Dietary Patterns and Human Reproduction

Voedingspatronen en de Menselijke Voortplanting

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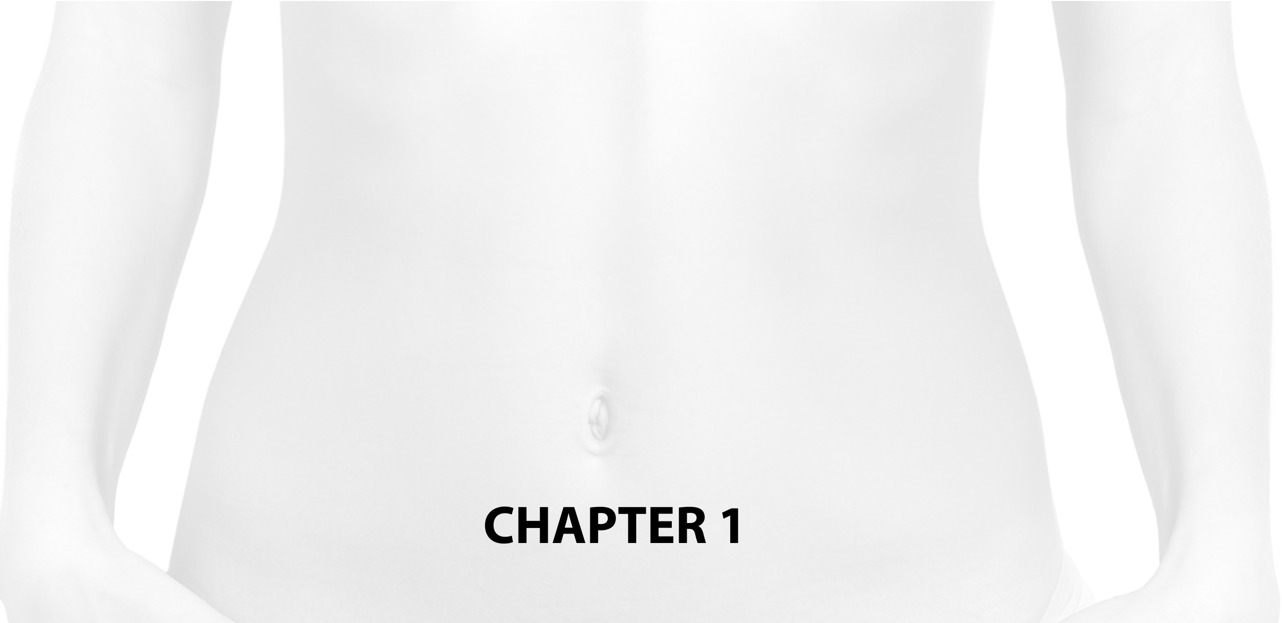
Contents



CHAPTER 1

General Introduction

'I wondered how something so small could grow to such immense dimensions.'

Nikola Tesla (1856 – 1943)

RATIONALE

An individual human life begins as a single cell at conception when an ovum and a sperm unite in the female uterine tube.[1] As the conceptus starts travelling to the uterus it will begin to rearrange, grow, and divide. This process is continuously repeated for 45 cell divisions until ultimately a multicellular body of approximately 30-100 trillion cells is reached in adulthood.[2] Thirty generations of cell divisions have already taken place when the embryo reaches its 8th week of existence, and by this time most internal organs and external body structures are formed.

Till the mid of the 20th century the placenta was believed to effectively protect the developing embryo *in utero* from all environmental influences. We know now that the developing embryo is not only vulnerable to environmental hazards, such as viruses and radiation, but to a harmful maternal modern lifestyle as well, such as the use of tobacco, alcohol, drugs, and unhealthy diet. Evidence is accumulating that nutritional depletions during pregnancy can permanently alter essential fetal developmental processes in order to survive.[3-5] These fetal adaptations can result in an irreversibly altered structure and function of some vital organs, leading ultimately to prematurity, birth defects, behavioural and learning disabilities in childhood, and illnesses later in life.[6-10]

In the three months prior to conception, sperm cells are forming in the testes, while oocytes are maturing in the ovaries. Despite the limited knowledge about these processes, environmental agents have shown to influence the quality of the maturation of gametes and the chances of conception.[11-13] Because nutrition is subject to imposed environmental or, in humans, deliberate change, effects on reproductive health are therefore also likely.[6,14] So far, most studies have been focusing on the effects of single nutrients with regard to health outcome. Scientific discoveries so far have allocated critical roles for nutrient deficiencies with respect to poor reproductive outcome. The 7 best documented nutrients are: iron, iodine, vitamin A, zinc, folate, vitamin D and calcium.[15]

Nutrition is growingly investigated with regard to disease risk in terms of dietary patterns rather than nutrients, for which three reasons are mainly responsible.[16-19] First, in everyday life people do not eat nutrients but meals, which consist of a variety of foods. Many patterns are observed among food intake ascribable to individual taste, neurophysiological impulses, attitude, economic resources, and cultural habits. Second, dietary patterns are able to include complex health effects resulting from interactions between nutrients. And third, the cumulative effect on health outcome of many foods as part of the overall diet is often greater than an isolated nutrient effect. Dietary patterns approximate real-world eating behaviour, and have the major advantage to provide valuable information for the design and realization of food-based dietary guidelines.

THESIS MOTIVATION, OBJECTIVES AND ANTICIPATED RESULTS

The communication of healthy food intake via food-based dietary guidelines has shown to be more straightforward and effective than traditional single nutrient advice.[20,21] Single nutrient analysis has been very successful in past in targeting specific health outcomes, well-illustrated by the discovery of folic acid's beneficial effects in preventing offspring with neural tube defects.[22,23] However, recent public health programs are aiming at improving overall dietary quality in order to prevent poor reproductive performance and adverse pregnancy outcome, and for this approach a single nutrient focus is insufficient. Therefore, the nutrition-disease relationship needs to be understood at the level of foods as well as dietary patterns.

The studies in the current thesis aim to delineate the impact of dietary patterns on pregnancy- and other reproductive outcomes, and the main objectives can be summarized as follows:

1. To identify patterns in food intake from food frequency questionnaire data by using modern empirical statistical methods, such as principal components factor analysis and reduced rank regression.
2. To examine the associations between dietary patterns and several biochemical markers, e.g. folate, vitamin B12, vitamin B6, homocysteine, S-adenosylmethionine, and S-adenosylhomocysteine.
3. To assess the relationship between preconceptional dietary patterns and reproductive performance in men and women, e.g. semen quality, fertility parameters, and chance of pregnancy.
4. To study the effects of periconceptional maternal dietary patterns on the risk of several congenital malformations in the offspring, e.g. spina bifida, congenital heart defects, and orofacial clefts.

With the findings described in this thesis we aim to provide a better understanding of specific dietary patterns that are associated with reproductive performance and chances of pregnancy in subfertile couples. Furthermore, new insights will be provided on to the degree in which maternal dietary patterns contribute to poor pregnancy outcomes, such as congenital malformations.

OUTLINE OF THE THESIS

Part 1 of the thesis focuses on dietary patterns and fatty acid intake in couples undergoing IVF/ICSI fertility treatment. The studies described in **Chapter 2, 3 and 4** are based on the FOod Lifestyle and Fertility Outcome study (FOLFO), a 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 University Medical Centre, Rotterdam, the Netherlands.[24]

Part 2 focuses on the role of the maternal dietary patterns periconception as a risk factor for congenital malformations in the offspring. The studies on risk factors for spina bifida **(Chapter 5)** and orofacial cleft **(Chapter 7)** offspring were conducted in a nationwide large-scale case-control triad study carried out between 1998 and 2004 at the Radboud University Nijmegen Medical Centre.[25, 26] The findings on associations with congenital heart malformations in **Chapter 6** are based on the HAVEN study (Hart

Afwijkingen, Vasculaire status, Erfelijkheid en Nutriënten).[7] The HAVEN study is an ongoing case-control triad study conducted in the Western part of the Netherlands. The study emphasizes nutrition, lifestyle and genes in the pathogenesis and prevention of congenital heart malformations and children and both parents were included. Data collected between March 2004 and August 2008 were used for analysis.

Finally, **Chapter 8** provides a general discussion of the thesis, a summary of the key findings, reflections on the strengths and limitations of the applied methods, implication for clinical practice and public health, and suggestions for future research.

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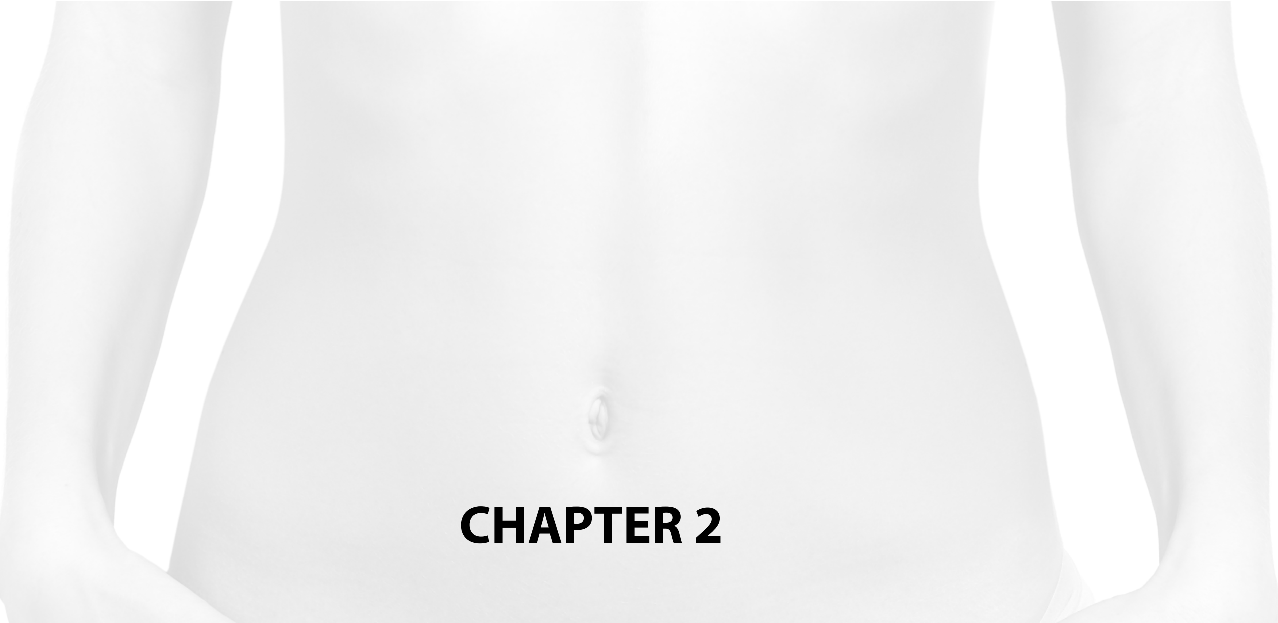
PART I

Dietary patterns and reproductive performance



CHAPTER 2

Associations between dietary patterns and semen quality in men undergoing IVF/ICSI treatment

Marijana Vujković, Jeanne H. de Vries, Gerard R. Dohle, Gouke J. Bonsel, Jan Lindemans, Nick S. Macklon, Peter J. van der Spek, Eric A. Steegers, and Régine P. Steegers-Theunissen

based on Hum Reprod 2009; 24: 1304 – 1312

ABSTRACT

Objective

This study investigates whether dietary patterns, substantiated by biomarkers, are associated with semen quality.

Methods

In 161 men of subfertile couples undergoing in vitro fertilization treatment in a tertiary referral clinic in Rotterdam, the Netherlands, we assessed nutrient intakes and performed principal component factor analysis to identify dietary patterns. Total homocysteine (tHcy), folate, vitamin B12, and vitamin B6 were measured in blood and seminal plasma. Semen quality was assessed by sperm volume, concentration, motility, morphology, and DNA fragmentation index (DFI). Linear regression models analyzed associations between dietary patterns, biomarkers and sperm parameters, adjusted for age, body mass index (BMI), smoking, vitamins and varicocele.

Results

The Health Conscious dietary pattern shows high intakes of fruit, vegetables, fish, and whole grains. The Traditional Dutch dietary pattern is characterized by high intakes of meat, potatoes and whole grains and low intakes of no-alcoholic drinks and sweets. The Health Conscious diet was inversely correlated with tHcy in blood (β –0.07, p 0.02) and seminal plasma (β –1.34, p 0.02) and positively with vitamin B6 in blood (β 0.217, p 0.01). An inverse association was demonstrated between the Health Conscious diet and DFI (β –2.81, p 0.05). The Traditional Dutch diet was positively correlated with red blood cell folate (β 0.06, p 0.04) and sperm concentration (β 13.25, p 0.01).

Conclusion

The Health Conscious and Traditional Dutch dietary pattern seem to be associated with semen quality in men of subfertile couples.

INTRODUCTION

Over the past decades, human fertility rates have declined dramatically in industrialized countries.[1] As a result of abnormal semen characteristics, ~30% of all subfertile couples today require artificial fertility treatment to address failed reproductive attempts.[2] The impact of risk factors like smoking and alcohol use is undisputed, and also manifest malnutrition is known to affect semen.[3]

As a reflection of global changes in dietary behaviour, the prevalence of unhealthy diets, characterized by low intakes of fruit and vegetables and high intakes of foods rich in saturated fats, has increased in women and men within the reproductive age range.[4] However, so far nutritional studies on semen quality in men have rather focused on investigating the role of zinc and the B-vitamin folate. Zinc is a trace element and acts as essential cofactor in metalloenzymes involved in many processes of spermatogenesis and a shortage can lead to oligospermia.[5] A folate deficiency results in homocysteine abundance and induces oxidative stress and apoptosis. Recent findings show associations between folate deficiency-dependent and sperm aneuploidy,[6] sperm DNA damage,[7] and low sperm count.[8] As expected, total homocysteine (tHcy) concentrations in seminal plasma appear to be associated with male subfertility and low embryo quality.[9]

The human diet contains a wide range of nutrients that act in concert on many biological pathways. Because nutrition is subject to imposed environmental or, in humans, deliberate change, effects on reproductive health can be expected. Our aim of the present study was to: 1) identify dietary patterns in men of subfertile couples undergoing in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) treatment; 2) relate dietary patterns to biomarker concentrations of the homocysteine pathway in blood and seminal plasma; and 3) relate dietary patterns as defined in 2) to semen quality, controlling for the risk factors of age, body mass index (BMI), smoking, vitamin supplement use and presence of varicocele. In our paper, the intake of alcohol is conceptually regarded as a nutriental exposure.

