge of the homocysteine pathway in human reproduction and subfertility, the objectives of this thesis are described in Chapter 1.

PART I

The objective of part I was to investigate the role of the homocysteine pathway in female subfertility. The biomarker concentrations and several covariates were determined and studied in association with fertility outcome parameters.

Chapter 2 describes the influence of the intake of folic acid supplementation on the follicular fluid concentrations of total homocysteine (tHcy) and folate. In an observational study concentrations of tHcy and folate in monofollicular and multifollicular fluid were determined in 37 women undergoing IVF or ICSI treatment. Folic acid supplementation significantly increased folate and decreased tHcy concentrations in multifollicular fluid. In monofollicular fluids the folate concentrations only were significantly increased in supplemented women. The tHcy concentration appeared to be positively correlated with the diameter of the follicle. Samples from single follicles appeared to be less prone to artefacts in the measurements of the folate and tHcy concentration.

Associations between tHcy, folate, cobalamin, and pyridoxine concentrations in multifollicular fluid and IVF-outcome parameters are investigated in Chapter 3. These biomarker

concentrations were determined in multifollicular fluids of 143 women undergoing IVF or ICSI treatment. Significant correlations were demonstrated between tHcy, folate, cobalamin, and pyridoxine concentrations in multifollicular fluid. Not any correlation was established between the biomarkers and fertilization. An inverse correlation, however, was determined between tHcy and the number of oocytes after adjustment for age and treatment. Moreover, the higher the tHcy concentration per follicle, the lower the chance of achieving biochemical pregnancy. These data suggest that a high follicular fluid tHcy concentration is detrimental for the maturation of oocytes and the chance of achieving biochemical pregnancy.

The FOod, Lifestyle and Fertility Outcome-project (FOLFO-project) was set up to study the influence of preconception nutrition and lifestyle on fertility and pregnancy outcome. Between September 2004 and October 2006, subfertile couples undergoing an IVF procedure with or without ICSI at the Erasmus MC, University Medical Centre, Rotterdam, The Netherlands, were invited to participate. Blood samples were collected on cycle day 2 and the day of hCG administration in 181 women undergoing ovarian stimulation for IVF. In each subject the diameter of the two leading follicles was measured and the corresponding monofollicular fluids were collected. In blood and monofollicular fluid samples tHcy, folate, cobalamin and pyridoxine were determined. According to the blood folate levels, women were classified as either folic acid supplemented (n=113, 67%) or non-supplemented (n=32, 19%). We conclude that even in highly motivated subfertile patients more education about folic acid supplementation seems necessary.

Chapter 4 aims to investigate whether ovarian stimulation deranges the homocysteine pathway thereby affecting the pre-ovulatory follicle. Ovarian hyperstimulation resulted in a significant decrease in blood tHcy and cobalamin levels. The blood concentrations of tHcy, folate, cobalamin and pyridoxine were significantly correlated with the corresponding monofollicular fluid concentrations. Monofollicular fluid tHcy concentrations were inversely correlated with the follicular diameter. In folic acid supplemented women, follicular fluid folate was inversely correlated with the follicular diameter as well. Therefore, it may be concluded that both high ovarian follicular fluid tHcy and high folate levels may have detrimental effects on follicular development. Moreover, ovarian hyperstimulation seems to derange blood and follicular fluid biomarkers of the homocysteine pathway.

In Chapter 5 associations between biomarkers of the homocysteine pathway and IVF outcome are investigated in the same study population. In blood, a significant correlation was established between high cobalamin and better embryo quality. In monofollicular fluid of non-supplemented women, high cobalamin correlated with better embryo quality whereas high tHcy resulted in poor embryo quality. In monofollicular fluid of supplemented women high tHcy correlated with better embryo quality. In the total group, a two-fold increase of monofollicular fluid folate corresponded with a 3.3 higher chance to achieve

biochemical pregnancy. These results suggest that an optimal homocysteine pathway in follicular fluid contributes to a better embryo quality and chance of pregnancy.

