Preconception Care:

The influence of Nutrition and Lifestyle on Fertility

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List of Abbreviations

AMH	Anti Mullerian Hormoone
ART	Artificial reproduction technique
BMI	Body mass index
BMR	Basal metabolic rate
CI	Confidence Interval
DFI	DNA fragmentation Index
DHA	docosahexaenoic acid
DRI	dietary reference intake
EPA	eicosapentaenoic acid
EDTA	plasma
FFQ	food frequency questionnaire
GnRH	gonadotropin releasing hormone
hCG	human chorionic gonadotropin
HPA	hypothalamic–pituitary–adrenal
ICSI	intracytoplasmic sperm injection
IVF	in vitro fertilization
kg	kilogram
LA	linoleic acid
LC	long chain
MUFA	mono-unsaturated fatty acid
PUFA	poly-unsaturated fatty acid
p	probaility
PAL	Physical Activity Level
r	correlation coefficient
RBC	red blood cell
rFSH	recombinant follicle stimulating hormone
SAH	s-adenosylhomocysteine
SAH	s-adenosylmethionine
SMS	short message service
SES	socioeconomic status
SD	standard deviation
SE	standard error
SHBG	sex hormone binding globulin
SPSS	Statistical Package Social Sciences
tHcy	total homocysteine
β	regression coefficient

Chapter 1

Introduction

"Without hardship, there is no treasure; he who worked is paid in full measure"

Rationale

In Western societies there has been a change in reproductive behaviour with a tendency towards more couples postponing childbearing.[1] As a consequence the number of couples experiencing impaired fertility in the Netherlands is rising. Female age in this respect is the commonly studied individual risk factor for subfertility.[2-3] Subfertility is clinically defined as 12 months of unprotected intercourse during the fertile period of the menstrual cycle without a resulting pregnancy.[4] Several modifiable factors exist that also affect fertility and the chance of having a healthy child.[5-6] Many studies underline the detrimental role of adverse lifestyles and dietary intake of both women and men on reproductive outcome.[6] During the periconceptional period – defined as the time span before and surrounding conception – these factors can detrimentally influence reproductive processes such as gametogenesis, fertilisation and implantation, through the induction of oxidative stress, chromosomal defects, interact with polymorphisms in detoxification enzymes and/or possible interfere with epigenetic mechanisms.[7-10]

Worldwide, there is a high prevalence of unhealthy lifestyle factors in women and men during their reproductive period. In the Netherlands 25% of the women and 35% of the men smoke, 80-90% of women and men use social alcohol , defined as the use of <14 units/week, and 40% of the women and 52% of the men have overweight or are obese.[11]

Therefore, research into potential modifiable risk factors may ultimately contribute to preventive and curative treatments. In the following paragraph the implication of adverse lifestyles on reproductive performance are outlined.

Smoking

Smoking has been recognized as one of the strongest risk factor for fertility and pregnancy outcome.[12] Women who smoke have a higher risk of being subfertile, experience a miscarriage and lower odds of pregnancy and life birth compared with non-smokers.[12]

Specifically, it takes smokers a longer time to conceive than non-smokers. It has been shown that an OR of 1.54 (95%CI 1.19-2.01) was found for delayed conception(> 12 months) in women who smoke compared to non-smokers and an OR of 1.14 (95CI 0.92-1.42) for passive smoking.[13] Fertility was most reduced in smokers who were exposed to cigarette smoke in utero.[14] In women undergoing in vitro fertilization (IVF) twice as many IVF cycles were needed to achieve pregnancy.[15-16]

Epidemiological data also point to a detrimental effect of smoking on semen parameters. Smoking in men is associated with higher sperm DNA damage, lower sperm count, motility and morphology and abnormal sperm fertilising capacity.[17-18] Male smoking is demonstrated to significantly decrease intracytoplasmic sperm injection (ICSI) and IVF success rates.[19]

Alcohol

Excessive alcohol consumption has been reported to decrease the fertility of both women and men. The amount of alcohol consumption associated with reproductive risk is not clear.[20-21] Less is known about the effects of social alcohol use. A 40% and 70% reduced fecundity has been reported in women with any alcohol intake and intakes above ten drinks per week, respectively.[22] Social alcohol use revealed an approximately 60% reduced fecundity.[23] Also, alcohol use during pregnancy is associated with an increased risk of poor pregnancy outcomes.[24-26]

In men, alcohol consumption can induce testicular atrophy, impotence, reduced libido and cause deterioration in sperm count.[27] It remains unclear from the available evidence what amount of alcohol consumption affects reproductive performance and pregnancy outcome.

Body Mass Index

The increasing use of an unhealthy diet (e.g. excessive energy intake) and lack of physical activity (e.g. low energy expenditure) disturb the energy balance, leading to increased fat storage. This trend has resulted in the worldwide epidemic of overweight (BMI ≤25–30 kg/m^2) and obesity (BMI≥30 kg/m^2).[28] Women with overweight or obesity have greater risks across the reproductive spectrum, including higher rates of subfertility as well as pregnancy complications. [29-30]

Increase in BMI reduces the chance of conception both in natural and assisted conception cycles.[31] It has been shown that pregnancy rate was 42% among women with BMI 20-24 kg/m^2, 30% for women with BMI 25-27 kg/m^2 and 21% for women with a BMI 28-36 kg/m^2.[32] Thus, pregnancy rates progressively decrease with increasing BMI. Additionally, obese women undergoing fertility treatment require higher doses of gonadotrophins**,** have significantly fewer and less quality oocytes retrieved, experience lower pregnancy rates and have an increased likelihood of miscarriage.[33-34] Weight loss of as little as 5%–10% can improve fertility outcomes, as a consequence of the improvement of endocrine parameters with return of normal menstrual cycles.[35-36]

Less is known about the effect of overweight and obesity on male fertility.[33] Several studies point to an increased chance of abnormal semen parameters and a higher incidence of male factor infertility.[37]

Nutrition

A nutritionally unbalanced diet characterized by low intakes of vitamins and minerals and excessive intake of sugars and fats has been associated with significantly high reproductive risks.[38-39] In this respect, folate is an important B vitamin, present in its natural form in green leafy vegetables, fruits and whole grains. In times of higher requirements, such as during the periconceptional period, women are recommended to take a folic acid supplement. Adequate folate levels at the time of pregnancy have been shown to decrease both the prevalence and incidence of neural tube defects (NTD) by 50% to 70%.[40] Folic acid supplementation may also decrease the risk of other congenital anomalies such as orofacial clefts and congenital cardiovascular defects.[41-42] Folate is also important for fertility: follicle-, oocyte quality and maturation, implantation.[43] It has been hypothesized that a low folate status reduces ovarian response and successful pregnancy in IVF patients.[44-45] Strong adherence to a dietary patterns supplying a high amount of folate, such as the Mediterranean diet (high in vegetables, fruit, whole grains and fish), is associated with a 40% increased chance of conception after fertility treatment and a 70% reduction of neural tube defects comparable to the use of folic acid supplements.[46]

Further studies suggest that folate is also important for male fertility and spermatogenesis. A randomized controlled trial showed that the use of folic acid and zinc sulphate supplements increased sperm count in subfertile men by 74%.[47] Strong adherence to the Traditional Dutch dietary pattern (high in whole grains, meat and potatoes), with high folate bioavailability, improved semen quality.[48]

Preconception Care

A new promising preventive approach – called Preconception Care – has been introduced to change unhealthy lifestyles and nutrition during the preconception period of the parents to be in order to increase reproductive performance and outcome. The objective of preconception care is preventing defects and disease in mother and child by detecting and, if possible, eliminating the identified risk factors before conception.[49] There is a need for greater clinician awareness for this concept. However, there is evidence that, in practice, only limited preconception counselling about unhealthy lifestyle and nutrition is provided to couples planning pregnancy.[50-51] Recognized barriers to providing this kind of preconception care include limited knowledge on the effectiveness, lack of standardized guidelines, lack of provider knowledge, lack of patient knowledge or demand for services, lack of provider time, and minimal to no insurance coverage.[52]

With the findings described in this thesis, we aim to provide more insights in the effectiveness of preconception programs by means of lifestyle and nutrition promotion directed both at women and men planning pregnancy.

Aims of the Thesis

Against this background the questions to be addressed in this thesis are:

- Effect of lifestyle and nutrition on fertility parameters in both women and men.
- To evaluate an outpatient clinic for preconception counselling on adverse lifestyle and nutrition in women and men planning pregnancy at a university hospital.

Outline of the thesis

Part 1 of the thesis focuses on lifestyle factors and fatty acid intake in couples undergoing an IVF or ICSI treatment The studies described in Chapter 2,3,4,5 are based on the Food Lifestyle and Fertility Outcome study (FOLFO), a periconceptional prospective cohort study, examining the influence of preconception lifestyle exposures in subfertile couples on fertility parameters and pregnancy outcome. This study was conducted between 2004 and 2007 in the Erasmus MC, University Medical Center Rotterdam.

Part 2 focuses on the effect of preconception counselling on adverse lifestyle and nutrition in women and men planning pregnancy and attending the outpatient clinic of Obstetrics and Gynecology of the Erasmus MC, University Center.

Finally, the main findings, implications for clinical practice and public health and suggestions for future research are discussed in Chapter 8.

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Chapter 2

Increased preconception omega-3 polyunsaturated fatty acid intake improves embryo morphology

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ABSTRACT

This study investigates associations between preconception dietary intake of omega-6 and omega-3 poly-unsaturated fatty acids (LC-PUFAs) on estradiol levels and IVF/ICSI outcome.

An observational prospective study was set up in a tertiary referral fertility clinic at the Erasmus University Medical Center, Rotterdam, the Netherlands. Two-hundred-thirty-five women undergoing IVF/ICSI treatment were included. Main outcome measures consisted of estradiol in blood, number of follicles and embryo morphology.

Estradiol on cycle day 2 was positively associated with a high intake of total omega-3 LC-PUFA (β 68.5, se 34.8, p≤0.05), in particular ALA (β 90.4, se 35.7, p ≤0.01). A lower estradiol response on the hCG day was observed in the groups with the highest EPA (β -1062, se 492, p≤0.03) and DHA (β -1006, se 485, p≤0.04) intakes. The number of follicles was inversely associated with high intakes of EPA (β -1.75, se 0.87, p≤0.05) and DHA (β -1.78, se 0.85, p≤0.04). Positive associations were established between embryo morphology and total omega-3 (linear β 0.63, se 0.26, p≤0.02), ALA (β 0.56, se 0.26, p≤0.03) and DHA (β 0.17, se 0.09, p≤0.05) LC-PUFAs intakes. Estradiol and fertility outcome parameters were not associated with omega-6 LA intake.

Omega-3 LC-PUFA intake in women undergoing IVF/ICSI treatment is associated with improved embryo morphology.

Introduction

Dietary intake of long chain poly-unsaturated fatty acids (LC-PUFAs) are beneficial in the prevention of cardiovascular disorders.[1,2] The role of LC-PUFAs in human fertility has received little attention thus far.[3] Several animal studies, however, reported that dietary fats influence oocyte maturation, corpus luteum function and embryo development.[4-6]

LC-PUFAs are essential of cell membranes and after activation by hormones and growth factors they become precursors of eicosanoids, such as prostaglandins, leukotrienes and tromboxanes, which are important mediators in inflammatory, trombogenic and vascular mechanisms.[3,4,7] Based on their chemical structure we distinguish omega-3 and omega-6 LC-PUFAs. Omega-3 LC-PUFAs comprise alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). ALA – present in green vegetables – can be converted in EPA and DHA.[7,8] However, this conversion is insufficient to meet daily EPA and DHA needs.

Therefore, the intake of fish as rich dietary source of these omega-3 LC-PUFAs is recommended. Its consumption, however, is rather low in Western countries and results in an increased ratio of omega-6 to omega-3 LC-PUFAs (10:1).[4] The most important omega-6 PUFA is linoleic acid (LA) serving as precursor of arachidonic acid (AA) and present in nearly all vegetable oils, while substantial amounts of AA are present in meat and eggs.[8] The effects of various amounts of individual omega-3 and omega-6 LC-PUFAs on human reproduction, however, is limited.[9] Therefore, the aim of this study was to investigate associations between the periconception maternal dietary intake of omega-3 and omega-6 LC-PUFAs on estradiol levels and reproductive outcome parameters in a periconception prospective observational study of women undergoing in vitro fertilization (IVF) or intracytoplasmatic sperm injection treatment (ICSI).

Materials and Methods

Study Population

The Food Lifestyle and Fertility Outcome Study (FOLFO Study) is a prospective preconception observational study which focuses on the influence of nutrition and lifestyle on fertility and pregnancy outcome. The design of the study has been described previously.[10] In summary, between September 2004 and January 2007 subfertile couples undergoing IVF/ICSI treatment at the Erasmus University Medical Center, Rotterdam, the Netherlands were invited to participate. Of the eligible IVF/ICSI population, 66% of the couples participated in the FOLFO study (n=251). We excluded couples who suffered from known conditions that may influence IVF/ICSI treatment outcome, such as oocyte donation, endometriosis and hydrosalpinx resulting in 235 women for this study.

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

General Questionnaire

All participants filled out a general questionnaire from which the following data were extracted: height, weight, ethnicity, education level, vitamin use, and other lifestyle factors. Ethnicity and education level were classified according to the definitions of Statistics Netherlands.[11] Education level was divided into three categories: low (primary, lower vocational, or intermediate secondary), intermediate (intermediate vocational or higher

secondary) and high (higher vocational, or university). Ethnicity was divided into Dutch Native, European other and Non-European.[11]

Food Frequency Questionnaire

All participants filled out a food frequency questionnaire (FFQ) to estimate habitual food intake over the previous four weeks. This FFQ was originally developed at the division of Human Nutrition, Wageningen University, the Netherlands and validated for intake of energy, B-vitamins and fats.[12,13] The FFQ was provided to the subfertile woman on the day of oocyte retrieval and was returned on the day of embryo transfer. The researcher verified the completeness of the FFQ. In case of missing or unclear information about type and amount of foods consumed, additional questions were asked by telephone. The intake of energy and fatty acids were compared with the Dietary Reference Intake (DRI) for the Netherlands.[14] To evaluate the existence of underreporting the ratio of total energy intake to basal metabolic rate (BMR) was calculated using the new Oxford equation for women aged 30-60 years: BMR (mJoule/day) = 0.0407 x weight (in kilogram) + 2.90.[15] This value is an estimation of the physical activity level (PAL) of a sedentary lifestyle. The physical activity level was then calculated by dividing the mean reported energy intake by the mean BMR. According to Goldberg et al. (1991) a cut-off point for underreporting for a sedentary lifestyle is a ratio of ≤1.35.[16]

In Vitro Fertilization Procedure

In our study population, three IVF stimulation treatments were used. In one group women started ovarian stimulation with daily injections of 150 IU recombinant Follicle Stimulating

Hormone (rFSH) subcutaneous on cycle day 2 (Puregon, Schering Plough, Houten, the Netherlands or Gonal-F, Merck Serono Benelux BV, Schiphol-Rijk, the Netherlands).

Administration of daily subcutaneous Gonadotropin Releasing Hormone (GnRH) antagonist (Orgalutran, NV Schering Plough, or Cetrotide, Merck Serono Benelux BV) was started when at least one follicle was ≥14 mm. In another treatment group women were randomized for either conventional ovarian stimulation or mild ovarian stimulation. Patients assigned to the conventional ovarian stimulation started the GnRH agonist 0.1 mg/day subcutaneous on cycle day 21 of the menstrual cycle. After two weeks of the GnRH regimen, co-treatment with rFSH 225 IU/day subcutaneous was started. Patients assigned to the mild ovarian stimulation were treated with a fixed dose of 150 IU/day rFSH subcutaneous started on cycle day 5. As soon as the leading follicle reached a diameter of 14 mm the GnRH-antagonist of 0.25 mg/day subcutaneous was added to the regimen. To induce final oocyte maturation a single dose of 10.000 IE human chorionic gonadotropin (hCG) subcutaneous was administered in all three regimens as soon as the leading follicle reached a diameter of at least 18 mm and at least one additional follicle reached a diameter of 15 mm.

Results

Data from 235 subfertile women were evaluated and the general characteristics are shown in Table 1. Women had a mean age of 35.0 years (sd 4.2) and a BMI of 23.7 kg/m^2 (sd 3.7). Furthermore, the majority was of Dutch origin (70.4%), highly educated (44.0%), consumed alcoholic drinks on a frequent basis (91.5%), and used folic acid and/or a folic acid containing multivitamin supplement (88.5%). Only 8.9% of the women smoked. In Table 2 total energy, macronutrient and LC-PUFA intakes are depicted and compared with the DRI for women between 19-40 years of age as reference.[19] The average total energy was slightly lower, fat, protein and carbohydrate intake were higher than the recommendations. The omega-3 LC-PUFA intake – in particular of EPA and DHA – was lower whereas the omega-6 LA LC-PUFA intake was higher than the recommendations for women in the age group of 19-40 years and pregnant women. The omega-6/omega-3 ratio was 12.1/1.14; this is higher than recommended. To evaluate whether the relatively low omega-3 intake was due to the general underreporting of food intake, we calculated the PAL measure.

This revealed that the PAL was 1.44, which is above the cut-off level of 1.35; therefore underreporting is not very likely. Table 3 shows associations, linear and after dichotomization in high (>p85) and low (<p15) LC-PUFA intakes, in relation to baseline and response estradiol levels and reproductive outcome parameters. Baseline estradiol on cycle day 2 was positively associated with high intakes of omega-3 LC-PUFA ALA (β 89.3(36.7) p≤0.05). Estradiol response was negatively associated with high intakes of EPA (β=-1100.2(498.4), p≤0.05) and DHA (β=-1065(492.6), p≤0.05) also substantiated with a linear association of DHA (β=-401.4(201.1), p≤0.05). Number of follicles was inversely associated with high intakes of total omega3 (β=-1.79(0.58), p≤0.01), EPA (β=-1.49(0.49), p≤0.01) and DHA (β=-1.60(0.49), p≤0.01). There were significant positive associations between embryo

morphology and total omega3 intake (β=0.16(0.08), p≤0.05), ALA (β=0.56(0.26), p≤0.05) and DHA (β=0.18(0.09), p≤0.05). Estradiol and fertility outcome parameters were not associated with omega6 intake. However, high omega6:omega3 ratio was positively associated with the number of follicles.

Discussion

To our knowledge this is the first study to evaluate omega-3 and omega-6 LC-PUFA intakes in association with estradiol status and response, number of follicles, and embryo morphology in women undergoing IVF/ICSI treatment. We demonstrate that in these women the dietary intake of omega-3 LC-PUFAs – in particular of EPA and DHA – is much lower than the dietary recommended intakes, in contrast to the adequate intakes of omega-6 LC-PUFA. Women with the highest intake of the omega-3 LC-PUFA ALA showed a higher baseline estradiol level, and in particular the high intakes of EPA and DHA reduced the estradiol response and number of follicles after ovarian stimulation treatment. This is in line with the improved embryo morphology by high intakes of omega-3 LC-PUFA, in particular ALA and DHA.

The high intake of the omega-3 LC-PUFA ALA was also associated with a higher baseline estradiol level. The importance of the baseline estradiol level for reproductive outcomes is controversial.[20] Omega-3 LC-PUFAs are an essential source for the synthesis of cholesterol, which acts as precursor of estradiol and other steroids. This is a possible pathway in which a high ALA intake increases follicular steroid synthesis.[4] Other studies support this finding by showing that trans fatty acids, mono-unsaturated fat and poly-unsaturated fat intake influence the levels of estradiol as well.[21,22] Two studies investigated potential associations between maternal omega-3 and omega-6 LC-PUFAs on estradiol levels during pregnancy, however, without significant results.[23,24] These negative results are suggested to be due to the type of fatty acid intake.

In this study, we also showed that a high intake of EPA and/or DHA reduced the estradiol response and number of follicles after ovarian stimulation treatment. In an animal study it has been shown that consumption of high levels of omega-3 LC-PUFAs resulted in elevated ova release, whereas consumption of moderate levels had no effect on ova release in rats.[25]

However, in this study fish oil was used, which included different omega-3 LC-PUFAs, therefore the enhancing effect couldn't be attributable to a specific omega-3 LC-PUFAs or their combination, as well as the dietary level. Our findings suggest a beneficial effect of omega-3 LC-PUFA intake on fertility outcomes, since a more physiological approach to ovarian stimulation, resulting in fewer dominant follicles, may allow only the healthiest follicles and oocytes to develop in competent embryos.[26] In addition, the existence of an estradiol window with an upper threshold at the time of hCG administration has been suggested.[20,27] An elevation above this threshold could be deleterious for embryonic implantation. It has been shown that uterine receptivity is affected in patients undergoing ovulation induction with high serum estradiol concentrations on the day of hCG administration, regardless of the number of oocytes retrieved and the progesterone concentration. It would be interesting to study the associations with number of implantations and pregnancies as well. However, due to the limited power this was not the aim of the current study.

Moreover, our finding is consistent with several studies in rats fed a diet high in EPA and DHA showing a decrease in the frequency of ovulations.[28-31] EPA and DHA have been reported to elicit a reduction of ovarian synthesis of prostaglandin $F_{2\alpha,}$ which is involved in follicle growth and ovulation and therefore may partly explain the inverse effect on number of follicles.[32-34] The mechanism, by which EPA and DHA inhibit secretion of prostaglandin $F_{2\alpha}$ is however not fully understood. Furthermore, the reduction of follicle numbers results in less estradiol synthesizing granulosa cells leading to a reduction of the estradiol level. In addition, omega-3 LC-PUFAs may affect the responses of the granulosa cells to gonadotropins due to interactions with transcription factors involved in the steroidogenic pathway.[35] The specific mechanism by which EPA and DHA affect the estradiol response and

number of follicles could not be determined in this study and more research needs to be carried out to fully understand underlying mechanisms.

In a previous study only omega-6 LC-PUFA intake seemed to increase the number of follicles.[4] This could not be confirmed by our findings and might be due to the fact that the omega-6 LC-PUFA intake in our study was within the range of the recommended daily intake.[36] In contrast, intakes of EPA and DHA were much lower than the dietary recommended intakes, which may be the reason of the demonstrated associations between EPA, DHA intake and fertility outcome parameters. These data, therefore, very much support the recommendation to increase fish and fish oil intakes to cover the needs of these omega-3 LC-PUFAs during the reproductive period.

In this study we also showed positive associations between the intake of omega-3 LC-PUFAs, specifically ALA and DHA, and embryo morphology. Other research groups showed beneficial effects of omega-3 LC-PUFA supplements on embryo morphology.[37,38] Although, Wakefield et al.[39] suggested that high dietary intakes of omega-3 LC-PUFA reduces normal embryo development by perturbation of mitochondrial metabolism. Their study population, however, was too small to show these effects.

The major strength of our study is its prospective design. Therefore, it is unlikely that recall bias has confounded the data on the dietary intakes of LC-PUFA, covariates and confounders. A limitation of our study is the lack of information on the omega-6 LC-PUFA arachidonic acid. This LC-PUFA could not be determined from the FFQ and therefore omega-6 intake was based on LA intake only. However, among Belgian women of reproductive age the arachidonic acid intake was only 0.6% of the total omega-6 LC-PUFA intake. Therefore, the effects of the slight underestimation of the total omega-6 LC-PUFA are considered to be minimal.[40] Furthermore, we assessed the LC-PUFA intakes with a FFQ and had no

opportunity to measure the biomarkers in blood or follicular fluid. The dietary intake of omega-3 LC-PUFA, however, results in increased concentrations in plasma and tissues and is therefore associated with the availability in tissues.[41-43] Additionally, we adjusted the nutrient intakes for total energy intake and included BMI and total energy intake as confounders, further eliminating extraneous variation which may cause spurious associations not attributable to the effect of short-term PUFA intake on fertility outcome parameters.

In conclusion, for the first time significant associations were observed between intakes of omega-3 LC-PUFAs and baseline and response estradiol serum levels after ovarian stimulation treatment, and on embryo morphology. We suggest that dietary LC-PUFAs significantly contribute to the estradiol levels and as such to the number of follicles and embryo morphology. Because the intake of omega-3 LC-PUFAs was relatively low, and the omega-6 LC-PUFA intake was according the recommendations, the dietary intake of especially fish and fish oils should be encouraged in women during their reproductive years and in particular in those undergoing IVF/ ICSI treatment.

Table 1

General characteristics of the study population

Characteristic	Participants (n 235)
age[1,2]	35.0 ± 4.2
BMI[1,3]	23.7 ± 3.7
Dutch ethnicity[4]	164 (70.1)
high education[4]	103 (44.0)
smoking[4]	21 (8.9)
medication[4]	36 (15.3)
alcohol[4]	215 (91.5)
folic acid use[4]	207 (88.5)
cause of subfertility[4]	
female	51(21.7)
male	86 (36.6)
male and female	15 (6.4)
idiopathic	83 (35.3)
fertilization by IVF[4]	146 (62.1)
stimulation scheme[4]	
P02-150	174 (76.7)
P05-150	32 (14.1)
DLP-225	21 (9.1)
estradiol[5,6,7]	138.5 (41.0 - 2051.0)
estradiol[5,6,8]	2484 (233 - 20018)

Data are presented as [1]means ± sd, [2]years, [3]kg/m^2, [4]n (%), [5]pmol/L, [6]median (min-max), [7]at baseline, [8]after stimulation.