METHODS

Study Population

Between September 2004 and January 2007, subfertile couples undergoing IVF/ICSI treatment at the Erasmus University Medical Centre in Rotterdam, the Netherlands, were included in the prospective FOod, Lifestyle and Fertility Outcome-study (FOLFO-study). This study was designed to study the influence of preconception nutrition and lifestyle on fertility and pregnancy outcome. Fertile and subfertile men were eligible for enrolment unless semen was cryopreserved or obtained by microsurgical or percutaneous epididymal sperm aspiration. Of the eligible IVF/ICSI population, 66% of the fertile and subfertile men participated in the FOLFO Study (n 251). Because of the influence of ethnicity on dietary habits and lifestyle, we included in the current analysis male participants of European origin only. This resulted in the evaluation of 161 male participants. The study protocol was approved by the Dutch Central Committee for Human Research and the Medical Ethical and Institutional Review Board of Erasmus University Medical Centre in Rotterdam, the Netherlands. All participants gave their written informed consent.

General Questionnaire

At the IVF intake visit, the couples were invited to participate in the study, and only the men were included in the current analysis. All participants filled out a general questionnaire at home comprising information on current lifestyle and demographic factors and returned it on the next hospital appointment. The extracted data comprised of age, weight, height, medical history, education, use of medication and lifestyle factors, such as smoking and the use of vitamin supplements. Between 2 weeks before and 2 weeks after oocyte retrieval, men visited the andrology outpatient clinic for fertility evaluation comprising semen analysis, blood sampling, and physical examination including varicocele detection. During physical examination, scrotal ultrasonography was performed using a Toshiba Nemio 20 with a 12 Hz transducer. A varicocele was diagnosed if at least two venous vessels with a diameter of 3 mm or more were present, in addition to reflux or diameter increase during Valsalva's manoeuvre. All laboratory analyses were performed by personnel blinded to the clinical diagnosis. Male subfertility was defined by a sperm concentration of $\leq 20 \times 10^6$ cells/ml.

Food Frequency Questionnaire

All participants filled out a food frequency questionnaire (FFQ) to estimate habitual food and alcohol intake of the previous 4 weeks. This semi-quantitative FFQ was originally developed at the division of Human Nutrition, Wageningen University, Wageningen, the Netherlands and validated for intake of energy, fatty acids and B-vitamins.[19,20] The FFQs were provided on the day of sperm sample collection and returned on the day of embryo transfer. A checklist was used to verify and check the completeness of the FFQ. Additional questions were asked by telephone interview. The FFQ consists of 195 food items and is structured according to meal pattern. Questions in the FFQ included frequency of consumption, portion size and preparation methods. Portion sizes were estimated according to Dutch household measures.[21] Nutritional values were determined with the use of the Dutch food composition table (NEVO) of 2001.[22]

From the original 248 questionnaires filled out by the men, 2 out of 23 (8.7%) with no embryo transfer did not respond. Five of 225 men (2.2%) with embryo transfer did not respond. Owing to these very small numbers, it is very unlikely that selection bias has occurred. To evaluate the existence of underreporting the ratio of energy intake divided by basal metabolic rate (BMR) was calculated. This value is an estimation of the physical activity level (PAL) of a sedentary lifestyle. The equations of Schofield were used for estimating BMR.[23] According to Goldberg et al.[24] a cut-off point for underreporting for a sedentary lifestyle is a ratio of ≤1.35.

Semen Analysis

Semen samples were produced via masturbation into polypropylene containers. Within half an hour, the samples were liquefied, and the semen parameters of volume, sperm concentration, sperm count, percentage progressive motility, and percentage normal morphology were assessed according to World Health Organization guidelines.[25] The 1999 World Health Organization reference values for normal sperm are fulfilled when sperm concentration exceeds 20×10^6 cells/ml, >50% of spermatozoa have forward progression and >30% a normal morphology. Subsequently an aliquot of semen was centrifuged at 2500g for 10 minutes. The supernatant seminal plasma was frozen without preservatives and stored at –20°C until assayed.

DNA Fragmentation Index

The principles and procedures of measuring sperm DNA damage by a FACScan flow cytometry SCSA have been described previously.[26] In short, semen samples were diluted with TNE buffer [0.01 M Tris–HCl, 0.15 M NaCl, 1 mM ethylenediamine tetraacetate (EDTA), pH 7.4] 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 (0.08 N HCl, 0.15 M NaCl, 0.1% Triton-X 100, pH 1.2) and then stained with 1.2 ml Acridine Orange (AO) staining solution (0.1 M citric acid, 0.2 M Na_2PO_4, 1 mM EDTA, 0.15 M NaCl, pH 6.0, containing 0.6 µl/L AO). A reference sample treated in the same way was run prior to the actual measurements and used to adjust the voltage gains of the flow cytometer FL3 and FL1 photomultipliers that detected red and green fluorescence, respectively. An aliquot of reference sample was stained and run again after every 5-10 samples. Data collection of the fluorescent pattern in 5000 cells was performed at 3 minutes after acid treatment. Each sperm sample was analyzed twice. The extent of DNA damage was expressed as the DNA fragmentation index (DFI), reflecting the red to total fluorescence ratio. Cell Quest Pro and WinList software (Becton Dickinson, San Jose, CA, US) were used to calculate the DFI of each sample.

Biomarker Assay

Venous blood samples were drawn into dry vacutainer tubes and allowed to clot. After centrifugation at 2000g, the blood serum was collected before being assayed for the concentrations of folate, vitamin B12 and testosterone. For the determination of red blood cell (RBC) folate and tHcy, venous blood samples were drawn into EDTA containing vacutainer tubes. The EDTA-blood samples were kept on ice, and plasma was separated by centrifugation within 1 hour for determination of tHcy. For the determination of vitamin B6, blood was drawn into lithium-heparin containing vacutainers. Blood serum and seminal plasma samples from each patient were analyzed during routine laboratory procedures for folate and vitamin B12 using an immuno-electro-chemoluminescence assay (E170; Roche Diagnostics GmbH, Mannheim, Germany). Directly after blood sampling, 0.1 ml of blood out of an EDTA tube 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 1000 g shortly before the folate measurement.

The folate concentration in the hemolysate was recalculated in RBC folate using the following formula: (nM hemolysate folate x 10 / hematocrit) – (nM serum folate x [1 – hematocrit] / hematocrit) = nM RBC folate. Vitamin B6 levels in whole blood and seminal plasma and tHcy levels in EDTA plasma and seminal plasma were determined during routine laboratory procedures using high-performance liquid chromatography with reversed-phase separation and fluorescence detection.[27,28] We determined whole blood vitamin B6 with pyridoxal-5'-phosphate (PLP) being the most common form. Testosterone was measured using the Coat-a-Count radioimmunoassay (Diagnostic Products Corp, Los Angeles, CA, US). Sex hormone binding globulin (SHBG) was determined, using an immunometric technique on an Immulite Analyzer obtained from the same supplier. Serum inhibin B was measured by immunoenzymometric assay (OBI, Oxford Bio-Innovation, Oxford, UK).

The between-run coefficient of variation for serum vitamin B12 was 5.1% at 125 pmol/L and 2.9% at 753 pmol/L; the coefficients of variation for serum folate were 9.5% at 8.3 nmol/L and 3.2% at 20.2 nmol/L; 3.3% at 14.55 µmol/L and 2.3% at 34.23 µmol/L for tHcy; 1.8% at 40 nmol/L and 1.3% at 115 nmol/L for PLP; for testosterone, these coefficients of variation were ≤7.5%, 6.1% at 11.6 nmol/L and 6.9% at 93 nmol/L for SHBG. For inhibin B, the coefficients of variation were ≤15%. The detection limit was 1.36 nmol/L for folate, 22 pmol/L for vitamin B12, 5 nmol/L for PLP, 4 µmol/L for tHcy, 0.1 nmol/L for testosterone, 5 nmol/L for SHBG and 10 ng/L for inhibin B.

Statistics

Because of the prior knowledge of high correlations among several food constituents, we used principal component factor analysis (PCA) to summarize dietary patterns from food consumption data.[4,29] In summary, all 195 food items from the FFQ data of men undergoing IVF/ICSI treatment were first reduced to 22 predefined food groups based on a nutrient content grouping, conform to other reported schemes.[30] Hereafter food groups were adjusted for the total intake of energy,[31] and subsequently PCA was performed. To keep rotated factors uncorrelated, the solution was rotated with the varimax method by maximizing the sum of the variance of the loading vectors.[32] The first two factors were extracted, and each represents a distinct dietary pattern. A factor consists of a selection of the initial variables, each with its own coefficient (here called loading), which defines the observed correlation of that variable with the latent constructed factor. As a weighted 'mix' of the initial variables, a factor explains a substantial amount of variation in the data set under study. In the preferable case, the statistically derived factor represents a recognizable pattern in the observable world. The Eigenvalue was used as indicator of the amount of variation explained by each factor. Each participant was assigned two personalized scores for the two factors, i.e. which represents a quantification of the similarity of the individual's diet with each of the two extracted factors. The factor loadings, i.e. the association of a factor with all measured food components, are presented for each factor separately; the association is calculated by Pearson's r correlation coefficient (Table 1).

After computation of the personalized scores, all 161 men were classified into tertiles according to their personal score for the respective dietary pattern. Additional explanatory variables were conventionally described. However, as age, body mass index (BMI, defined as kg/m^2) and energy intake showed skewed distributions even if log-transformed, these variables are described by the median. For the same reason, we displayed medians and inter-quartile ranges for the biomarkers. Differences in general characteristics between tertiles were evaluated in an unconditional linear regression model. The prevalence of causes of subfertility, ethnic background, smoking and vitamin use were related to each tertile of the respective dietary pattern score, and the dietary associations with this prevalence were tested with chi-square test for linear association.

Next, the diet was used to predict the logarithmically transformed biomarkers of the homocysteine pathway and semen quality parameters in a multivariable linear regression model, additionally adjusted for the potential confounding variables age, BMI, smoking, intake of multivitamin and/or folic acid supplements and the presence of a varicocele. Individual food groups were also analyzed in a linear regression model to predict semen parameters and investigate which food groups of the dietary pattern contribute most to the observed associations with semen parameters.

Table 1 | Food group factor loadings for two identified dietary patterns from food-frequency questionnaire data of 160 men undergoing IVF/ICSI treatment

food group	*Health Conscious diet*	*Traditional Dutch diet*
alcohol	– 0.10	– 0.19[1]
cereals	– 0.09	– 0.28[2]
butter	0.00	0.03
dairy	– 0.04	0.04
eggs	0.00	0.12
fish	– 0.56[2]	– 0.14
fruit	0.80[2]	– 0.16[1]
legumes	0.16[1]	0.05
margarine	– 0.13	0.18[1]
mayonnaise	– 0.20[1]	0.22[2]
meat	– 0.17[1]	0.47[2]
non-alcoholic drinks	0.06	– 0.71[2]
nuts	0.12	0.00
refined grains	– 0.16[1]	– 0.13
potatoes	– 0.01	0.56[2]
sauces	0.11	0.05
snacks	– 0.08	– 0.03
soup	0.08	– 0.23[2]
sweets	– 0.17[1]	– 0.46[2]
vegetable oil	0.09	0.03
vegetables	0.74[2]	0.12
whole grains	0.43[2]	0.48[2]
variance explained	*11.7%*	*9.5%*

[1]p ≤0.05, [2]p ≤0.01

Covariates were age, BMI, smoking, intake of multivitamin and/or folic acid supplements. The food groups with all β estimates and 95% confidence intervals (95%CI) described. All analysis was performed with SPSS software, release 15.0.0 for Windows (SPSS Inc., Chicago, IL, US).

RESULTS

The characteristics of the dietary intake of 161 male participants, in terms of the two selected factors (dietary patterns), are shown in Table 1. The first factor explained 11.7% of the total variance. After inspection of the associative pattern, it was labelled the Health Conscious diet; it comprises high intakes of fruit, vegetables, fish, whole grains, and legumes and low intakes of mayonnaise and other fatty sauces, meat, refined grains, and sweets. The second factor explained 9.5% of the total variance of dietary intake, and in our interpretation represented the Traditional Dutch diet; it is characterized by high intakes of potatoes, meat, whole grains, margarine, mayonnaise and other fatty sauces and low intakes of non-alcoholic and alcoholic drinks, cereals, fruit, soup, and sweets. Neither subgroups of the Health Conscious nor the Traditional Dutch diet showed differences in general characteristics or endocrine parameters (Table 2).

Initial significant correlations were present between the Health Conscious diet and serum and RBC folate, tHcy, vitamin B6 and vitamin B12, but after adjusting for confounders, significant correlations only remained for tHcy (β –0.07, p 0.02) and vitamin B6 (β 0.22, p 0.01) as shown in Table 3. In seminal plasma, the adjusted linear model revealed an inverse association between tHcy and the Health Conscious diet (β –1.34, p 0.02). The Traditional Dutch pattern was positively correlated with RBC folate (β 0.06, p 0.05, p_{adj} 0.04), and no significant associations were observed with biomarkers in seminal plasma. Table 4 reveals that the use of the Health Conscious diet is inversely associated with sperm DNA damage (β –2.81, p 0.05). This effect seems to be explained by the high intakes of fruit (β –0.13, 95%CI –0.25; –0.02) and vegetables (β –0.25, 95%CI –0.49; –0.01) (Table 5). The Traditional Dutch diet showed an increase in sperm concentrations (β 13.25, p 0.01) (Table 4). The high intake of potatoes (β 0.82, 95%CI 0.24; 1.4) and low intake of non-alcoholic drinks (β –0.29, 95%CI –0.49; –0.1) seem to explain this finding (Table 5). All significance levels denoted have been adjusted for age, BMI, smoking, vitamin use and presence of varicocele at the study moment.