PART II

The objective of part II was to investigate the role of the homocysteine pathway in male subfertility. This was investigated by collecting blood and semen samples from male participants of the FOLFO project. In blood and seminal plasma folate, cobalamin, pyridoxine, and tHcy were determined. Semen analysis included sperm concentration, motility and morphology according to WHO criteria.

In Chapter 6 data from 73 men are analyzed. Concentrations of tHcy and B-vitamins were significantly correlated in blood, but not in seminal plasma. The serum and RBC folate concentrations were also significantly correlated with the folate concentration in seminal plasma. Likewise, the cobalamin concentration in serum and seminal plasma was significantly correlated. Of interest is that the cobalamin concentration in seminal plasma was significantly correlated with the sperm concentration. This is in contrast to the absence of significant associations between the other B-vitamins and tHcy in blood and seminal plasma and any of the semen parameters.

In chapter 7 a larger group of 251 participants is analyzed. Subgroups of fertile (n = 70) and subfertile men (n = 63) were defined according to semen concentration and proven fertility. In addition to the semen parameters used in Chapter 6, the DNA fragmentation index (DFI) as a marker of sperm DNA damage was determined using the Sperm Chromatin Structure Assay (SCSA). No correlation was found between the biomarkers in blood and the semen parameters. In seminal plasma both tHcy and cobalamin positively correlated with sperm count. Folate, cobalamin, and pyridoxine were inversely correlated with ejaculate volume. In fertile men seminal plasma folate showed an inverse correlation with DFI. This may indicate that low concentrations of folate in seminal plasma are detrimental for sperm DNA stability.

In conclusion, the findings of chapter 6 and 7 emphasize the importance of B-vitamins during spermatogenesis in human.

In the general discussion (Chapter 8) we consider the significance of these findings. Furthermore, implications for clinical practice and future research are discussed. The results of this thesis confirm the role of the homocysteine pathway in human subfertility. Moreover, our findings support the importance of periconception folic acid supplementation and suggest that the additional intake of cobalamin may also be beneficial. Moreover, these recommendations would appear to apply to both men and women. Clearly, further studies are now required to confirm these data and to find the best and safest B-vitamin dosages,

because the present results may suggest that very high concentrations of folate may be detrimental for gametogenesis and fertility as well.

Samenvatting

Naar schatting tien tot vijftien procent van alle paren krijgt te maken met subfertiliteit. Subfertiliteit wordt gedefinieerd als het uitblijven van een zwangerschap na tenminste een jaar onbeschermde coïtus met dezelfde partner. De oorzaak is bij de meeste paren multifactorieel. Het gebruik van geassisteerde voortplantingstechnieken zoals in vitro fertilisatie (IVF) vergroot de kans op bevruchting en zwangerschap. Daarnaast kan het ook informatie geven over de directe omgeving van gameten en het jonge, zich ontwikkelende embryo. Het toepassen van deze technieken heeft onze kennis over de factoren die een rol spelen in de periconceptie periode sterk vergroot. Het is duidelijk geworden dat zowel genetische factoren als omgevingsfactoren, in het bijzonder leefstijl en voeding, bijdragen aan de uitkomst van een vruchtbaarheidsbehandeling.

Genetische factoren zijn moeilijk te veranderen, maar leefstijl en voeding zijn potentieel aan te passen en kunnen worden gebruikt bij de behandeling en preventie van subfertiliteit. Voeding is een belangrijke, behandelbare factor die nog steeds onderbelicht blijft tijdens de behandeling van subfertiele paren. De homocysteïne stofwisseling zou een belangrijke rol kunnen spelen bij de vruchtbaarheid, omdat verschillende vitaminen en tussenproducten uit deze stofwisseling direct betrokken zijn bij processen die essentieel zijn bij de gametogenese zoals de aanmaak van eiwitten en DNA.

In hoofdstuk 1 wordt de rol van de homocysteïne stofwisseling in het voortplantingsproces beschreven en worden de doelstellingen van dit proefschrift geformuleerd. Hierna wordt de rol van de homocysteïne stofwisseling bepaald in de vrouwelijke (deel I) en mannelijke subfertiliteit (deel II).