Table 2

Nutrient Intakes

Nutrient	Unit	Participants	DRI[4]
Energy intake	*kJoule/day*	7861 (1999 - 29814)	8100
Total fat	*g/day*	70.0 (13.4 - 28.7)	50
	adjusted	81.2 (30.5 - 124.7)	
Total protein	*g/day*	70.8 (24.2 - 175.3)	50-52
	adjusted	74.7 (43.7 - 100.7)	
Total carbohydrates	*g/day*	223 (59 - 980)	270
	adjusted	247 (136 - 386)	
Omega 6 PUFA[1]			
LA	*g/day*	12.1 (1.5 - 49.8)	12.0
	adjusted	13.6 (5.6 - 33.5)	
Omega 3 PUFA[1]			
ALA	*g/day*	0.98 (0.18 - 6.61)	1.1
	adjusted	1.06 (0.47 - 5.37)	
EPA	*g/day*	0.04 (0.00 - 0.38)	EPA + DHA > 0.4
	adjusted	0.05 (0.00 - 0.39)	
DHA	*g/day*	0.07 (0.00 - 0.51)	EPA + DHA > 0.4
	adjusted	0.08 (0.00 - 0.52)	
Total omega 3[2]	*g/day*	1.14 (0.28 - 7.42)	
	adjusted	1.26 (0.49 - 1.63)	
omega-6: omega-3 ratio[3]		10.1 (3.5 - 20.7)	

Data are presented as medians (min-max).
[1]PUFA= poly unsaturated fatty acid.
[2]EPA + DHA + ALA. [3]LA / (ALA + EPA + DHA).
[4]Dietary Reference Intakes: energy, The Hague: Health Council of the Netherlands 2001/19R (corrected edition June 2002)

Table 3

Associations between omega LC-PUFA and fertility outcome

Nutrient		Estradiol cycle day 2[1]	Estradiol hCG Day[2]	no. of follicles[1]	Embryo morphology[3]
LA		*(n 188)*	*(n 178)*	*(n 194)*	*(n 175)*
linear	*β (se)*	–49.1 (40.1)	–662.9 (667)	–0.50 (0.67)	0.50 (0.29)
	p	0.22	0.32	0.45	0.08
>p85 (17.9 g)	*β (se)*	3.5 (41.7)	–415.2 (679.8)	–0.33 (0.66)	0.54 (0.32)
	p	0.93	0.54	0.61	0.10
<p15 (10.6 g)	*β (se)*	–10.7 (33.9)	472.9 (574.8)	–0.12 (0.57)	–0.22 (0.24)
	p	0.75	0.41	0.84	0.36
ALA					
linear	*β (se)*	–5.0 (35.5)	–672.2 (582.5)	–0.19 (0.45)	0.56 (0.26)
	p	0.89	0.25	0.67	0.03
>p85 (1.5 g)	*β (se)*	89.3 (36.7)	–709.4 (622.9)	–0.23 (0.65)	0.09 (0.27)
	p	0.02	0.26	0.73	0.75
<p15 (0.8 g)	*β (se)*	–14.9 (38.5)	-21.5 (639)	0.05 (0.66)	–0.42 (0.26)
	p	0.70	0.97	0.94	0.11
EPA					
linear	*β (se)*	8.1 (11.3)	–301.9 (182.6)	–6.29 (2.93)	0.09 (0.08)
	p	0.48	0.10	0.03	0.26
>p85 (0.1 g)	*β (se)*	–23.4 (30.7)	–1100.2 (498.4)	–1.49 (0.49)	0.11 (0.22)
	p	0.45	0.03	0.00	0.62
<p15 (0.01 g)	*β (se)*	–27.2 (39.5)	746.7 (637.2)	–0.23 (0.67)	–0.19 (0.27)
	p	0.49	0.24	0.73	0.50

Adjusted for [1]ethnicity, age, BMI, smoking, alcohol, total energy intake, stimulation scheme, folic acid , IVF/ICSI treatment; [2]estradiol on cycle day 2, ethnicity, age, BMI, smoking, alcohol, total energy intake, stimulation scheme, folic acid, IVF/ICSI treatment; [3]age, BMI, ethnicity, total energy intake, IVF/ICSI, stimulation scheme.

Table 3 continued

Associations between omega LC-PUFA and fertility outcome

Nutrient		*Estradiol cycle day 2*[1]	*Estradiol hCG Day*[2]	*no. of follicles*[1]	*Embryo morphology*[3]
DHA		*(n 188)*	*(n 178)*	*(n 194)*	*(n 175)*
linear	β (se)	8.4 (12.2)	–401.4 (201.1)	–4.02 (2.07)	0.18 (0.09)
	p	0.49	0.05	0.05	0.04
>p85 (0.2 g)	β (se)	–24.7 (30.2)	–1065.0 (492.6)	–1.60 (0.49)	0.15 (0.22)
	p	0.42	0.03	0.00	0.50
<p15 (0.02 g)	β (se)	–29.7 (35.8)	1167.5 (584.7)	0.81 (0.65)	–0.44 (0.26)
	p	0.41	0.05	0.21	0.09
Total omega 3					
linear	β (se)	9.0 (11.0)	–373.2 (182.7)	–2.48 (1.22)	0.16 (0.08)
	p	0.42	0.04	0.04	0.05
>p85 (1.7 g)	β (se)	-7.28 (36.2)	–550.5 (608.3)	–1.79 (0.58)	0.21 (0.26)
	p	0.84	0.37	0.00	0.41
<p15 (1.0 g)	β (se)	–29.8 (36.5)	1183.1 (596.5)	0.46 (0.65)	–0.45 (0.26)
	p	0.42	0.05	0.48	0.09
O6:O3 ratio					
linear	β (se)	–31.3 (43.0)	620.2 (707.6)	0.04 (0.07)	–0.30 (0.31)
	p	0.47	0.38	0.61	0.33
>p85 (16.2)	β (se)	–39.6 (39.4)	590.0 (643.1)	1.67 (0.76)	–0.45 (0.30)
	p	0.32	0.36	0.03	0.13
<p15 (7.3)	β (se)	–30.4 (33.1)	–629.1 (556.2)	0.35 (0.61)	0.19 (0.24)
	p	0.36	0.26	0.56	0.43

Adjusted for [1]ethnicity, age, BMI, smoking, alcohol, total energy intake, stimulation scheme, folic acid, IVF/ICSI; [2]estradiol on cycle day 2, ethnicity, age, BMI, smoking, alcohol, total energy intake, stimulation scheme, folic acid, IVF/ICSI treatment; [3]age, BMI, ethnicity, total energy intake, IVF/ICSI, stimulation scheme.

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Chapter 3

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

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Abstract

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

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

Folic acid use modified the ovarian response to ovarian stimulation treatment. The estradiol response was higher in non-folic acid users receiving conventional treatment ($\beta^{interaction}$=0.52, [0.07-0.97]; p=0.03), this effect was independent of serum AMH levels and preovulatory follicle count. In the conventional treatment the mean follicle number was also greater in non-users compared to the users group (14.1 vs. 8.9, p=0.03**)**

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

Introduction

In women undergoing controlled ovarian hyperstimulation (COH) during assisted reproductive treatment (ART) there is still unpredictable inter- and intraindividual variability in the ovarian response to COH.[1] Ovarian response is defined as 'the endocrine and follicular reaction of the ovaries to a stimulus'.[1] There is an optimum quantitative ovarian response to COH, where both a poor and high ovarian response to COH are associated with unfavorable treatment outcome.[2] Knowledge of which determinants influence ovarian response to gonadotrophins would improve the predictions of the response to COH. This would reduce the number of treatment cycles required to achieve pregnancy, reduce the incidence of treatment complications or offer the possibility to tailor the treatment protocol according to patient characteristics. Accurately predicting ovarian response to gonadotrophins is currently not possible.

Folate deficient women undergoing COH have lower oocyte quality, lower pregnancy rates and impaired ovarian function.[3-7] The underlying mechanism, however, is not known. The natural B-vitamin folate is involved in numerous metabolic pathways including cell cycle regulation, amino acid biosynthesis and protein processing.[8] Folate primarily serves as a methyl-group donor for these reactions. In a recent study in ewes fed a methyl deficient diet, low methyl-group availability increased mRNA expression of genes involved in mediating the ovarian response to gonadotrophins.[9]

Epigenetic mechanisms aim to maintain the gene expression profile of cells after mitotic division. Epigenetic mechanisms are essentially post-replication modifications superimposed on the genome, regulating gene expression without causing changes in DNA sequence. In contrast to the genetic background of the individual, the epigenetic composition of the genome is sensitive to environmental influences, including nutrition, which effectively

modify gene expression.[10-11] DNA methylation is an epigenetic mechanism, dependent on the availability of methyl groups. In experimental animal and retrospective human studies, in utero exposure to low levels of methyl group donors lead to an altered DNA methylation profile and phenotype in offspring.[12-13]

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

Materials and methods

Study design

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

Eligible couples visiting the Erasmus MC, University Medical Center in Rotterdam, The Netherlands, with an indication for IVF or ICSI treatment were invited to participate in the FOLFO II study. Exclusion criteria for the FOLFO II study where: oocyte donation, endometriosis, hydrosalpinx, a priori indication for ICSI treatment, age >37 years old, BMI <18 or >29 kg/m^2, irregular menstrual cycle, previous IVF treatment without embryo transfer, recurrent abortion, abnormal karyotype of man/woman and/or uterus anomalies. These criteria served to select only those patients with unexplained subfertility in order to be able to asses the role of food and lifestyle factors. A higher cancellation rate before oocyte retrieval and fewer embryos were expected following mild ovarian stimulation.[14] Therefore, randomization to one of the two treatment groups was performed according to a computer generated randomization schedule in a ratio of 2:3 (conventional group : mild group), assigned via numbered sealed envelopes. After the patient agreed to participate, the treating physician opened the next available numbered envelope on entry into the study during the preparatory IVF consultation. Of all eligible couples (n=161), 54 participated in the FOLFO II study and 49 participated in other clinical studies (Figure 1). Fifty-eight couples did

not participate in any of the studies because the extra effort for participation relative to normal treatment was considered too great or they did not see a clear benefit in participating. At the end of the inclusion period, 24 couples were randomized to the conventional protocol and 30 couples to the mild protocol. After randomization 4 women with exclusion criteria appeared to be included into the study, they were therefore excluded from the final analysis.

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

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

IVF procedure

After randomization, patients assigned to conventional ovarian stimulation treatment were treated with the GnRH agonist Triptorelin (Decapeptyl®, Ferring BV, Hoofddorp, The Netherlands) at 0.1 mg/day s.c., starting on CD21 of the menstrual cycle preceding the actual stimulation cycle. After two weeks of the GnRH-agonist regimen, co-treatment with rFSH 225 IU/day s.c. (Puregon®, Schering-Plough, Houten, The Netherlands) was initiated. Patients assigned to mild ovarian stimulation treatment were treated with a fixed dose of 150 IU/day rFSH s.c. (Puregon®, Schering-Plough, Oss, The Netherlands) from CD 5 onwards. As soon as

the leading follicle reached a diameter of 14mm, a GnRH-antagonist (Orgalutran®, Schering-Plough, Houten, The Netherlands) was administered at 0.25 mg/day s.c.. To induce final oocyte maturation a single s.c. dose of 10,000 IE hCG (Pregnyl®, Schering-Plough, Houten, The Netherlands) was administered in both regimens as soon as the leading follicle reached a diameter of at least 18 mm and at least one additional follicle reached a diameter of 15 mm or more. Oocytes were retrieved 35 hours after hCG injection by transvaginal ultrasound-guided aspiration of follicles.

Sample collection and analysis

Isolated oocytes were washed and transferred to a separate droplet of medium in order to monitor their quality. The monofollicular fluid samples were centrifuged for 10 min at 1,700 x 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 CD2, i.e. the early follicular phase of the menstrual cycle preceeding the treatment cycle and on the day of hCG administration. For the determination of folate, cobalamin, pyridoxine and hormones, venous blood samples were drawn into dry vacutainer tubes and allowed to clot. After centrifugation at 2,000 x g, serum was collected for assay. Anti-Müllerian hormone (AMH) levels were measured using an enzyme-linked immunosorbent assay (ELISA) (Immunotech-Coulter, Marseille, France). Folate and cobalamin were analysed using an immunoelectrochemoluminescence assay (Roche Modular E170, Roche Diagnostics GmbH, Mannheim, Germany). These assays are calibrated to detect the folate form 5-methyl-tetrahydrofolate most effective. Serum concentrations of FSH were measured by luminescence-based immunometric assay (Immulite 2000, Siemens Diagnostics, Los Angeles, CA, USA). Estradiol was determined using

a coated tube radioimmunoassay obtained from the same supplier. For the determination of plasma total homocysteine (tHcy) and pyridoxine in whole blood, venous blood samples were drawn into ethylenediamine tetra-acetate (EDTA) containing vacutainer tubes. The EDTA-blood samples were placed on ice and within 1 hour, plasma was separated by centrifugation. Total homocysteine in EDTA plasma and pyridoxine as pyridoxial'5-phosphate in whole blood was determined using high-performance liquid chromatography with reversed phase separation and fluorescence detection.

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

Statistical analysis

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

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

To allow for adjustment, linear regression methods were used to investigate the relation between baseline biomarkers and endocrine responses after COH. Regression parameters are reported with their 95% confidence intervals. Collinearity was assessed using the VIF statistic, a VIF of ≥4 was considered to indicate collinearity. In the final model the highest observed VIF was 3.4. In figures, line fitting to data points was conducted using the least squares estimate.

Folic acid supplement use was confirmed by serum folate levels. Patients were considered folic acid supplement users when the serum folate level was ≥22.5 umol/L .[15]

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

Results

Study population

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

Biochemical parameters

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

Baseline anti-Müllerian hormone (AMH) levels predict the ovarian response after COH (β^{AMH} = 0.43, [0.14-0.72]; p<0.01). On the day of hCG administration treatment groups showed considerable differences with respect to estradiol concentrations, with the response in the conventional protocol being the highest (4,293 pmol/L vs. 2,706 pmol/L; p=0.03). Also, within each treatment group we observed a decline of tHcy levels relative to basal concentrations, where the median decline was more profound in the conventional stimulation treatment arm than that observed in the mild stimulation treatment arm (-1.20 umol/L vs. -0.7 umol/L; p<0.01 and p=0.01, respectively). This observation was further analysed using a linear regression which showed a differential tHcy decline between the stimulation protocols ($\beta^{protocol}$ = 0.15, [0.06-0.24]; p<0.01). Furthermore, AMH levels declined after stimulation treatment, which is different between the stimulation protocol ($\beta^{protocol}$ = 0.57 [0.34-0.79], p<0.001).

After stratification for folic acid use and stimulation treatment, women in the conventional stimulation protocol, who did not use folic acid supplements, had an increased ovarian

response to COH (users: 3,482 pmol/L (conventional) vs. 2,978 pmol/L (mild); p=0.55 and non-users: 6,190 pmol/L (conventional) vs. 1,996 pmol/L (mild); p<0.001) (Table III.)) To confirm effect modification, this finding was further investigated in a linear regression model, which showed an interaction between baseline serum folate levels and stimulation protocol with regard to the estradiol response after COH ($\beta^{interaction}$ =0.52, [0.07-0.97]; p=0.03) (Figure II). The effect of folate on the ovarian response was independent of preovulatory follicle count and AMH levels. Including baseline concentrations of pyridoxine, cobalamin and homocysteine into the model did not alter the effect of serum folate on the outcome.

Clinical outcome parameters after IVF or ICSI treatment

As presented in Tables III and IV, the mean number of preovulatory follicles (10.6 vs. 7.4; p<0.01) and the median number of retrieved oocytes (12.3 vs. 6.7; p=0.001) differed between treatment groups. The number of follicles was positively correlated with the estradiol response (r=0.78; p<0.001). Furthermore, in the non-users stratum the conventional group had a higher number of preovulatory large antral follicles (14.1 vs. 6.9; p<0.01), an effect which was not observed in the low dose users stratum (8.9 vs. 7.6; p=0.29) (Table III). Fertilization rates where comparable (0.56 vs. 0.49; p=0.36) and the number of transferred embryos did not differ (Table IV.) The number of ongoing pregnancies was not different (29.2% vs. 15.4%; p=0.34).

Discussion

The results of our study suggest that the ovarian response to gonadotrophins is subject to the availability of the methyl donor folate. After conventional stimulation treatment, women who did not use a low dose folic acid supplement had a higher ovarian response to stimulation treatment than those who did use a folic acid containing supplement. The effect of folate on the ovarian response is independent of follicle count and AMH levels. Kanakkaparambil et al. [9] have previously observed the interplay between COH and low methyl group availability in ewes fed a methyl deficient (MD) diet. They reported a higher ovarian response after rFSH administration in MD ewes. Further in vitro analysis of granulosa cells revealed higher FSH receptor (*FSHR*) mRNA expression as homocysteine levels increased, reflecting low methyl group availability.

The ovarian response to mild stimulation treatment seems not affected by folic acid use. Incidentally, ovarian dynamics differ considerably between conventional- and mild ovarian stimulation treatment. After pituitary desensitizing using a GnRH-agonist, as is done in the conventional stimulation treatment arm, the proportion of immature, FSH-responsive follicles has increased in size.[16] In addition to the higher doses of rFSH, this can partially underlie the overall higher ovarian response after conventional stimulation treatment. The mild stimulation protocol does not interfere with the initial follicle recruitment by the natural menstrual cycle, and the lower dose of rFSH only stimulates more mature follicles for which the FSH-threshold is higher.[17] Nevertheless, despite a higher oocyte yield, clinical outcome was comparable after conventional and mild stimulation treatment.[18] This suggests that the absolute number of competent oocytes is not different, but only the proportion of competent oocytes is higher after mild stimulation treatment. It seems that preconception folic acid use attenuates the ovarian response; by affecting only less mature follicles that are

stimulated after GnRH-agonist treatment. Therefore, this finding can help improve oocyte quality after COH.

Clinical, animal and in vitro studies permit speculation on potential mechanisms underlying the observed effect. The intertwined folate-methionine cycle is the main route of utilization for folates. In the folate cycle, folates primarily serve as a substrate for DNA nucleotide synthesis, where deficiencies in folate can result in faulty DNA-repair and nucleotide synthesis.[19-20] In the methionine cycle, folates serve as a substrate for the re-methylation of homocysteine into methionine. Thereafter, methionine-adenosyltransferase metabolizes methionine into S-Adenosyl-Methionine (SAM), which is the substrate for virtually all methylation reactions. Transmethylation of SAM forms S-Adenosyl-Homocysteine (SAH). Deficiencies in folate can result in accumulation of homocysteine and SAH.[21] Although we have not assessed SAM and SAH, the concentrations of these biomarkers of methylation are more direct measures of the methylation potential than serum folate. Furthermore, deficiencies in co-factors for these reactions, like cobalamin and pyridoxine, can also aggravate derailment of the methionine-cycle. In our study, however, cobalamin and pyridoxine levels did not affect the results. Additionally, when deliberating the folate- and methionine cycle as an underlying mechanism, it is necessary to take into account the influence of some polymorphisms in genes coding for enzymes of these cycles. Some attenuate the efficiency of the folate- and methionine cycle, most notably the C677T polymorphism in the 5,10-methylenetetrahydrofolate reductase enzyme, which also associates with the ovarian response.[7,22] Homocysteine is a reactive metabolite. Homocysteine metabolites can be wrongfully incorporated in protein instead of methionine, affecting protein function [23] and thereby possibly cellular function. Also, homocysteine can generate reactive oxygen species (ROS).[24] Although ROS function as second messengers, an

excess of ROS results in oxidative stress. Oxidative stress affects female fertility and ART success.[25] In this study, there was no significant effect of homocysteine on the outcome of interest. Also, folate availability affects the DNA methylation pattern and thereby the gene expression profile of a cell.[21] The reduction in SAM levels and accumulation of homocysteine due to folate deficiency inhibit the activity of DNA-methylases.[26] Investigation of the mouse genome indicates that the *FSHR* gene contains CpG repeats, which when methylated inhibit *FSHR* gene transcription.[27] In addition, Kanakkaparambil et al.[9] showed that incubating granulosa cells with homocysteine increases *FSHR* mRNA levels. Similarly, the aromatase enzyme, which converts androgens into estrogens, expressed in bovine granulosa cells is regulated by DNA methylation.[28] Finally, steroidogenesis by the ovarian follicle is augmented by the Insulin-like Growth Factor (IGF) family [29], Insulin-like Growth Factors mainly elicit their effect through the type 1 IGF receptor [29], which is over expressed in folate deficient cells.[30] In proliferating tissues, shortages of methyl groups can inhibit the full methylation of hemi-methylated DNA strands by DNA methylases, eventually leading to hypomethylation of DNA in progeny cells of originally methylated gene-loci.[10] Such a process of passive demethylation is also seen in the early zygote.[31] In addition, oxidative DNA products inhibit effective (re)methylation of DNA or induce loss of methylation.[32-34]

Folate status is associated with the quality of many parameters in human reproduction, from gamete- and embryo quality to the occurrence of congenital malformations.[3] In the current study, folates also attenuate the ovarian response to ovarian stimulation treatment. Studies in human show that there is an optimal ovarian response with regard to the number of retrieved oocytes [18] and attained estradiol levels after stimulation treatment [35] with respect to clinical outcome after ovarian stimulation treatment. High hormone levels affect oocyte competency [36] and endometrial receptivity to the embryo.[37] Possibly, folates affect ovarian

stimulation treatment outcome by mediating the ovarian response gonadotrophins through interference with FSH receptor, aromatase availability and an increase in ROS.

We also need to address strengths and limitations of our study. Given the small sample size, it will not be justified to draw strong conclusions on the current data. Even so, statistical analyses show no complications of small numbers and our findings are supported by earlier observations by Kanakkaparambil et al. 2009.[9] At the moment of study initiation, the effect estimates where unknown and therefore sample size estimation was not possible. Although, not primarily designed as a folic acid intervention study, folic acid use across the treatment groups was similar. Nevertheless, we cannot exclude selective non-participation. Because of the randomized design, however, confounding factors associated with folic acid supplement use will have an equal distribution over the two treatment groups.

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

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

Table 1
Baseline characteristics of women undergoing ovarian stimulation treatment (n=50)

	Conventional (n=24)		Mild (n=26)	
Age (years) mean (IQR)	32.7	(31.0-35.7)	34.0	(33.0-36.0)
Body Mass Index (kg/m^2) mean (IQR)	21.8	(19.5-23.5)	22.7	(21.0-24.0)
Ethnicity:				
Dutch % (n)	57.1	(12)	66.7	(16)
Non-Dutch European % (n)	9.5	(2)	16.7	(4)
Non-European % (n)	33.3	(7)	16.7	(4)
Education:			26	
Low % (n)	22.7	(5)	11.5	(3)
Intermediate % (n)	31.8	(7)	38.5	(10)
High % (n)	45.5	(10)	50.0	(13)
Fertilization procedure:				
IVF % (n)	91.7	(22)	87.5	(21)
ICSI % (n)	8.3	(2)	12.5	(3)
Folic acid containing supplement, yes % (n)	66.7	(14)	80.8	(21)
Smokers % (n)	13.4	(3)	3.8	(1)
Duration of subfertility (months) median (range)	41.0	(16.0-101.0)	42.0	(3.0-135.0)

Table 2
Biochemical markers of women undergoing ovarian stimulation treatment (n=50)

	Conventional (n=24)		Mild (n=26)		p
Follicles	10.6	(8.0-15.7)	7.4	(8.7-9.2)	<0.01
Baseline serum					
FSH (U/L)	7.7	(6.5-8.8)	8.0	(6.8-9.7)	ns
Estradiol (pmol/L)	141.6	(103.5-203.6)	160.1	(119.2-199.0)	ns
AMH (ug/L)	4.4	(2.9-6.1)	5.6	(3.3-10.1)	ns
Cobalamin (pmol/L)	351.0	(259.5-480.0)	309.3	(256.8-387.0)	ns
Pyridoxine (nmol/L)	81.5	(66.0-91.0)	83.2	(61.0-108.0)	ns
Folate (nmol/L)	27.3	(17.9-37.4)	37.1	(19.1-72.5)	ns
Homocysteine (umol/L)	9.5	(8.1-11.2)	9.5	(7.7-10.7)	ns
HCG-day serum					
Estradiol (pmol/L)	4,293	(2,889-6,646)	2,706	(1,716-3,558)	0.03
AMH (ug/L)	1.8	(1.4-3.0)	3.5	(2.4-5.3)	0.01
Cobalamin (pmol/L)	333.4	(262.7-481.5)	300.0	(221.7-381.8)	ns
Pyridoxine (nmol/L)	76.9	(65.0-84.0)	82.1	(56.0-106.0)	ns
Folate (nmol/L)	31.5	(18.2-59.4)	35.7	(19.1-65.4)	ns
Homocysteine (umol/L)	7.9	(6.7-9.8)	8.9	(7.0-10.7)	ns

Variables are depicted: Geometric Mean (Inter Quartile Range)