DISCUSSION

This study demonstrates that human nutrition affects semen quality in men undergoing IVF/ICSI procedures. We observed that men who consume a Health Conscious diet have lower sperm DNA damage. Furthermore, sperm concentrations are much higher in men who strongly adhere to the Traditional Dutch dietary pattern. These findings suggest that the two dietary patterns indeed have beneficial effects on semen quality. Our findings also represent important epidemiological information as it provides a specific explanation and empirical evidence on the putative relation between the increased unhealthy food availability and the decline of semen quality in industrialized countries.

We were surprised by the observation that the Health Conscious dietary pattern is inversely related to tHcy in blood and seminal plasma, but positively related to vitamin B6 in blood. This may imply that this dietary pattern prohibits sperm DNA from being damaged by regulating the flow of tHcy concentrations.

Table 2 | Baseline characteristics of the Health Conscious and Traditional Dutch diet

	Health Conscious diet			
	low	*intermediate*	*high*	
characteristic	*(n 53)*	*(n 55)*	*(n 53)*	*p*
age[1,2]	35.3 (29.1- 53.9)	35.8 (29.8 - 46.6)	37.3 (28.6 - 50.5)	0.13
BMI[1,3]	25.0 (18.4 - 37.9)	26.0 (19.6 - 37.1)	24.9 (18.8 - 34.8)	0.38
cause of subfertility[4]				0.69
male factor	20 (37.7)	20 (36.4)	17 (32.1)	
female factor	13 (24.5)	11 (20.0)	7 (13.2)	
both	3 (5.7)	4 (7.2)	4 (7.5)	
unexplained	17 (32.1)	20 (36.4)	25 (47.2)	
presence of varicocele[5]	10 (20.8)	8 (17.8)	8 (17.4)	0.90
Dutch ethnicity	46 (86.8)	45 (81.8)	44 (83.0)	0.77
smoking	14 (26.4)	14 (25.5)	6 (11.3)	0.10
vitamin use	12 (22.6)	13 (23.6)	17 (32.1)	0.27
energy intake[1,6]	10.8 (4.5 - 19. 4)	9.2 (3.6 - 18.1)	10.5 (5.3 - 15.5)	0.86
testosterone[7,8]	16.0 (14.4 - 17.7)	14.6 (13.3 - 16.2)	14.9 (13.8 - 16.1)	0.96
SHBG[7,8]	28.2 (25.0 - 31.8)	23.1 (20.7 - 25.8)	28.0 (24.6 - 31.7)	0.35
inhibin B[7,9]	172 (150 - 195)	171 (151 - 191)	175 (152 - 199)	0.79
	Traditional Dutch			
	low	*intermediate*	*high*	
characteristic	*(n 54)*	*(n 54)*	*(n 53)*	*p*
age[1,2]	36.3 (30.0 - 50.5)	36.3 (28.6 - 46.8)	35.6 (28.7 - 53.9)	0.91
BMI[1,3]	24.8 (18.4 - 37.9)	24.7 (18.8 - 33.8)	26 (19.9 - 37.1)	0.07
cause of subfertility[4]				0.11
male factor	14 (25.9)	27 (50.0)	16 (30.2)	
female factor	14 (25.9)	9 (16.7)	8 (15.1)	
both	5 (9.2)	2 (3.7)	4 (7.5)	
unexplained	21 (38.9)	16 (29.6)	25 (47.2)	
presence of varicocele[5]	12 (26.1)	4 (8.2)	10 (22.7)	0.06
Dutch ethnicity	44 (81.5)	46 (85.2)	45 (84.9)	0.84
smoking	12 (22.2)	9 (16.7)	13 (24.5)	0.59
vitamin use	16 (29.6)	14 (25.9)	12 (22.6)	0.41
energy intake[1,6]	10.6 (5.7 - 19.4)	9.6 (3.6 - 18.1)	10.8 (5.8 - 18.6)	0.36
testosterone[7,8]	15.2 (13.9 - 16.5)	14.9 (13.5 - 16.4)	15.5 (14.1 - 17.2)	0.99
SHBG[7,8]	28.0 (24.8 - 31.6)	26.2 (23.6 - 29.2)	24.4 (21.4 - 27.9)	0.19
inhibin B[7,9]	174 (153 - 194)	174 (151 - 198)	170 (148 - 193)	0.86

Data are presented by [1]median (range), [2]years, [3]kg/m^2, [4]n (%), [5]presence of varicocele was missing in 22 men, [6]mJoule/day, [7]mean (95% CI), [8]nmol/L, [9]ng/L.

We also found that the beneficial effects of the Health Conscious pattern seem to be determined in particular by vegetable and fruit intake, as has been further substantiated by the single food group analyses. The high consumption of folate- and vitamin B6-rich foods may stimulate tHcy to be re-methylated into methionine and trans-sulfurated into cystathionine and cysteine.

In males, the regulation of homocysteine is mediated partially through the testosterone-dependent cystathionine-β-synthase pathway.[33] Other studies support the importance of the homocysteine pathway on semen parameters. A Spanish study revealed positive associations between the intake of folate-rich food sources, such as fruit and vegetables, and semen quality.[34] Furthermore, Young et al.[6] observed inverse relationships between folate, zinc and antioxidant intake in healthy non-smoking men in the US and sperm aneuploidy. Suggested mechanisms by which elevated tHcy may exert its effects are excessive induction of oxidative stress, defective methylation of proteins, lipids, and DNA, altered nitric oxide bioavailability, induction of vascular inflammation, and activation of apoptosis.[3] However, these mechanisms in reproductive tissues should be studied in much more detail in future studies, both experimentally in animals and observationally in human cohorts.

Table 3 | Concentration of biomarkers in blood and seminal plasma in two dietary patterns[1]

			Health Conscious diet		
		low	*intermediate*	*high*	
	biomarker	*(n 32)*	*(n 21)*	*(n 26)*	*p*[5]
blood	serum folate[2]	16.1 (5.6)	15.3 (6.5)	18.5 (8.9)	0.15
	RBC folate[2]	1006 (350)	1063 (536)	1107 (542)	0.10
	vitamin B6[2]	81.5 (36.8)	77.0 (35.6)	94.0 (48.3)	0.01
	vitamin B12[3]	288 (94)	289 (188)	361 (155)	0.07
	tHcy[4]	12.2 (3.2)	11.7 (4.1)	10.8 (4.4)	0.02
semen	folate[2]	26.5 (11.6)	25.7 (16.4)	25.0 (15.5)	0.70
	vitamin B6[2]	30.5 (39.0)	30.0 (23.5)	28.5 (30.2)	0.30
	vitamin B12[3]	467 (608)	424 (450)	493 (339)	0.51
	tHcy[4]	4.1 (3.1)	4.4 (3.2)	3.7 (4.5)	0.02
			Traditional Dutch diet		
	biomarker	*low*	*intermediate*	*high*	*p*[5]
blood	serum folate[2]	17.3 (7.7)	16.2 (6.7)	15.7 (9.1)	0.84
	RBC folate[2]	990 (554)	986 (382)	1110 (511)	0.04
	vitamin B6[2]	99.0 (48.0)	78.5 (31.0)	77.0 (36.5)	0.81
	vitamin B12[3]	329 (172)	293 (148)	282 (158)	0.41
	tHcy[4]	11.9 (4.7)	11.2 (3.1)	12.3 (3.7)	0.84
semen	folate[2]	28.3 (14.8)	24.8 (10.5)	22.6 (15.8)	0.69
	vitamin B6[2]	33.0 (32.0)	29.0 (21.3)	29.0 (33.5)	0.42
	vitamin B12[3]	409 (406)	438 (400)	680 (440)	0.44
	tHcy[4]	3.7 (3.9)	4.0 (2.5)	4.7 (5.7)	0.62

[1]mean(sd), [2]nmol/L, [3]pmol/L [4]µmol/L. [5]Adjusted for age, BMI, smoking, vitamins, varicocele.

Unfortunately, no data on seminal poly-unsaturated fatty acid (PUFA) concentrations were collected, which would have enabled us to measure whether the increased fish intake resulted in higher levels of unsaturated fats that might enhance the fluidity of the plasma membrane. A fluid membrane, however, also makes spermatozoa more vulnerable to ROS attack, which may explain why oxidative stress-inducing lifestyle factors may have led to reduced human fertility in the past decades.

The second identified dietary pattern being the Traditional Dutch comprises high intakes of meat, potatoes, and whole grains. This resembles a typically Dutch dietary tradition up to the 19th century when agriculture and domestic education were widely implemented in the Netherlands. This dietary pattern is positively correlated with folate in RBC and shows a significant positive association with sperm concentrations. Because of the substantial amount of potatoes eaten in one serving, they provide a rich source of folate. Furthermore, the high intake of meat, a natural source of zinc elements, influences the bioavailability of dietary folate. The zinc-dependent enzyme γ-glutamylhydrolase in the jejunum efficiently converts dietary folate as poly-glutamates into mono-glutamates, which are the only absorbable form of folate. Furthermore, zinc is a cofactor of methionine synthase involved in the remethylation of tHcy into methionine thereby reducing tHcy. The beneficial effects of zinc are supported by a randomized, placebo-controlled intervention study conducted in the late nineties, in which administration of zinc and/or folic acid to subfertile patients led to a significant increase in semen concentration ranging from 18% to 74%.[9,12,17]

Table 4 | Semen parameters according to the tertiles of the two major dietary patterns[1]

		Health Conscious diet		
	low	*intermediate*	*high*	
semen parameter	*(n 40)*	*(n 44)*	*(n 42)*	*p*[5]
DFI[2]	25.2 (21.1 - 29.2)	25.0 (20.2 - 29.8)	20.6 (17.5 - 23.6)	0.05
volume[3]	3.4 (2.9 - 3.8)	2.8 (2.4 - 3.2)	3.1 (2.6 - 3.5)	0.41
concentration[4]	46.2 (30.8 - 61.5)	39 (27.6 - 50.4)	48.2 (33.1 - 63.2)	0.89
motility[2]	35 (30 - 40)	33 (28 - 39)	37 (32 - 42)	0.06
morphology[2]	5 (4 - 6)	5 (4 - 6)	5 (4 - 6)	0.74

		Traditional Dutch diet		
	low	*intermediate*	*high*	
semen parameter	*(n 44)*	*(n 43)*	*(n 39)*	*p*[5]
DFI[2]	23.5 (20.1 - 26.9)	25.0 (20.0 - 30.1)	22.0 (18.5 - 25.6)	0.53
volume[3]	3.1 (2.6 - 3.5)	3.1 (2.7 - 3.5)	3.0 (2.6 - 3.5)	0.89
concentration[4]	36.7 (26.8 - 46.6)	36.4 (23.1 - 49.8)	61.7 (44.5 - 78.9)	0.01
motility[2]	35 (31 - 39)	34 (29 - 40)	37 (31 - 43)	0.98
morphology[2]	5 (4 - 6)	5 (4 - 6)	6 (5 - 7)	0.34

Data are presented by [1]median with range, [2]percentages, [3]ml, [4]x10^6 cells/ml. [5]Adjusted for age, BMI, smoking, vitamin use and presence of a varicocele.

In the same FOLFO study, but in a much smaller study group of men, we measured the biomarkers of homocysteine metabolism in blood and seminal plasma and investigated the associations with semen parameters. A positive association was observed between seminal plasma vitamin B12 and sperm concentration.[35] In the current analysis, we demonstrate a significant association between the Traditional Dutch dietary pattern and semen concentrations (increase from $36.7x10^6$ to $61.7x10^6$ cells/ml in the highest tertile, p 0.01). We also observe positive but not statistically significant associations between the Traditional Dutch dietary pattern and seminal vitamin B12 concentrations, which are very likely to be due to the high meat intake. This supports the previous observed association between seminal vitamin B12 and sperm concentration.

In a recent study, Mendiola et al.[34] suggested that meat intake negatively affects semen quality. They assumed this detrimental effect to be due to natural and synthetic estrogens in meat, hormones that are used in cattle to stimulate growth and development.[36] However, their study size was rather small (n 61) and only single food items were investigated. There was no possibility of comparing dosage effects because only reports of frequency were used to assess the dietary intake, and portion sizes were not reported. We can only speculate that the favourable effect of the meat-rich Traditional Dutch diet on semen quality might be due to the relative lower amounts of meat used in our country, or perhaps a lower exposure of Dutch cattle to hormones and endocrine disruptors.

Some concerns regarding the study design apply. We cannot exclude the possibility that the dietary patterns identified are confounded by lifestyle factors other than the adjusted covariates age, energy intake, BMI, smoking and use of vitamin supplements. We observed little or no association of these factors with the Health Conscious and Traditional Dutch dietary pattern, but cannot rule out a possible role of other residual confounding variables, e.g. exercise, psychological stress, and exposure to environmental pollutants.[3] We have reduced the possibility of multiple testing by summarizing the overall nutrient intake with PCA into two dietary patterns. By limiting our analysis to only two exposure variables and taking all nutrients into account, we have avoided the issue of selective testing. These two exposure variables were associated with biomarkers of the homocysteine metabolism and semen parameters, whereby we have attempted to minimize the probability of chance-finding.

All methods of dietary assessment are prone to error. Nutritional intake was assessed with an FFQ and individually checked for completeness and accuracy. However, self-reported dietary intake tends to habitually underestimate energy intake.[37] Therefore, we evaluated the presence of underreporting by estimating the PAL. The mean PAL for all participants in our study was 1.28, and similar in fertile and subfertile men indicating an underestimation of energy intake in both groups according to Goldberg standard (PAL $\leq$1.35).[24] We judge it therefore to be unlikely that underestimation has distorted the associations between semen parameters and energy.