DEEL I

In dit eerste gedeelte was het doel het bepalen van de rol van de homocysteïne stofwisseling in de vrouwelijke subfertiliteit. Biomarkers van deze stofwisseling werden gemeten in bloed en follikelvloeistof. Vervolgens werden correlaties tussen de biomarker concentraties en uitkomstparameters van vruchtbaarheid bestudeerd.

Hoofdstuk 2 beschrijft de invloed van de inname van foliumzuurtabletten in de preconceptie periode op de concentraties homocysteïne (tHcy) en foliumzuur in follikelvloeistof bij 37 vrouwen die behandeld werden met IVF met of zonder Intracytoplasmatische Sperma Injectie (ICSI). Homocysteïne en foliumzuur werden bepaald in monofolliculaire en gepoolde multifolliculaire vloeistof. Extra foliumzuur inname verhoogde de foliumzuurconcentratie en verlaagde de tHcy concentratie in follikelvloeistof significant. In de

monofolliculaire vloeistof van vrouwen die foliumzuur gebruikten, was alleen de foliumzuurconcentratie verhoogd. Er werd een positieve correlatie gevonden tussen de tHcy concentratie en de diameter van de follikel. Tot slot bleken de metingen van foliumzuur en tHcy in de monsters van aparte follikels minder gevoelig voor meetfouten.

In hoofdsuk 3 worden associaties bestudeerd tussen de concentraties tHcy, foliumzuur, vitamine B12 en B6 in gepoolde multifolliculaire vloeistof en enkele uitkomstparameters van de IVF-behandeling. De biomarker concentraties werden gemeten in gepoolde multifolliculaire monsters van 143 vrouwen tijdens een IVF/ICSI behandeling. De concentraties tHcy, foliumzuur, vitamine B12 en B6 waren significant met elkaar gecorreleerd. Er werden geen significante correlaties gevonden tussen deze concentraties en het percentage bevruchte eicellen. Na correctie voor leeftijd en het behandelingstype werd er wel een negatieve correlatie gevonden tussen tHcy en het aantal verkregen eicellen. Daarnaast was een hoge concentratie tHcy per follikel geassocieerd met een lage kans op het ontstaan van een zwangerschap. Deze data suggereren dat een hoge tHcy concentratie in follikelvloeistof slecht is voor de uitrijping van eicellen en de kans om zwanger te worden verlaagd.

Het 'FOod, Lifestyle and Fertility Outcome-project' (FOLFO-project) is geïnitieerd om de invloed van preconceptie voeding en leefstijl op de IVF-uitkomst te bestuderen. Tussen september 2004 en oktober 2006 werden paren geïncludeerd die een IVF of ICSI behandeling kregen in het Erasmus MC, Universitair Medisch Centrum in Rotterdam. Op cyclusdag 2 en de dag van hCG-toediening werd bloed afgenomen bij 181 vrouwen die een ovariële hyperstimulatie in het kader van een IVF/ICSI behandeling ondergingen. Bij iedereen werd in beide ovaria de diameter van de grootste voorliggende follikel gemeten en monofolliculaire vloeistoffen en eicellen werden apart verzameld. In de bloed en de monofolliculaire monsters werd de concentratie tHcy, foliumzuur, vitamine B12 en B6 bepaald. Op basis van de foliumzuurconcentratie in de bloedmonsters werden twee subgroepen gedefinieerd van foliumzuurgebruikers (n = 113, 67%) en niet-gebruikers (n = 32, 19%). We concluderen dat zelfs in een populatie van sterk gemotiveerde, subfertiele patiënten meer voorlichting over het gebruik van foliumzuur nodig is.

In hoofdstuk 4 wordt bestudeerd of ovariële hyperstimulatie de homocysteïne stofwisseling verstoord en daardoor de pre-ovulatoire follikel beïnvloed. Ovariële hyperstimulatie bleek geassocieerd te zijn met een daling van tHcy en vitamine B12 concentraties in het bloed. Er werden significante correlaties gevonden tussen de concentraties tHcy, foliumzuur, vitamine B12 en B6 in bloed en monofolliculaire vloeistof. De tHcy concentratie in monofolliculaire vloeistof was negatief gecorreleerd met de diameter van de bijbehorende follikel. Bij vrouwen die foliumzuur slikten was ook de monofolliculaire foliumzuurconcentratie negatief gecorreleerd met de follikel diameter. Daarom kan worden geconcludeerd dat zowel hoge tHcy concentraties als hoge foliumzuurconcentraties schadelijk kunnen

zijn voor de follikel ontwikkeling. Bovendien lijkt ovariële hyperstimulatie de homocysteïne stofwisseling te verstoren.