Figure 1 Flowchart for eligible couples

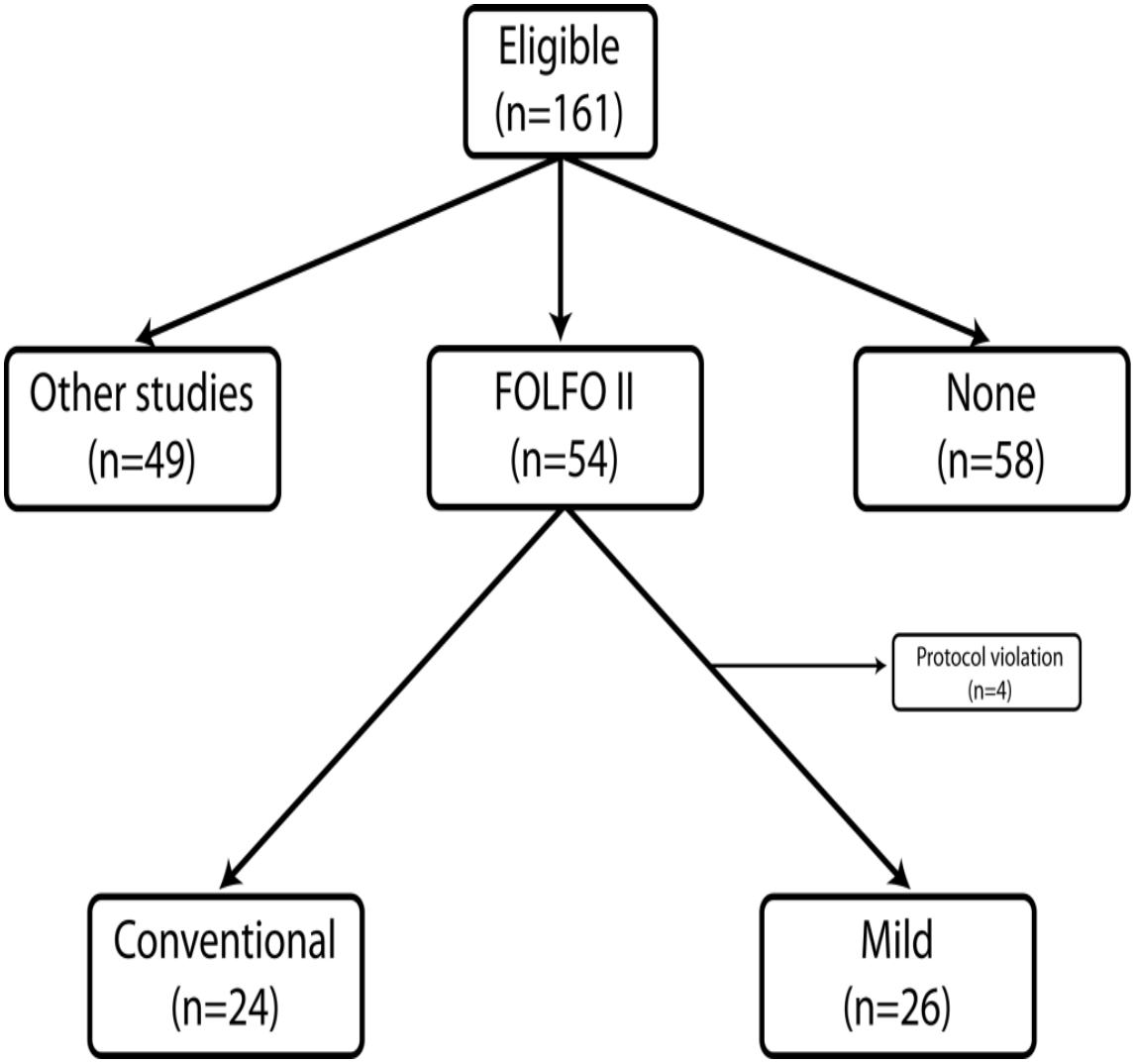
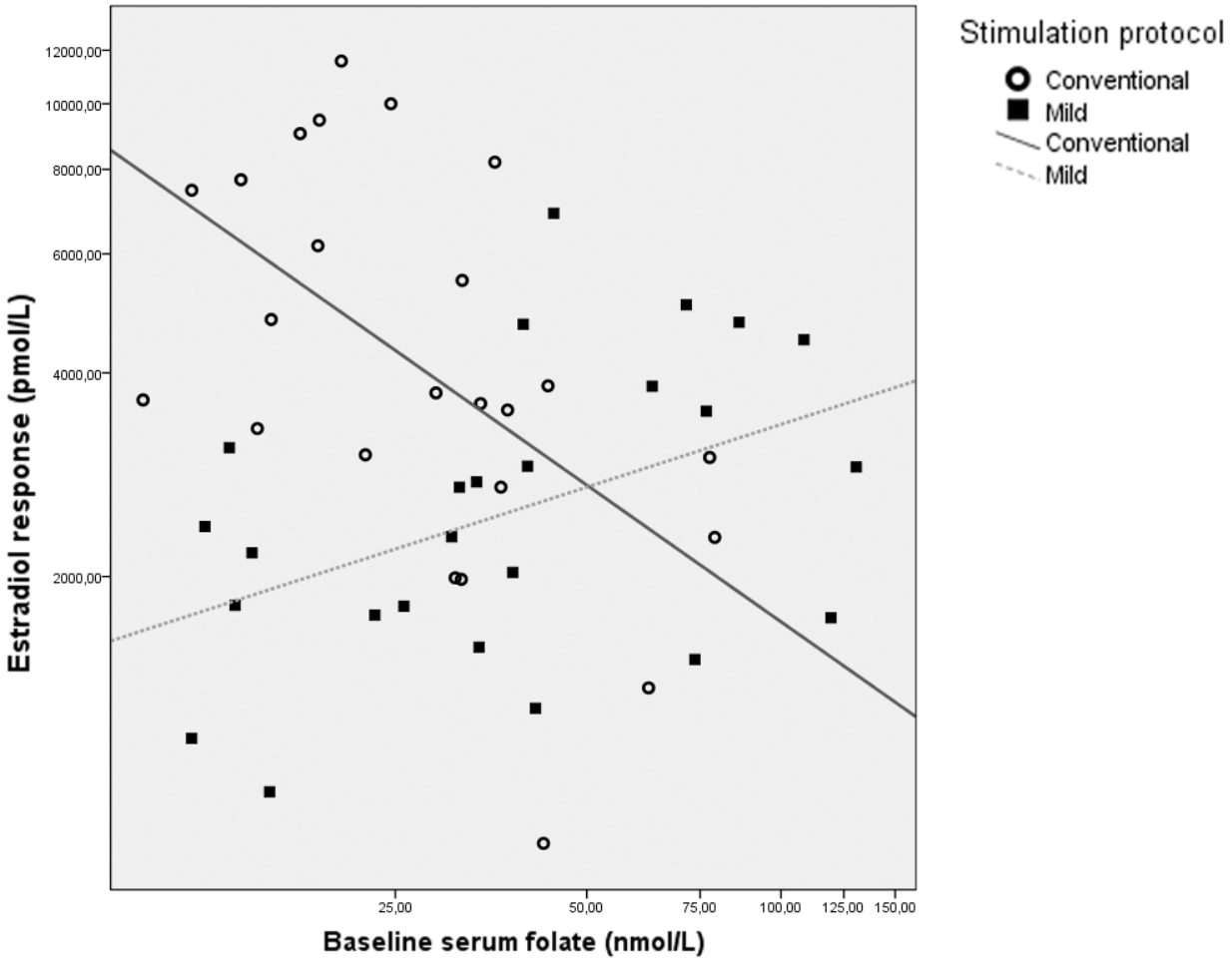


Figure 2 Interaction between serum folate and stimulation protocol on estradiol response



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Chapter 4

Body Mass Index mediates AMH response after ovarian hyperstimulation treatment

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Abstract

Poor nutrition, in particular low folate intake, affects ovarian function and subsequent fertility. Moreover, low folate intake and a high body mass index (BMI) result in global hypomethylation. Recently, Anti-Müllerian hormone (AMH) has emerged as a valuable novel biomarker for ovarian reserve. Therefore, the goal of this study was to investigate in women undergoing fertility treatment whether BMI is associated with AMH levels and whether the association can be modified by the folate status. This study was performed in a tertiary referral reproductive medicine unit at the Erasmus University Medical Center, Rotterdam, the Netherlands.

We included 163 women undergoing ovarian hyperstimulation in a periconceptional prospective cohort. Height (m) and weight (kg) were measured according to a standardised protocol and BMI was calculated (kg/m^2). Blood samples were taken at cycle day 2 (CD 2) prior to ovarian hyperstimulation and on the day of human chorionic gonadotropin (hCG) administration for determination of serum AMH levels. On CD 2 folate and hormone levels were also measured. The association between BMI and AMH was assessed using Spearman correlation and linear regression analysis. Age, cause of subfertility, serum folate and baseline AMH on CD 2 were included as potential confounding variables. After ovarian hyperstimulation the serum AMH level was significantly reduced (3.9 µg/L vs. 2.7 µg/L, p=0.000). BMI was not correlated with baseline serum AMH level (r=0.125, p=0.105), but significantly correlated with post stimulation AMH levels (r=0.208, p=0.009). However, BMI did correlate significantly with post stimulation AMH serum level (r=0.208, p=0.009). After adjustment for potential confounders BMI remained positively associated with AMH levels after ovarian hyperstimulation (β=0.04(0.014), p=0.007). Furthermore, the serum folate level on CD 2 was inversely associated with both baseline AMH (β=-0.002(0.001), p=0.04) and AMH after ovarian hyperstimulation (β=-0.001(0.001), p=0.01). The increased AMH level after ovarian hyperstimulation in subfertile overweight or obese women is suggested to be due to hypomethylation of the AMH gene in which folate seems to act as modifier.

Introduction

The high prevalence of overweight and obesity has become one of the greatest burdens for public health worldwide, including in women of reproductive age.[1] In the Netherlands, around 41% of women during their reproductive life span do suffer from overweight or obesity.[2] Besides the long term risks associated with overweight or obesity, such as type 2 diabetes, cardiovascular disease and several cancers, epidemiological studies have demonstrated the adverse effects of overweight and obesity on reproductive health of women.[3-4] Hence, women of reproductive age with a high body mass index (BMI) have a higher risk to suffer from ovulatory dysfunction and consequently reduced fertility and tend to respond less favourable to fertility treatment.[5] It has been shown that the relative risk of anovulatory dysfunction is 1.3 in women with a BMI between 24 and 29.9, and 2.7 in women with a BMI >30 kg/m^2.[6] Furthermore, women with a high BMI seem to have a 4% lower pregnancy rate per unit increase in BMI [hazard ratio: 0.96 (95% CI 0.91–0.99).[4]

Additionally, overweight or obesity is due to a combination of a poor diet and reduced physical exercise. Poor nutrition, in particular low folate intake, has been shown to hamper reproductive function and performance.[7-8] Folate status is associated with the quality of many parameters involved in human reproduction ranging from gamete- and embryo quality to the occurrence of congenital malformations.[9] The folate status is suggested to be negatively associated with BMI, but this association is not completely clear.[10] Recently, we showed an inverse association between BMI and global methylation, being an important determinant of genome programming by DNA methylation.[11] Moreover, Twigt et al., demonstrated that folic acid supplement use attenuates the follicular and endocrine responses to ovarian hyperstimulation.[12] Therefore, we hypothesize that BMI and folate influence the regulation of the tissue specific epigenome of the ovary by affecting its global

methylation state.

Anti-Müllerian hormone (AMH) has emerged as an important novel marker of ovarian reserve .[13] AMH - a member of the transforming growth factor-β superfamily - is involved in ovarian function and is produced by granulosa cells from pre-antral and small antral follicles.[13] AMH is involved in both the regulation of primordial follicle recruitment and the follicular responsiveness to follicle stimulating hormone (FSH) in an inhibitory manner.[13-14] As a result, serum AMH levels reflect the primordial follicle pool in the ovaries and are indicative of a woman's reproductive capacity.[13] Because the number of primordial follicles decreases with age, AMH is considered a marker for ovarian aging.[15] A high BMI and a diet poor in folate may stimulate aging processes.[16-17] Data on the association between overweight, obesity, folate and AMH, however, are lacking.

Therefore we investigated in a prospective periconceptional cohort study the association between BMI and AMH and the modification by folate in women undergoing assisted reproductive treatment (ART).

Materials and methods

Study design

The periconceptional Food Lifestyle and Fertility Outcome (FOLFO) cohort study was designed to investigate the influence of periconceptional nutrition and lifestyle factors on biochemical, clinical fertility and pregnancy outcome parameters following in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) treatment. The design of the study has been described in detail previously.[18] In summary, eligible couples visiting the Erasmus MC, University Medical Center in Rotterdam, The Netherlands, with an indication for IVF or ICSI treatment were invited to participate in the FOLFO study. We excluded couples who suffered from known conditions that may influence IVF/ICSI treatment outcome, such as oocyte donation, endometrioma and hydrosalpinx resulting in 163 women for this study. These restrictions were applied because these conditions might influence IVF/ICSI success rate much stronger than BMI, nutrition and lifestyle factors compared to other fertility disorders.

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

Questionnaires

All participants filled out a general questionnaire on the day of the oocyte pick-up from which the following data were extracted: height (m), weight (kg), ethnicity, education level, folic acid use and smoking habits. BMI was calculated by dividing weight (kg) by height

squared (m^2). Ethnicity and educational level were classified according to the definitions of Statistics Netherlands.[19-20] Education level was divided into three categories: low (primary / lower vocational / intermediate secondary), intermediate (intermediate vocational / higher secondary) and high (higher vocational / university). Ethnicity was divided into Dutch Natives, European others and Non-European.

IVF procedure

All women started the ovarian stimulation treatment with daily injections of 150 IU recombinant follicle stimulating hormone (rFSH) s.c. on cycle day 2 (CD2) (Puregon®, Schering-Plough, Houten, The Netherlands or Gonal-F®, Serono Benelux BV, Schiphol-Rijk the Netherlands). Administration of daily s.c. gonadotrophin releasing hormone antagonist (Orgalutran®, Schering-Plough, Houten, The Netherlands) was started when at least one follicle was ≥14 mm. To induce final oocyte maturation, a single dose of 10,000 IU hCG s.c. (Pregnyl®, Schering-Plough, Houten, The Netherlands) was administered as soon as the largest follicle reached at least 18 mm in diameter and at least one additional follicle of ≥15 mm was observed. Oocyte retrieval was carried out 35 h after hCG injection by transvaginal ultrasound-guided aspiration of follicles.

Sample collection and analysis

Venous blood samples were drawn from each woman on CD2, i.e., the early follicular phase of the menstrual cycle proceeding the treatment cycle, and on the day of hCG administration. For the determination of folate 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 to be assayed. Serum Anti-Müllerian hormone (AMH) levels were measured

using an in-house double-antibody enzyme-linked immunosorbent assay (ELISA).[21] To estimate the short term (3-5 days) and long term (2-4 months) folate state serum and red blood cell (RBC) levels, respectively, were measured. Folate was analysed using an immunoelectrochemolu-minescence assay (Roche Modular E170, Roche Diagnostics GmbH, Mannheim, Germany). Serum concentrations of FSH were measured by luminescence-based immunometric assay (Immulite 2000, Siemens Diagnostics, Los Angeles, CA, USA). Serum estradiol was determined using a coated tube radioimmunoassay obtained from the same supplier.

Inter-assay coefficients of variation for folate were 4.5% at 13 nmol/L and 5.7% at 23 nmol/L; for AMH this coefficient was <10%; for FSH < 5.8%; and for estradiol, <8.8%. The detection limit for folate was 1.36 nmol/L, for AMH 0.1 mg/L, for FSH 0.1 U/L and for estradiol 10 pmol/L.

Statistical analysis

Prior to statistical analyses, all continuous variables were log-transformed, to obtain a normal distribution of data suitable for parametric statistical testing. Normality was assessed using histograms and Q-Q plots. When a normal distribution was not attained after transformation, the variable is presented as median (range).

Spearman correlation was used to investigate the correlations between several covariates and BMI and AMH. Adjustments were made in a multivariable linear regression model. Age, cause of subfertility, serum folate and baseline AMH (at cycle day 2) were included as potential confounding variables. Regression coefficients (β) are reported with standard error (s.e.). A p-value <0.05 was considered statistically significant. All statistical analyses were done using SPSS 15.0 for Windows software (SPSS Inc., Chicago, IL, USA).

Results

Study population characteristics

In total we included 163 women. The age of the women in the study group ranged from 23.2 to 43.7 years with a median of 36.1 years. The median BMI of the women was 23.3 (16.1-36.3), 66.3% had a normal weight (BMI<25) and 33.7% were overweight or obese (BMI≥25). Overall women were highly educated (39.7%) and 61% were of Dutch ethnicity. There was no significant difference in folic acid supplement use in normal weight, and overweight and obese women, respectively 75% and 71%.

Biochemical parameters and ovarian hyperstimulation treatment

In the total study group the baseline AMH level was 3.9 μg/ L (0.1-19.5), which significantly declined after ovarian hyperstimulation to 2.7 μg/L (0.07-32.4) (p=0.000).

Women were stratified into normal weight (BMI <25) and overweight or obese women (BMI≥25). Women with a BMI≥25 kg/m^2 had a significantly higher AMH level after ovarian hyperstimulation compared to normal weight women (p=0.006) (Table 1). There was no difference in the number of follicles and oocytes after ovarian hyperstimulation between these groups. Furthermore, significantly lower RBC folate levels were found in women with a BMI≥25 (1145 (488-2676)) compared to women with a normal BMI (1539 (549-3611)) (p=0.001).

BMI was not correlated with baseline AMH (r=0.125, p=0.105), but correlated significantly with AMH after ovarian hyperstimulation (r=0.208, p=0.009). This observation was further analysed using a multivariable linear regression model with adjustment for age, cause of subfertility, folic supplement use and baseline serum AMH level (Table 2). After adjustment BMI remained positively associated with AMH after ovarian hyperstimulation

(β=0.040(0.014), p=0.007). Furthermore, weight was the component in BMI that was associated with AMH after ovarian hyperstimulation (β=0.012(0.005), p=0.01).

Serum folate at CD 2 was both inversely associated with baseline AMH (β=-0.002(0.001), p=0.04) and AMH after ovarian hyperstimulation (β=-0.001(0.001), p=0.01).

Discussion

Our study shows that serum AMH levels significantly decline in subfertile women who underwent ovarian hyperstimulation. Most interesting is that in subfertile women with overweight or obesity compared to normal weight women the AMH levels were higher before, albeit not significantly, and after ovarian hyperstimulation (p=0.006). These effects were independent of age, cause of subfertility, and baseline folate and AMH levels. In the total group of women, we also observed a significant inverse association between baseline serum folate and AMH before and after ovarian hyperstimulation suggesting effect modification.

A novel finding is that overweight or obese women do exhibit higher AMH levels before, but especially after ovarian hyperstimulation compared to normal weight women, despite the comparable total number of follicles and oocytes. Fanchin and associates showed improved responsiveness to ovarian hyperstimulation in women with high AMH levels on the day of hCG administration indicated by a reduced gonadotrophin requirement, a large number of antral follicles and oocytes.[22] However, this study only included women with a normal BMI ranging from 18-25 kg/m^2. AMH levels are positively correlated with ovarian dysfunction meaning that higher levels reflecting greater impairment in follicular development and granulosa cell function.[23-25] Previous studies suggested an inverse association between obesity and baseline AMH levels, which is in line with our findings in overweight and obese women, albeit not significantly.

The first explanation for this novel finding is that the high AMH level after ovarian hyperstimulation in overweight or obese women may be due to an altered follicle dynamic in overweight and obese women. This might be related to pre-existing differences in early antral follicles with more small antral, i.e., AMH producing follicles, in these women.

Furthermore, the higher AMH level in the same overweight and obese women after ovarian hyperstimulation treatment suggests that there are more small AMH producing follicles which seem less sensitive to ovarian hyperstimulation by exogenous FSH. The mechanism, however, underlying the heterogeneity in characteristics of antral follicles remains unclear. However, it has been shown that during the late luteal phase there is a gradual FSH elevation occurring to preserve antral follicles from atresia and ensure their subsequent growth.[26-27] It is possible that in overweight or obese women there is an impairment of this early follicular development due to a lower endogenous FSH secretion during the luteal phase or a lower expression of the FSH receptor. In addition, increased body size is associated with increased estradiol and lower FSH and LH levels. This is in line with our results, since we showed a lower FSH level at baseline in these women compared to their normal weight counterparts.[28-29]

AMH inhibits the initial and cyclic processes of follicular recruitment and the response to exogenous FSH, yet the aspects involved in its regulation are still poorly understood. However, because of the significant inverse association between also baseline serum folate and AMH before and after ovarian hyperstimulation treatment, comparable with the association between folate and estradiol.[12] We suggest that folate as methyl donor influences the expression of the AMH receptor gene by affecting the methylation of its promoter.[30] Folate acts as a methyl donor in the remethylation of homocysteine to methionine. Deficiencies in folate can result in accumulation of homocysteine, which is associated with DNA hypomethylation and could potentially result in changes in gene expression.[31] DNA methylation is an epigenetic mechanism involved in the regulation of a wide variety of biological processes, including gene expression.[32] Several genes involved in follicle development are regulated by DNA methylation. Investigation of the mouse genome

indicates that the *FSHR* gene contains CpG repeats, which when methylated inhibit *FSHR* gene transcription.[33] In addition, Kanakkaparambil et al. showed that incubating granulosa cells with homocysteine increases *FSHR* mRNA levels.[34] Similarly, the aromatase enzyme, which converts androgens into estrogens, expressed in bovine granulosa cells is regulated by DNA methylation.[35] Environmental factors, such as a poor diet and obesity can influence global and DNA methylation with consequences for aging processes.[11, 30] In our study women with a BMI≥25 kg/m^2 showed a significantly lower baseline RBC folate used as proxy of the long term tissue methylation state, from which we can postulate that the folate state might modulate the higher AMH response after ovarian hyperstimulation in these women. This is substantiated by the study of van Driel et al. showing a significant association between an increased BMI and a state of global DNA hypomethylation.[11] Thus, our explanation for the observed high AMH levels is that overweight and obesity stimulate epigenetic processes in the ovary related to aging, which seems to be modified by folate.[11] To substantiate this finding, we showed in an additional multivariable linear regression analysis that especially weight was positively associated with the AMH level after ovarian hyperstimulation, whereas no association was found with height. The further assessed inverse association between weight and serum folate (r=-0.228; p<0.01) is further substantiating a possible explanatory role for DNA hypomethylation.

The significant decrease of AMH levels after ovarian hyperstimulation is in accordance with previous studies.[23, 36] The decrease in AMH after ovarian stimulation is consistent with the reduction in the number of small antral follicles due to stimulation by exogenous FSH. These data further support the hypothesis that maturing or aging follicles progressively loose their ability to produce AMH. [36-38] In addition, it has been shown that FSH treatment significantly reduces AMH expression in cultured granulosa cells.[25, 38] Finally, it has been suggested that

increased serum estradiol levels during ovarian hyperstimulation could have an inhibitory effect on AMH secretion, which we couldn't establish.[26]

Some strengths and limitations have to be addressed. Folate, cobalamin and total homocysteine were measured as biomarkers of global methylation. Although, we are aware that total homocysteine and SAH are highly correlated, we were not able to measure the SAM/SAH ratio as a potentially better biomarker of global methylation. Nevertheless, the evidence of the link between folate, homocysteine and the global methylation status is strong.[39] Mild to moderate folate depletion increases total homocysteine and decreases DNA methylation, which can be reversed by folic acid supplement use.[40] Body size may affect also other predictors of ovarian reserve, such as antral follicle count and Inhibin B. In our study these measurements were lacking. A strength of our study is that all women were on the same ovarian hyperstimulation treatment in which the measurements were carried out in a standardized fashion.

In conclusion, we show that AMH levels significantly decline after ovarian hyperstimulation and that women with a higher BMI have higher AMH levels especially at the end of the ovarian hyperstimulation protocol. In line with the inverse association between BMI and a global DNA methylation, the increased AMH response in overweight or obese women may be due to hypomethylation of the AMH gene receptor resulting in increased expression. Moreover, it seems that the folate state acts herein as 'aging' modifier. In vitro studies may help to further understand the causal relationship between BMI and AMH by investigating DNA methylation patterns in human and animal granulosa cells.

TABLE 1
Biochemical parameters in 163 women of subfertile couples before (cycle day 2) and after ovarian stimulation treatment (hCG day)

	BMI <25 (n=108)	BMI ≥25 (n=55)	p
Biochemical Parameters (cycle day 2)			
AMH (µg/L)	3.9 (0.1-18.8)	4.7 (0.2-16.7)	0.1
Estradiol (pmol/L)	148 (41-691)	130 (53-295)	0.052
FSH (U/L)	8.6 (0.4-30.3)	7.5 (4.4-16)	0.051
Folate, serum, (nmol/L)	32.6 (11.5-908)	29.4 (9.8-95.2)	0.3
Folate RBC (nmol/L)[a]	1539 (549-3611)	1145 (488-2676)	0.001
Total homocysteine, plasma, (µmol/L)	9.0 (5.5-16.6)	9.3 (6.1-75.3)	0.4
Cobalamin, serum, (pmol/L)	351 (140-1856)	298 (74-863)	0.1
Pyridoxine, plasma (nmol/L)	84 (39-310)	75 (39-310)	0.1
Biochemical Parameters (hCG day)			
AMH (µg/L)	2.2 (0.07-32.5)	3.2 (0.1-21.5)	0.006
Estradiol (pmol/L)	2173 (233-8982)	2272 (291-11978)	0.9
LH (pmol /L)	1.2 (0.1-34)	1.3 (0.3-17.6)	0.3
Folate, serum (nmol/l)	33.9 (7.7-174.3)	32.7 (8.0-95.3)	0.3
Folate RBC (nmol/L)	1491 (530-4413)	1204 (599-2524)	0.054
Total homocysteine, plasma, (µmol/L)	8.4(5.4-16.8)	8.4(4.3-71.6)	0.9
Cobalamin, serum, (pmol/L)	319 (133-1046)	285 (75-1019)	0.3
Pyridoxine, plasma, (nmol/L)	78 (35-310)	74 (40-310)	0.2
Number of follicles	7 (1-23)	6 (1-20)	0.9
Number of oocytes	6 (1-25)	6 (1-23)	0.7

RBC folate= red blood cell folate
Results are shown as number (%) or median (range).