We recognize that the findings from this Dutch sample and from comparable European subfertile samples may not apply to other populations, particularly if patterns in dietary intake are dissimilar or foods are differently produced and processed. Still our results apply to a substantial part of the globe with westernized dietary patterns and lifestyle. The strength of the study is its prospective design and the relatively large study sample size of 161 men. This gave us the opportunity to produce reliable dietary patterns which were comparable to studies conducted in the Dutch general population.[38]

General Conclusion

The Health Conscious and the Traditional Dutch dietary pattern seem to be associated with semen quality. More studies are needed to show the causation of these associations and their effects on human reproduction.

Table 5 | Contribution of individual food groups to semen parameters

			semen parameter		
food group	*DFI*	*volume*	*concentration*	*motility*	*morphology*
alcohol	0.0 (-0.1; 0.1)	0.0 (-0.1; 0.1)	0.0 (-0.1; 0.2)	– 0.0 (–0.1; 0.1)	0.0 (-0.1; 0.1)
cereals	– 0.4 (-1.1; 0.4)	– 0.1 (-0.1; 0.1)	1.0 (-1.3; 3.2)	0.5 (0.2; 1.3)	0.0 (-0.2; 0.2)
butter	– 4.1 (-13; 4.8)	– 0.4 (-1.3; 0.4)	2.8 (–24; 30)	7.6 (-1.7; 17)	– 1.4 (-3.2; 0.4)
dairy	0.1 (-0.3; 0.4)	0.0 (0.0; 0.1)	0.5 (–0.6; 1.6)	– 0.1 (-0.5; 0.3)	0.0 (-0.1; 0.1)
eggs	– 0.3 (-0.9; 0.3)	0.1 (0.0; 0.1)[1]	– 0.2 (–2.1; 1.7)	– 0.1 (-0.7; 0.5)	0.0 (-0.1; 0.1)
fish	– 0.7 (-2.0; 0.6)	– 0.1 (-0.2; 0.1)	0.9 (–3.3; 5.1)	1.3 (0.0; 2.6)[1]	0.1 (-0.1; 0.4)
fruit	– 0.1 (-0.3; -0.0)[1]	0.0 (-0.1; 0.1)	– 0.1 (–0.5; 0.3)	0.2 (0.1; 0.3)[1]	0.0 (-0.1; 0.1)
legumes	– 0.5 (-1.2; 0.1)	– 0.0 (-0.1; 0.1)	0.2 (-1.7; 2.1)	0.1(-0.5; 0.8)	0.0 (-0.1; 0.1)
margarine	4.4 (-2.3; 11)	0.5 (-0.2; 1.2)	– 3.9 (-26; 18)	– 9.1 (-16; -1.5)[1]	0.2 (-1.3; 1.7)
mayonnaise	0.1 (-1.3; 1.6)	– 0.2 (-0.3; -0.1)[1]	– 0.1 (-4.3; 4.2)	– 0.8 (-2.3; 0.6)	– 0.1 (-0.4; 0.2)
meat	1.0 (-0.1; 2.0)	0.1 (-0.1; 0.2)	– 2.2 (-5.7; 1.4)	– 1.0 (-2.2; 0.2)	– 0.1 (-0.4; 0.1)
non-alc. drinks	0.0 (-0.1; 0.1)	0.0 (-0.1; 0.1)	– 0.3 (-0.5; -0.1)[1]	– 0.0 (-0.1; 0.1)	0.0 (-0.1; 0.1)
nuts	– 0.8 (-1.6; -0.1)[1]	– 0.1 (-0.2; 0.1)	– 1.0 (-3.4; 1.3)	0.2 (-0.6; 1.0)	0.1 (-0.1; 0.2)
refined grains	– 0.1 (-0.5; 0.2)	– 0.0 (-0.1; 0.1)	0.3 (-0.7; 1.3)	– 0.1 (-0.5; 0.2)	0.0 (-0.1; 0.1)
potatoes	– 0.1 (-0.2; 0.1)	0.0 (-0.1; 0.1)	0.8 (0.2; 1.4)[1]	0.1 (-0.1; 0.3)	0.0 (-0.1; 0.1)
sauces	– 0.8 (-1.8; 1.6)	– 0.1 (-0.3; 0.1)	0.2 (-5.3; 5.7)	– 0.8 (-2.6; 1.0)	– 0.0 (-0.4; 0.3)
snacks	0.1 (-0.4; 0.5)	0.0 (-0.1; 0.1)	– 0.6 (-2.1; 1.0)	0.2 (-0.3; 0.7)	– 0.1 (-0.2; 0.0)
soup	0.0 (-0.1; 0.1)	0.0 (-0.1; 0.1)	– 0.2 (-0.4; -0.0)[1]	– 0.0 (-0.1; 0.1)	0.0 (-0.1; 0.1)
sweets	0.4 (-0.4; 1.3)	– 0.0 (-.01; 0.1)	0.3 (-2.5; 3.1)	– 0.1 (-0.9; 0.8)	– 0.1 (-0.2; 0.1)
vegetable oil	– 1.1 (-5.2; 3.0)	– 0.3 (-0.7; 0.2)	– 3.3 (-17; 10)	1.1 (-3.4; 5.6)	– 0.2 (-1.1; 0.7)
vegetables	– 0.3 (-0.5; -0.0)[1]	– 0.0 (-0.1; 0.1)	0.8 (0.0; 1.6)[1]	0.3 (0.1; 0.6)[1]	0.0 (-0.1; 0.1)
whole grains	– 0.2 (-0.3; 0.0)	0.0 (-0.1; 0.1)	0.4 (-0.2; 1.0)	0.3 (-0.0; 0.4)	0.0 (-0.1; 0.1)

Data are presented by mean with 95% CI. [1]p ≤0.05, after adjustments for age, BMI, smoking and vitamin use.

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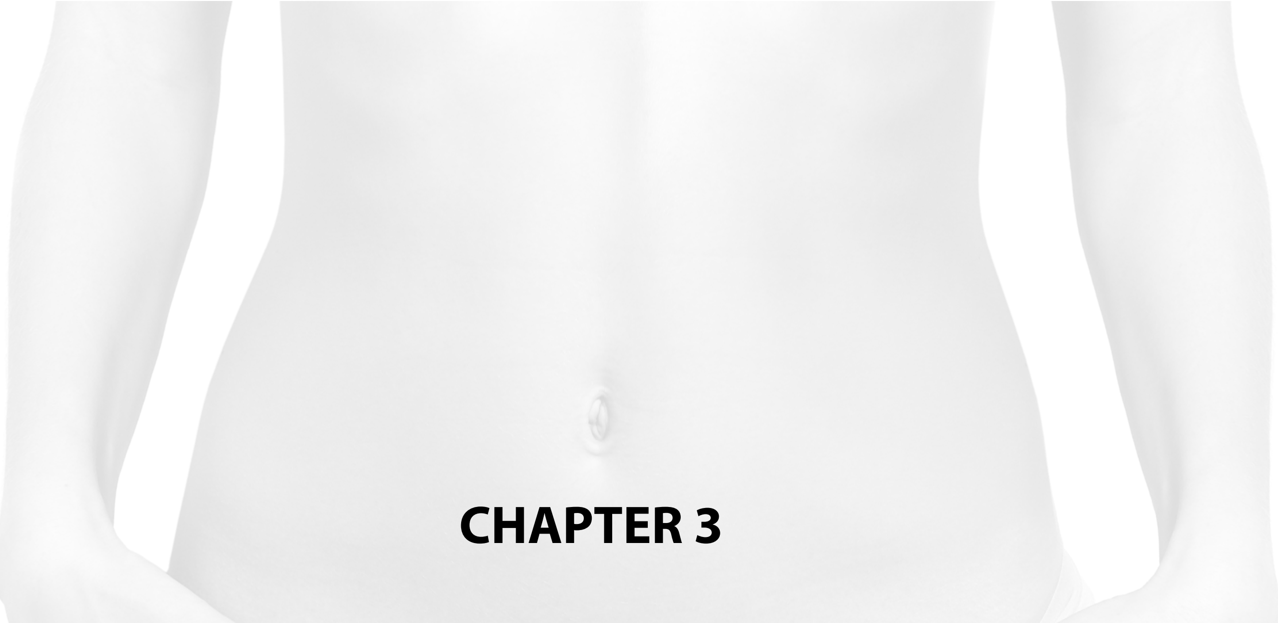

CHAPTER 3

The preconception Mediterranean dietary pattern in couples undergoing IVF/ICSI treatment increases the chance of pregnancy

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ABSTRACT

Objective

To investigate associations between preconception dietary patterns and IVF/ICSI outcomes validated by biomarkers of the homocysteine pathway.

Methods

An observational prospective study was set up in a tertiary referral fertility clinic at the Erasmus University Medical Centre, Rotterdam, the Netherlands. One hundred sixty-one couples undergoing IVF/ICSI treatment were included. Main outcome measures consisted of dietary patterns, blood and follicular fluid concentrations of folate, vitamin B12, vitamin B6, and homocysteine, fertilization rate, embryo quality, and pregnancy.

Results

Two dietary patterns were identified in women. The Health Conscious – Low Processed dietary pattern (variance explained 12.1%) was characterized by high intakes of fruit, vegetables, fish, and whole grains and low intakes of snacks, meats, and mayonnaise, and positively correlated with red blood cell folate (β 0.07). The Mediterranean dietary pattern (variance explained 9.1%), that is, high intakes of vegetable oils, vegetables, fish, and legumes and low intakes of snacks, was positively correlated with red blood cell folate (β 0.13), and vitamin B6 in blood (β 0.09) and follicular fluid (β 0.18). High adherence by the couple to the Mediterranean diet increased the probability of pregnancy with approximately 40%, odds ratio 1.4 (95%CI1.0; 1.9).

Conclusion

The adherence to a preconception Mediterranean diet in couples undergoing IVF/ICSI treatment contributes to the success of achieving pregnancy.

INTRODUCTION

Subfertility is an increasing problem in the reproductive population of industrialized countries, primarily because of postponed childbearing.[1] This problem is accentuated by unhealthy lifestyles, such as smoking, alcohol use, and malnutrition.[2] A nutritionally unbalanced diet characterized by low intakes of minerals and vitamins has previously been associated with adverse fertility outcomes.[3] Especially the B-vitamins folate, vitamin B6, and vitamin B12, are important because of their role in the homocysteine pathway.[4-6] A deficiency of these vitamins may cause an accumulation of homocysteine concentrations, which can ultimately lead to hyperhomocysteinemia.[3,5-7] This biochemical derangement seems to be detrimental for reproductive outcome as elevated total homocysteine (tHcy) concentrations in follicular fluid have been inversely associated with in vitro fertilization (IVF) / intracytoplasmic sperm injection (ICSI) outcomes, that is, number of preantral follicles, the number of retrieved oocytes, and embryo quality.[8-10]

Thus, it seems biologically plausible that the homocysteine pathway is at least one of the intermediate mechanisms between nutritional intake and reproductive outcome. Given that most micronutrients are present in common food sources, such as fruit, vegetables, and cereals, we are interested in the impact of the overall diet on reproductive outcome after fertility treatment.

Therefore, our aims were to 1) identify patterns in food consumption that explain the largest proportion of variation, i.e., dietary patterns, in women of subfertile couples undergoing IVF/ICSI treatment; 2) validate these dietary patterns with biomarkers of the homocysteine pathway in blood and follicular fluid; and 3) determine associations between the dietary patterns in subfertile couples and IVF/ICSI outcomes.

METHODS

Study Population

The FOod, Lifestyle and Fertility Outcome study (FOLFO) was designed to investigate the relationship between nutrition and lifestyle and IVF/ICSI outcome.[11] In short, between September 2004 and January 2007 subfertile couples undergoing IVF/ICSI treatment at the Erasmus University Medical Centre, Rotterdam, the Netherlands, were invited to participate. Sixty-four percent (n 161) of the couples were included in the analysis. Of the eligible 251 couples 15 couples dropped out because of oocyte donation, endometrioma, hydrosalpinx, medication error, or pregnancy before start of the treatment. Couples of whom no oocytes could be retrieved were also excluded (n 20). Because lifestyle factors, including dietary patterns, are culturally determined, we excluded couples of non-European origin (n 55) as well.[12] Ethnicity was categorized into Dutch native, European other, and non-European according to the definitions of Statistics Netherlands (http://www.cbs.nl/). The study protocol was approved by the Dutch Central Committee for Human Research and the Medical Ethical and Institutional Review board of the Erasmus University Medical Centre in Rotterdam. Participants provided written informed consent and the obtained materials and questionnaires were processed anonymously.

Questionnaires

All participants filled out a general questionnaire that generated the following information: age, educational level, medical history, body mass index as kg/m^2 (BMI), ethnicity, medication use, smoking, folic acid, and vitamin supplement use. All participants filled out a food frequency questionnaire (FFQ) to estimate food intake of the previous 4 weeks. This FFQ was developed by the division of Human Nutrition, Wageningen University, Wageningen, the Netherlands and validated for intake of energy, B-vitamins, and fatty acids.[13,14] The FFQ was provided on the day of oocyte retrieval or semen sample collection and returned on the day of embryo transfer.

In Vitro Fertilization Procedure

We used three stimulation treatments. Women were assigned either to one of two types of conventional ovarian stimulation, or a mild ovarian stimulation treatment as previously described.[11] In all three regimens a single dose of 5,000 or 10,000 IU human chorionic gonadotropin subcutaneously (hCG, Pregnyl, NV Organon, the Netherlands) was administered to induce oocyte maturation 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. Oocyte retrieval was performed 35 hours after hCG injection by transvaginal ultrasound-guided puncture of the follicles. Intravaginal luteal phase supplementation of 600 mg/day progesterone was started on the evening following oocyte pickup, and was continued for 12 days thereafter. On day 3 after oocyte pickup a maximum of two embryos was transferred.