In hoofdstuk 5 worden associaties tussen biomarkers van de homocysteïne stofwisseling en IVF-uitkomst bestudeerd in dezelfde studiepopulatie. Een hoge vitamine B12 concentratie in bloed correleerde met een betere embryokwaliteit. Een hoge vitamine B12 concentratie in monofolliculaire vloeistof van vrouwen die geen foliumzuur gebruikten correleerde ook met een betere embryokwaliteit terwijl een hoger tHcy correleerde met een slechtere kwaliteit. Bij foliumzuur gebruiksters was een hoger tHcy juist geassocieerd met een betere embryokwaliteit. In de totale groep was een verdubbeling van de foliumzuurconcentratie in monofolliculaire vloeistof geassocieerd met een 3.3 maal hogere kans om zwanger te worden. Deze resultaten suggereren dat er voor een betere embryokwaliteit en grotere kans op een zwangerschap, een optimale balans van de homocysteïne stofwisseling in follikel vloeistof bestaat.

DEEL II

In dit tweede gedeelte was het doel het bepalen van de rol van de homocysteïne stofwisseling in de mannelijke subfertiliteit. Hiervoor werden bloed en semen monsters verzameld van de mannelijke deelnemers aan het FOLFO-project. In bloed en seminaal plasma werden de concentraties tHcy, foliumzuur, vitamine B12 en B6 gemeten. Analyse van het semenmonster bestond uit het bepalen van de concentratie zaadcellen, de motiliteit, en morfologie volgens de WHO criteria.

In hoofdstuk 6 worden de gegevens van 73 mannen geanalyseerd. De concentraties tHcy en B-vitaminen waren in bloed wel significant gecorreleerd, maar in seminaal plasma niet. De foliumzuurconcentraties in bloed en rode bloedcellen correleerden significant met foliumzuur in seminaal plasma. Ook de vitamine B12 concentraties in bloed en seminaal plasma correleerden significant. De vitamine B12 concentratie in seminaal plasma correleerde ook met de concentratie zaadcellen. De andere B-vitaminen en tHcy in bloed en seminaal plasma correleerden met geen enkele semen parameter.

In hoofdstuk 7 wordt een grotere groep van 251 deelnemers geanalyseerd. Hierbij werden subgroepen van fertiele (n = 70) en subfertiele mannen (n = 63) gedefinieerd op basis van de concentratie zaadcellen en bewezen fertiliteit. In aanvulling op de semenparameters die werden gebruikt in hoofdstuk 6, werd in hoofdstuk 7 ook de DNA schade in zaad gemeten door middel van de Sperm Chromatin Structure Assay (SCSA). De biomarkerconcentraties in bloed correleerden met geen van de semenparameters. Homocysteïne en vitamine B12 in seminaal plasma correleerden allebei positief met het aantal zaadcellen. Foliumzuur, vitamine B12 en B6 waren negatief gecorreleerd met het volume van het ejaculaat. Bij fertiele mannen bleek een lage foliumzuurconcentratie in

seminaal plasma geassocieerd te zijn met meer DNA schade. Dit zou kunnen betekenen dat een lage concentratie foliumzuur in seminaal plasma schadelijk is voor de stabiliteit van DNA.

Er kan worden geconcludeerd dat de resultaten van hoofdstuk 6 en 7 het belang van B-vitaminen in de menselijke spermatogenese benadrukken.