TABLE 2
Associations between height, weight, BMI and folate before ovulation stimulation treatment and AMH before and after ovulation stimulation treatment

		Baseline AMH [a]	AMH hCG day [b]
Height			
	β(s.e.)	0.002(0.010)	0.004(0.007)
	p-value	0.8	0.5
Weight			
	β(s.e.)	0.003(0.005)	0.012(0.005)
	p-value	0.515	0.01
BMI			
	β(s.e.)	0.014(0.016)	0.040(0.014)
	p-value	0.4	0.007
Folate			
	β(s.e.)	-0.002(0.001)	-0.001(0.001)
	p-value	0.04	0.01

[a] Linear regression analysis with adjustment for age, BMI, cause of subfertility and folic acid use.
[b] Linear regression analysis with adjustment for age, BMI, cause of subfertility, folic acid use and baseline AMH.

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Chapter 5

Sperm quality decline among men below 60 years of age undergoing IVF or ICSI treatment

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Abstract

Due to changes in the society, couples in Western countries are increasingly delaying reproduction. This is accompanied by unhealthy lifestyles that may not only be detrimental to general health but also for reproductive capacity. It is well-known that maternal age has detrimental effects on fertility; the paternal influence on this outcome is largely unknown. This study aims to investigate associations between a paternal age below 60 years of age, lifestyles and sperm quality.
In a periconceptional prospective cohort study we included two hundred twenty-seven men undergoing in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) treatment. Age at sperm collection, lifestyles, cause of subfertility, ethnicity, sperm DNA fragmentation index (DFI), as marker of sperm DNA damage) and sperm parameters were determined. Linear regression analyses showed a positive association between a rising age from 26 to 59 years and DFI ($P \leq 0.01$) and an inverse association with ejaculate volume ($P \leq 0.05$). Inverse associations were determined between DFI and all conventional sperm parameters (all $P \leq 0.01$).
There were no associations between smoking, alcohol use, BMI and DFI and sperm parameters. Dutch men compared to migrants, however, showed a higher DFI ($P \leq 0.05$) independent of lifestyles. We conclude that the trend of delaying fatherhood in men undergoing IVF or ICSI treatment is detrimental to sperm quality.

Introduction

Approximately 10-15% of all couples in the Western world do not conceive within one year and 4-5% remains involuntarily childless. This has a great influence on the quality of life of the couples and their families. Male factor subfertility plays a role in 20-26% of subfertile couples.[1] Concerns about the worldwide decline in sperm quality over the past 50 years, however, are increasing, in particular in western countries. Furthermore, there is a trend that the age at which men are reproducing is rising. From literature reveals that spermatogenesis in men above 55 years of age declines resulting in a reduced reproductive capacity.[2-3] A rising age is often accompanied with changes in more unhealthy lifestyles, such as smoking and social alcohol use which both affect biological processes associated with sperm quality.[4-5] Excessive exposure to smoking and alcohol consumption significantly diminishes sperm quality.[6-7] Smoking reduces sperm production, motility and morphology and also seems to interfere with the chromatin integrity of spermatozoa thereby inducing DNA damage.[8] It is worrisome that in the Netherlands around 31 % of men in reproductive ages smoke cigarettes and approximately 85 % of them also consume alcohol. [9] This is not different in other Western countries.

Conventional sperm analysis, including ejaculate volume, sperm concentration, motility, and morphology determined according to World Health Organization (WHO) criteria is used to discriminate between fertile and subfertile males.[10] However, these parameters are of limited predictive value for fertility outcome.[11] Human and animal studies have recently indicated that the integrity of sperm DNA might be a more accurate and precise predictor of fertility.[12-13] The sperm chromatin structure assay (SCSA) determines the chromatin integrity of the sperm genome from which the DNA fragmentation index (DFI) and high DNA stability (HDS) is calculated.[14] These parameters reflect the fraction of damaged or defective sperm

either due to the presence of DNA breaks or poorly condensed chromatin. Studies directed to investigate associations between the effects of age up to 60 years, unhealthy lifestyles, and sperm quality in men undergoing IVF or ICSI treatment are scarce and inconclusive.[15-16] From this background, this study aims to examine in men undergoing IVF or ICSI treatment the effects of: 1) rising age and unhealthy lifestyles on sperm quality, and 2) ethnicity on these associations.

Materials and Methods

Patients

The study was included in the Food, Lifestyle and Fertility Outcome-study (FOLFO-study) at the Erasmus MC, University Medical Center in the Netherlands. This prospective periconception study is focused on the effects of nutrition and lifestyles during the periconception period on fertility and has previously been described.[17] From September 2004 to January 2007, all subfertile couples undergoing IVF or ICSI were invited to participate in the FOLFO- study at the first intake visit for fertility treatment. For this study men were only included unless sperm was cryopreserved or obtained by microsurgical or percutaneous epididymal sperm aspiration (MESA or PESA).

The study protocol was approved by the Central Committee for Human Research and the local Medical Ethical and Institutional Review Board of the Erasmus MC, University Medical Center in Rotterdam, the Netherlands. Written informed consent was obtained from all men before participation.

Men visited the Andrology outpatient clinic for standard fertility evaluation consisting of physical examination, blood sampling and sperm analysis. Male factor subfertility was defined as a sperm concentration of $<20 \times 10^6$ cells/mL, failure to conceive after 1 year of regular unprotected intercourse with the same partner, duration of child wish of more than 1 year with the same partner, and no prior conception. 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. They filled out a general questionnaire from which the following data were extracted: age, medical history, height, weight, ethnicity, educational level, use of medication, and the lifestyle factors social use of alcohol and smoking. Body mass index (BMI) was calculated by weight divided by squared height. Educational level was assessed by

the highest completed education and classified into three categories: 1) low education: no education, primary school, lower vocational training, intermediate general school, or 3 years or less general secondary school; 2) intermediate education: more than 3 years general secondary school, intermediate vocational training, or first year of higher vocational training; 3) high education: higher vocational training, university or PhD degree.[18] Ethnic background was classified according to the definitions of Statistics Netherlands.[18] For the present analyses we included only men with information on ethnic background and availability of one sperm analysis (n=175). Because lifestyle factors are related to ethnicity we stratified the total group of men into Dutch men (n=148) and migrant men (n=27). The obtained materials were processed anonymously.

Sperm collection and preparation

Sperm samples were obtained after a standardized abstinence period of 3 to 5 days by masturbation. The sperm samples were processed within 1 hour after ejaculation. After liquefaction, the sperm parameters volume, concentration, count, percentage progressive motility and percentage normal morphology were assessed according to World Health Organization guidelines (WHO, 2001). We used the following WHO normal reference values: sperm concentration > 20 x 10^6 numbers/ml, 50% or more motile sperm or 25% or more progressive motile sperm and 30% normal morphology. Sperm concentration was determined with an improved Neubauer Hemacytometer® counting chamber. Morphology was evaluated using the Diff-Quick staining method. At least 200 spermatozoa per patient were evaluated at a magnificent of x100 according to the guidelines of the WHO. An aliquot of unprocessed sperm was stored at -80ºC until the determination of DFI. Subsequently, the remainder of sperm was centrifuged at 2,500 x g for 10 minutes.

Sperm Chromatin Structure Assay (SCSA)

The principles and procedures of measuring sperm DNA damage by FAC Scan flow cytometry SCSA have been described in detail previously.[19] Briefly, sperm samples were diluted with Tris-NACL-EDT buffer (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 a reference sample was stained and run again after every 5 to 10 samples. Data collection of the fluorescent pattern in 5,000 cells was performed at 3 minutes after acid treatment. Each sperm sample was analyzed in duplicate.

The percentage 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.

Statistical analysis

Normality of the variables was tested using the Kolmogorov-Smirnov tests. Because of skewed distributions, age, BMI, DFI, sperm parameters are presented as medians (range). The categorical variables are displayed in numbers with percentages. To test differences between the subgroups Mann-Whitney U and Chi-Square test were used.

To investigate the influence of age and lifestyles on DFI and conventional sperm parameters, we used linear regression models on log transformed sperm parameters. The

regression analyses were adjusted for age, BMI, ethnicity, smoking, and alcohol use. The associations are presented by the adjusted regression coefficient (β). A *P*-value 0≤.05 was considered statistically significant. Statistical analyses were performed with Statistical Package of Social Sciences version 15.0 for windows (SPSS Inc., Chicago, IL, USA).

Results

Characteristics

The characteristics of the total group of men undergoing IVF/ICSI treatment and stratified into Dutch and migrant men are presented in Table 1. The median duration of subfertility is 37 (4-121) months.

Age, BMI, cause of the subfertility, educational level, alcohol use and cigarette smoking were comparable between Dutch and migrant men. Dutch men compared with migrants showed a higher DFI ($P \leq 0.05$).

Age, lifestyles, conventional sperm parameters and DFI

Associations between age, lifestyles, and DFI and sperm parameters, adjusted for confounders are presented in Table 2. Male age was significantly associated with ejaculate volume (adjusted β=-0.64, $P<0.05$) and DFI (adjusted β=0.08, $P<0.01$). In figure 1 the association between age and volume, and in figure 2 the correlation between age and DFI are also presented. Dutch men showed a higher ejaculate volume (adjusted β=0.04, $P \leq 0.01$) and a higher DFI (adjusted β=0.004, $P \leq 0.05$) compared to migrants. No associations were found between BMI, smoking, alcohol use, and DFI and conventional sperm parameters.

After adjusting for potential confounders, i.e., age, BMI, smoking, alcohol use and ethnicity, positive associations were found between DFI and sperm volume (adjusted β=2.03, $P \leq 0.01$). Statistically significant inverse associations were observed between DFI and sperm concentration (adjusted β=-0.07, $P \leq 0.01$), percentage progressive motility (adjusted β=-36.84, $P \leq 0.01$), and sperm morphology (adjusted β=-97.57, $P \leq 0.01$).

Discussion

This study shows that in men between 26 and 59 years of age and undergoing IVF or ICSI treatment the rising age is detrimental for sperm DNA integrity and ejaculate volume. Furthermore, Dutch men showed a significantly poorer sperm quality based on a higher DFI compared to migrants.

The increase in DFI in this group of men with an average age of 37 year is worrisome but in line with a previous study showing that men with an average age of 40 show more DNA damaged spermatozoa due to increased oxidative stress as a consequence of the aging process.[20] In addition, studies in rats revealed that a decrease in epididymal antioxidant capacity occurs with rising age thereby disrupting germ-cell differentiation and sperm quality.[21] This is consistent with the observations that in older men the apoptotic functions of spermatogenesis seem to be less efficient resulting in the production of more spermatozoa with defragmented DNA.[20] The age related increase in DFI at lower ages has never been investigated before but the reduced sperm quality seems to be comparable with that of older men.[22-23] The absence of significant effects of smoking, alcohol use and BMI on sperm quality may suggest that other factors may have masked the relatively weak effects of these lifestyles.[24-25] Studies of others, however, showed detrimental effects of smoking on sperm quality in man. We investigated the effects of social alcohol use of which the accuracy of the measurement is difficult, which may explain the absence of an association with sperm quality. Not many studies have been performed on the association between BMI and sperm quality. Compared to the study of Aggerholm *et al* (2008) the absence of an association between BMI and sperm quality in our study might be due to the relatively small group of overweight and obese men.[26] Other explanations are the differences and sizes of the populations investigated, the methods used to assess smoking and alcohol use, and the

definition used for overweight and obesity. It cannot be excluded that the lack of association may also be due to some misclassification of exposure status, since data on smoking and alcohol use were obtained from questionnaires and have not been validated by measuring biomarkers of smoking, e.g., cotinine, and alcohol use, e.g., ethanol, in serum or seminal plasma.[7, 27-28] On the other hand sperm quality may be considered a biomarker of overall health.[29] A new finding was that Dutch men showed more DNA damage, substantiated by a higher DFI, in their sperm compared to migrants. This could not be explained by differences in age, BMI, smoking and alcohol use between the groups, suggesting that these migrant men may have adapted to the Dutch lifestyle. It is possible that several unmeasured lifestyle factors, such as diet, occupational hazards, exercise, psychosocial stress and genetic factors, and overall health may also play a role.[30] It has been reported that an adequate intake of unsaturated fatty acids present in fish improves sperm quality. Because in the general Dutch population the consumption of fish is very much compromised this dietary factor may have contributed to the poorer sperm quality in Dutch men.[31-32] Exposures to environmental toxicants, such as pesticides and heavy metals are detrimental for spermatogenesis.[33] Animal and human studies have suggested that male reproductive disorders might be due to harmful in utero exposures disrupting embryonic programming and gonadal development.[34-35] This is demonstrated in a study in male mice exposed to polycyclic aromatic hydrocarbons in utero, a major mutagenic of tobacco smoke, showing a reduced fertility.[36] This is in line with the in utero exposure to DES (diethylstilbestrol) or tobacco smoke with detrimental effects on fertility.[34, 37-38] Thus, it can be hypothesized that Dutch men may have been more exposed to toxic agents than migrants, an issue of interest to be investigated in future studies.

Besides ageing and lifestyle factors also genetic variations affect spermatogenesis.[30, 39] This is supported by the reported ethnical as well as geographical differences in sperm quality. Genetic variations in the Y chromosome, CAG repeats of the androgen receptor (AR), and endocrine and metabolic pathways also contribute to differences in susceptibility to adverse environmental exposures and as such influence sperm quality.[30,39] The effects of genetic influences become particularly clear from the studies performed in the Baltic countries, in which despite a similar lifestyle a higher sperm concentration was reported in Finland, Estonia, Lithuania and Latvia than in Denmark and Norway.[40-41]

Some limitations of our study have to be addressed. Due to the feasibility of the study, only one standardized sperm sample per participant was obtained. Furthermore, the group of migrant men was relatively small due to less participation as a consequence of language problems which is a known problem in this kind of research. In addition, we cannot exclude that migrants prefer doing fertility treatments in their home country. This group was heterogeneous with regard to the different ethnicities. Nevertheless, the results are in our opinion interesting and warrant more research in which ethnicity is taken into account.

In conclusion, the age related decrease in sperm quality below 59 years of age - based on increased sperm DNA damage and decreased ejaculate volume - suggests that delaying childbearing not only in women but also in men, contributes to a reduced reproductive capacity. The significantly higher DFI in Dutch men compared to migrants could not be explained by differences in age and the most prominent unhealthy lifestyles. Further studies on other (un)healthy lifestyles, overall health and interventions to improve male factor subfertility in different ethnic populations should be stimulated.

Table 1
General characteristics of the study participants

Characteristics		Total group		Dutch men		Migrant men	P*
	N	N=175	N	N = 148	N	N = 27	
Age(y)	175	36.9 (25.8-59.1)	148	36.4 (25.8-59.1)	27	36.7 (28.6-53.9)	0.57
BMI(kg/m^2)	175	25.5 (18.8-37.9)	148	25.6 (18.8-37.9)	27	25.0 (19.8 – 34.3)	0.70
Cause of the subfertility (%)	187		148		27		0.7
Male factor		89 (47.6%)		69 (46.6%)		13 (48.1%)	
Male and female factor		15 (8%)		12 (8.1%)		1 (3.7%)	
Unexplained		83 (344.4%)		67 (45.3%)		13 (48.1%)	
Educational Level (%)	187		148		27		0.91
Low		30 (16%)		26 (17.6%)		4 (14.8%)	
Intermediate		61 (32.6%)		52 (35.1%)		9 (33.3%)	
High		84 (44.9%)		70 (47.3%)		14 (51.9%)	
Alcohol (units/week)	173	5.9 (0 – 54.9)	146	6.2 (0-55.0)	26	3.7 (0-28.1)	0.09
Smoking, Yes (%)	175	35 (18.7%)	148	29 (19.6%)	27	6 (22.6%)	0.75
1-10 cigarettes/day		16 (8.6 %)		12(8.1%)		4(14.8%)	
10-25 cigarettes/day		17 (9.1%)		16(10.8%)		1(3.7%)	
25 cigarettes/day		2 (1.1%)		1(0.7%)		1(3.7%)	
Conventional Sperm parameters †							
Volume (ml)	162	2.9 (0.3 – 8.1)	128	2.9 (0.6-8.1)	22	2.8 (0.3-6.0)	0.06
Concentration (x10^6 cells/ml)	162	23.0 (0.7 – 278.0)	128	23.0 (0.7-278.0)	22	34.5 (1.4-167.0)	0.12
Count (x10^6 cells)	162	46.2 (0 – 1556.8)	128	46.5 (2.0-1556.8)	22	73.8 (0-690.0)	0.40
Progressive motility (%)	162	29.0 (0 – 74.0)	128	29.0 (0-74.0)	22	37.0 (1.0-61.0)	0.49
Normal morphology (%)	162	4.0 (0 – 15.0)	128	4.0 (0-15.0)	22	6.0 (0-12.0)	0.39
DFI (%)	162	24.4 (1.9 – 74.8)	128	24.3 (3.6 -74.8)	22	22.7 (1.9-50.5)	<0.05

Data are either presented as median (range) or as percentages (%). Numbers do not count up to 100 percent due to missings.
* Mann- Whitney U and Chi-Square test were used to test differences between Dutch men and Migrant men.
†Determination according to World Health Organization (1999) criteria. Normal reference values are >20× 10^6 cells/ml sperm concentration, >50% motility, and ≥30% normal morphology.

Table 2

Associations between general characteristics and semen parameters

	Volume	Concentration	Motility	Count	Morphology	DFI
	β	β	β	β	β	β
Age, years	-0.64‡	0.01	-3.35	0	1.72	0.08§
BMI, kg/m^2	0.07	0.01	-2.70	0	-4.52	0.01
Ethnicity †	0.04‡	0	0.17	0	0.40	0.004‡
Smoking	0.01	0	-0.30	0	-1.11	0
Alcohol	-0.67	0.03	3.0	0	-10.8	-0.06

Linear regression analysis is used to test independent associations. The regression coefficient (β) indicates the increase or decrease (-) change per unit of age (per year), BMI (per 1.0 kg/m^2), smoking (yes versus no) and alcohol (glasses per week).

† Dutch men versus migrant men

‡ P≤0. 05 and § P≤0.01

Figure 1 The correlation between age (years) and volume (ml) (r = -0.17; p = < 0.05).

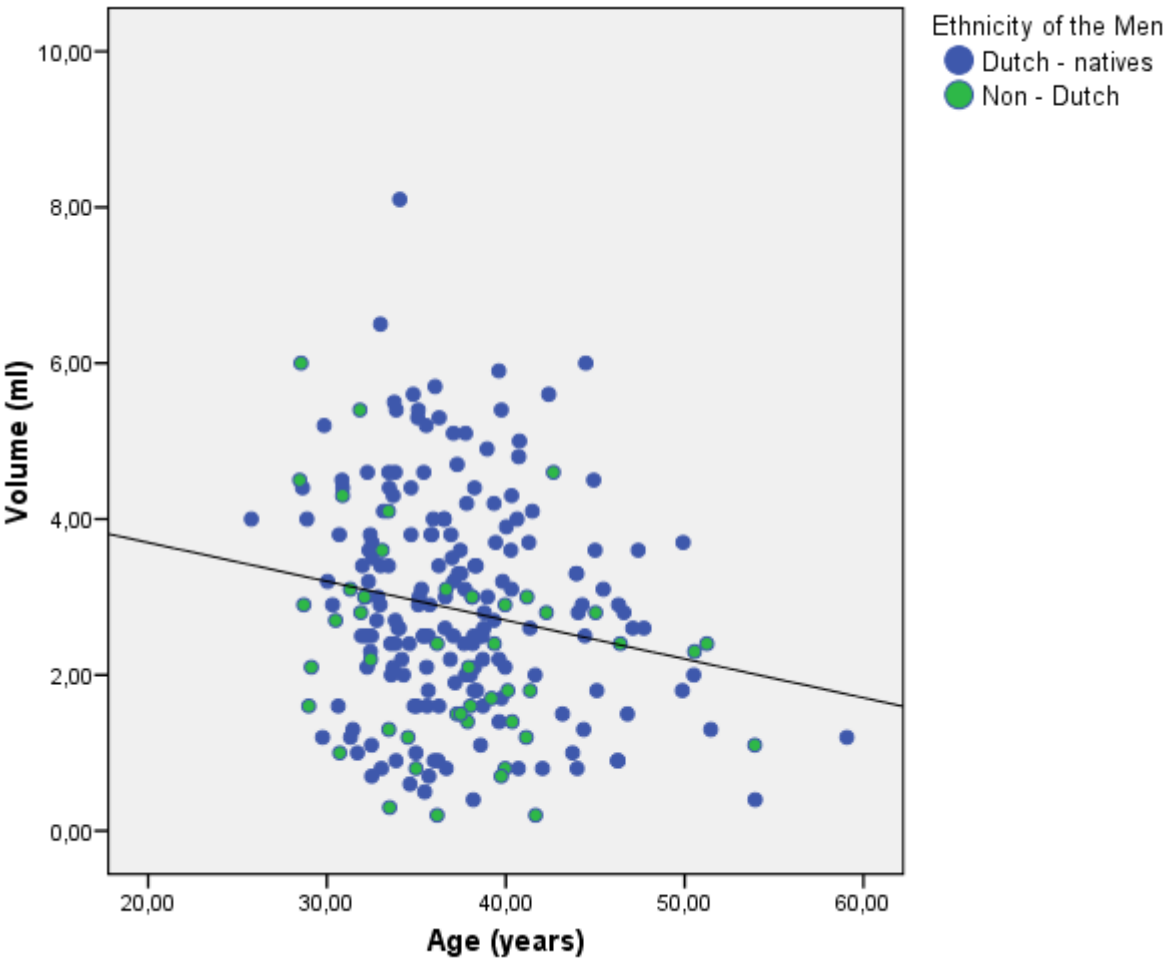
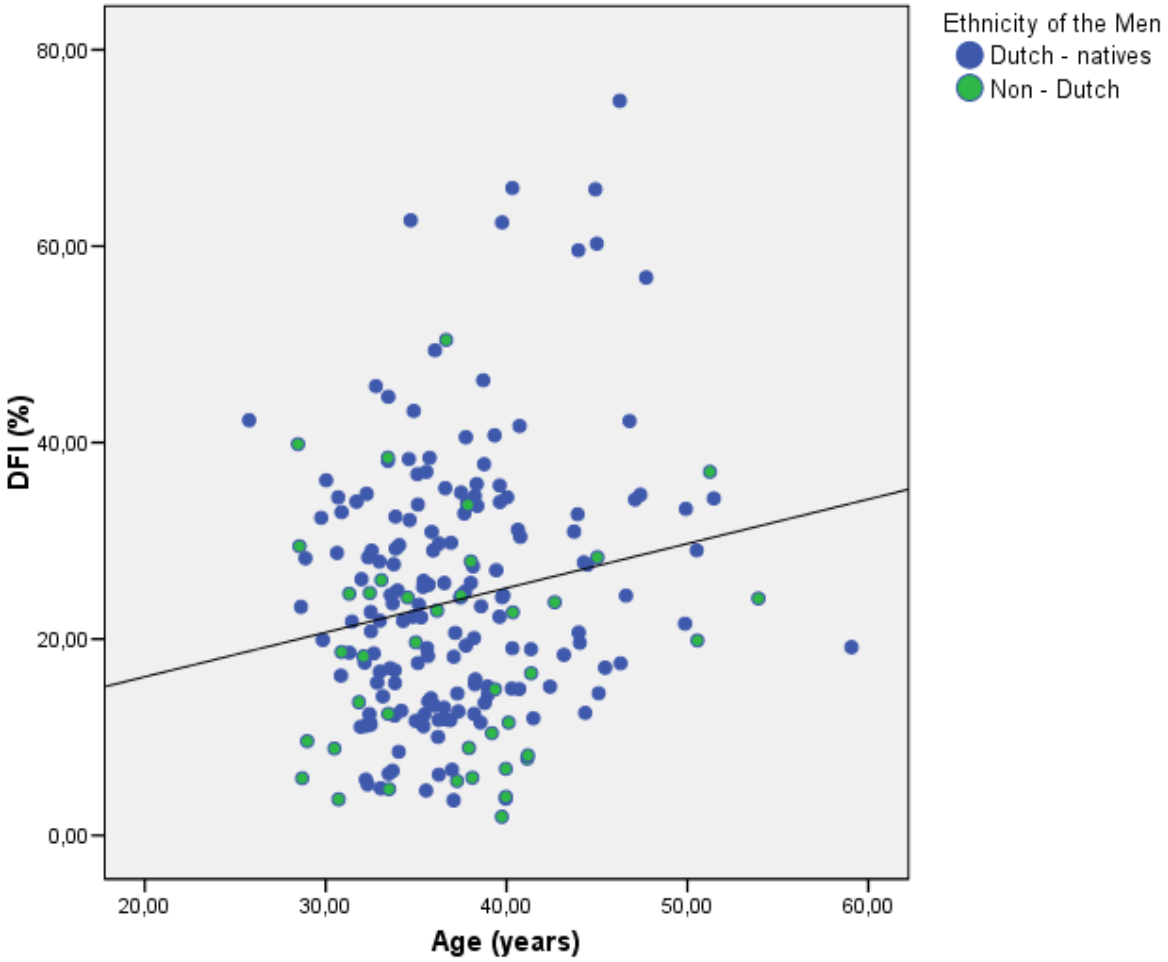


Figure 2 The correlation between age (years) and DFI (%) (r = 0.19; p = < 0.05).



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Chapter 6

Body Mass Index and central adiposity are associated with sperm quality in men of subfertile couples

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Abstract

The objective of this study was to investigate the effect of body mass index (BMI) and waist circumference on semen parameters in men of subfertile couples visiting a tertiary fertility clinic. We included 455 male partners of subfertile couples in a retrospective preconception cohort study .

Semen Volume (mL), semen concentration (millions per mL), percentage of progressive motile and non-motile spermatozoa and total motile sperm count (in millions) were included as main outcome measures.