Follicular Fluid and Blood Sample Collection

During oocyte retrieval, follicular diameters were measured and monofollicular fluid from the largest follicle was aspirated from each ovary and collected separately.[11] The oocytes were washed and transferred to a separate droplet of medium to monitor embryo quality. The monofollicular fluids were centrifuged for 10 minutes at 1,700 × 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 on cycle day 2 before the first injection of recombinant FSH and on the day of hCG administration. At both time points folate, vitamin B6, vitamin B12, tHcy, and estradiol were determined. Baseline FSH levels were determined on cycle day 2 only.[11] We previously demonstrated that folate concentrations increase on average to 22.5 nmol/L when women of reproductive age use 250 µg folic acid daily for 4 weeks.[16] A blood concentration above this value was therefore classified as regular folic acid use and below as no folic acid use.

The primary endpoints of the study were the biomarker concentrations in blood and follicular fluid and the IVF/ICSI treatment outcomes fertilization rate, embryo quality, and positive pregnancy test. Fertilization was determined on day 1 after the IVF/ICSI procedure, and was calculated as the number of fertilized oocytes divided by the total number of oocytes retrieved. On day 3 post-oocyte retrieval embryo quality scores were assigned ranging from 1 (best quality) to 5 (to poorest quality without transfer).[17] Pregnancy confirmation was assessed by a biochemical pregnancy test in urine 15 days after oocyte retrieval.

Statistics
All 195 food items from the FFQ data of all participants were classified into 22 food groups and adjusted for total energy intake.[15,18] This was followed by principal component factor analysis (PCA) applied on the energy-adjusted food groups of the women to construct overall dietary patterns by explaining the largest proportion of variation in the food group intake.[19,20] The two most prevalent dietary patterns were selected by rotating the solution with varimax method.[21]

Table 1 | Characteristics of two dietary patterns in 161 women undergoing IVF/ICSI treatment

food group	*Health Conscious – Low Processed diet*	*Mediterranean diet*
alcoholic drinks	– 0.15	0.13
cereals	0.13	– 0.05
butter	0.04	– 0.04
dairy	– 0.02	0.07
eggs	– 0.02	0.16
fish	0.39[2]	0.53[2]
fruit	0.62[2]	– 0.07
legumes	0.23[1]	0.53[2]
margarine	0.15	– 0.17
mayonnaise	– 0.61[2]	– 0.05
meat	– 0.23[2]	– 0.15
non-alcoholic drinks	0.19	– 0.11
nuts	– 0.05	0.15
refined grains	0.03	– 0.10
potatoes	0.03	0.04
sauces	0.01	0.06
snacks	– 0.49[2]	– 0.30[2]
soup	0.13	0.07
sugar	0.02	0.19
vegetable oil	– 0.14	0.75[2]
vegetables	0.45[2]	0.57[2]
whole grains	0.43[2]	– 0.02
explained variance	*12.1%*	*9.1%*

Data are presented by correlation coefficients.[1]p ≤0.05, [2]p ≤0.01

Each woman was assigned two personal scores for the two respective factors, calculated as the product of the food group value and its factor loadings summed across foods. According to their personal score the group of 161 women was divided into tertiles and classified as low, intermediate, or high adherence to the respective dietary pattern. The strength of adherence indicates the resemblance of the woman's diet compared with the respective dietary pattern identified by PCA. To elucidate the relative contribution of each food group to the two dietary patterns Pearson's r correlation coefficients were calculated (Table 1). The associations between lifestyle characteristics and the identified dietary patterns are described in Table 2.

Table 2a | Characteristics of the 161 women undergoing IVF/ICSI treatment

	Health Conscious – Low Processed diet			
	low	*intermediate*	*high*	
characteristic	*(n 54)*	*(n 54)*	*(n 53)*	*p*
age[1,2]	34.2 (23.2 - 43.7)	35.6 (23.7 - 41.8)	36.4 (26.6 - 41.6)	0.06
BMI[1,3]	24.1 (16.1 - 33.7)	23.0 (18.1 - 36.3)	22.0 (18.0 - 29.8)	0.03
high education[4]	20 (37.7)	26 (48.1)	26 (49.1)	0.15
female subfertility[4]	17 (31.5)	13 (24.1)	12 (22.7)	0.79
Dutch ethnicity[4]	48 (88.9)	46 (85.2)	41 (77.4)	0.26
smoking[4]	5 (9.3)	6 (11.1)	2 (3.8)	0.32
alcohol use[1,5]	0.9 (0.0 - 17)	1.5 (0.0 - 32.2)	1.1 (0.0 - 18.3)	0.44
folic acid use[4]	42 (77.8)	39 (72.2)	43 (81.1)	0.50
multivitamin use[4]	24 (44.4)	29 (53.7)	25 (47.2)	0.61
energy intake[6,7]	8083 (2198)	7649 (2146)	8614 (3537)	0.21
FSH[8,9]	8.2 (4.2 - 18.5)	7.5 (4.1 - 30.3)	8.1 (0.4 - 14.1)	0.20
estradiol[8,10]	142 (72 - 273)	132 (63 - 338)	146 (41 - 443)	0.17
duration of subfertility[1]	33 (9 - 115)	40 (3 - 135)	36 (6 - 121)	0.94
fertilized oocytes[6,11]	0.7 (0.3 - 1.0)	0.6 (0.1 - 1.0)	0.6 (0.2 - 1.0)	0.68
biochemical pregnancy[12]	14 (34.1)	10 (26.3)	11 (26.2)	0.66
stimulation scheme[4]				0.95
PO2-150	40 (75.5)	35 (68.6)	36 (70.6)	
PO5-150	6 (11.3)	4 (7.8)	7 (13.7)	
DLP225	7 (13.2)	12 (23.5)	8 (15.7)	
fertilization by IVF[4]	32 (66.7)	33 (68.8)	38 (76.0)	0.31

Data are presented by [1]median (range) [2]years, [3]kg/m^2, [4]n (%), [5]units/week, [6]mean (sd), [7]kJoule/day, [8]cycle day 2, [9]U/L, [10]pmol/L, [11]percentage, [12]per started treatment.

Continuous variables with skewed distributions are displayed as median with range. Normally distributed variables are presented by mean and standard deviation. P values were estimated from a linear regression model. Categorical variables are presented in frequencies with percentages and tested with chi-square test.

The associations between dietary patterns and biomarkers are shown in Table 3. Because the biomarkers showed skewed distributions they are displayed as median with 95% confidence intervals (95%CI). P values were estimated from a multivariable linear regression model in which logarithmically transformed biomarkers were chosen as the dependent, whereas dietary pattern, age, BMI, and vitamin use were included as covariates. Biomarkers in follicular fluid were adjusted for gram of protein to eliminate potential confounding of the oocyte maturation status. Statistically significant β estimates are given textually in the Results section. Finally we investigated whether the couple's dietary pattern was associated with IVF/ICSI outcome parameters (data not shown). The personal scores for the two dietary patterns were now calculated for both the woman and man and analyzed as the couple's dietary pattern score by taking the average of these personal factor scores. Linear and logistic regression analyses were used in which fertilization rate, average embryo quality, and pregnancy, were chosen as the dependent. Covariates included woman's age, smoking, vitamin use, and type of fertility treatment. In the final regression model BMI, alcohol use and stimulation scheme were added as covariates. Statistical analysis was performed using SPSS 15.0 for Windows software (SPSS Inc., Chicago, IL, US).

RESULTS

Two major dietary patterns are identified with PCA in 161 women. The first is labelled Health Conscious – Low Processed, containing high intakes of fruit, vegetables, whole grains, fish, and legumes, but low intakes of mayonnaise, snacks, and meat products. The second dietary pattern, called Mediterranean, comprises of high intakes of vegetable oil, fish, legumes, and vegetables but low intakes of snacks (Table 1). The Health Conscious – Low Processed and Mediterranean diet explain proportions of 12.1% and 9.1%, respectively, of the total variation in the nutritional intake of the women.

Table 2 depicts the general characteristics for women with low, intermediate, or high adherence to both diets. Women with high adherence to the Health Conscious – Low Processed diet show a lower BMI compared with women with low adherence. Women with high adherence to the Mediterranean diet are generally older, higher educated, consume more alcoholic drinks (e.g., wine), are more frequently of non-Dutch origin, and undergo more often IVF treatment. An increase in RBC folate is observed among women with a high adherence to the Health Conscious – Low Processed diet (β 0.07, p 0.05) and Mediterranean diet (β 0.13, p ≤0.01) (Table 3). Furthermore, a high adherence to the Mediterranean diet is positively associated with vitamin B6 in blood (β 0.09, p 0.04) and follicular fluid (β 0.16, p 0.02). Neither the Health Conscious – Low Processed nor the Mediterranean diet are associated with fertilization rate (β 0.00, p 0.44; β 0.00, p 0.31, respectively) and embryo quality (β –0.03, p 0.95, β 0.01, p 0.35, respectively).

However, high adherence of the couple to the Mediterranean diet substantially increases the probability of pregnancy, odds ratio (OR) 1.4 (95%CI 1.0; 1.9). This association is not present in couples with high adherence to the Health Conscious – Low Processed diet, OR 0.8 (95%CI 0.6; 1.0). All Ors have been adjusted for the confounders age, BMI, smoking, alcohol use, IVF/ICSI treatment, and stimulation scheme. All associations are not significantly affected by the characteristics of the men, i.e., age, BMI, smoking, and alcohol use (data not shown).

Table 2b | Characteristics of the 161 women undergoing IVF/ICSI treatment

		Mediterranean diet		
characteristic	*low (n 54)*	*intermediate (n 54)*	*high (n 53)*	*p*
age[1,2]	35.2 (23.2 - 43.7)	33.9 (23.7 - 40.6)	37.2 (29.3 - 42.1)	≤0.01
BMI[1,3]	23.5 (18.5 - 36.3)	22.8 (18.1 - 34.4)	22.3 (16.1 - 29.8)	0.34
high education[4]	18 (33.3)	21 (39.6)	33 (62.3)	0.03
female subfertility[4]	17 (31.5)	9 (16.7)	16 (30.2)	0.07
Dutch ethnicity[4]	47 (87.0)	49 (90.7)	39 (73.6)	0.04
smoking[4]	4 (7.4)	4 (7.4)	5 (9.6)	0.89
alcohol use[1,5]	0.3 (0.0 - 13.4)	0.7 (0.0 - 32.2)	2.1 (0.0 - 18.3)	0.02
folic acid use[4]	38 (70.4)	44 (81.5)	42 (79.2)	0.33
multivitamin use[4]	23 (42.6)	26 (48.1)	29 (54.7)	0.45
energy intake[6,7]	8040 (1922)	8087 (2234)	8212 (3707)	0.86
FSH[8,9]	8.1 (4.5 - 30.3)	7.6 (2.4 - 18.4)	8.6 (0.4 - 18.5)	0.53
estradiol[8,10]	139 (49 - 338)	142 (68 - 307)	140 (41 - 443)	0.78
duration of subfertility[1]	42 (3 - 135)	36 (10 - 121)	34 (6 - 115)	0.52
fertilized oocytes[6,11]	0.6 (0.1 - 1.0)	0.7 (0.2 - 1.0)	0.6 (0.2 - 1.0)	0.22
biochemical pregnancy[12]	11 (25)	13 (32.5)	11 (29.7)	0.75
stimulation scheme[4]				0.09
PO2-150	34 (63)	36 (73.5)	41 (78.8)	
PO5-150	8 (14.8)	3 (6.1)	6 (11.5)	
DLP225	12 (22.2)	10 (20.4)	5 (9.6)	
fertilization by IVF[4]	30 (61.2)	28 (60.9)	45 (88.2)	≤0.01

Data are presented by [1]median (range) [2]years, [3]kg/m2, [4]n (%), [5]units/week, [6]mean (sd), [7]kJoule/day, [8]cycle day 2, [9]U/L, [10]pmol/L, [11]percentage, [12]per started treatment.

DISCUSSION

This study demonstrates that Dutch subfertile couples with high adherence to the Mediterranean dietary pattern have a 40% increased probability of achieving pregnancy after IVF/ICSI treatment. The adherence to this diet is reflected by relatively high concentrations of folate and vitamin B6 in blood and follicular fluid. The Health Conscious – Low Processed dietary pattern was positively associated with RBC folate, but did not affect IVF/ICSI outcomes.

Labelling the extracted factors as the Health Conscious – Low Processed and Mediterranean dietary pattern is part of a continuing effort to achieve consistency in the use of dietary patterns in medical research. Northstone et al.[22] recently investigated dietary patterns during pregnancy in a large cohort study and identified a Health Conscious, *Traditional*, and *Processed* dietary pattern. Our first factor consists of high intakes of fruit, vegetables, fish, and whole grains and low intakes of snacks, meat, and mayonnaise, and resembles a combination of Northstones Health Conscious and *Processed* diet, and was therefore labelled as Health Conscious – Low Processed.

Table 3a | Biochemical determinants of the homocysteine pathway in blood in women undergoing IVF/ICSI treatment[1]

	Health Conscious – Low Processed diet			
	low	*intermediate*	*high*	
biomarker	*(n 32)*	*(n 25)*	*(n 28)*	*p*[9]
folate[2,3]	1553 (488 - 2461)	1149 (531 - 3116)	1672 (783 - 3611)	0.05
folate[2,4]	32.1 (9.8 - 82.4)	38.0 (11.7 - 82.3)	37.0 (15.8 - 119.5)	0.09
vitamin B6[2,5]	78.5 (43 - 231)	75 (51 - 173)	81 (46 - 250)	0.33
vitamin B12[6,7]	316 (74 - 1856)	325 (140 - 724)	302 (143 - 863)	0.55
tHcy[8]	9.7 (6.1 - 75.3)	9.2 (5.5 – 37.0)	8.4 (5.8 - 12.6)	0.72
		Mediterranean diet		
	low	*intermediate*	*high*	
biomarker	*(n 27)*	*(n 27)*	*(n 31)*	*p*[9]
folate[2,3]	1078 (531 - 3293)	1462 (488 - 2244)	1665 (909 - 3611)	≤0.01
folate[2,4]	32.6 (11.7 - 119.5)	33.9 (9.8 - 108.5)	37.3 (17.1 - 92.0)	0.28
vitamin B6[2,5]	72 (50 - 231)	79 (43 - 173)	89 (43 - 250)	0.04
vitamin B12[6,7]	297 (74 - 724)	333 (183 - 688)	353 (140 - 1856)	0.14
tHcy[8]	10.1 (6.1 - 75.3)	8.9 (5.5 - 18.3)	8.7 (5.8 - 12.9)	0.48

Data are presented by [1]median with range, [2]nmol/L, [3]red blood cell, [4]serum, [5]whole blood, [6]pmol/L, [7]plasma, [8]μmol/L. [9]Adjusted for age, BMI, and use of vitamin supplements.