In de algemene discussie (hoofdstuk 8) wordt het belang van de bevindingen besproken. Daarnaast worden de consequenties voor de praktijk en toekomstig onderzoek bediscussieerd. De resultaten van dit proefschrift suggereren dat de homocysteïne stofwisseling een rol speelt bij de voortplanting van de mens. Bovendien ondersteunen de bevindingen het belang van foliumzuur suppletie in relatie tot zwangerschap. Ook wordt gesuggereerd dat aanvullende vitamine B12 suppletie een positief effect op de vruchtbaarheid heeft. Deze aanbevelingen zouden voor zowel vrouwen als mannen gelden. Het is duidelijk dat er meer onderzoek nodig is om deze bevindingen te bevestigen en de optimale dosering te vinden, want de huidige resultaten suggereren ook dat een zeer hoge concentratie foliumzuur schadelijk kan zijn voor de spermatogenese en vruchtbaarheid.

List of publications

PUBLICATIONS INCLUDED IN THIS THESIS

Boxmeer JC, Smit M, Weber RF, Lindemans J, Romijn JC, Eijkemans MJ, Macklon NS, Steegers-Theunissen RP. Seminal plasma cobalamin significantly correlates with sperm concentration in males undergoing IVF or ICSI procedures. J Androl. 2007;28(4):521-7

Boxmeer JC, Brouns RM, Lindemans J, Steegers EAP, Martini E, Macklon NS, Steegers-Theunissen RPM. Preconception folic acid treatment affects the micro-environment of the maturing oocyte in human. Fertil Steril. 2008;89(6):1766-70

Boxmeer JC, Smit M, Utomo E, Romijn JC, Eijkemans MJC, Lindemans J, Laven JSE, Macklon NS, Steegers EAP, Steegers-Theunissen RPM. Low folate in seminal plasma is associated with increased sperm DNA damage. Fertil Steril. 2008 Aug 21. [Epub ahead of print]

Boxmeer JC, Steegers-Theunissen RPM, Lindemans J, Wildhagen MF, Martini E, Steegers EAP, Macklon NS. Homocysteine metabolism in the pre-ovulatory follicle during ovarian stimulation. Hum Reprod. 2008 Jul 29. [Epub ahead of print]

Boxmeer JC, Macklon NS, Lindemans J, Beckers NGM, Eijkemans MJC, Laven JSE, Steegers EAP, Steegers-Theunissen RPM. IVF outcomes are associated with biomarkers of the homocysteine pathway in monofollicular fluid. *Submitted for publication*

Boxmeer JC, Brouns RM, Lindemans J, Steegers EAP, Martini E, Macklon NS, Eijkemans MJC, Steegers-Theunissen RPM. Homocysteine in follicular fluid of women undergoing IVF/ICSI treatment is inversely correlated with the number of oocytes and the occurrence of pregnancy. *Submitted for publication*

OTHER PUBLICATIONS

Reijnierse M, van Buchem MA, Boxmeer J, Zwinderman AH, Breedveld FC, Bloem JL. Distribution and cause of cervical spinal canal stenosis in patients with rheumatoid arthritis. Gepubliceerd in het proefschrift van M. Reijnierse. 13 Sep 2000

Boxmeer JC, Balk AH, Steegers EA, Visser W. Twee zwangere vrouwen met kortademigheid op grond van peri-partumcardiomyopathie. Ned Tijdschr Geneeskd. 2006;150(5):255-8

Boxmeer JC, Fauser BCJM, Macklon NS. Effect of B vitamins and genetics on succes of in-vitro fertilisation. Lancet 2006; 368:200

ABSTRACTS AND PRESENTATIONS RELATED TO THE PRESENT THESIS

Boxmeer JC, Brouns MM, Lindemans J, Martini E, Macklon NS, Steegers-Theunissen RPM. Folic acid treatment affects oocyte environment. Poster Presentation at the Annual meeting of the Society for Gynecologic Investigation, Los Angeles. J Soc Gynecol Investig. Suppl 2005;12(2):24A

Boxmeer JC, Smit M, Weber RFA, Lindemans J, Romijn JC, Eijkemans MJC, Macklon NS, Steegers-Theunissen RPM Seminal plasma cobalamin significantly correlates with sperm concentration. Poster Presentation at the Annual meeting of the Society for Gynecologic Investigation, Toronto. J Soc Gynecol Investig. Suppl 2006;13(2):145A