Overweight was inversely associated with ejaculate volume (β-0.161(s.e.0.07); p=0.02) and the percentage of progressive motility type A (β-0.365(s.e.0.14); p=0.01), and positively associated with the percentage of immotility type C (β 0.210(s.e.0.06); p=0.001). Obesity was inversely associated with ejaculate volume (β-0.228(s.e.0.10); p=0.02), sperm concentration (β-0.678(s.e.0.26); p=0.009) and total motile sperm count (β -0.755(s.e.0.30); p=0.01). Waist circumference ≥102 cm, as measure for central adiposity, was inversely associated with sperm concentration (β-0.615(s.e.0.22); p=0.01) and total motile sperm count (β-0.645(s.e.0.26); p=0.01). All associations remained significant after adjustment for age, ethnicity, active and passive smoking, alcohol and medication use, folate status and a history of andrological surgery.

This study shows that especially sperm concentration and total motile sperm count in men of subfertile couples are detrimentally affected by increasing BMI and central adiposity. The effect of weight loss on sperm quality and fertility needs further investigation.

Introduction

In the Western countries subfertility is a serious health problem affecting 10-15% of all couples trying to conceive. Male factor subfertility accounts for 25-30% of all cases.[1] In the majority of men no apparent cause for the impaired fertility can be found.[1-2] This has drawn attention to the impact of poor lifestyles, such as smoking, alcohol consumption, and a high body mass index (BMI) on sperm quality.[3] In recent decades, the prevalence of overweight and obesity in men of reproductive age has increased dramatically in the Netherlands, of which the trends are similar to other countries.[4-5] Overweight is defined as a BMI between ≥25 and <30 kg/m^2 and obesity as a BMI≥30 kg/m^2.[5]

The adverse effects of a high BMI on female fertility, such as an increased time to conception and menstrual irregularities, are well known.[6-7] Additionally, central adiposity, expressed by waist circumference and waist-hip ratio (WHR), has been shown to independently influence the reproductive potential in women.[8] Evidence about the disadvantages of a high BMI on male fertility are conflicting.[9] This is partially due to studies in which no adjustment are made for confounding variables, such as lifestyle factors. It has been shown that poor nutrition, smoking and alcohol use impair sperm function.[10-11] These lifestyles are associated with excessive oxidative stress, which has been related to male subfertility due to its damaging effects on spermatozoa.[12-13]

Since the increasing prevalence of overweight and obesity in man of reproductive age, this study aims to assess the association between BMI, central adiposity and sperm quality in men visiting the preconception outpatient clinic of the Erasmus University Medical Center in Rotterdam, the Netherlands.

Materials and Method

Study design

Between October 2007 and October 2010 couples planning pregnancy and visiting the outpatient clinic of the department of Obstetrics and Gynaecology of the Erasmus University Medical Center Rotterdam, were offered preconception counselling at the outpatient clinic "Achieving a Healthy Pregnancy".[14] The couples filled out questionnaires from which the following data were extracted: age, ethnicity, educational level, smoking, alcohol consumption, and the use of medication, folic acid and multivitamins. Ethnicity and educational level were classified according to the definitions of Statistics Netherlands.[15] At the preconception counselling visit the questionnaires were checked by the counsellor in detail. Height (m) and weight (kg) were standardized measured to calculate the body mass index (BMI=kg/m^2). The waist circumference (WC) was measured at the narrowest point between the lower border of the rib cage and the iliac crest. Subsequently, venous blood samples were drawn to measure serum and red blood cell (RBC) folate, serum cobalamin and plasma total homocysteïne (tHcy) concentrations. All study participants assigned an informed consent form before participation. The study was approved by the Medical Ethical and Institutional Review Board of the Erasmus University Medical Center in the Netherlands.

Sperm Collection and Analysis

The sperm collection was done within a timeframe of 0-70 days prior to the preconception counselling. Sperm specimens were produced via masturbation after a required abstinence period of 3 to 5 days. After liquefaction, ejaculate volume, sperm concentration, percentage progressive (type A+B) and immotile spermatozoa (type C+D) were assessed according to World Health Organization guidelines.[17] Total sperm count was calculated as the product

between ejaculate volume and sperm concentration. Total motile sperm count was calculated as the product between ejaculate volume, sperm concentration and progressive motile spermatozoa (type A+B). From a clinical relevance point of view we looked at percentage progressive (type A+B) and immotile spermatozoa (type C+D). However, we were also interested in the individual sperm motility parameters.

In addition, the laboratory participates in the external quality control scheme of the Dutch Foundation for Quality Assessment in Clinical Laboratories (SKML).

Laboratory determinations

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 for the concentrations of folate and cobalamin. For the determination of RBC folate and plasma tHcy, venous blood samples were drawn into ethylenediamine tetraacetate (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. Serum samples from each patient were analyzed during routine laboratory procedures for folate, cobalamin, and tHcy using an immunoelectrochemoluminescence assay (E170; Roche Diagnostics GmbH, Mannheim, Germany). Directly after blood sampling, 0.1 ml EDTA-blood was hemolyzed with 0.9 mL of freshly prepared 1.0% ascorbic acid. Subsequently the hematocrit of the EDTA-blood was determined on an ADVIA 120 Hematology Analyzer (Bayer Diagnostics, Leverkusen, Germany). The hemolysate was centrifuged for 5 minutes at 1,000 x g after which the folate concentration was measured in the hemolysate. RBC folate was calculated using the following formula: (nM hemolysate folate x 10/hematocrit) - (nM serum folate x [1 - hematocrit]/hematocrit) = nM RBC folate. tHcy in EDTA plasma was determined using high-

performance liquid chromatography with reversed phase separation and fluorescence detection.[16] Inter-assay coefficients of variation for serum folate were 4.5% at 13 nmol/L and 5.7% at 23 nmol/L, for serum cobalamin 3.6% at 258 pmol/L and 2.2% at 832 pmol/l, for plasma total homocysteine 4.8% at 14.6 mmol/L. The detection limit for serum folate was 1.36 nmol/L, for serum cobalamin 22 pmol/L and for plasma tHcy 4 mmol/L.

Analysis

Men were categorized into three BMI groups: 1) <25 kg/m^2, 2) ≥25 and <30 kg/m^2, and 3) ≥30 kg/m^2, according to the World Health Organization.[5] We dichotomized waist circumference (WC) into high-risk and low-risk groups on the basis of the gender specific cut off point of ≥102 cm for the risk of cardiovascular disease according to the National Institutes of Health.[18] As the distribution of the sperm parameters is skewed, these were log transformed before further analysis. The Kruskal-Wallis test was applied to test differences between the three BMI strata and the various sperm parameters. Spearman's correlation coefficient was calculated between BMI and WC.

The relationships between BMI categories, WC and sperm parameters were studied using a linear regression analysis with adjustment for potential confounders age, ethnicity, active and passive smoking, alcohol and medication use, folate status and history of andrological surgery. The regression coefficient (β) describes how the change of one unit affects each sperm parameter. All statistics were performed by using the SPSS 17 software package (SPSS, Inc., Chicago, IL). A two-tailed p≤.05 was considered statistically significant.

Results

The general characteristics are depicted in Table 1 and stratified according to the three BMI categories. In total 455 men participated, from which 158 (34.7%) had a BMI<25 kg/m^2, 225 (49.5%) a BMI between ≥25 and <30 kg/m^2 and 72 (15.8%) a BMI ≥30 kg/m^2. Men with overweight or obesity were significantly older compared to normal weight men (p≤0.05). Overweight was significantly more present in Dutch men (75.1%) and obesity in non-Western men (37.5%), (p≤0.05). Additionally, obese men consumed significantly less alcohol compared to normal weight and overweight men (p≤0.001). Last, folate RBC is significantly lower in obese men (p=0.03).

Correlation analysis revealed that waist circumference is significantly correlated with BMI (r=0.829; p≤0.01).

Association between BMI, waist circumference and sperm parameters

Table 1 show that overweight and obese men showed a significantly lower ejaculate volume, sperm count (p≤0.05). Interestingly, although total motile sperm count didn't show any significant difference between the groups, overweight and obese men showed a significantly lower percentage progressive motility type A (p=0.04). Furthermore, overweight men showed a significantly higher percentage immotility type C (p=0.003).

We further analysed these associations between BMI and sperm parameters in a multivariable linear regression analysis with adjustment for the potential confounders age, ethnicity, active and passive smoking, alcohol and medication use, history of andrological surgery and folate status (Table 2). BMI analysed as linear variable (BMI linear) and all three BMI categories were inversely associated with ejaculate volume (all p≤0.05). The association was most pronounced in men with BMI≥30 (adjusted β -0.237 (s.e. 0.10), p=0.02). Both BMI

linear and BMI≥30 were inversely associated with sperm concentration (adjusted β -0.056 (s.e. 0.02), p=0.01; adjusted β -0.873 (s.e. 0.27), p=0.001, respectively). Inverse associations were estimated between BMI linear, BMI≥30 and total sperm count, (adjusted β -0.056 (s.e. 0.02), p=0.002) and (adjusted β -1.086 (s.e. 0.28), p=0.000), respectively. In a similar manner BMI linear and BMI≥30 were inversely associated with total motile sperm count (adjusted β -0.068 (s.e. 0.03), p=0.01) and (adjusted β -0.892 (s.e. 0.33), p=0.01), respectively. Furthermore, BMI ≥25-<30 was inversely associated with percentage progressive motility type A (adjusted β-0.295 (s.e. 0.15), p=0.05). BMI linear and the BMI category ≥25-<30 were positively associated with the percentage immotility type C, (adjusted β 0.015 (s.e. 0.01), p=0.05) and (adjusted β 0.187 (s.e. 0.07), p=0.006), respectively.

Association between waist circumference and sperm parameters

Men with a waist circumference ≥102 cm had a significantly lower sperm concentration compared to men with a waist circumference<102 cm, respectively 30.0 (0-661) and 20.0 (0-350); p≤0.05. Table 3 shows the multivariate linear regression analysis with adjustment for confounders. After adjustment a waist circumference ≥102 cm remained inversely associated with sperm concentration (adjusted β -0.623 (s.e. 0.22), p=0.01). Furthermore, waist circumference ≥102 cm was also negatively associated with total sperm count (adjusted β -0.750 (s.e. 0.23), p=0.001) and total motile sperm count (adjusted β -0.603 (s.e. 0.27), p=0.03). To investigate whether fat distribution explained these associations, we additionally adjusted for BMI in the linear regression analysis after which all associations disappeared.

Discussion

This study demonstrates that BMI and waist circumference - independent of other lifestyle factors - affect sperm quality in men of subfertile couples attending an outpatient preconception clinic. Being overweight is associated with a significantly lower ejaculate volume, a lower percentage of progressive motility type A and a higher percentage of motility type C. Furthermore, obesity is associated with an even significantly lower ejaculate volume, lower sperm concentration, lower total sperm count and a lower total motile sperm count. A waist circumference ≥102 cm, a marker for central adiposity, was associated with a lower sperm concentration, lower total sperm count and a lower total motile sperm count. Due to the high correlation between BMI and waist circumference, these associations, disappeared after adjustment for BMI.

Thus, body weight and waist circumference are especially associated with ejaculate volume, sperm concentration and sperm motility. These associations have also been investigated and described by others.[9] Our findings, however, are in contrast to a recent Dutch study[19] which observed no significant association between BMI and sperm parameters. This lack of an association may be a statistical power issue, given that a smaller proportion of obese men (10.4%) compared to the 15.8% in our study has been investigated. In addition, it is not clear whether the anthropometric features were standardized measured or self-reported. The latter could have induced a differential misclassification of the exposure of interest, which may have led to an underestimation of obesity resulting in a non-significant estimate. This is supported by others showing that the prevalence of obesity based on self-reported data underestimates the true prevalence.[20]

In line with the study of Chavarro et al, we also found different effects of the BMI strata on sperm parameters.[21] They reported a similar inverse association between BMI and ejaculate

volume and total sperm count. However, they didn't find an association between BMI and sperm concentration, which is the most consistent finding across studies.[3,22-24] Furthermore, opposite to our results, the group of Chavarro showed that men with overweight had a higher percentage of progressive motile sperm.

Our findings of the association between a high BMI and sperm parameters strengthen previously reported studies in Europe and the United States.[3,22-23] The majority of these studies focused only on BMI as the predominant measure of adiposity and not on waist circumference. The sensitivity of BMI in estimating individuals body fat mass suffers from the inability to distinguish between variability in body composition and body fat mass distribution.[25] Recent studies indicated that abdominal obesity is more strongly associated with obesity-related health problems than adiposity measured by BMI.[26] In women it has been shown that differences in fat mass distribution exist between subfertile women and normal controls. The different fat mass patterns were accompanied by different prognoses of fertility.[27] We have shown that men with a waist circumference of ≥102 cm have lower sperm concentrations, total sperm count and total motile sperm count. However, after additional adjustment for BMI in the linear regression analysis the association attenuated, which may indicate that BMI and WC are intermediates in the same pathway. BMI and WC are intermediates in the same pathway. BMI and WHR are highly correlated, and it can be speculated that they are both involved in the same causal pathway. Therefore, it is not unlikely that BMI can be simultaneously a confounder and an intermediate variable in the causal pathway of WC and semen quality. Therefore including BMI in the regression model may underestimate the true effect of WC.

Several mechanisms might account for the harmful effects of a high BMI on sperm parameters. Numerous studies have noted that obesity and several of its causes, such as

insulin resistance and dyslipidaemia, are associated with increased oxidative stress.[28-29] Oxidative stress is an independent marker for male factor subfertility since it impairs sperm quality.[30] An animal study showed that obesity increases oxidative stress and as a result reduced sperm motility and increased DNA damage.[31]

It has also been suggested that the detrimental influence of a high body weight on sperm quality is partially driven by an altered reproductive hormonal profile.[3,32] Overweight and obesity, particularly when central, have been shown to affect the GnRH-LH/FSH pulse, which may impair Leydig and Sertoli cell functions and thus interfere with the release of sex hormones and production and maturation of sperm.[33] Consequently, a high BMI is associated with lower levels of total testosterone, SHBG and inhibin B and higher levels of serum estradiol.[3, 21] Additionally, serum leptin, which is higher in overweight and obese men, inhibits testosterone synthesis which is a cause of impaired sperm quality.[34] However, the levels across which alterations of these hormones have a deleterious effect on sperm quality are unknown. In our study we were not able to substantiate our findings with changes in male sex hormonal levels. While weight loss normalizes testosterone and inhibin B levels in obese men, it is unknown whether this also restores sperm quality.[35] A previous study concluded that associations between male BMI and sperm quality were found to be statistically significant even after adjustment for reproductive hormones.[36] This suggests that a hormonal explanation as the sole mechanism is unlikely. Future studies are needed to investigate this finding in more detail.

Finally, overweight and obesity are often associated with a diet characterised by foods containing excessive amounts of macronutrients and poor micronutrient concentrations, which can ultimately lead to essential nutrient deficiency involved in male fertility. Folate and zinc play an important role in male reproduction.[37-39] In an RCT Wong et al. showed a

significant 76% increase in sperm count after the use of folic acid and zinc supplements for a period of 26 weeks.[38] This is supported by the observation that a strong adherence to the traditional Dutch diet, comprising of potatoes, whole grains and meat as a rich source of folate and zinc, was significantly associated with a higher sperm concentration.[10]

The strengths and weaknesses of the study design need to be addressed. Strengths of our study are the prospective design and the assessment of standardized anthropometric measures and potential confounders in a relatively large homogenous group of men in subfertile couples. This has never been performed in previous studies. To prevent selection bias we included men of subfertile couples planning pregnancy visiting one tertiary center between October 2007 and October 2010. BMI was measured in a standardized way, as well as semen parameters and biomarkers. Semen parameters and biomarkers were also measured at one single center and laboratory. A limitation might be that only one single sperm analysis was performed in this study. However, we do not believe that this poses a major threat to the validity, whilst a population based study showed that analyzing multiple sperm samples per subject does not seem superior to a single sperm sample analysis.[40] Finally, this study was performed in men of subfertile couples which limit its external validity and the result can not be extended to the general population.

A high BMI and a high waist circumference detrimentally affect sperm quality. Increased awareness of the target population of men, gynecologists, urologists, andrologists and general practitioners is needed to address the importance of this relationship. Future preventive interventions should be developed and directed at men to loose weight especially during the window of planning pregnancy. However, this emphasizes the need of intervention studies directed on the effects of loosing weight on sperm quality. Future

studies are also needed to gain insight into the underlying mechanisms and the effects on fertility outcome.

Table 1
Characteristics of men of subfertile couples (n=455)

	BMI<25 (n= 158)	BMI≥25-<30 (n=225)	BMI≥30 (n=72)	*p*
Age (years), median	33.4 (22.7-60.5)	35.4 (24.3-56.7)	35.3 (21.8-52.3)	0.03
Waist Circumference(cm)	85.0 (65-106)	95.0 (81-110)	113.0 (95-135)	≤0.001
Hip Circumference	100 (78-112)	107 (76-120)	118 (97-154)	≤0.001
Waist-Hip ratio	0.85 (0.64-1.22)	0.90 (0.76-1.20)	0.95 (0.86-1.15)	≤0.001
Ethnicity n (%)				0.04
Dutch	108 (68.4)	169 (75.1)	39 (54.9)	
Other – Western,	13 (8.2)	12 (5.3)	5 (7.0)	
Non-western	36 (22.8)	43 (19.1)	27 (38.0)	
Educational level n (%)				0.07
High	67 (42.4)	85 (37.8)	17 (23.9)	
Intermediate	65 (41.1)	89 (39.6)	36 (50.7)	
Low	26 (16.5)	51 (22.7)	18 (25.4)	
Subfertility n (%)				0.9
Primary	101 (72.1)	138 (70.4)	47 (73.4)	
Secondary	39 (27.9)	58 (29.6)	17 (26.6)	
Lifestyles n (%)				
Smoking (yes)	38 (24.4)	64 (28.8)	22 (31.0)	0.5
Smoking of Partner (yes)	31 (19.6)	52 (23.1)	13 (18.3)	0.4
Alcohol (yes), n (%)	124 (78.5)	170 (75.6)	39 (54.9)	≤0.001
Folic acid supplement use (yes)	19 (12)	17 (7.5)	7 (9.8)	0.71
Multivitamin supplement use (yes)	44 (27.8)	53 (23.7)	16 (22.5)	0.81
Medication use (prescribed and over the counter) (yes)	40 (25.6)	53 (23.7)	24 (33.8)	0.24
History of andrological surgery [a], n (%)	25 (16.0)	47 (21.2)	9 (13.2)	0.23
Biochemical Parameters				
Folate (nmol/L)	18.5 (6-64)	17.3 (7-45)	16.3 (8-33)	0.28
Folate RBC (nmol/L)	874 (64-2247)	948 (153 -2194)	869 (474-1622)	0.03
Cobalamin (pmol/L)	319 (122-1130)	290.5 (141-844)	281 (114-1475)	0.15
tHcy (µmol/L)	11.5 (5-44)	11.4 (6-35)	11.0 (7-26)	0.27
Sperm parameters (p25-p75)				
Ejaculate volume(mL)	3.0 (1.8-4.0)	2.7 (1.5-3.5)	2.4 (1.6-3.4)	0.02
Sperm concentration (10^6/mL)	34 (9.2-62.3)	23 (6.8-51.5)	18 (1.1-60.3)	0.08
Sperm count	67.9 (20.6-186.7)	49.6 (14-124.8)	45.9 (2.8-147.5)	0.02
Total motile sperm count (10^6/mL)	27.1 (4.1-84.6)	17.2 (2.8-50.0)	15.8 (6.4-73.3)	0.05
Progressive motility (A+B) (%)	38.5 (22.0-48.3)	37.0 (21.0-47.0)	39.5 (23.0-49.0)	0.53
Immotile sperm (C+D) (%)	61.5 (51.8-78.0)	63.0 (53.0-79.0)	60.5 (51.0-77.0)	0.53

Note: p≤.05 was considered statistically significant. Values are expressed as median (range), median (p25-p75) or as number (%) per BMI stratum. Not all percentages count up to 100% due to missings. Total sperm count=ejaculate volume x sperm concentration. Total motile sperm count=ejaculate volume x sperm concentration x progressive motile spermatozoa (type A+B).

[a] Surgery for varicocele, orchidopexy, vasovasostomy and testis carcinoma.

Table 2
Associations between BMI and sperm parameters

		BMI Crude	BMI Adjusted [a]	BMI<25 Crude	BMI<25 Adjusted [a]	BMI≥25-<30 Crude	BMI≥25-<30 Adjusted [a]	BMI≥30 Crude	BMI≥30 Adjusted [a]
Sperm Parameters									
Ejaculate volume (mL)	*β (s.e.)*	-0.026(0.01)	-0.024(0.01)	0.178(0.07)	0.167(0.07)	-0.161(0.07)	-0.146(0.07)	-0.228(0.10)	-0.237(0.10)
	p	0.001	0.003	0.01	0.02	0.02	0.05	0.02	0.02
Sperm concentration (10^6/mL)	*β (s.e.)*	-0.039(0.02)	-0.056(0.02)	0.273(0.18)	0.273(0.19)	-0.137(0.19)	-0.088(0.19)	-0.678(0.26)	-0.873(0.27)
	p	0.06	0.01	0.13	0.14	0.47	0.65	0.009	0.001
Total sperm count (10^6/mL)	*β (s.e.)*	-0.056(0.02)	-0.080(0.02)	0.437(0.18)	0.422(0.19)	-0.280(0.19)	-0.218(0.20)	-0.902(0.26)	-1.086(0.28)
	p	0.002	0.000	0.02	0.03	0.15	0.28	0.001	0.00
Total motile sperm count (10^6/mL)	*β (s.e.)*	-0.057(0.02)	-0.068(0.03)	0.375(0.21)	0.309(0.22)	-0.247(0.22)	-0.133(0.23)	-0.755(0.30)	-0.892(0.33)
	p	0.02	0.01	0.08	0.16	0.27	0.57	0.01	0.01
Progressive motility (A+B)(%)	*β (s.e.)*	-0.001(0.01)	-0.002(0.01)	0.074(0.08)	0.045(0.09)	-0.102(0.08)	-0.065(0.09)	0.014(0.11)	0.024(0.13)
	p	0.90	0.85	0.34	0.60	0.21	0.47	0.90	0.85
Immotile sperm (C+D)(%)	*β (s.e.)*	0 (0.003)	-0.001 (0.004)	-0.025(0.03)	-0.013(0.03)	0.036(0.03)	0.023(0.03)	-0.006(0.04)	-0.022(0.05)
	p	0.98	0.88	0.37	0.67	0.24	0.46	0.89	0.62

Note: p≤.05 was considered statistically significant. All data in the table are presented as unstandardised adjusted linear regression coefficients (ß) (standard error (s.e.)) which reflect the relative effect per 1 point of BMI on the respective sperm parameter. Total sperm count=ejaculate volume x sperm concentration. Total motile sperm count=ejaculate volume x sperm concentration x progressive motile spermatozoa (type A+B).

[a] p-values are adjusted for the following covariates: age (in years), ethnicity, active and passive smoking, alcohol , medication use, history of andrological surgery and folate status.

Table 3
Associations between waist circumference and sperm parameters

		Waist Circumference <102 cm Crude	Waist Circumference <102 cm Adjusted [a]	Waist Circumference ≥102 cm Crude	Waist Circumference ≥102 cm Adjusted [a]
Sperm Parameters					
Ejaculate volume (mL)	*β (s.e.)*	-0.047 (0.08)	-0.022 (0.09)	-0.133 (0.08)	-0.149 (0.09)
	p	0.56	0.80	0.11	0.09
Sperm concentrations (10^6/mL)	*β (s.e.)*	0.100 (0.21)	0.152 (0.22)	-0.615 (0.22)	-0.623 (0.22)
	p	0.63	0.48	0.01	0.01
Total sperm count (10^6/mL)	*β (s.e.)*	0.041 (0.22)	0.125(0.22)	-0.728 (0.22)	-0.750(0.23)
	p	0.85	0.58	0.001	0.001
Total motile sperm count (10^6/mL)	*β (s.e.)*	-0.158 (0.25)	-0.039 (0.26)	-0.645 (0.26)	-0.603 (0.27)
	p	0.53	0.88	0.01	0.03
Progressive motility (A+B) (%)	*β (s.e.)*	-0.159 (0.09)	-0.123 (0.10)	-0.122 (0.10)	-0.067 (0.11)
	p	0.09	0.23	0.21	0.52
Immotile sperm (C+D) (%)	*β (s.e.)*	0.049 (0.034)	0.035 (0.04)	0.030 (0.036)	0.003 (0.04)
	p	0.16	0.34	0.40	0.95

Note: p≤.05 was considered statistically significant. All data in the table are presented as unstandardised adjusted linear regression coefficients (ß) (standard error (s.e.)) which reflect the relative effect of waist circumference on the respective sperm parameter. Total sperm count=ejaculate volume x sperm concentration. Total motile sperm count=ejaculate volume x sperm concentration x progressive motile spermatozoa (type A+B).

[a] p-values are adjusted for the following covariates: age (in years), ethnicity, active and passive smoking, alcohol, medication use, history of andrological surgery and folate status.

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Chapter 7

Tailored preconceptional dietary and lifestyle counselling in a tertiary outpatient clinic in the Netherlands

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Abstract

Adverse reproductive performance has been linked to unhealthy dietary intake and lifestyles. Our objectives were to investigate the prevalence of unhealthy dietary intake and lifestyles before conception and to evaluate whether tailored preconception counselling modifies these behaviours.

Between October 2007 and April 2009 419 couples received tailored preconception dietary and lifestyle counselling at the outpatient clinic of Obstetrics and Gynaecology of the Erasmus University Medical Center Rotterdam, the Netherlands. A subgroup (n=110 couples) was counselled twice with a fixed time interval of 3 months. Self-administered questionnaires were used for tailored dietary and lifestyle counselling. A cumulative score based on six Dutch dietary guidelines was displayed in the personal Preconception Dietary Risk score (PDR-score). In a similar manner the Rotterdam Reproduction Risk score (R3-score) was calculated from lifestyle factors (women: 13 items, men: 10 items). Univariate and paired tests were used.