The second factor shares many features with the classical Mediterranean diet,[23] defined by high intakes of vegetable oils, vegetables, fruit, nuts, fish, and legumes, low dairy intake, and moderate intake of alcohol. We examined the magnitude of comparability by scoring the factor to the 10-point Mediterranean diet scale as introduced by Trichopoulou et al.[23] Analysis revealed that women with high adherence to the second factor scored positively in 7 of 10 dietary criteria. This indicates good comparability with the traditional definition of Mediterranean, justifying the labelling as the Mediterranean diet.

The two dietary patterns in our study show a remarkable overlap in foods, for example, high intakes of vegetables, fish, and legumes and low intakes of snacks. However, only the Mediterranean diet seems to increase the chance of pregnancy after IVF/ICSI treatment. There are two major differences between these two diets that may explain this finding.

First, when comparing differences in food intakes, the high intake of vegetable oils in the Mediterranean diet is outstanding. Vegetable oils are generally rich in linoleic acid and belong to the family of omega-6 fatty acid molecules, which can only be obtained by the diet. They are precursors of different prostaglandins, important for the initiation of the menstrual cycle, growth, and development of preantral follicles and ovulation. Prostaglandins are also involved in the maintenance of pregnancy by optimizing endometrial receptivity.[24-27] This may imply that a higher intake of linoleic acid perhaps positively affects the implantation of the fertilized ovum.

Table 3b | Biochemical determinants of the homocysteine pathway in follicular fluid in women undergoing IVF/ICSI treatment[1]

	Health Conscious – Low Processed diet			
	low	*intermediate*	*high*	
biomarker	*(n 36)*	*(n 32)*	*(n 39)*	*p*[8]
folate[2,3]	29.9 (7.3 - 84)	32.7 (8.9 - 200)	35.5 (12.5 - 190)	0.23
vitamin B6[2,4]	68.0 (17.5 - 310)	86.4 (14.5 - 310)	84 (22.5 - 310)	0.97
vitamin B12[5,6]	193 (59 - 3695)	247 (123 - 31013)	209 (88 - 1673)	0.59
tHcy[6,7]	6.3 (3.8 - 70.2)	6.6 (3.3 - 27.2)	6.1 (3.6 - 14)	0.72

	Mediterranean diet			
	low	*intermediate*	*high*	
biomarker	*(n 34)*	*(n 37)*	*(n 36)*	*p*[8]
folate[2,3]	30.5 (8.9 - 199.6)	32.3 (7.3 - 69)	35.3 (12.0 - 190)	0.14
vitamin B6[2,4]	70.5 (14.5 - 310)	74.0 (17.5 - 310)	91.5 (22.5 - 310)	0.02
vitamin B12[5,6]	206 (59 - 31013)	196 (104 - 4199)	230 (88 - 2959)	0.89
tHcy[6,7]	6.3 (3.3 - 70.2)	6.4 (3.8 - 15.5)	6.2 (3.6 – 14.0)	0.20

Data are presented by [1]median with range, [2]nmol/L, [3]serum, [4]whole blood, [5]pmol/L, [6]plasma, [7]µmol/L. [8]Adjusted for age, BMI, use of vitamin supplements, and total protein in follicular fluid.

The second difference between the two dietary patterns is found in their effect on the biomarkers of the homocysteine pathway. Both diets increase folate concentrations, but the Mediterranean diet shows an additional rise in vitamin B6 in blood and follicular fluid. Vitamin B6 is a versatile coenzyme involved in many biochemical pathways. Research has shown that giving vitamin B6 to subfertile women increases reproductive performance, that is, a 40% increased chance of conception and a 30% lower risk of miscarriage early in pregnancy.[28] The positive association between vitamin B6 and the success of IVF/ICSI treatment is herewith in line.

Thus, this study has provided novel insights in the relationship between dietary patterns and IVF/ICSI outcomes, in which vitamin B6 and fatty acids may play an important role. Further research, however, is needed to validate the current findings and investigate optimal dosage effects before any dietary preparation can be generally recommended.

Self-reported dietary assessment methods are susceptible to measurement errors, where habitual energy intake tends to be underestimated.[29] However, we consider it unlikely that these measurement errors would have produced unvalid associations. Because of the prospective study character, exposure determinants were measured before the outcomes were known. Therefore, energy and nutritional intake are unlikely to be differentially underreported or differently reported between couples with and without successful IVF/ICSI treatment outcome, e.g. ongoing pregnancy. The findings from this Dutch subfertile study group cannot be equally generalized to the overall reproductive population because couples seeking fertility treatment generally have a higher age and education. These results should also not be extrapolated to non-Europeans for which studies in different ethnic populations are required.

General Conclusion

A high adherence to the Mediterranean dietary pattern by the couple may improve the chance of pregnancy after IVF/ICSI treatment. These findings are important with regard to the development of nutritional interventions to further improve fertility treatment and success rates.

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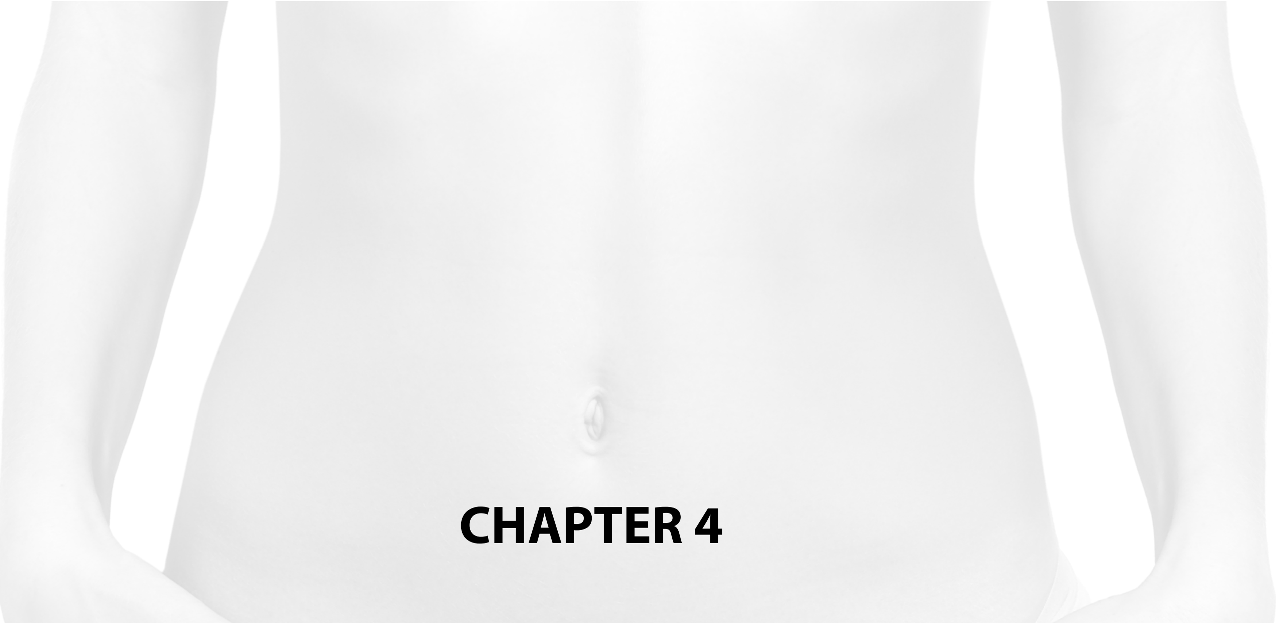

CHAPTER 4

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

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ABSTRACT

Objective

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.

Methods

An observational prospective study was set up in a tertiary referral fertility clinic at the Erasmus University Medical Centre, 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.

Results

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.

Conclusion

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 individual and various amounts of omega-3 and omega-6 LC-PUFAs on human reproduction, however, are 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 injection treatment (ICSI).

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 Centre, 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

Centre 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.

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

Oocytes were retrieved 35 hours after hCG administration by transvaginal ultrasound-guided puncture of follicles. Prior to ovum pick up the total number of follicles were counted by transvaginal ultrasound. Seventy two hours after oocyte retrieval embryo morphology scores were assigned according to previously described criteria.[17] These scores ranged from one (best quality) to five (poor quality and inadequate for embryo transfer). Embryos with an assigned score of 1 or 2 are classically denoted as perfect and respectively good embryos.

Estradiol Assessment

Venous blood samples were drawn from each woman on cycle day 2 before the first injection of rFSH and at the day of hCG administration for the determination of estradiol. Venous blood samples were drawn into dry vacutainer tubes and allowed to clot. After centrifugation at 2000 g, serum was collected before being assayed. Estradiol was determined by using coated radioimmunoassay (RIA, Diagnostic Products Corporation). Intra- and inter-assay coefficients of variation were 5% and 7% for estradiol, respectively.

Statistics

Dietary intakes of the LC-PUFAs were log-transformed to obtain normal distributions. The log-transformed intakes were adjusted for energy intake according to the residual method of Willet et al.[18] Multivariable linear regression models were used to assess the relationship between the logarithmically transformed LC-PUFA intakes and the estradiol levels on cycle day 2 and hCG day, number of follicles and embryo morphology. In the linear regression model the potential confounders considered comprised ethnicity, age, BMI, smoking, alcohol use, folic acid supplement use, IVF/ICSI treatment, and ovarian stimulation regimen (n 3). Using a stepwise backward regression model covariates were included when they reached significance defined as a $p \leq 0.1$. The associations between LC-PUFAs with estradiol level on cycle day 2 and number of follicles are adjusted for all covariates. Estradiol on hCG day, i.e., estradiol response after ovarian stimulation treatment, was also adjusted for estradiol on cycle day 2. The LC-PUFA associations with embryo morphology are adjusted for ethnicity, BMI, age, IVF/ICSI treatment, and ovarian stimulation treatment. All data in the table are presented as standardized adjusted regression coefficients (ß) with standard error (se) both linear and after dichotomization of the LC-PUFA intake into 15^{th} (p15) and 85^{th} (p85) percentiles, representing respectively low and high intake. The latter was conducted to evaluate possible threshold values for the effects on the fertility outcome parameters. A $p \leq 0.05$ was considered statistically significant. All statistical analyses were performed using SPSS 15.0 for Windows (SPSS Inc, Chicago, Il, US).

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), 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 is 12.1/1.14; this is higher than recommended. To evaluate whether the relatively low omega-3 intake were due to the general underreporting of food intake, we calculated the PAL measure.

Table 2 | Nutrient Intakes

nutrient	*unit*	*participants*	*DRI*[3]
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)	
LA	*g/day*	12.1 (1.5 - 49.8)	12.0
	adjusted	13.6 (5.6 - 33.5)	
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[1]	*g/day*	1.14 (0.28 - 7.42)	
	adjusted	1.26 (0.49 - 1.63)	
omega-6: omega-3 ratio[2]		10.1 (3.5 - 20.7)	

Data are presented by median (min-max). [1]EPA + DHA + ALA. [2]LA / (ALA + EPA + DHA). [3]Dietary Reference Intakes: energy, The Hague: Health Council of the Netherlands 2001/19R (corrected edition June 2002)

It revealed that the PAL was 1.44 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 (β 68.5, se 34.8, p 0.05). An inverse estradiol response on the day of hCG administration was associated with highest intakes of EPA (β -1062, se 492, p 0.03) and DHA (-1006, se 485, p 0.04) also substantiated with a linear association of DHA (β -395, se 200, p 0.03).

The number of follicles was inversely associated with high intakes of EPA (β -1.75, se 0.87, p 0.05) and DHA (-1.78, se 0.85, p 0.04). Positive associations were established between embryo morphology and intakes of omega-3 LC-PUFA (linear β 0.63, se 0.26, p 0.02), in particular ALA (β 0.56, se 0.26, p 0.03) and DHA (β 0.17, se 0.09, p 0.05). Estradiol and fertility outcome parameters were not associated with omega-6 LA intake and the omega-6:omega-3 ratio.

Table 3a | 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)*	–49.2 (39.9)	–647 (665)	–0.71 (1.14)	0.48 (0.28)
	p	0.22	0.33	0.54	0.09
>p85 (17.9 g)	*β (se)*	0.9 (38.1)	–243 (620)	–0.51 (1.08)	0.48 (0.3)
	p	0.98	0.70	0.64	0.11
<p15 (10.6 g)	*β (se)*	–11.2 (33.1)	500 (561)	–0.18 (0.95)	–0.17 (0.23)
	p	0.74	0.37	0.85	0.47
ALA					
linear	*β (se)*	–2.7 (35.3)	–647 (578)	–0.45 (1.02)	0.56 (0.26)
	p	0.94	0.27	0.66	0.03
>p85 (1.5 g)	*β (se)*	90.4 (35.7)	–637 (609)	–0.33 (1.05)	0.07 (0.27)
	p	0.01	0.30	0.76	0.79
<p15 (0.8 g)	*β (se)*	–11.3 (38.1)	5.79 (633)	–0.01 (1.1)	–0.39 (0.26)
	p	0.77	0.99	1.00	0.13
EPA					
linear	*β (se)*	9.1 (11.3)	–294 (181)	–0.28 (0.32)	0.1 (0.08)
	p	0.42	0.11	0.38	0.21
>p85 (0.1 g)	*β (se)*	–19.7 (30.4)	–1062 (492)	–1.75 (0.87)	0.13 (0.22)
	p	0.52	0.03	0.05	0.54
<p15 (0.01 g)	*β (se)*	–27.4 (39.4)	746 (635)	–0.2 (1.09)	–0.19 (0.27)
	p	0.49	0.24	0.85	0.50

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

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.