Boxmeer JC, Brouns RM, Lindemans J, Steegers EAP, Martini E, Macklon NS, Steegers-Theunissen RPM. Preconceptional folic acid treatment affects the micro-environment of the oocyte. Poster Presentation at the World Congress on Hyperhomocysteinemia - 6th Conference on Homocysteine Metabolism. Saarbruecken, Germany, June 5-9, 2007

Boxmeer JC, Brouns RM, Lindemans J, Steegers EAP, Martini E, Macklon NS, Eijkemans MJC, Steegers-Theunissen RPM. Homocysteine in follicular fluid lowers the number of oocytes and the chance of pregnancy. Poster Presentation at the World Congress on Hyperhomocysteinemia - 6th Conference on Homocysteine Metabolism. Saarbruecken, Germany, June 5-9, 2007

Boxmeer JC, Macklon NS, Lindemans J, Beckers NGM, Eijkemans MJC, Laven JSE, Steegers EAP, Steegers-Theunissen RPM. Follicular fluid folate, cobalamin and total homocysteine levels are affecting IVF outcomes. Oral presentation at the Reinier de Graaf symposium 13-14 March 2008, Groningen, the Netherlands

Boxmeer JC, Steegers-Theunissen RPM, Lindemans J, Wildhagen MF, de Jong FH, Martini E, Steegers EAP, Macklon NS IVF Treatment Significantly Affects the Homocysteine Pathway in the Maturing Follicle. Poster Presentation at the Annual meeting of the Society for Gynecologic Investigation, San Diego. J Soc Gynecol Investig. Suppl 2008;15(2):159A

Boxmeer JC, Macklon NS, Lindemans J, Beckers NGM, Eijkemans MJC, Laven JSE, Steegers EAP, Steegers-Theunissen RPM. Levels of the Biomarkers of the Homocysteine Pathway in Follicular Fluid Affect Oocyte Quality. Poster Presentation at the Annual meeting of the Society for Gynecologic Investigation, San Diego. J Soc Gynecol Investig. Suppl 2008;15(2):160A

Boxmeer JC, Smit M, Utomo E, Romijn JC, Eijkemans MJC, Lindemans J, Laven, JSE Macklon NS, Steegers EAP, Steegers-Theunissen RPM. Significant Influences of Derangements in the Homocysteine Pathway on Human Sperm Quality. Poster Presentation at the Annual meeting of the Society for Gynecologic Investigation, San Diego. J Soc Gynecol Investig. Suppl 2008;15(2):245A

Dankwoord

Promoveren doe je niet alleen en daarom wil ik hierbij een aantal mensen persoonlijk bedanken.

Allereerst wil ik alle paren die hebben deelgenomen aan de FOLFO-studie heel hartelijk danken. Zonder jullie was dit onderzoek niet mogelijk geweest. Door jullie bijdrage kan een beter advies over B vitaminen gegeven worden aan paren die graag zwanger willen worden.

Co-promotor dr. Régine Steegers-Theunissen, hoewel je vele promovendi en studenten begeleidt bij hun onderzoek, probeer je toch altijd tijd te maken om resultaten en manuscripten tot in detail door te nemen en mee te denken hoe het beter kan. Ik bewonder de manier waarop je dat voor elkaar krijgt.

Professor Macklon, beste Nick, door je vertrek naar Utrecht kreeg je vele nieuwe tijdrovende taken, maar je betrokkenheid bij het onderzoek bleef. Helaas zat je niet meer bij me op de gang, maar gelukkig bestaan de fax, de e-mail en telefoon zodat we toch contact konden houden. Naast het meedenken over de inhoud, gaf je als 'native-english-speaker' vele nuttige taalkundige tips. Dankjewel.

Professor Steegers, beste Eric, als AGNIO had ik het al eens met je gehad over onderzoek en aan kleine projecten gewerkt, maar dit was toch anders. Hartelijk dank voor je begeleiding.

Bij de sub-afdeling Voortplantingsgeneeskunde voelde ik me direct thuis. Beste Joop Laven, jij hebt me de kans gegeven om meer tijd in het onderzoek te steken en als het nodig was, kon ik altijd bij je terecht. Bedankt voor je steun. Als fertiliteits-arts kon ik altijd laagdrempelig overleggen en er waren vele leerzame besprekingen. Joop, Evert, Jits en Marjan, het was fijn om met jullie samen te werken. Zonder mijn collega-fertiliteitsartsen Nicole, Cezanne, Neomar, Berthe, Elisabeth en Hjalmar was er bovendien niks van mijn onderzoek terecht gekomen. Bedankt voor de extra tijd en energie die jullie in het FOLFO-onderzoek hebben gestoken.