Most couples (93.8%) were subfertile. At the second counselling, the percentage consuming the recommended intake of fruit had increased from 65 to 80 in women and from 49 to 68 in men and the percentage of women getting the recommended intake of fish increased from 39 to 52. As a consequence the median PDR-score was decreased (women: 2.6 (95% CI 2.4-2.9) to 2.4(95% CI 2.1-2.6), men: 2.5(95% CI 2.3-2.7) to 2.2 (95% CI 1.9-2.4), both $p<0.05$). The median R3-scores were also lower (women: 4.7(95% CI 4.3-5.0) to 3.1 (95% CI 2.8-3.4), men: 3.0 (95% CI 2.8-3.3) to 2.0 (95% CI 1.7-2.3), both $p<0.01$) due to less alcohol use (-14.6%), more physical exercise and folic acid use in women, and less alcohol use in men (-19.4%) (all $p<0.01$). The R3-scores in women and men were decreased in all ethnicity, educational level, neighbourhood and BMI categories. However, low educated women appeared to show a larger reduction than better educated women and men with a normal BMI to show a larger decrease than overweight men. The reduction in the PDR-score of women was similar in both ethnic groups. More than 85% women and men found the counselling useful and around 70% recommends it to others.

Tailored preconception counselling about unhealthy dietary and lifestyle behaviours of subfertile couples in an outpatient tertiary clinic is feasible and seems to decrease the prevalence of harmful behaviours in the short term. These results with subfertile couples are promising and illustrate their opportunities to contribute to reproductive performance and pregnancy outcome.

Introduction

The high prevalence rates of unhealthy diets and lifestyles in the reproductive population in industrialized countries are worrisome.[1-3] Current evidence indicates that unhealthy preconceptional diets and lifestyles of both women and men significantly contribute to impaired reproduction with long-term consequences for parental health and health of their offspring. [4-7] Health professionals and parents-to-be generally are unaware of these adverse effects[8-9], and adjustment of such habits is generally not perceived as beneficial for reproduction. The available evidence justifies reorganization and redefining obstetrical care such that it includes preconceptional screening and informing of parents-to-be, and support to those who intend to change unhealthy diets and lifestyles. [10-11]

The public recommendation of periconceptional maternal folic acid use is an example of the introduction of a preconceptional measure to prevent adverse pregnancy outcomes, in particular neural tube defects[12]. It may also positively influence follicular-, oocyte-, embryonic-, placental- and fetal growth.[13-14] Despite its obvious benefits, compliance is moderate and therefore public health efforts should be reinforced by systematic individual preconceptional care to all parents-to-be. The preconceptional window allows for a personal contribution to a successful reproductive career and seems suitable to include lifestyle modification too.[15] Organized preconceptional care programmes to stimulate a healthy dietary intake and lifestyle behaviours, however, are scarce.[16] Therefore, the department of Obstetrics and Gynaecology of the Erasmus University Medical Center in Rotterdam started an outpatient clinic on preconceptional tailored dietary and lifestyle counselling “Achieving a Healthy Pregnancy”. In the current evaluation we investigated the prevalence of unhealthy diet and lifestyles in mainly subfertile couples planning pregnancy, the effects of preconception counselling on the improvement of these behaviours and the influence of personal characteristics on these determinants.

Materials and Method

Study design

Between October 2007 and April 2009 couples planning pregnancy and visiting the outpatient clinic of the department of Obstetrics and Gynaecology of the Erasmus University Medical Center Rotterdam were offered preconception counselling at the outpatient clinic "Achieving a Healthy Pregnancy". At the first gynaecological visit couples were referred for the preconceptional counselling tailored on dietary intake and lifestyle. They received a flyer with information and a self-administered questionnaire to be filled out at home. The questionnaires were used for individual tailored counselling during the outpatient visit of the couple.

From the questionnaire we extracted the following data: age, ethnicity, educational level, indication for referral, dietary intake, lifestyle factors (smoking, alcohol and drug use), medication and vitamin use. Ethnicity and educational level were classified according to the definitions of Statistics Netherlands.[17] Educational level was divided into three categories: low (primary/lower vocational/intermediate secondary), intermediate (intermediate vocational/higher secondary) and high (higher vocational/university).[17]

Preconception counselling on dietary intake and lifestyle

At the first outpatient preconception counselling (PC1) visit, the filled out questionnaires were checked by the counsellor, and height and weight were measured, to calculate the body mass index (BMI=weight in kilograms divided by squared height in centimetres). Additionally, waist-hip circumference and blood pressure were measured. During the counselling the questionnaire data were discussed in detail for tailored dietary and lifestyle advice. For example if the woman and/or man smoke, they receive the following comment and advice:

"You urgently have to quit smoking, because in both women and men who smoke the time to conceive is much longer than in non-smokers. Tobacco smoke contains compounds that detrimentally affect the female and male gametes. Moreover, women who smoke have a higher risk of experiencing a miscarriage and pregnancy-related complications, such as intrauterine growth restriction".

Laboratory determinations

Venous blood samples were drawn to measure sensitive biomarkers of the homocysteine pathway to obtain unbiased information on the intake of foods related to this pathway, i.e., serum and red blood cell (RBC) folate, serum cobalamin and plasma total homocysteine (tHcy). Venous blood samples were drawn into dry vacutainer tubes and allowed to clot. After centrifugation at 2000 g, serum was collected before being assayed for the concentrations of folate and cobalamin. For the determination of RBC folate and plasma tHcy, venous blood samples were drawn into ethylenediamine tetraacetate (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. Serum samples from each patient were analysed during routine laboratory procedures for folate, cobalamin, and tHcy using an immunoelectrochemoluminescence assay (E170; Roche Diagnostics GmbH, Mannheim, Germany). Directly after blood sampling, 0.1 ml EDTA tube was haemolysed with 0.9 ml of freshly prepared 1.0% ascorbic acid. Subsequently the hematocrit of the EDTA-blood was determined on an ADVIA 120 Hematology Analyzer (Bayer Diagnostics, Leverkusen,Germany). The hemolysate was centrifuged for 5 minutes at 1000 g after which the folate concentration was measured in the haemolysate. RBC folate was calculated using the following formula: (nM haemolysate folate x 10/haematocrit) - (nM serum folate x [1 - haematocrit]/haematocrit) = nM RBC folate. tHcy in EDTA plasma was determined using high-performance liquid

chromatography with reversed phase separation and fluorescence detection [18]. Inter-assay coefficients of variation for serum folate were 4.5% at 13 nmol/L and 5.7% at 23 nmol/L, for serum cobalamin 3.6% at 258 pmol/L and 2.2% at 832 pmol/L, for plasma tHcy 4.8% at 14.6 mmol/L. The detection limit for serum folate was 1.36 nmol/L, for serum cobalamin 22 pmol/L and for plasma tHcy 4 mmol/L.

Tailored preconception dietary and lifestyle counselling

Within the infrastructure of the Dutch Preconception Center of Excellence Rotterdam we developed and provided individual tailored preconception dietary and lifestyle counselling using the attitude social influence efficacy (ASE) model.[19] The ASE model has been frequently used for the development of health education and prevention and is based on the interplay of attitudes, social influences and self-efficacy of an individual. Attitudes are the opinions of a person based on knowledge, experience and examples of others. Social influences include social norms, perceived behaviours of others, and direct pressure or support to perform a behaviour. Finally, self-efficacy includes confidence in one's ability to perform a behaviour intention and progression through the stages of change. Together these factors determine the intention to perform or change certain behaviour. Whether or not the behavioural intention actually is performed depends in the ASE model from thresholds and positive incentives. Following the ASE structure we intended to modify intentions towards a healthier diet and lifestyle in terms of improved reproductive performance. A specific feature was that change was aimed in both women and men.

The couple filled out an informed consent form and an evaluation form about their experiences of the preconception counselling. Moreover, they were offered a voluntary second counselling after 3 months. Within 3 weeks after the first counselling couples received a letter in which the identified (un)healthy dietary and lifestyle factors, biomarker concentrations and advises are reported.

Preconception Dietary Risk score (PDR-score)

Six questions about dietary intake were filled out by the couple and estimate the general personal intake of six main food groups, with responses defined according to the food-based dietary guidelines of the Dutch Nutrition Center in the Netherlands.[20] The guidelines included: at least four slices of brown bread daily, the use of monounsaturated or polyunsaturated oils/fats, at least 200 grams of vegetables daily, at least two pieces of fruit daily, at least three to four servings of meat a week, and at least one to two servings of fish a week. Each person received one point for every food group where they consumed less than the recommended amount; subsequently the total score was calculated and expressed by the individual the Preconception Dietary Risk score (PDR-score). We based the PDR-score on the unweighted summation of affirmative compliant responses. Consequently, the range of the PDR score was 0 - 6, where 6 implies a highly inadequate diet.

Rotterdam Reproduction Risk score (R3-score)

The Rotterdam Reproduction Risk Score (R3-score) was created and based on the current scientific evidence of harmful effects of modifiable lifestyle risk factors (see addendum1). A similar approach has been used by the United States 'Special Supplemental Food Program for Women, Infants and Children' (WIC).[21] Each person received one point for every risk factor; subsequently the total score was calculated and expressed by the individual R3-score. The R3-score comprises of the following risk factors: no folic acid supplement use, use of medication (over the counter), smoking (yes, no), alcohol use (yes, no), caffeine use (≥6 cups a day), drug use (yes, no), physical exercise (yes, no), infection risk (yes; including Rubella or Toxoplasmosis or Listeriosis, no), BMI (<20 of ≥30 kg/m^2), waist circumference (woman:≥88 cm and man: ≥102 cm), waist-to-hip-ratio (≥0.8), blood pressure (systolic ≥160 or diastolic ≥90 mmHg) and deranged homocysteine pathway: folate: serum <15 nmol/L or (RBC) <500 nmol/L), or vitamin

B12 serum <160 pmol/l or tHcy>15 µmol/L. To reduce infection risk, we informed and advised women about the risks of consuming foods, such as raw meat/fish, and raw milk cheeses. Thus, they could change this risk by avoiding the intake of potentially contaminated foods with Toxoplasmosis and/or Listeriosis. Furthermore, when the women were not vaccinated for Rubella we indicated the need for vaccination to the woman and treating gynaecologist.

For women the maximum score was 13. For men the maximum score was 10 because of excluding: folic acid supplement use, infection risk and waist-hip ratio since those factors are not related to reproductive performance and pregnancy outcome in men. Furthermore, age, ethnicity, educational level, marital status and parity are not modifiable and therefore not included in the R3-score.

Statistical analysis

The Kolmogorov Smirnov test was used to test for normality of the continuous variables. The variables that were not normally distributed were presented as medians with ranges and all other variables with numbers and percentages. The Wilcoxon signed rank test was used to analyse differences between paired continuous variables, the McNemar test for paired dichotomous variables, the Mann-Whitney U test for non-paired continuous variables and the Chi-Square for non-paired categorical variables. A p-value of <0.05 was considered statistically significant. All statistical analyses were performed using SPSS 15.0 for Windows (SPSS Inc, Chicago, Il, USA).

Results

General characteristics

In Table I, the non-modifiable and 6 modifiable dietary and 12 lifestyle risk factors are presented of the 419 couples and stratified into couples receiving preconception counselling (PC1) once (n=309) or twice after a fixed interval of 3 months (n=110, PC2).

The median age of the total group of couples at PC1 was about 31 years, 56% had a Dutch ethnicity, 35% were high educated, and the main indication for referral to the outpatient preconception clinic was subfertility (93.8%). These characteristics were not significantly different between couples counselled once or twice.

The non-modifiable and modifiable dietary risk factors were not significantly different between women or men who came for preconception counselling once or twice. Modifiable risk factors were also comparable between the two groups of women and men. However, more women who came for a second counselling were more often obese, had a higher waist circumference, waist-hip ratio and did not exercise.

None of the couples showed a highly adequate diet that conformed to the guidelines, i.e., PDR-score of 0. Most (>50%) women showed inadequate intakes of bread, vegetables, and fish but adequate intakes of butter/oils, fruit and meat. Most men had inadequate intakes of vegetables and fish but adequate intakes of bread, butter/oils, fruit and meat. In the total groups, overweight (BMI 25-30) or obesity (BMI ≥30) was present in 46.1% of the women and in 58.1% of the men. The median waist circumference was 90 cm (65-126) in women and 95 cm (78-137) in men. For the waist-hip ratio this was 0.86 (0.67-1.41) and 0.92 (0.78-1.20), respectively. Both median systolic and diastolic blood pressure were within normal ranges in women and men. A pregnancy related infection risk was present in 38.7% of the women and 29.1% of the women used medication. In men 22.4% used medication. In women and men 49.6% and 59.7% consumed caffeine beverages, 11.7% and 29.3% smoked, 41.8% and 65%

used alcohol, 2.1% and 7.3% used drugs respectively, and 65.9% of the women and 57.7% of the men did not physically exercise. In women 63.5% used folic acid supplements.

Dietary intake and lifestyle risk factors

In Table II, the effects after 3 months of preconceptional tailored dietary and lifestyle counselling are depicted. The median PDR score decreased significantly in the total group of women and men, 2.6(95% CI 2.4-2.9) to 2.4(95% CI 2.1-2.6) and 2.5(95% CI 2.3-2.7) to 2.2(95% CI 1.9-2.4), respectively, both $p<0.05$. This indicates that they better meet the food-based dietary guidelines. In women this effect is mainly due to a higher percentage taking at least guideline amounts of fruit (64.5% to 80%, $p<0.05$) and fish (39.1% to 51.8%, $p<0.05$), respectively. The percentage of men eating sufficient fruit increased (48.5% to 68%, $p<0.05$).

R3-score decreased from 4.7(95% CI 4.3-5.0) to 3.1(95% CI 2.8-3.4) in women and from 3.0 (95% CI 2.8-3.3) to 2.0 (95% CI 1.7-2.3) in men, both $p<0.01$ reflecting an improved lifestyle. In women, this was due to decreases in the percentages using alcohol (-14.6%), at risk of infection (-34.5%), and to increases in the percentage taking physical exercise (+ 43.7%) and starting to use a folic acid supplement (+ 17.2%) (all $p<0.01$). In men the prevalence of alcohol users decreased 19.4% ($p<0.01$). Reductions in the R3 and PDR scores were seen in all groups, but the reduction in R3 appeared to be larger in low educated women and in men with a normal BMI.

Biomarkers

As shown in Table III, in women and men attending for one or two sessions, the median concentrations of serum and RBC folate, serum vitamin B12 and plasma tHcy were within the normal range. Biomarker concentrations were not significantly different between women and men who were counselled once or twice, except a lower RBC folate in men who underwent counselling twice. At the second visit in women tHcy decreased from 8.6 mmol/L (95% CI 8.3-8.8) to 7.7 mmol/L (95% CI 7.4-8.0), $p<0.05$. In men serum folate and RBC folate increased from 16.6 nmol/L (95% CI 15.0-18.2) to 19.4 nmol/L (95% CI 17.8-21.0) and from 657 nmol/L (95% CI 626-688) to 739 nmol/L (95% CI 689-789), respectively, both $p<0.05$.

Evaluation of preconceptional tailored dietary and lifestyle counselling

Table IV shows the assessment of the preconception counselling by the women and men. Most couples were referred to the preconception counselling clinic by the gynaecologist (women 74.7% and men 57.5%). Most women and men found the preconception counselling very useful (64% and 58.7%), understood all information (90.7% and 83.1%), did not feel pressure to change their diet and lifestyle risk factors (81.1% and 75.2%), felt happy about the counselling (81.6% and 75.2%), and recommended the counselling to others (75.4% and 68%) respectively. There was no significant difference in the rating between women and men who visited the preconception counselling once or twice. In the subgroup that was counselled twice, men found the second counselling less useful.

Discussion

The results of this study suggest that tailored preconceptional dietary and lifestyle counselling is effective in subfertile couples to change unhealthy behaviours within 3 months. In women and men the improvement in dietary intake (PDR-score) was achieved independent of ethnicity. The strongest effects were observed in women with low education, normal weight and living in a non-deprived neighbourhood, and in normal weight men with intermediate/high education. The significant improvement in the lifestyle risk factors (R3-score) was in both women and men independent of ethnicity, education, neighbourhood and BMI. The differences in R3-scores between the subgroups of women and men at baseline disappeared after 3 months except for BMI. These data very much encourages tailored preconception dietary and lifestyle counselling, because it is known that ethnic minorities and populations with a low education and living in deprived neighbourhoods are very difficult to reach and motivate to change unhealthy behaviours.[22] Despite intensive health care efforts, low socioeconomic groups still have a poorer health and shorter life expectancy and higher risk of adverse pregnancy outcome compared with high socioeconomic groups.[23] This is caused amongst others by a higher prevalence of unhealthy dietary and lifestyle behaviours, such as a low intake of vegetables and fruits, obesity, smoking, and poor living and working conditions.[23] This is substantiated in our study with a higher PDR- and R3-score among couples with low education used as proxy of low socioeconomic class.

We realize that these changes were achieved in a selective group of motivated mainly subfertile couples who voluntarily returned for a second preconception counselling. Since the given advices were offered without obligations, it is likely that even more health benefits can be achieved if the preconception counselling is mandatory and has consequences for the accessibility of fertility treatment.

In The Netherlands as well as in other countries the prevalence of unhealthy dietary intake and lifestyles is high.[22] Our study clearly showed that the frequency of those factors is similar in subfertile couples planning pregnancy and that the knowledge about these risk factors is lacking despite the wish to be informed. This is in line with our observation that 93.4% of the women and 86.6% of the men found the preconception counselling useful and underscore the importance of using the preconception period as 'window of opportunity' to optimize dietary and lifestyle behaviours.[10]

Furthermore, we established an increase in folic acid supplement use in women 3 months after counselling. A Dutch study showed that 50% of pregnant women used folic acid after an intensive mass media campaign for the entire advised period.[24] A proactive intervention of Dutch pharmacists at informing and motivating women taking oral contraceptives to start taking folic acid supplements before pregnancy showed a significantly increase in folic acid supplement use.[25] This is in line with our study since tailored preconception counselling was effective to increase folic acid supplement use up to 84.5%. This may suggest that tailored personalized counselling is more effective than anonymous public campaigns.

The major strength of this study is that we implemented preconception counselling in a clinical setting, offered this to both women and men planning pregnancy, and included a follow-up period to examine changes in behaviours. This is unique as most studies obtained retrospective information in women only.[26-28] Additionally, the effectiveness of counselling of the couple is assumed to be higher than that of the woman only.[29] We validated the questionnaire data on dietary intake, i.e., PDR-score, and folic acid supplement use by measuring some of the biomarkers of the homocysteine pathway in which the B vitamins in fruit, vegetables and vitamin preparations play an important role. In this clinical evaluation the higher B vitamin and lower tHcy, albeit not always significant, are reflected by the higher intake of folic acid supplement use and fruit. In the clinical setting when using instruments we always have to

consider the time constraints. That was the rational for using a six-item food questionnaire and not a time consuming food frequency questionnaire (FFQ). Recently, Crozier et al (2009) developed a 20-item FFQ to assess a prudent dietary pattern. This could be a useful instrument for future preconception dietary counselling. We developed the PDR-score as a novel tool to predict an (in)adequate dietary intake of the women and men. Although our data, i.e., questionnaires and biomarkers, are in line with previous findings on the dietary intake of couples in reproductive age, the PDR-score should be further evaluated with regard to its measure of overall healthy nutrient intake.[30] Furthermore, since it is very difficult to give a valid weight to each of the R3-risk factors in association with outcome, we have given the same weight to each factor. We realize, however, that some risk factors should be weighted more than others, such as smoking.

Finally, in our study, 46% of the women and 38% of the men had a non-Dutch ethnicity, which is a good reflection of the multi ethnic composition of the urban population of the city of Rotterdam in the Netherlands. For that reason, all couples received counselling from health professionals apprehending the Dutch and/or Moroccan, Turkish and English language. However, information bias due to language problems cannot be ruled out completely.

Since, only 26% of the couples returned for a second preconception counselling, this may have led to selection bias. Therefore, the results do not apply to all couples with fertility problems and to the general population of couples planning pregnancy. On the other hand, in case no effects would have been shown in this motivated group, this would certainly apply and in a stronger degree to less motivated groups. Additionally, most couples don not visit their obstetrician/gynaecologist before conception. The issue to be addressed in the next years is how can we make the reproductive population aware of the needs and benefits of preconception counselling and what are the best manners to reach this target group. Thus, this evaluation shows that the way seems open to offer preconception counselling to other

populations as well and to investigate its effectiveness thereafter. The high percentage of non-responders for the second counselling may have contributed to confounding by a “healthy cohort effect”. Therefore, we have performed a non-response analysis showing that women who were counselled twice were more often obese and had more physical exercise (Table I). However, we cannot totally rule out desirable answers at the second visit.

There was no difference in the evaluation of the usefulness, quality and understanding of the given information and the feeling of pressure in responders and non-responders. Both were very happy and satisfied about the first counselling. Therefore, we assume that the low compliance of the second counselling may be due to the fact that these couples were already satisfied after the first counselling. Finally, we are aware that this evaluation is not designed as a randomized controlled trial. Therefore, the results should be interpreted carefully. If ethically allowed, the time seems right to further investigate preconception care initiatives in randomized controlled trials.

Our results confirm the very high prevalence of unhealthy dietary and lifestyle risk factors even in subfertile couples planning pregnancy, in one of the largest urban cities in the Netherlands. Couples with low education seem to benefit most from tailored personalized preconception dietary and lifestyle counselling. Therefore, we emphasize that the period of planning pregnancy should be used as ´window of opportunity´ to change unhealthy behaviours. In future it must be shown whether this new preventive care also applies to the general population planning pregnancy, whether the results improve reproductive performance and pregnancy outcome and reduce the costs for fertility treatment and care and treatment of pregnancy complications and adverse outcome. Future studies should also elaborate on the predictive value of the PDR and R3-score for reproduction.