Table 3b | 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)	8.8 (12.1)	−395 (200)	−0.42 (0.35)	0.17 (0.09)
	p	0.47	0.05	0.22	0.05
>p85 (0.2 g)	β (se)	−21.1 (29.9)	−1006 (485)	−1.78 (0.85)	0.13 (0.22)
	p	0.48	0.04	0.04	0.53
<p15 (0.02 g)	β (se)	−27.4 (35.6)	1177 (582)	0.82 (0.99)	−0.42 (0.26)
	p	0.44	0.04	0.41	0.11
total omega-3					
linear	β (se)	4.9 (35.4)	−871 (576)	−0.65 (1.02)	0.63 (0.26)
	p	0.89	0.13	0.52	0.02
>p85 (1.7 g)	β (se)	68.5 (34.8)	−847 (572)	−1.31 (1.02)	0.36 (0.26)
	p	0.05	0.14	0.20	0.17
<p15 (1.0 g)	β (se)	−23.5 (31.8)	34.6 (525)	−0.56 (0.92)	−0.41 (0.23)
	p	0.46	0.95	0.54	0.08
O6:O3 ratio					
linear	β (se)	−29.7 (42.4)	562.8 (698)	0.15 (1.23)	−0.38 (0.31)
	p	0.48	0.42	0.91	0.22
>p85 (16.2)	β (se)	−39.7 (39.3)	596 (641)	1.49 (1.14)	−0.43 (0.29)
	p	0.31	0.35	0.19	0.14
<p15 (7.3)	β (se)	−30.5 (32.9)	−609 (553)	0.07 (0.96)	0.20 (0.24)
	p	0.36	0.27	0.94	0.42

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

Furthermore, the reduction of follicles 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 intakes 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 intakes in our study were 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. This 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]

General Conclusion

In conclusion, for the first time significant associations are 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.

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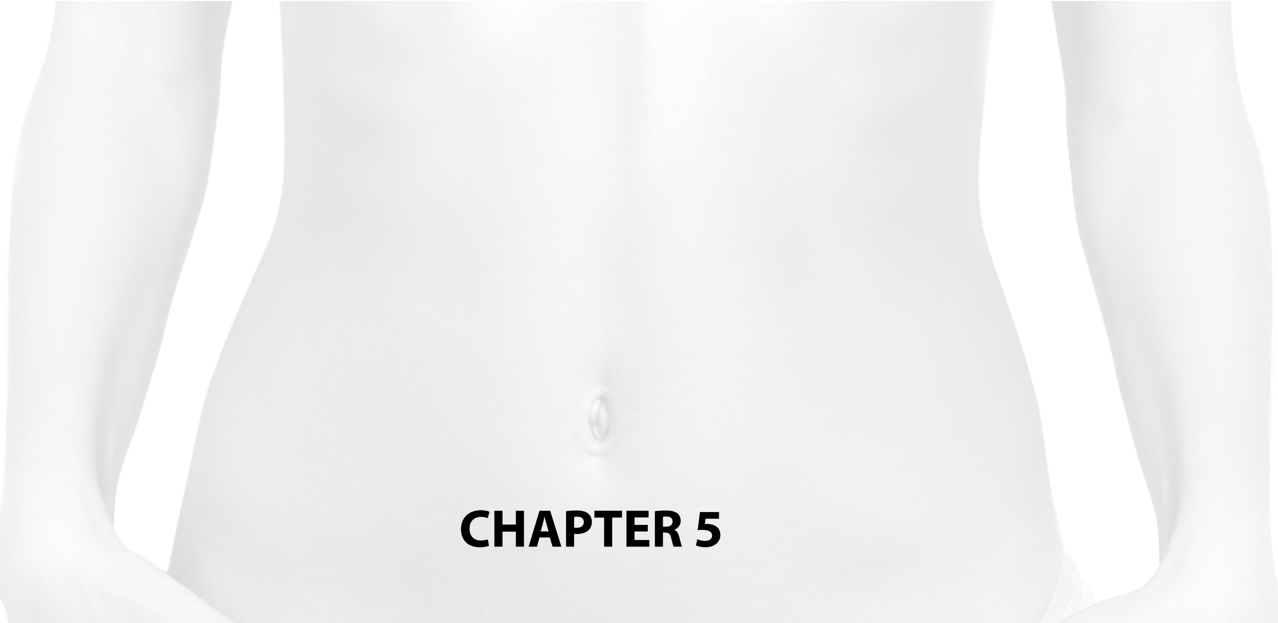
PART II

Dietary patterns and pregnancy outcome



CHAPTER 5

The maternal Mediterranean dietary pattern is associated with a reduced risk of spina bifida in the offspring

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ABSTRACT

Objective

This study investigates the hypothesis whether a maternal dietary pattern is associated with the risk of spina bifida (SB) in the offspring.

Methods

In a case–control study a total of 50 mothers of children with SB and 81 control mothers were recruited from eight clinic sites in the Netherlands between 1999 and 2001. Maternal food intakes were obtained by food frequency questionnaires at the standardized study moment of 14 months after the birth of the index child. Principal component factor analysis (PCA) and reduced rank regression (RRR) were used to identify dietary patterns. Maternal biomarkers were used as response measures in the RRR analysis and composed of serum and red blood cell (RBC) folate, serum vitamin B12 and total plasma homocysteine. The strength of the use of the dietary pattern in association with SB risk was estimated by odds ratios (OR) and 95% confidence intervals (95%CI) with the highest quartiles of the dietary pattern as reference.

Results

A predominantly Mediterranean dietary pattern was identified by both PCA and RRR. Those dietary patterns were highly correlated (r 0.51, p ≤0.001) and characterised by joint intakes of fruit, vegetables, vegetable oil, alcohol, fish, legumes, and cereals and low intakes of potatoes and sweets. We observed a significantly increased risk of SB offspring in mothers with a low adherence to the Mediterranean dietary pattern, OR 2.7 (95%CI 1.2; 6.1) and OR 3.5 (95%CI 1.5; 7.9). The Mediterranean dietary pattern was correlated with higher levels of serum and RBC folate, serum vitamin B12 and lower plasma homocysteine.

Conclusion

A high maternal adherence to the Mediterranean dietary pattern seems to be associated with a reduction in the risk of offspring affected by SB.

INTRODUCTION

Spina bifida (SB) belongs to the neural tube defects and is a severe malformation of the central nervous system. In most cases, SB is considered to have a multifactorial origin, and during the past decades, the role of maternal nutrition – as an environmental factor – has been emphasised, in particular that of the B-vitamin folate.[1,2] The discovery that maternal folic acid supplementation protects against SB has stimulated the introduction of folic acid fortification and supplementation programs in many countries worldwide.[3-7] It has been shown that low maternal dietary intakes of other nutrients, such as a vitamin B12, iron, magnesium and niacin, are associated with SB offspring as well.[8,9] Furthermore, there is a large variation in the occurrences of SB in regions and countries worldwide. The prevalence is generally lower in Southern European countries and in countries with mandatory folic acid fortification.[10] This leads to the hypothesis that the overall maternal diet may confer supplementary protection against maldevelopment of the neural tube.

Nutrients from natural food sources are generally consumed in meals and not as isolated components. They serve as substrates, transcription factors and modifiers of gene expression and thereby influence complex biological pathways involved in embryogenesis. Dietary patterns have been derived successfully from food frequency questionnaires (FFQs) by data-driven dimension reduction techniques, such as principal component factor analysis (PCA)[11] and reduced rank regression (RRR).[12] To date, dietary patterns have been associated with biomarker concentrations and complex diseases, such as cardiovascular disease.[13-15] Therefore, the aims of this study were 1) to identify dietary patterns in case and control mothers, and 2) to examine whether associations exist between these dietary patterns and the risk of SB risk in the offspring. This new approach is important because it could result in recommendations for preconception counselling and dietary interventions.

METHODS

Study Population

A case–control study was conducted between 1999 and 2001 in which 77 mothers of children with SB and 151 control mothers have been included as described previously.[8,9] In summary, Dutch Caucasian mothers and their child with a nonsyndromic meningo(myelo)cele were eligible to participate at around 15 months after the index pregnancy. The meningo(myelo)cele, referred to as SB, was diagnosed by a neuropediatrician at birth. Controls were Dutch Caucasian mothers of children without congenital abnormalities of the same age as the cases. Exclusion criteria were 1) pregnancy at the study moment, 2) consanguinity, 3) a familial relationship between the case and the control families and 4) maternal diabetes mellitus. Informed consent was obtained from every subject, and the study was approved by the Central Committee on Research in Human and the Medical Ethics Committees of all participating hospitals.

General Questionnaire

Questionnaires were filled out at home at the study moment for information on current lifestyle, demographic factors and other. From the questionnaire, data were extracted concerning age, body weight, education and lifestyle factors, such as smoking and vitamin intake. The maternal body mass index (BMI) was calculated as the ratio of weight per height squared (kg/m^2). Education was categorized into low (primary, lower vocational, intermediate, or secondary intermediate vocational), intermediate (higher secondary) and high education (higher vocational or university). Mothers were considered smokers when any smoking (cigarettes, cigars or pipe) was reported. Data on vitamin intake comprised information on the contents (folic acid only or multivitamins), dosage and frequency of intake.

Food Frequency Questionnaire

For quantification of the diet, mothers filled out a validated FFQ developed for the Dutch cohorts of the European Prospective Investigation into Cancer and Nutrition study at around 14 months after the index pregnancy covering the nutrient intake 3 months before the study moment.[16,17] The frequency with which mothers consumed the foods could be indicated per day, week, month, year or never. Participants were also asked about the methods of preparation, additions and portion sizes. All FFQs were individually checked for completeness and consistency at the hospital or by telephone by the researcher. The average daily nutrient intakes were estimated by multiplying the frequency of consumption of food items by the portion size and nutrient content per gram based on the 1998 electronic version of the Dutch Food Composition Table (NEVO). The exceptions to this were folate and cobalamin, which were based on the 2001 electronic version.[18,19]

As stated by the expert nutritionist Willett and confirmed by others, in general, the individual dietary pattern is rather constant and is influenced only by episodes of temporary dieting, illnesses, nausea and increased needs due to excessive growth, such as during pregnancy and breastfeeding.[20-23] Therefore, in this analysis, mothers were excluded 1) who were pregnant, 2) who were breastfeeding, 3) who reported a changed diet at the study moment compared with the periconception period, 4) who reported to have had severe nausea and/or vomiting starting after the first week of pregnancy, resulting in a changed or decreased food intake and 5) whose nutritional intake data and/or biomarkers were incomplete or lacking.

Periconception Period

The periconception period was defined as the period from 4 weeks before through 8 weeks after the conception of the index pregnancy. Women who took a a folic acid or folic acid containing supplement in the periconception period and women who experienced mild nausea and/or vomiting during the first trimester of pregnancy were included in the analysis. The periconception intake of supplements was specified according to which weeks the supplements were taken on a daily basis before and during pregnancy.

Biomarker Assay

Appointments were made at the University Medical Centre in Nijmegen, the Netherlands, for blood sampling. Mothers fasted from 10 pm the day before the blood sampling. Venous blood of the mother was drawn into anticoagulant-free vacutainer tubes for folate and vitamin B12 measurements according to the procedures of Kuemmerle et al.[24] and Molloy and Scott.[25] The intra-assay and interassay coefficients of variation for folate and vitamin B12 were 6.1 and 10.2% and 5.7 and 6.3%, respectively. For vitamin B6 determination, venous blood was drawn into lithium heparin vacutainer tubes and routinely analysed according to the methods of Schrijver et al.[26] The intra-assay and interassay coefficients of variation were 1.4 and 7.3%, respectively.

Venous blood was drawn into vacutainer tubes that contained ethylenediaminetetraacetic acid for the measurement of total homocysteine (tHcy). The tubes were immediately placed on ice and processed within 2 hours after blood sampling and analysed according to routine methods of Te Poele-Pothoff et al.[27] Both intra-assay and interassay coefficients of variation were ≤6.5%. The remaining blood was used to determine the haematocrit level. All laboratory analyses were performed without knowledge of the identity of the participants. Not all blood parameters were available for every participant. Missing results occurred in approximately 5% of the participants because of failures in blood sampling or laboratory testing.

Table 1 | Characteristics of mothers of children with SB and controls

characteristic	*cases (n 50)*	*controls (n 81)*	*p*
age[1,2]	30.3 (3.9)	31.7 (3.5)	0.09
BMI[1,3]	25.4 (5.3)	22.9 (4.5)	≤0.01
high education[4]	8 (1.0)	44 (54.3)	≤0.001
male child[4]	19 (3.0)	38 (46.9)	0.32
periconception period			
use of folate[4]	19 (3.0)	38 (47.5)	0.29
smoking[4]	4 (2.0)	15 (18.5)	0.63
alcohol[4]	18 (3.0)	44 (54.3)	0.04
14 months postpartum			
use of folate[4]	4 (2.0)	7 (8.6)	0.03
smoking[4]	12 (2.0)	15 (18.5)	0.45
alcohol[4]	26 (5.0)	60 (74.1)	0.01
energy intake[5,6]	8.8 (6.2 - 15.8)	9.1 (5.2 - 13.6)	0.70

Data are presented by [1]mean (sd), [2]at delivery in years, [3]kg/m^2, [4]n %, [5]median (range), [6]mJoule/day.

Statistics

A detailed description of the PCA and RRR procedure for extracting dietary patterns from food consumption data can be found elsewhere.[12] In short, PCA works only by extracting successive linear combinations of predicting variables to explain a maximal variance occurring within these predicting variables. The RRR method also extracts linear combinations (called factors) from predicting variables; however, the goal of this method is to maximize the variance explained within a set of response measures. The predicting variables are the food groups, and biomarkers are the response measures.