Wat is een polikliniek zonder secretaresses? Het FOLFO-onderzoek zou in ieder geval niet mogelijk zijn geweest zonder de IVF-secretaresses: Beate, Ria en Annemarie en de andrologie-secretaresses: Chris en Frouke. Chris wil ik trouwens ook nog hartelijk danken

voor de stoelmassages. Die kon ik goed gebruiken! Ook de hulp van de fertiliteits-verpleegkundigen was nodig bij het FOLFO-onderzoek. Jullie moesten bij alle deelnemers bloed afnemen en tijdens de punctie extra zorg dragen voor het 'linker' en 'rechter' potje. Daarnaast heb ik veel met jullie te maken gehad door mijn baan als fertiliteitsarts. Ik wil jullie hartelijk danken voor de prettige sfeer en alle hulp.

Op het IVF-laboratorium, onder leiding van Elena Martini en Wouter van Inzen, moest er ook heel wat gebeuren om FOLFO-samples en data te verzamelen. Bedankt IVF-analisten, jullie hebben vele, vele buisjes en ebjes gevuld met follikelvloeistof en zijn er heel veel 'FOLFO-oocyten' apart vervolgd.

Door dit onderzoek kwam ik ook uitgebreid in contact met de afdeling andrologie. Allereerst wil ik Marij Smit bedanken voor haar inzet voor de FOLFO-mannen. Zonder jou hulp waren de twee mannen-artikelen niet mogelijk geweest. Hopelijk duurt het niet lang meer voordat ook jou boekje af is! Niels van Casteren, jij nam het stokje van Marij over en zag daarom ook vele FOLFO-mannen op je spreekuren. Dankjewel! Op het andrologie-laboratorium was het al erg druk toen er ook nog eens een heleboel semen analyses bij in het kader van het FOLFO onderzoek bij kwamen. Beste analisten, bedankt voor jullie extra inspanningen om alle samples te verwerken. Dr. Hans Romijn, het was fijn om feedback van je te krijgen op de andrologie stukken. Ook wil ik dr. Weber bedanken voor zijn enthousiasme voor het onderzoek. Helaas heeft hij het verschijnen van dit proefschrift niet meer mee mogen maken.

Op het Klinisch Chemisch laboratorium zijn onder leiding van professor Lindemans vele samples geanalyseerd. Vooral de seminaal plasma samples kostten zeer veel tijd. Beste Monique en Martin, dankzij jullie verliep de logistiek perfect. Ik wil Montsy Brouns bedanken voor het uitvoeren van de pilot FOLFO-studie. Ook de analisten van het Endocrinologie laboratorium onder leiding van professor de Jong wil ik danken voor de analyses. Professor de Jong wil ik daarnaast bedanken voor zijn beoordeling van het manuscript en zijn nuttige commentaar.

Statistiek is best leuk, maar ook reuze ingewikkeld. Rene Eijkemans, je hebt het talent om ingewikkelde analyses rustig en duidelijk uit te leggen. Ik heb veel van je geleerd en daardoor veel meer zelf kunnen analyseren dan ik van tevoren had verwacht. Door de hulp van Mark Wildhagen werden mijn Access database en tabellen met aangeleverde uitslagen samengevoegd tot een professioneel geheel. Mark, met jou datadumps werden de analyses een stuk overzichtelijker.

In de loop van de tijd heb ik vele collega-promovendi gehad. Het was fijn om te merken dat ik niet de enige was die z'n stappen in onderzoeksland zette. Het was ook leerzaam om te zien hoe onderzoekstrajecten kunnen verschillen. Onderzoek met PGS vind ik fascinerend en ik vond het geweldig dat mijn onderzoek voor een deel mocht aansluiten op de al lopende PGS-studie. Beste Esther Baart, hopelijk kunnen we in de toekomst nog eens samenwerken. Fatima, wat fijn dat jij het FOLFO-stokje van me over wilde nemen. Het is niet gemakkelijk om een onderzoek waarvan je niet alle ins en outs kent voort te zetten. Heel veel succes! Ook alle anderen die nog bezig zijn met hun onderzoek wil ik heel veel succes wensen.