Table 1

Baseline characteristics of couples at the first preconception counselling (PC1)

	Total women (n=419)				Total men (n=409)			
	Total PC1 (n=419)	**PC1 only (n=309)**	**Two PCs PC1 (n=110)**	***p*** [a]	**Total PC1 (n=409)**	**PC1 only (n=306)**	**Two PCs PC1 (n=103)**	***p*** [a]
Non-Modifiable factors								
Age (years) median(range)	31 (19-44)	31.2 (19-44)	32 (19-42)	0.9	34 (22-63)	34.1 (22-63)	34.5 (22-60)	0.4
Ethnicity; n(%)				0.8				0.1
Dutch	223 (53.2)	167 (54.0)	56 (50.9)		245 (59.9)	190 (62.1)	55 (53.4)	
European-others	40 (9.5)	29 (9.4)	11 (10)		30 (7.3)	21 (6.9)	9 (8.7)	
Non-European	151(36)	109 (35.3)	42 (38.2)		129 (31.5)	91 (29.7)	38 (36.9)	
Educational level; n(%)				0.4				0.6
Low	64 (15.3)	41 (13.3)	23 (20.9)		90 (21.5)	68 (21.5)	22 (21.4)	
Intermediate	199 (47.5)	157 (50.8)	42 (38.2)		151 (36.0)	116 (37.9)	35 (34)	
High	145 (34.6)	111 (35.9)	34 (30.9)		150 (35.8)	113 (36.9)	37 (35.9)	
Indication for referral; n(%)				0.7				
Subfertility	393 (93.8)	289 (93.5)	104 (94.5)					
High obstetrical risk	11 (2.6)	10 (3.2)	1 (0.9)					
Recurrent miscarriages	14 (3.3)	10 (3.2)	5 (4.5)					
Modifiable factors:								
All items of PDR-Score [b]								
Bread; n(%)	268 (64)	198 (64.1)	70 (63.6)	0.9	129 (31.5)	93 (30.4)	36 (35)	0.4
Butter/Oils; n(%)	55 (13.1)	39 (12.6)	16 (14.5)	0.6	54 (13.2)	41 (13.4)	13 (12.6)	0.8
Vegetables; n(%)	313 (74.7)	231 (74.8)	82 (74.5)	1.0	327 (80)	244 (79.7)	83 (80.6)	0.9
Fruit; n(%)	140 (33.4)	101 (32.7)	39 (35.5)	0.6	199 (48.7)	146 (47.7)	53 (51.5)	0.5
Meat; n(%)	68 (16.2)	51 (16.5)	17 (15.5)	0.8	53 (13)	39 (12.7)	14 (13.6)	0.8
Fish; n(%)	224 (53.5)	157 (50.8)	67 (60.9)	0.07	215 (52.6)	158 (51.6)	57 (55.3)	0.5
Rotterdam Reproduction Risk Score Items (R3-score)								
BMI (kg/m^2); median (range)	24.6 (17-43.2)	24.4 (17-43.2)	25.3 (18.4-42.4)	0.2	26.1 (17.4-46.8)	26.0 (17.4-46.8)	26.7 (18.5-42.5)	0.9
25-30 (kg/m^2); n(%)	96 (22.9)	75 (24.3)	21 (19.1)	0.3	156 (38.1)	116 (37.9)	40 (38.8)	0.8
>30 (kg/m^2); n(%)	97 (23.2)	63 (20.4)	34 (30.9)	<0.05	82 (20)	60 (19.6)	22 (21.4)	0.9
Waist circumference (cm); median(range)	90 (65-126)	84 (64-135)	90 (65-126)	<0.05	95.0 (78-137)	95 (71-138)	95 (78-137)	0.6
Waist - Hip ratio (cm)	0.86 (0.67-1.41)	0.83 (0.65-1.43)	0.86 (0.67-1.41)	<0.01	0.92 (0.78-1.20)	0.91 (0.75-1.22)	0.92 (0.78-1.20)	0.1
Systolic blood pressure (mmHg)	112 (90-152)	112 (88-180)	112 (90-152)	0.4	124 (90-165)	120 (90-178)	124 (90-165)	0.8
Diastolic blood pressure (mmHg)	75 (40-96)	70 (50-106)	75 (40-96)	0.06	80 (60-110)	78 (50-110)	80 (60.0-110.0)	0.5
Infection risk; n(%)	162 (38.7)	116 (37.5)	46 (41.8)	0.4	-	-	-	-

Medication use; n(%)	122 (29.1)	83 (26.9)	39 (35.5)	0.09	94 (22.4)	66 (21.6)	28 (27.2)	0.2
Caffeine use; n(%)	208 (49.6)	159 (51.5)	49 (44.5)	0.2	250 (59.7)	192 (62.7)	58 (56.3)	0.7
Smoking; n(%)	49 (44.5)	69 (22.3)	18 (16.4)	0.2	120 (29.3)	95 (31)	25 (24.3)	0.2
Alcohol use ; n(%)	175 (41.8)	136 (44)	39 (35.5)	0.1	266 (65)	198 (64.7)	68 (66)	0.4
Drug use; n(%)	9 (2.1)	5 (1.6)	4 (3.6)	0.1	30 (7.3)	23 (7.5)	7 (6.8)	0.7
Physical exercise (no); n(%)	276 (65.9)	193 (62.5)	83 (75.5)	<0.05	236 (57.7)	171 (55.9)	65 (63.1)	0.1
Folic acid supplement use; n(%)	266 (63.5)	192 (62.1)	74 (67.3)	0.3	-	-	-	-

[a] p-values show differences in characteristics of women and men who visited the preconception counselling only once (PC1 only) or twice (PC1 and 2) with a three months interval.
[b] Dietary intake of six food groups not according to the Dutch guideline (Nutrition Center the Netherlands, 2009).[20]

Table 2

Preconceptional dietary and lifestyle risk factors in couples visiting the preconception counselling clinic twice

	Women			Men		
	PC1 (n=110)	**PC2 (n=110)**	***p*** [a]	**PC1 (n=103)**	**PC 2 (n=103)**	***p*** [a]
Preconceptional Dietary Risk Score Items (PDR-score) [b]						
Total PDR-score Median (95% CI)	2.6 (2.4-2.9)	2.4 (2.1-2.6)	<0.05	2.5 (2.3-2.7)	2.2 (1.9-2.4)	<0.05
Bread n(%)	70 (63.6)	65 (59.1)	0.3	36 (35.0)	35 (34.0)	1.0
Butter/Oils n(%)	16 (14.5)	18 (16.4)	0.7	13 (12.6)	17 (16.5)	0.5
Vegetables n(%)	82 (74.5)	80 (72.7)	0.7	83 (80.6)	80 (77.7)	0.7
Fruit n(%)	39 (35.5)	22 (20)	<0.05	53 (51.5)	33 (32.0)	<0.05
Meat n(%)	17 (15.5)	21 (19.1)	0.4	14 (13.6)	13 (12.6)	1.0
Fish n(%)	67 (60.9)	53 (48.2)	<0.05	57 (55.3)	47 (45.6)	0.06
Rotterdam Reproduction Risk Score Items (R3-score)						
Total R3-score Median (95% CI)	4.7 (4.3-5.0)	3.1 (2.8-3.4)	<0.01	3.0 (2.8-3.3)	2.0 (1.7-2.3)	<0.01
BMI (kg/m^2); median(range)	25.3 (18.4-42.4)	25.3 (18.8-40.3)	0.4	26.7(18.5-42.5)	26.8(19.1-41.9)	0.8
25-30 (kg/m^2); n(%)	21 (19.1)	25 (22.7)	0.2	40 (38.8)	40 (38.8)	0.1
>30 (kg/m^2); n(%)	34 (30.9)	31 (28.2)	0.4	22 (21.4)	17 (16.5)	0.3
Waist circumference (cm); median(range)	90 (65-126)	94 (64-120)	0.7	95.0 (78-137)	96.5 (71-137)	0.9
Waist-Hip ratio (cm)	0.86 (0.67-1.41)	0.87 (0.67-1.46)	0.9	0.92 (0.78-1.20)	0.93 (0.73-1.08)	0.9
Systolic Blood pressure (mmHg)	112 (90-152)	110 (90-150)	0.2	124 (90-165)	120 (92-160)	0.5
Diastolic Blood pressure (mmHg)	75 (40-96)	74 (48-94)	0.2	80 (60-110)	80 (55-100)	0.4
Infection risk; n(%)	46 (41.8)	8 (7.3)	<0.01	-	-	
Medication use; n(%)	39 (35.5)	39 (35.5)	1.0	28 (27.2)	24 (23.3)	0.5
Cafeïne use; n(%)	49 (44.5)	48 (43.6)	1.0	58 (56.3)	54 (52.4)	1.0
Smoking; n(%)	18 (16.4)	17 (15.4)	0.9	25 (24.3)	21 (20.4)	0.4
Alcohol use; n(%)	39 (35.5)	23 (20.9)	<0.01	68 (66.0)	48 (46.6)	<0.01
Drug use; n(%)	4 (3.6)	4 (3.6)	1.0	7 (6.8)	4 (3.0)	0.3
Physical Exercise (no); n(%)	83 (75.5)	35 (31.8)	<0.01	65 (63.1)	67 (65.0)	0.6
Folic acid supplement use; n(%)	74 (67.3)	93 (84.5)	<0.01	-	-	

[a] p-value shows the difference after 3 months between PDR-score, R3-score, dietary and lifestyle items in women and men who visited the preconception counselling twice.

[b] Dietary intake of food groups not according to the recommendations of daily allowances (Nutrition Center the Netherlands, 2009).[20]

Table 3
Biomarkers of couples visiting the preconception outpatient clinic once or twice

	Women				Men			
	Total PC1 (n=419)	**PC1 only (n=309)**	**Two PCs PC1 (n=110)**	**PC2 (n=110)**	**Total PC1 (n=409)**	**PC1 only (n=306)**	**Two PCs PC1 (n=110)**	**PC2 (n=110)**
Folate, serum (nmol/L)	26.9 (25.0-28.8)	27.1 (24.9-29.3)	26.3 (21.7-30.9)	32.4 (29.3-35.5)	17.0 (16.3-17.6)	17.2 (16.4-18.0)	16.6 (15.0-18.2)	19.4 (17.8-21.0)
Folate, RBC (nmol/L)	806 (775-837)	818 (784-852)	742 (682-802)	877 (827-928)	705 (683-727)	724 (696-752)	657 (626-688)	739 (689-789)
Vitamin B12, serum (pmol/L)	316 (304-328)	322 (305-338)	312 (289-336)	311 (293-329)	307 (293-321)	309 (290-328)	304 (2801-327)	312 (277-347)
tHcy, plasma (µmol/L)	8.4 (8.0-8.7)	8.2 (7.8-8.5)	8.6 (8.3-8.8)	7.7 (7.4-8.0)	10.7 (10.5-10.9)	10.8 (10.5-11.0)	10.7 (10.0-11.4)	10.5 (9.9-11.1)

Folate RBC = red blood cell folate
Results are presented as median (95% Confidence Interval)

Table 4

Assessment of the preconception counselling by the couples

	Women						Men					
	Total PC1 (n=419)	**PC1 only (n=309)**	**Two PCs PC1 (n=110)**	**PC2 (n=110)**	***p*** [a]	***p*** [b]	**Total PC1 (n=419)**	**PC1 only (n=309)**	**Two PCs PC1 (n=110)**	**PC2 (n=110)**	***p*** [a]	***p*** [b]
Reason for preconception counselling; n(%)*					0.5	0.8					0.2	0.05
I wanted to go	161 (38.4)	113 (36.6)	48 (43.6)	59 (53.6)			154 (36.7)	121 (39.2)	33 (30)	76 (73.8)		
Gynaecologist told me to go	313 (74.7)	235 (76.1)	78 (70.9)	62 (56.4)			241 (57.5)	177 (57.3)	64(58.2)	5 (4.9)		
Partner told me to go	3 (0.7)	3 (1.0)	0	1 (0.9)			37 (6.4)	26 (8.4)	10 (9.1)	6 (5.8)		
Friends and family told me to go	0	0	0	1 (0.9)			1 (0.2)	0	0	0		
Usefulness of the counselling; n(%)*					0.2	0.06					0.2	0.03
Yes, very useful	268 (64.0)	191 (61.8)	77 (70.0)	56 (50.9)			246 (58.7)	179 (57.9)	67 (60.9)	50 (48.5)		
Yes, a bit useful	123 (29.4)	99 (32.0)	24 (21.8)	39 (35.5)			117 (27.9)	93 (30.1)	24 (21.8)	33 (32.0)		
No, not useful	7 (1.7)	7 (2.3)	0	4 (3.6)			6 (1.4)	5 (1.6)	1 (0.9)	3 (2.9)		
Understanding of the information; n(%)*					0.9	0.4					0.9	0.2
Yes, everything was clear	380 (90.7)	282 (91.3)	98 (89.1)	92 (83.6)			348 (83.1)	262 (84.8)	86 (78.2)	76 (73.8)		
Yes, most was clear	12 (2.9)	10 (3.2)	2 (1.8)	5 (4.5)			19 (4.5)	14 (4.5)	5 (4.5)	10 (9.7)		
No, some was not clear.	3 (0.7)	3 (1.0)	0	1 (0.9)			2 (0.5)	2 (0.6)	0	0		
No, everything was not clear	1 (0.2)	0	1 (0.9)	0								
Feeling pressure to change nutritional and lifestyle risk factors; n(%)*					0.7	0.5					0.9	0.5
No	340 (81.1)	251 (81.2)	89 (80.9)	84 (76.4)			315 (75.2)	236 (76.4)	79 (71.8)	76 (73.8)		
Yes, by gynaecologist	18 (4.3)	12 (3.9)	6 (5.5)	9 (8.2)			14 (3.3)	10 (3.2)	4 (3.6)	5 (4.9)		
Yes, during counselling	34 (8.1)	28 (9.1)	6 (5.5)	7 (6.4)			33 (7.9)	24 (7.8)	9 (8.2)	6 (5.8)		
Yes, by partner	6 (1.4)	4 (1.3)	2 (1.8)	2 (1.8)			17 (4.1)	15 (4.9)	2 (1.8)	0		
Yes, by family and friends	8 (19.1)	5 (1.6)	3 (2.7)	0			9 (2.1)	5 (1.6)	4 (3.6)	0		
Feeling happy about the counselling; n(%)					0.6	0.7					0.1	0.9
Yes	342 (81.6)	249 (80.6)	93 (84.5)	85 (77.3)			315 (75.2)	232 (75.1)	83 (75.5)	78 (75.7)		
No	8 (1.9)	6 (1.9)	2 (1.8)	1 (0.9)			8 (1.9)	6 (1.9)	2 (1.8)	2 (1.9)		
Don't know	29 (6.9)	27 (8.7)	2 (1.8)	11 (10.0)			30 (7.2)	26 (8.4)	4 (3.6)	7 (6.8)		
Recommendation of counseling to others? n(%)					0.2	0.9					0.2	0.4
Yes	316 (75.4)	226 (73.1)	90 (81.8)	84 (76.4)			285 (68.0)	211 (68.3)	74 (67.3)	68 (66.0)		
No	13 (3.1)	13 (4.2)	0	4 (3.6)			16 (3.8)	11 (3.6)	5 (4.5)	4 (3.9)		
Don't know	48 (11.5)	41 (13.3)	7 (6.4)	10 (9.1)			51 (12.2)	42 (13.6)	9 (8.2)	13 (12.6)		

[a] p-values show difference in rating of the preconception counselling of women and men who were counselled once (PC1 only) or twice (PC1 and 2) with a three months interval

[b] p-value shows the difference in preconception consultation between PC1 and PC2 in women and men.

* Numbers and percentages may exceed 100% because multiple answers were possible to the question.

Addendum 1 Rotterdam Reproduction Risk Score (R3-Score)									
	Risk factor	**Score woman**	**Score man**	**Fertility**	**Miscariage**	**Fetal growth restriction**	**Premature birth**	**Pre-eclampsia**	**Congenital malformation**
Health	Medication; Yes	1	1	*Dunlop et al., 2008*	*Siberstein et al.,2004*	*Koren et al.,1998*	Reis et al.,2010; *Calderon-Margalit et al.,2009*	*Saftlas et al., 2004*	*Koren et al.,1998;*
Lifestyle	Folic acid use; No	1	-	*Tamura et al., 2006*	*Tamura et al., 2006*	*Timmermans et al, 2008; Tamura et al., 2006*	*Tamura et al., 2006*	*Tamura et al., 2006*	*Tamura et al., 2006;Czeizel et al.,2009*
	Exercise; No	1	1	*Homan et al., 2007*	-	*Takito et al.,2010*	*Takito et al.,2010*	-	-
	Infection risk; yes	1	-	*Coonrod et al., 2008*	-	-	-	-	*Elsheikha et al., 2008*
Intoxication	Smoking; yes	1	1	*Hassan et al.,2004*	*Rasch et al,2003*	*Bada et al,2005; Aliyu et al., 2009*	*Kolas et al., 2000*	-	*Lorente et al,2000*
	Alcohol use; yes	1	1	*Windham et al., 1992; Grodstein et al., 1994;Hassan et al.,2004*	*Rasch et al,2003*	*Bada et al.,2005; O'Leary et al.,2009; Aliyu et al., 2009*	*O'Leary et al.,2009*	-	*Lorente et al,2000*
	Drug use; yes	1	1	*Hassan et al., 2004*		*Slutsker et al., 1992*	*Slutsker et al., 1992*		*Slutsker et al., 1992*
	>6 cups of coffee; yes	1	1	*Jensen et al., 1998*	*Rasch et al,2003*	*Weng et al., 2008*	-	-	-
Physical examination	BMI <20/ ≥30	1	1	*Hassan et al., 2004*	*Micali et al., 2007;Landres et al.,2010*	*Micali et al., 2007*	*Jensen et al.,2003*	*Siega-Riz et al.,2006*	*Siega-Riz et al.,2006; Stothard et al., 2009*
	Systolic≥160mmHg Diastolic ≥90 mmHg blood pressure	1	1	-	-	*Chappell et al.,2008*	*Chappell et al., 2008*	*Duckitt et al.,2005*	-
	Waist circumference Woman; ≥88 cm Man; ≥102 cm	1	1	-	-	*Berends et al.,2009*	-	*Berends et al.,2009*	-
	Waist to Hip ratio ≥0.8	1	-	*Zaadstra et al., 1993*	-	*Berends et al.,2009*	-	*Berends et al.,2009*	-
Biomarkers	*Deviating biomarkers value:*	*maximum 1*	*maximum 1*	*Wong et al., 2001; Boxmeer et al., 2009*	*de la Calle et al., 2003*	*Timmermans et al, 2008*	*de la Calle et al., 2003*	*de la Calle et al., 2003*	*Tamura et al., 2006;Czeizel et al.,2009*
	B12 total <160 pmol	1	1						
	B12 active <20 pmol/l	1	1						
	folate serum <8 nmol/l	1	1						
	folate erythrocytes <350 nmol/l	1	1						
	homocysteine >15 umol	1	1						
Note: The maximum R3-score for women is 13 and for men 10.									

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Chapter 8

General Discussion

Introduction

The main objective of this thesis was to study the effects of nutrition and lifestyle factors on fertility parameters in both women and men in the preconception period (part I) and to evaluate the implementation of preconception counselling tailored on adverse nutrition and lifestyle in women and men planning pregnancy at the Erasmus MC in Rotterdam, The Netherlands, a tertiary university medical center (part II).

The focus in this thesis is on:

1. The effects of folic acid supplement use and dietary omega-3 polyunsaturated fatty acid consumption on fertility outcome parameters in subfertile couples who underwent ovarian hyperstimulation treatment;
2. The associations between Body Mass Index (BMI) and AMH response in women who underwent ovarian hyperstimulation treatment;
3. The associations between BMI and sperm quality in man of subfertile couples;
4. The short term effectivity of tailored preconceptional nutrition and lifestyle counselling of subfertile couples.

These research objectives were investigated in the FOLFO study and the preconceptional outpatient clinic “Achieving a Healthy Pregnancy”, which both have a prospective preconception cohort design.

Main Findings

In part I we found that folic acid supplement use attenuates estradiol and follicular and endocrine responses to conventional ovarian hyperstimulation treatment, independent of AMH and antral follicle count (chapter 3). Next, we observed that dietary omega-3 LC-PUFA intake in women undergoing IVF/ICSI treatment is associated with improved embryo morphology (chapter 2). We also demonstrated that AMH levels after ovarian hyperstimulation remained elevated in women with a BMI≥25 compared to normal weight women (chapter 4).

In Part II, we found that sperm quality in men of subfertile couples is significantly affected by BMI and central adiposity (chapter 6). Additionally, we showed that tailored preconceptional counselling on unhealthy dietary and lifestyle behaviours of subfertile couples in an outpatient tertiary clinic is effective. Three months after preconceptional counselling this has led to a significant improvement in fruit (women: +15.5% and men: +19.5%) and fish (women: +13%) consumption and a decrease in alcohol use (women:-14.6% and men: -19.4%). In women also use of folic acid supplementation improved (+17.2%). These improvements were in both women and men independent of ethnicity, education, neighbourhood and BMI.

Inferences of the findings

There is a body of literature showing that nutrition is important in human reproduction.[1] Especially, in the preconception period, which represents a sensitive window during which nutritional status of both the woman and man play a critical role in their reproductive performance. In this critical time episode of 3 months, gamete development and maturation takes place. This involves cell growth and differentiation.[1] Malnutrition and lifestyle and

demographic factors related to nutrition, such as smoking and education, can have adverse effects on these biological processes.[1]

Folic acid

As we described in Chapter 3 we observed that moderate folic acid use modifies the ovarian response after conventional ovarian hyperstimulation treatment. The estradiol response and the mean follicle number after ovarian hyperstimulation was higher in women not using a folic acid supplement compared to women using folic acid supplementation. Supraphysiological estradiol levels and a high number of follicles retrieved after ovarian hyperstimulation are associated with a higher frequency of embryo aneuploidy.[2] These data suggest that folate interferes with ovarian physiology, which might lead to the generation of oocytes with a better quality of the genome. This suggestion is further reinforced by the fact that folate has a major role in one-carbon metabolism, important in epigenetic processes. It provides methyl groups for various macromolecules like DNA, RNA, proteins and membrane phospholipids. Our results suggest that the ovarian response to gonadotropins is subject to the availability of the methyl donor folate. Others also reported a higher ovarian response after rFSH administration in methyl-deficient ewes.[3] An additional in vitro analysis of granulosa cells revealed higher FSH receptor (FSH) mRNA expression as homocysteine levels increased, reflecting low methyl group availability.

Long-chain polyunsaturated fatty acids (LC-PUFAs)

We also showed that women undergoing IVF/ICSI treatment with high intakes of the long-chain polyunsaturated fatty acids (LC-PUFA) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have a lower estradiol (E2) response and number of follicles after ovarian hyperstimulation. Additionally, we demonstrated that total omega-3 intake, in particular

ALA and DHA intake, improves embryo morphology (chapter 2). Animal studies have reported beneficial effects on cow reproductive performance following supplementation with omega-3 PUFA. However, human studies are scarce.[4-5] These fatty acids may act in the regulation of some key reproductive processes including ovarian function, steroidogenesis and suppression of uterine prostaglandin F2α synthesis.[6-8] LC-PUFAs are an indispensable component of all cell membrane phospholipids and precursors of eicosanoids, such as prostaglandins, leukotrienes and tromboxanes.[8]

Similar to folate, omega-3 PUFAs are also known to be regulators of gene transcription in many tissues.[9-11] LC-PUFAs may alter the function of transcription factors controlling gene expression and can thus affect cellular concentrations of enzymes regulating both the prostaglandin and steroidogenic synthetic pathways. Namely, membrane phospholipids are major methyl group acceptors and reduced DHA levels may result in diversion of methyl groups towards DNA ultimately resulting in DNA methylation as was recently described in the one-carbon metabolic pathway.[12] DNA methylation is critical for developmental changes in gene regulation, and changes that take place during this critical period may result in altered imprinting of genes which might be transferred to the next generation.[13]

Additionally, the consumption of omega-3 PUFAs has been associated with lower homocysteine (tHcy) levels.[14] A possible mechanism is that omega-3 PUFAs as transcription factors modulate gene expression of enzymes that are involved in the formation and metabolism of tHcy.[15] High concentrations of tHcy are associated with reduced embryo quality.[16]

PRECONCEPTIONAL INTERVENTIONS

Thus, maternal folate and omega-3 LC-PUFAs status in the preconception period of women planning pregnancy is important for the outcome of IVF/ICSI treatment. Humans do not have

the ability to endogenously synthesize folate and omega-3 PUFAs. Therefore, the demand for these nutrients has to be met entirely by dietary intake. Therefore, periconceptional folic supplement use has been recommended to women since the early 1990's to reduce the risk of neural tube defects.[17]

Yet, in the Netherlands only 51% of the women in the general population use folic acid for the entire advised period.[18] Although the majority of women know about the beneficial effects of folic acid, its use in the advised period is not guaranteed, and there is still a large gap between women of different educational levels. Our data from the FOLFO study and the preconceptional outpatient clinic "Achieving a Healthy Pregnancy"; involving couples planning pregnancy, revealed that respectively 67% and 67.3% used folic acid according to the recommendations. One would also expect that these women are well aware of this measure and that the percentage using folic acid is higher. We showed in chapter 7 that 3 months after tailored preconception counselling an increase in folic acid supplement use from 67.3% to 84.5% was established in women. This was also achieved in women of low socio-economic class.

At present, the Dutch debate specifically surrounds the issue whether folate intake should be increased by mandatory folic acid fortification, which will affect the entire population.[19] Folate has beneficial effects in prevention of several diseases. However, on the other hand there is concern that exposure of the total population might promote the growth of pre-neoplastic lesions.[20-21] Additionally, also with food fortification it is very difficult to reach the daily recommended intake of 0.4 mg folate in every women of childbearing age.[22] Therefore, more studies are needed to address this issue. Until then the intake of excessive folate should be regarded with caution and more public health measures should be taken to increase information regarding folic acid supplement use in women of reproductive age.

Furthermore, it is recommended that good sources of omega-3 PUFAs, namely pelagic fish, are included in the diet. The Dutch Nutrition Health Center recommends to consume at least two portions of fish per week, one of which should be oily (equivalent to about 3 g EPA + DHA per week).[23] Current intakes, especially of oily fish, are considerably lower than this. We also demonstrated in women undergoing IVF/ICSI treatment that the intake of omega-3 LC-PUFAS is much lower than the recommended intake, in contrast to the adequate intakes of omega-6 LC-PUFA. The question then rises, whether women planning pregnancy should also be recommended to take PUFA supplements. Many women of reproductive age already take PUFA supplements for various health reasons, such as rheumatoid arthritis.[24] It appears that LC-PUFAs are a two-edged sword—some are essential, but too much is potentially harmful. We remain largely ignorant as to the best balance to take at different points in our life in order to achieve optimum fertility. Therefore, just as with regard to folic acid fortification one should be cautious in taking PUFA supplements without proven beneficial effects. We showed, however, that tailored preconception counselling is also an effective intervention to increase dietary PUFA consumption by more fish consumption in women planning pregnancy (chapter 7).

Body Mass Index (BMI)

A high body mass index (BMI≥25) is a good phenotype for unhealthy nutrition and lifestyles. There is mounting evidence on the effect of a high BMI on female fertility. However, data on the effect of BMI on AMH - a marker for ovarian reserve - is scarce. A negative correlation between BMI and AMH levels has been found among late reproductive-age women and young women using oral contraceptives.[25-26] Another study, however, failed to demonstrate a similar association.[27] To date, there is still controversy about the relationship between BMI and AMH, and possible mechanisms underlying this association have not been elucidated. In Chapter 4

we have demonstrated that overweight or obese women undergoing ovarian hyperstimulation have higher AMH levels after ovarian hyperstimulation. We also suggest that folate acts herein as an intermediate.

AMH inhibits the initial and cyclic processes of follicular recruitment and the response to exogenous FSH, yet the aspects involved in its regulation are still poorly understood. However, because of the significant inverse association between baseline serum folate and AMH before and after ovarian hyperstimulation, comparable with the association between folate and estradiol (chapter 3), we suggest that folate as methyl donor influences the expression of the AMH receptor gene by affecting the methylation of its promoter.[28] Folate acts as a methyl donor in the remethylation of homocysteine to methionine. Deficiencies in folate can result in accumulation of homocysteine, which is associated with DNA hypomethylation and could potentially result in changes in gene expression [29].

Unlike the well established inverse association between obesity and female fertility, little is known about what effect male obesity has on semen quality. In this thesis we have investigated the effects of lifestyle factors on semen parameters in both the FOLFO and "Achieving a Healthy Pregnancy" study (chapter 5 and 6). We could not demonstrate associations between smoking, alcohol use, BMI and the conventional sperm parameters and DNA fragmentation index (DFI) in the FOLFO study. The absence of significant effects of these lifestyle factors on sperm parameters may be due to a power problem. Additionally, it cannot be excluded that the lack of association may also be due to some misclassification of exposure status, since data on smoking and alcohol use were obtained from questionnaires and have not been validated by measuring biomarkers of smoking, e.g., cotinine, and alcohol use, e.g., ethanol, in serum or seminal plasma. Nevertheless, we showed a positive association between age and DFI and an inverse association with ejaculate volume (chapter 5). This suggests that

delaying childbearing not only in women but also in men can contribute to a reduced reproductive capacity.

Our finding is consistent with the observation that older men show more DNA damaged spermatozoa due to increased oxidative stress as a consequence of the aging process.[30-31] In addition, studies in rats revealed that a decrease in epididymal antioxidant capacity occurs with rising age thereby disrupting germ-cell differentiation and sperm quality.[32]

In the study "Achieving a Healthy Pregnancy", we demonstrated an inverse association between a high BMI and sperm quality (chapter 6). Being overweight was associated with a significantly lower ejaculate volume, percentage of progressive motile sperm and higher percentages of immotile sperm. Furthermore, obesity was associated with an even significantly lower ejaculate volume, sperm concentration, total sperm count and total motile sperm count. Furthermore, we showed that a waist circumference ≥102 cm, a marker for central adiposity, was associated with a lower sperm concentration, total sperm count and total motile sperm count. Due to the high correlation between BMI and waist circumference, these associations, disappeared after adjustment for BMI.

Thus, in addition to the importance of maternal lifestyle and nutrition, this thesis stresses the significance of a healthy lifestyle and nutritional behaviour in reproductive active men in order to improve their fertility capacity.

Methodological considerations

FOLFO study

One of the strengths of the FOLFO study is its prospective preconception design, the relative large sample size and the fact that information of both women and men were collected. However, in this study only couples undergoing IVF/ICSI treatment were included.

Misclassification could have occurred in our study on semen since we only used one semen sample. However, in daily clinical practice only one semen sample is used to differentiate between fertile and subfertile men. By using a questionnaire involving lifestyle and dietary factors, recall bias and underreporting could have occurred which also could have led to misclassification.

"Achieving a Healthy Pregnancy"

The major strength of this study is that we implemented preconception counselling in a clinical setting, offered this to both women and men planning pregnancy, and included a follow-up period to investigate changes in behaviours. This is unique as most studies obtained retrospective information in women only.[33-34] Additionally, the effectiveness of counselling of the couple is assumed to be higher than that of the woman only.[35] In this study, couples returned voluntary for a second visit to evaluate to which extent they adapted the preconceptional advices. Since only 26% of the couples returned for a second preconception counselling, this may have led to selection bias. Therefore, the question arises whether the results can be applied to all couples with fertility problems and to the general population of couples planning pregnancy. Further research should be carried out in this regard and to address the effects on reproductive outcome

Future perspectives

It has become increasingly clear that reproductive disorders have their origin in the pre- and early postconception period, in which lifestyle and nutrition play an essential role. In this thesis we have added to this knowledge and also emphasize the importance of the man. Reproductive disorders are complex multifactorial diseases involving both genetic and environmental factors in the reproductive span of both the woman and the man.

Polymorphisms in genes can lead to differences in the level of susceptibility of individuals to potentially adverse effects of unhealthy lifestyles and nutrition, such as obesity (western lifestyle and diet), on female or male reproduction[36]. This depends not only on the dose and potency of a given toxicant, but also on the occurrence of exposure during critical developmental time periods, such as the gametogenesis, a time of rapid growth and development. Disruption of processes during gametogenesis can cause permanent functional deficits, as well as delayed effects, such as diseases in later life.[37] However, there is little known about this genetic variability. Therefore, differences in genetic traits should be investigated to elucidate the critical level of susceptibility to adverse effects of environmental influences in relation to reproductive outcomes. It is also becoming clear that distinct epigenetic marks are essential for normal germ cell formation and during early embryonic development. Therefore, studies focusing on epigenetic control of gene expression should be encouraged as a potential mechanism. For example, the link between reproductive outcome, lifestyle, nutrition and epigenetic modifications.

We showed in this thesis that multiple risk factors exist in the preconception period in women and men of reproductive ages (Rotterdam Reproduction Risk score (R3-score) and Preconception Dietary Risk score (PDR-score)). Also there is a strong correlation between different lifestyles and nutritional habits. Therefore it would be interesting to study the effects of different risk factors together in relation to reproductive outcome. The department of Obstetrics and Gynaecology of Erasmus MC Rotterdam has started a prospective cohort study built-in in a clinical setting, e.g., Predict Study, which investigates the effects of periconceptional lifestyle, nutritional and biomarker status on reproductive outcome. In this design the cumulative effect of multiple risk factors and underlying (epigenetic) mechanisms in association with reproductive outcome can be studied.

There is little evidence on the effects of preconceptional health promotion on reproductive outcome and much more research is needed in this area. Ideally, a large randomized controlled trial is needed to study these effects.

One should, however, realize that for some topics, especially withdrawing the use of folic acid, a randomized clinical trial for ethical reasons is not feasible.

Public health relevance

In 2007 the department of Obstetrics and Gynaecology of the Erasmus MC Rotterdam initiated the preconception counselling program "Achieving a Healthy Pregnancy" for its patients and employees.[38] This implicated that health behaviour changes might be initiated and sustained with tailored personalised counselling during the preconception period (chapter 6). Promotion of a healthy diet and lifestyle should therefore be implemented in the current preconception care and should be targeted to all women and men planning pregnancy. Special attention should be paid to immigrants and people from low socioeconomic class, since they are often not reached in the provision of information about the consequences of unhealthy behaviours on fertility and pregnancy. Additionally, most couples don't visit their general practitioners, midwife or obstetrician/gynaecologist before conception. The issue to be addressed in the next years is how can we make the reproductive population aware of the needs and benefits of preconception counselling and what are the best manners to reach this target group. An important medium like the internet can be used for health education and behaviour change applications.[39-40] Moreover, the mobile phone can be used, to send a tailored short message service to prospective parents.[41-42] These technologies allow for the provision of timely information to consumers as well as individual tailored intervention at distance.

Final conclusions

The studies described in this thesis emphasize the importance of nutrition and lifestyle for women and men planning pregnancy and that tailored preconception counselling on these factors is necessary and feasible. General practitioners, gynaecologists and other care givers should be aware of the importance of preconception counselling and focus on this.

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Summary/Samenvatting

English Summary

It has been estimated that 10-15% of couples experience impaired fertility at some point in reproductive life. However, in 30% of these couples no medical cause is identified. Differences in the prevalence of putative lifestyle and nutrition risk factors have been suggested to play a role. The aims of this thesis were to investigate the effects of lifestyle and nutrition on fertility parameters in both women and men, and to evaluate the effects of preconception counselling tailored on lifestyle and nutrition in women and men planning pregnancy at the outpatient fertility clinic of the Erasmus MC Rotterdam. The studies described in this thesis are based on a prospective preconception cohort study the "Food, Lifestyle and Fertility Outcome" (FOLFO) study and the outpatient preconception clinic "Achieving a Healthy Pregnancy".

Part I

The first part of this thesis focused on the associations between preconception nutrition and lifestyle factors on fertility parameters in both women and men. After the general introduction in **Chapter 1** we describe **i**n **Chapter 2** the associations between preconception nutritional intake of long-chain polyunsaturated fatty acids (LC-PUFA) and estradiol and fertility outcome parameters in women undergoing IVF or ICSI treatment. We showed that women with the highest intake of the omega-3 LC-PUFA ALA showed a higher baseline estradiol level. Furthermore, high intakes of EPA and DHA reduced the estradiol response and number of follicles after ovarian hyperstimulation treatment. High intakes of omega-3 LC-PUFA, in particular ALA and DHA, were associated with improved embryo morphology. In **Chapter 3**, we hypothesized that the ovarian response to gonadotrophins is affected by the availability of one-carbon-donors, like folate. In a randomized clinical trial, comparing a mild- and conventional

ovarian hyperstimulation protocol, we aimed to study the effect of low dose folic acid supplement use on specific biomarkers of the folate dependent homocysteine pathway and estradiol concentrations following conventional- and mild ovarian hyperstimulation treatment. After conventional hyperstimulation treatment, women who did not use a low dose folic acid supplement had a significantly higher ovarian response after hyperstimulation treatment than those who did use a folic acid containing supplement. Therefore, low dose folic acid use attenuates follicular and endocrine responses to conventional stimulation, which occurred independent of AMH and follicle count. In **Chapter 4**, we examined the association between BMI and AMH and the modification by folate in women undergoing assisted reproductive treatment. Subfertile women with overweight or obesity exhibit higher AMH levels after ovarian hyperstimulation compared to normal weight women despite the comparable total number of follicles and oocytes. The effect on AMH before and after stimulation was inversely modified by baseline serum folate.

In **Chapter 5**, we evaluated the effects of increasing age and unhealthy lifestyles on sperm quality in men attending a fertility clinic. We observed in men of couples undergoing IVF or ICSI treatment that the rising age between 26 and 59 years is detrimental for sperm DNA integrity and ejaculate volume.

Part II

In **Chapter 6** we demonstrated that a high body weight as marker of poor nutrition and lifestyle, independently affects sperm quality in men of subfertile couples. We observed that overweight is associated with a significantly lower ejaculate volume, a lower percentage motility type A and a higher percentage immotility type C. Furthermore, obesity is associated with a significantly lower ejaculate volume, lower sperm concentration and lower total motile

sperm count. Waist circumference, a marker for central adiposity, was also associated with lower sperm concentration and total motile sperm count.

In **Chapter 7**, we evaluated whether tailored preconception counselling modifies unhealthy behaviours with respect to nutrition and lifestyle. For this purpose we created the Preconception Dietary Risk score (PDR-score) and Rotterdam Reproduction Risk score (R3-score). In subfertile couples the improvement in dietary intake (PDR-score) was achieved independent of ethnicity. In women and men the PDR-score decreased by respectively, 8% and 12%. The R3-score in women and men decreased by respectively, 34% and 33%. The R3-scores in these couples decreased independent of ethnicity, educational level, neighbourhood and BMI. However, low educated women appeared to show a larger reduction than those with a higher education. Furthermore, in men with a normal BMI a larger decrease was shown than in overweight men. More than 85% of the subfertile couples indicated the counselling as useful and around 70% recommends it to others.

The general discussion in **Chapter 8** elaborates on the strengths and weaknesses of the studies and reflects on the clinical implications of our results. The relatively high prevalence of poor nutrition and lifestyle factors in subfertile couples planning pregnancy emphasis the need to raise more awareness on these issues in fertility treatment. Therefore, targeted preconception health educational programmes should be developed and applied in order to improve reproductive outcome.

Nederlandse Samenvatting

De schatting is dat ongeveer 10-15% van de paren een verminderde vruchtbaarheid heeft op enig moment in de reproductieve levensfase. Echter, bij 30% van deze paren wordt geen medische oorzaak gevonden. Er komen wel steeds meer aanwijzingen dat ongezonde voedings- en leefstijlfactoren hieraan een bijdrage leveren. Het identificeren van deze risicofactoren en het bestuderen van biologische mechanismen zal in de toekomst bijdragen aan de preventie van vruchtbaarheidsstoornissen.

Het doel van dit proefschrift was om de effecten te bestuderen van voedings- en leefstijlfactoren op een aantal vruchtbaarheids parameters bij zowel vrouwen als mannen. Daarnaast werd het in 2007 gestarte speciale preconceptie spreekuur, gericht op de screening en counseling van voedings- en leefstijlfactoren, van paren met kinderwens geëvalueerd. Dit proefschrift is gebaseerd op gegevens die verzameld zijn in het prospectieve cohort onderzoek; “Food, Lifestyle and Fertility Outcome” (FOLFO) en het preconceptie spreekuur "Gezond Zwanger Worden".

Deel I

Het eerste deel van dit proefschrift beschrijft de associaties tussen voedings- en leefstijlfactoren in de preconceptie periode van zowel vrouwen als mannen in relatie tot vruchtbaarheids parameters. Na de algemene introductie in **Hoofdstuk 1** beschrijven we in **Hoofdstuk 2** de positieve associatie tussen de preconceptionele inname van meervoudig onverzadigde vetzuren (Omega3), oestradiol concentraties en de vruchtbaarheids parameters van vrouwen die een IVF of ICSI behandeling hebben ondergaan. Vrouwen met de hoogste inname van ALA, een van de omega3 vetzuren, vertoonden een hoger gehalte van het vrouwelijke hormoon oestradiol in het bloed voorafgaand aan de ovariële hyperstimulatie voor

de IVF/ICSI behandeling. Daarnaast werd een afname vastgesteld van de oestradiol respons en het totale aantal follikels verkregen na ovariële hyperstimulatie door een hoge inname van de omega-3 vetzuren EPA en DHA. Een opvallende bevinding was dat een hoge inname van omega3 vetzuren, in het bijzonder van ALA en DHA, geassocieerd was met een verbeterde morfologie van het embryo. In **Hoofdstuk 3** werd de invloed bestudeerd van de inname van foliumzuur in tabletvorm op de ovariële respons na ovariële hyperstimulatie behandeling. Hiervoor werden in de gerandomiseerde FOLFO II trial de biomarkers van de foliumzuur afhankelijke homocysteine pathway en oestradiol gehalten vergeleken tussen vrouwen die het milde en het conventionele ovariële hyperstimulatie protocol kregen. Hierbij werd een verdere onderverdeling gemaakt in vrouwen die al dan niet een laag gedoseerd foliumzuur supplement gebruikten. Na de conventionele ovariële hyperstimulatie behandeling hadden vrouwen die geen foliumzuur supplement gebruikten een significant hogere ovariële respons in vergelijking met vrouwen die wel foliumzuur gebruikten. Dit is een eerste aanwijzing dat het gebruik van een laag gedoseerd foliumzuur supplement de folliculaire en endocriene respons na conventionele behandeling beïnvloed. Dit effect was onafhankelijk van het anti- mullerian hormoon (AMH) gehalte en het aantal follikels.

In **Hoofdstuk 4** onderzochten we de associatie tussen de body mass index (BMI) en het AMH gehalte en de invloed hierop van het foliumzuurgehalte in het bloed bij vrouwen die een ovariële hyperstimulatie behandeling ondergingen. De subfertiele vrouwen met overgewicht of obesitas bleken een hoger AMH gehalte te hebben na ovariële hyperstimulatie in vergelijking met vrouwen met een normaal gewicht. Dit was ondanks het feit dat het totaal aantal verkregen follikels en oocyten vergelijkbaar was. Opvallend was de bevinding dat het foliumzuurgehalte in het bloed het effect op het AMH gehalte voor en na ovariële hyperstimulatie ook lijkt te beïnvloeden.

In **Hoofdstuk 5**, bestudeerden we de effecten van de leeftijd en een ongezonde leefstijl op de zaadkwaliteit van mannen die een fertiliteitskliniek bezochten in verband met een IVF of ICSI behandeling. Uit deze studie bleek dat met het toenemen van de leeftijd van mannen (26 tot 59 jaar) het volume van het ejaculaat en de kwaliteit van het erfelijk materiaal (DNA) in het zaad afneemt.

Deel II

In **Hoofdstuk 6** werd de bevinding gedaan dat overgewicht en obesitas bij mannen die een fertiliteitskliniek bezochten, als proxy voor ongezonde voedings- en leefstijlgewoonten, onafhankelijk van andere leefstijlfactoren nadelig is voor de zaadkwaliteit. Overgewicht bij mannen bleek geassocieerd te zijn met een lager volume van het ejaculaat, een verminderde motiliteit (type A) en een hogere immotiliteit (type C). Bovendien hadden mannen met obesitas ook een lager volume van het ejaculaat, een lagere zaadconcentratie en een verminderde totale beweeglijkheid van de zaadcellen. De middelomtrek van deze mannen, als marker voor centrale adipositas, bleek eveneens geassocieerd te zijn met een lagere zaadconcentratie en een verminderde totale beweeglijkheid van de zaadcellen.

In **Hoofdstuk 7** hebben we de behoefte, patiëntvriendelijkheid en effectiviteit geëvalueerd van het speciale preconceptie spreekuur Gezond Zwanger Worden (GZW), dat tot doel heeft het screenen van paren met kinderwens op (on)gezonde voedings- en leefstijlfactoren en het bevorderen van gezonde gewoonten. Voor dit doel hebben we de “Preconceptie Dieet Risico Score” (PDR-score) en de “Rotterdam Reproductie Risico Score” (R3-score) ontwikkeld. Het spreekuur werd grotendeels bezocht door paren met een vruchtbaarheidsprobleem, waarvan bij de meerderheid een of meerdere ongezonde voedingsgewoonten en een of meerdere leefstijlrisicofactoren werden vastgesteld. Drie maanden na het GZW consult vertoonden 18% van de vrouwen en 12% van de mannen een verbetering van de voedingsgewoonten (PDR-

score). Een resultaat dat onafhankelijk was van etniciteit. Hiernaast lieten ook de leefstijlgewoonten een verbetering zien. De R3-score van vrouwen verbeterde met 34% en die van mannen met 33%. Deze verbetering was bij zowel vrouwen als mannen onafhankelijk van BMI, etniciteit, opleidingsniveau en woonomgeving. Echter, laag opgeleide vrouwen vertoonden een grotere verbetering in vergelijking met hoger opgeleide vrouwen. Bovendien waren de effecten bij mannen met een normale BMI groter dan bij degenen met overgewicht. Opvallend was dat meer dan 85% van de vrouwen en mannen het GZW consult als nuttig beoordeelden en ongeveer 70% het anderen zou aanraden. Deze bevindingen ondersteunen zowel de behoefte als eerste effectiviteit van een speciale preconceptie spreekuur gericht op voeding en leefstijl.

In **Hoofdstuk 8** worden de resultaten van dit proefschrift in een breder perspectief geplaatst en worden aanbevelingen gedaan voor toekomstig onderzoek. De relatieve hoge frequentie van ongezonde voedings- en leefstijlgewoonten onder paren met een vruchtbaarheidsprobleem en kinderwens alsmede de biologische effecten die hiervan werden aangetoond op vruchtbaarheidsparameters, ondersteunen het belang van deze gewoonten voor de eigen gezondheid maar ook voor de vruchtbaarheid en zwangerschap.
Hieruit spreekt de behoefte en noodzaak voor het uitrollen en ontwikkelen van preconceptie spreekuren gericht op het screenen van voedings- en leefstijlrisicofactoren en het coachen bij het aanleren en onderhouden van gezonde gewoonten. Dit is de investering die nu moet worden gedaan voor de gezondheid van de huidige en toekomstige generaties.

groter bewustzijn voor de nadelige effecten van deze factoren voor de fertiliteit. Daarom is het ontwikkelen en toepassen van gerichte op maat gemaakte preconceptie gezondheidsprogramma's noodzakelijk om de reproductie uitkomsten te verbeteren.

Authors and Affiliations

List of Publications

PhD Portfolio

Authors and Affiliations

From the Department of Obstetrics and Gynaecology, Erasmus MC, Rotterdam, the Netherlands
R.P.M. Steegers-Theunissen, J.S.E. Laven, E.A.P. Steegers, M.Vujovic, G.Bonsel, N. van Mil

From the Department of Obstetrics and Gynaecology, Division of Reproductive Medicine Erasmus MC, Rotterdam, the Netherlands
J.S.E. Laven, N. Beckers

From the Department of Epidemiology, Erasmus MC, Rotterdam, the Netherlands
R.P.M. Steegers-Theunissen

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R.P.M. Steegers-Theunissen

From the Department of Bioinformatics, Erasmus MC, Rotterdam, the Netherlands
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From the Department of Clinical Chemistry, Erasmus MC, Rotterdam, the Netherlands
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From the Department of Human Nutrition, Wageningen University, Wageningen, the Netherlands
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From the Department of reproductive Medicine and Gynacology, University Medical center Utrecht, Utrecht, the Netherlands
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From the Department of Andrology and Urology, Erasmus University Medical Center, Rotterdam, the Netherlands
G.R. Dohle, W.P.A. Boellaard

From the department of Internal Medicine, Erasmus MC, Rotterdam, the Netherlands
F.H. de Jong

List of Publications

Articles included in this thesis

Hammiche F, Laven JS, Boxmeer JC, Dohle GR, Steegers EA, Steegers-Theunissen RP. Sperm quality decline among men below 60 years of age undergoing IVF or ICSI treatment. J. Androl. 2011;32:70-6

Twigt JM, **Hammiche F**, Sinclair KD, Beckers NG, Visser JA, Lindemans J, de Jong FH, Laven JS, Steegers-Theunissen RP. Preconception folic acid use modulates estradiol and follicular responses to ovarian stimulation. J Clin Endocrinol Metab. 2011;96:322-9

Hammiche F, Vujkovic M, wijburg W, de Vries JH, Macklon NS, Laven JS, Steegers-Theunissen RP. Increased preconception omega-3 polyunsaturated fatty acid intake improves embryo morphology.Fertil Steril. 2011;95:1820-3

Hammiche F, Laven JS, van Mil N, de Cock M, de Vries JH, Lindemans J, Steegers EA, Steegers-Theunissen RP. Tailored preconceptional dietary and lifestyle counselling in a tertiary outpatient clinic in the Netherlands. Hum Reprod. 2011; Jul 12: Epub ahead of print

Hammiche F, Steegers-Theunissen RP, Beckers NG, de Jong FH, Laven JS. Body Mass Index mediates AMH response after ovarian hyperstimulation teratment. Submitted for publication.

Hammiche F, Laven JS, Boellaard WPA, Steegers EA, Steegers-Theunissen RP. Body Mass Index and central adiposity are associated with semen quality in men of subfertile couples.
Submitted for publication

Other articles

Passchier J, Erdman J, **Hammiche F**, Erdman RA. Adrogenetic alopecia: stress of discovery. Psychol Rep. 2006;98:226-8

Hammiche F, Temel S, Laven JS, Verhagen-van den Graaf MJ, Steegers EA, Steegers-Theunissen RP. Vruchtbare Adviezen. Medisch Contact. 2008;41:1672-1674

Summary of PhD training and teaching activities

Name PhD student:	Fatima Hammiche
Erasmus MC Department:	Obstetrics and Gynaecology
Research School:	NIHES
PhD period:	August 2007- August 2011
Promotors	Prof. dr. R.P.M. Steegers-Theunissen
	Prof. dr. J.S.E. Laven

	Year	Workload: ECTS
General academic skills		
- Methodologie van patient gebonden onderzoek en voorbereiding subsidie aanvragen, Erasmus MC Rotterdam	2010	0.7
- Introduction to statistics and working with SPSS	2009	2.0
- English Medical writing course	2008	2.0
- PhD introduction day, Erasmus MC	2008	0.3
- NIHES: Classical Methods for Data Analysis	2008	5.7
- NIHES: Maternal and Child Health	2008	1.4
- NIHES: Summer Programme	2007	5.0
National and International conferences(presentations), seminars, and workshops		
- Wetenschapsdag gynaecologie en urologie. Erasmus MC Rotterdam	2010	0.3
- VFS meeting, Leuven, Belgium	2010	1.0
- SGI 57th Annual Scientific Meeting Orlando, Florida, USA	2010	0.7
- RCOG onderzoeksdag / Wladimiroff Symposium. Erasmus MC Rotterdam	2010	0.3
- First European Preconception Congres. Brussel, Belgium	2009	1.4
- Preconception Congress, Nieuwegein	2009	1.0
- Kennispoort symposium, Preconception Care, Utrecht	2009	0.7
- DOHAD, Santiago, Chile	2009	1.0
- RCOG onderzoeksdag / Wladimiroff Symposium. Erasmus MC Rotterdam	2009	0.3
- Wetenschapsdag gynaecologie en urologie. Erasmus MC Rotterdam	2009	1.4
- Nederlandse Vereniging Obstetrie en Gynaecologie. Gynaecongres Arnhem	2009	0.3
- Achieving a Healthy Pregnancy: 9 maanden beurs Rai, Utrecht	2009	0.7
- Nederlandse Vereniging voor Obstetrie en Gynaecologie. Gynaecongres Utrecht	2008	0.9
- Symposium “New imaging and developmental concepts in early pregnancy”. Erasmus MC Rotterdam	2008	0.3
- SGI 56th Annual Scientific Meeting Glasgow, Scotland	2008	0.7
- RCOG onderzoeksdag / Wladimiroff Symposium. Erasmus MC Rotterdam	2008	0.3
- Wetenschapsdag gynaecologie en urologie. Erasmus MC Rotterdam	2008	0.3
- Symposium De Jonge Zwangerschap. Erasmus MC Rotterdam	2008	0.7
- Generation R Symposium. Imaging and early brain development. Erasmus MC Rotterdam	2008	0.7
- ABCD Study Symposium. Een gezonde start voor een gezond leven. Vumc, Amsterdam	2008	0.3
- VFS meeting, Leiden, LUMC	2008	0.3

	Year	Workload: ECTS
National and International conferences, seminars, and workshops		
- RCOG onderzoeksdag / Wladimiroff Symposium. Erasmus MC Rotterdam	2008	1.0
- Nederlandse Vereniging Toxicologie voorjaarsvergadering. Erasmus MC Rotterdam	2008	0.3
- Epigenetic epidemiology: lecture Rob Waterland. LUMC	2008	0.3
- Wetenschapsmiddag. Erasmus MC Rotterdam	2008	0.3
- Wetenschapsdag gynaecologie en urologie. Erasmus MC Rotterdam	2007	0.3
- Generation R Study Symposium. Fetal Growth and Development, Erasmus MC Rotterdam	2007	0.7
Lecturing, Supervising practicals		
- Ithar Alghanam	2011	2.0
- Marieke de Cock, student Human Nutrition Wageningen University	2010	3.0
- Nina van Mil, Medical student Erasmus MC Rotterdam	2010	2.4
- Tamara Sterkenburg, student Human Nutrition Wageningen University	2010	1.0
- Supervising pratical, Course Basic Introduction Course to SPSS, Molmed	2010	0.7
- John Twigt, PhD student Erasmus MC Rotterdam	2009	2.0
- Inge Granneman, student Human Nutrition Wageningen University	2008	2.0
- Marieke van Oversteeg, student Human Nutrition Wageningen University	2008	2.0
- Sevilay Temel, Medical student Erasmus MC Rotterdam	2007	2.0
		51.7