Er zijn verschillende studenten geweest die in het kader van een stage hebben bijgedragen aan de FOLFO-studie. Eline, Marloes, Willeke, Leonie en Elaine, ik wil jullie bedanken en succes wensen met jullie verdere carrière. Jeanne de Vries en Saskia Meijboom wil ik bedanken voor de begeleiding vanuit Wageningen. Helaas zijn er geen resultaten van de voedingsvragenlijsten in dit proefschrift terecht gekomen.

In eerste instantie hulp op afstand, maar na de verhuizing naar de staftoren werden we kamergenoten. Lizka, dankjewel voor je hulp bij het vullen van de vele studie-enveloppen en natuurlijk ook voor je gezelschap op Hs 423.

Nog voor dit proefschrift was afgerond ben ik in het Sint Franciscus Gasthuis begonnen met de opleiding tot gynaecoloog. Ik wil alle gynaecologen, collega-assistenten, verpleegkundigen en andere medewerkers bedanken voor de prettige werksfeer waarin ik terecht ben gekomen.

Beste Nicole, van jou heb ik het fertiliteits-artsen-vak geleerd. Daarnaast ben jij altijd de spin in het web van het wetenschappelijk onderzoek bij de fertiliteits-afdeling geweest. Jij weet precies hoe je onderzoek in 'Jip-en-Janneke-taal' kan overbrengen aan potentiële deelnemers. Ik vond het erg gezellig dat we uiteindelijk op dezelfde kamer kwamen. Geweldig dat je als paranimf naast me wil staan. Beste Heleen, samen studeren, samen op reis, samen leuke dingen doen…Omdat we beide hetzelfde pad volgen, is het er de laatste jaren helaas wat minder van gekomen, want promoveren en medisch specialist worden kost veel tijd. Zullen we nog eens een reisje boeken? Fijn dat jij op 14 januari naast me wil staan als paranimf.

Lieve familie en vrienden. Jullie hebben mij de afgelopen jaren minder vaak gezien, dan de bedoeling was. Nu kan ik jullie eindelijk laten zien waar ik al die tijd zo druk mee bezig ben geweest. Bedankt voor jullie gezelligheid en steun op momenten dat ik het nodig had.

Robert, ik hou van jou! Jij was er altijd met een luisterend oor en goede raad en met jouw technische inzicht werd elk figuur net een beetje mooier. Zonder jou liefde en steun was dit boekje er niet gekomen. Ik hoop dat we nog heeeeel lang gelukkig blijven met elkaar.

Curriculum Vitae

Jolanda Boxmeer was born on the 6th of June 1976 in Delft, The Netherlands. After graduating from secondary school at College 't Loo in Voorburg in 1994, she studied 'Sociaal Kunstzinnige Therapie' at the Leidse Hogeschool in Leiden for one year and passed the first year's exams. In 1995 she attended Medical School at Leiden University Medical Center (LUMC) from which she graduated Cum Laude in June 2002. Meanwhile, in the period of 1997 to 1999, she passed the first year's exams of the study Psychology at the Faculty of Social and Behavioural Sciences of Leiden University. Furthermore, she worked in a hospital in Bulawayo, Zimbabwe, for three months in 1999. From June 2002 until March 2004, she worked as a junior resident at the Department of Obstetrics and Gynaecology of the Erasmus MC, University Medical Centre in Rotterdam. Subsequently, she worked as a junior researcher at the Department of Obstetrics and Gynaecology, division of Reproduction of Erasmus MC, University Medical Centre in Rotterdam where she performed the studies that are described in this thesis. During this period she combined her research with a part-time function as fertility doctor at the same department. In November 2007, she started her training in Obstetrics and Gynaecology at the Sint Franciscus Gasthuis in Rotterdam. She is married to Robert Bakker and they live in Delft.