In summary, all FFQ data of case and control mothers were pooled into one data set, and factor analysis was performed on all mothers. This enabled us to examine whether the magnitude of adherence of the respective identified dietary pattern differed between cases and controls to estimate risk of SB, and enable associations with biomarker levels. The 200 food items from the FFQ were reduced to 16 predefined food groups based on similar nutrient content, which are comparable with grouping schemes in literature.[28] The food groups were adjusted for the total intake of energy.[29] The first factor, e.g., dietary pattern, was extracted from the PCA solution. In the RRR method, the 16 predefined food groups were chosen as predicting variables and the maternal biomarkers as response measures. The overall p for the explained variance of the factor solution was tested with the permutation method. Each mother received a personal score for PCA as well as RRR to describe the agreement of her individual dietary behaviour compared to that of the extracted factor.

Table 2a | Characteristics of mothers (pooled) stratified in quartiles from low (Q1) to high (Q4) dietary pattern scores from PCA solution[1]

	Mediterranean diet				
	q1	*q2*	*q3*	*q4*	
PCA	*(n 33)*	*(n 33)*	*(n 33)*	*(n 32)*	*p*
age[1,2]	29.1 (3.4)	31.1 (3.5)	31.8 (3.1)	33.0 (3.7)	≤0.001
BMI[1,3]	26.0 (6.3)	25.2 (5.0)	25.0 (4.7)	22.9 (3.0)	0.08
high education[4]	3 (9.4)	4 (33.3)	16 (48.5)	22 (66.7)	≤0.001
male child[4]	16 (5.0)	10 (30.3)	18 (54.5)	13 (39.4)	0.86
use of folate[4]	15 (46.9)	12 (36.4)	19 (57.6)	13 (40.6)	0.94
smoking[4,5]	10 (31.3)	4 (12.1)	6 (18.2)	6 (18.2)	0.30
alcohol[4,5]	3 (9.4)	16 (48.5)	20 (60.6)	23 (69.7)	≤0.001
energy intake[6,7,8]	9.3 (5.7 - 15.8)	8.5 (5.5 - 13.2)	8.9 (5.2 - 13.9)	9.2 (6.2 - 13.7)	0.86
tHcy[6,9,10]	4.7 (7.5 - 85.8)	4.9 (3.7 - 59.3)	10.3 (4.5 - 18.7)	10.3 (4.9 - 22.3)	0.05
folate[6,10,11]	12.7 (3.4 - 183)	4.0 (5.0 - 36.5)	15.9 (2.4 - 179)	19.9 (6.6 - 680)	0.01
folate[6,10,12]	600 (139 - 1758)	617 (345 - 1545)	629 (147 - 3143)	815 (352 - 3999)	0.04
vitamin B6[6,10,13]	51 (35 - 90)	57 (35 - 140)	60 (35 - 225)	60 (37 - 140)	0.16
vitamin B12[6,10,11]	219 (66 - 426)	239 (131 - 449)	281 (54 - 741)	320 (48 - 680)	≤0.01

Data are presented by [1]at delivery in years [2]mean (sd), [3]kg/m^2, [4]n (%), [5]periconception al, [6]median (range), [7]kJoule/day, [8]at 14 months postpartum, [9]adjusted for vitamin use, [10]plasma, [11]serum, [12]red blood cell, [13]whole blood.

PCA was performed with SPSS software, release 11.0.1, (SPSS Inc., Chicago, IL, US) and RRR analysis with CANOCO (Canonical Community Ordination), version 4.5 (Microcomputer Power, Ithaca, NY, US) for Windows.[30] Differences in baseline characteristics between case and control mothers were tested with analysis of variance for maternal age and BMI and Mann–Whitney U test for the remaining continuous variables. Dietary characteristics were obtained using Pearson r correlation coefficients between maternal factor scores and food groups. Subsequently, cases and controls were pooled and divided into quartiles according to their factor score (e.g. adherence to the respective dietary pattern).

Maternal age at the study moment and BMI are presented as mean with standard deviation (sd), and energy intake and biomarkers are presented as median with range for each quartile of the respective dietary pattern. The associations between dietary patterns and continuous variables were evaluated in a linear regression model. Differences in educational level, smoking, alcohol use and vitamin supplement intake between the quartiles were tested using the chi-square test for linear association. Differences in biomarker concentrations between quartiles of the dietary patterns were evaluated in a linear regression model, adjusted for the periconception use of folic acid and/or multivitamins. The percentiles were also used for risk estimation by odds ratios (ORs) and 95% confidence interval (95%CIs). The distribution of case and control mothers in the highest quartiles (q2 + q3 + q4) of the respective diet was used as a reference. The estimates were adjusted for potential confounders in a logistic regression model based on significant baseline periconception characteristics between cases and control and significant characteristics of each dietary pattern (p ≤0.05).

RESULTS

The characteristics of case and control mothers are shown in Table 1. At the study moment, case mothers had a slightly higher BMI (p 0.08), lower education (p ≤0.001) and used less folic acid supplements (p 0.03) and alcohol (p 0.01). Alcohol use of case mothers was also lower in the periconception period (p 0.04).

Table 2b | Characteristics of mothers (pooled) stratified in quartiles from low (Q1) to high (Q4) dietary pattern scores from RRR solution[1]

	Mediterranean diet				
	q1	*q2*	*q3*	*q4*	
RRR	*(n 33)*	*(n 33)*	*(n 33)*	*(n 32)*	*p*
age[1,2]	30.5 (3.7)	31.0 (3.2)	33.1 (3.9)	30.4 (3.3)	0.09
BMI[1,3]	25.4 (6.1)	25.0 (4.7)	24.5 (4.8)	24.2 (4.3)	0.76
high education[4]	6 (18)	12 (36)	21 (64)	13 (41)	≤0.01
male child[4]	9 (27)	18 (55)	15 (46)	15 (47)	0.20
use of folate[4]	20 (61)	24 (73)	15 (46)	14 (45)	0.16
smoking[4,5]	9 (27)	5 (15)	6 (18)	6 (19)	0.47
alcohol[4,5]	10 (30)	4 (33)	22 (67)	19 (59)	≤0.01
energy intake[6,7,8]	8.6 (5.5 - 13.6)	8.9 (5.2 -15.8)	8.8 (5.5 -12.2)	9.3 (6.2 - 13.9)	0.67
tHcy[6,9,10]	12.8 (5.2 - 85.8)	10.3 (8.2 -16.3)	11.0 (6.9 -16.7)	19.2 (5.9 - 679.5)	≤0.001
folate[6,10,11]	11.7 (2.36 - 183)	13.8 (4.5 - 37.2)	17.9 (4.6 - 179)	19.2 (5.9 - 698)	≤0.01
folate[6,10,12]	564 (139 - 1757)	587 (159 - 1498)	706 (291 - 3142)	834 (394 - 3998)	≤0.001
vitamin B6[6,10,13]	50 (35 - 120)	58 (35 - 225)	55 (37 - 100)	61(36 - 140)	0.43
vitamin B12[6,10,11]	221 (48 - 433)	240 (54 - 550)	284 (138 - 680)	305 (182 - 741)	≤0.001

Data are presented by [1]at delivery in years [2]mean (sd), [3]kg/m^2, [4]n (%), [5]periconception al, [6]median (range), [7]kJoule/day, [8]at 14 months postpartum, [9]adjusted for vitamin use, [10]plasma, [11]serum, [12]red blood cell, [13]whole blood.

The first factor quantified the most prominent diet in the study group as a continuous variable. It explained 14.7% of the total variance and was labelled the Mediterranean dietary pattern comprising high intakes of vegetables, fruit, legumes, vegetable oil, cereals, alcoholic drinks, and fish and low intakes of potatoes and sweets.

This dietary pattern was characterised by a higher maternal age at birth of the index child, higher education and more alcohol consumption in the periconception period and at the study moment ($p \leq 0.001$ for all comparisons). We used the magnitude of adherence to these maternal dietary patterns to assess risk of SB in the offspring. Table 3 reveals that mothers in the lowest quartile of the Mediterranean dietary pattern, i.e., lowest adherence, extracted by both PCA and RRR, showed a significantly increased risk of SB compared with mothers with a high adherence, OR 2.7 (95%CI 1.2; 6.1) and OR 3.5 (95%CI 1.5; 7.9), respectively.

Adjustment for confounders including maternal BMI, age at the index pregnancy and periconception folic acid supplement use did not significantly affect the risk estimates. Thus, mothers with a low adherence to the Mediterranean dietary pattern in the periconception period were over two times as likely to give birth to SB offspring when compared with high adherers to the Mediterranean diet.

Table 3 | Low (<p25) versus high (≥p25) percentiles of the maternal dietary pattern and the association with SB risk[1]

	Mediterranean diet			
	cases (n 50)	*controls (n 81)*	*OR (95% CI)*	*OR[2] (95% CI)*
PCA				
<p25	18 (36.0%)	14 (17.3%)	2.7 (1.2 - 6.1)	2.3 (0.9 - 5.6)
≥p25	32 (64.0%)	67 (82.7%)	1.0 (Ref)	1.0 (Ref)
RRR				
<p25	20 (40.0%)	13 (16.0%)	3.5 (1.5 - 7.9)	3.5 (1.5 - 8.2)
≥p25	30 (60.0%)	68 (84.0%)	1.0 (Ref)	1.0 (Ref)

[1]Calculated from the summation of intake food groups weighted by their factor loadings. [2]Odds ratios for SB been adjusted for maternal age, periconception folic acid/multivitamin supplement use and BMI in a logistic regression model.

DISCUSSI0N

For the first time, a maternal dietary pattern is associated with the risk of having SB offspring independent of periconception folic acid supplementation. This is considered to be clinically relevant because low maternal intake of vegetable oil, vegetables, fruit, fish and whole grains was associated with a more than two-fold increased SB risk. This observation may suggest that a healthy diet contains enough natural folate to reach a similar protective effect as supplementation with a synthetic folic acid tablet.[6] Latest findings in literature have reported higher bioavailability of folate in foods than previously was suggested.[31] Of interest is that the results also provide a plausible explanation for the relatively low occurrence of SB in Southern European countries where the Mediterranean diet is commonly used.[10] Nevertheless, this association should be interpreted cautiously, and further studies are needed to substantiate this finding.

The PCA and RRR methods have identified a comparable dietary pattern, which was labelled Mediterranean as it was characterised by high intakes of fruit, vegetables, vegetable oils, legumes, fish, alcoholic drinks, and cereals and low intakes of potatoes and sweets.[32] The dietary pattern from PCA differs by the higher intakes of butter and lower intakes of meat. The diet from RRR contained more dairy products but less non-alcoholic drinks, sauces, and condiments.

Alcohol intake was positively correlated with the Mediterranean diet. In general alcohol intake was very low among all mothers in the periconception period, and the intake was usually restricted to one or occasionally two glasses of wine per week. Nevertheless, the observed association may be explained by the well-known relationship between educational level and alcohol intake in the Netherlands. The drinking of wine is part of social behaviour in the socio-economic privileged households, who also demonstrate a remarkable interest in the Mediterranean style of food preparation and cooking. This is substantiated by the significantly lower education in case mothers compared with controls. Education is a known risk factor for SB and could be considered a proxy for the quality of the diet and lifestyle. This is also in agreement with the higher BMI observed in case mothers.

There was also a strong association between education and use of the Mediterranean diet. This was the rationale for not adjusting the odds ratios for educational level. Finally, the Mediterranean dietary pattern was also associated with higher maternal age and folic acid supplementation, which may give rise to the future identification of a maternal lifestyle and thus phenotype with regard to the risk for SB offspring.

It is important to note that the Mediterranean dietary pattern revealed positive associations with blood concentrations of folate and vitamin B12 and an inverse association with tHcy. This may suggest that the Mediterranean diet reduces hyperhomocysteinemia and related oxidative stress. Oxidative stress is also involved in cardiovascular disease and, not surprisingly, the Mediterranean diet has been associated with a decreased risk of cardiovascular diseases and increased lifespan.[14,15] These findings are in line with the fact that intrauterine nutritional programming has long-term consequences and is associated with adult diseases in adulthood.[33]

The use of dietary pattern analysis is commonly accepted as it provides essential and complementary insights into the overall dietary behaviour and, to date, has successfully been related to disease risk. The PCA analysis describes interactions between a set of correlated predicting variables, for example maternal food intakes, independent of a certain outcome measure. Therefore, it is possible that certain combinations of food intakes are overlooked that may be better related to disease status. Hoffmann et al.[12] addressed this issue by introducing RRR, in which predicting variables, e.g., food groups, are used to predict certain response measures, such as the biomarkers that are known to be linked to a disease or phenotype.

However, the use of specific biomarkers as response measures also has some disadvantages because biomarkers serve as intermediary variables in biochemical pathways that may not be important for all individuals. Other pathways that may be involved in the chain from diet to disease have, however, not been considered. We addressed these issues by performing both PCA and RRR. Both statistical methods have identified a comparable Mediterranean dietary pattern predominantly present in the study group.

Our study was conducted at a fixed study moment of 14 months after the birth of the index child to reflect the maternal dietary intake and lifestyle during periconception period. While a prospective study focused on maternal exposures during periconception period would be a preferable study design, this would require enormous financial and logistic resources. Therefore, we standardized the study design as much as possible and excluded women from analysis if their dietary intake might have been different between the periconception period and the time of the study due to illnesses, a reported change in diet, pregnancy or lactation. This is in agreement with the rational of Willet[23] and others that the individual dietary intake is rather stable during life, except for periods of dieting, breastfeeding and extreme growth. The use of such strict exclusion criteria may potentially result to a degree of selection bias. However, the additional analysis showed that there were almost no differences in the general characteristics, biomarkers, and micronutrient and macronutrient intakes of the included and excluded mothers, which makes such bias unlikely. Nevertheless, our findings should be carefully interpreted and future studies with larger samples sizes are needed for further validation.

We considered the power of the study by performing an additional power calculation based on estimated odds ratios between 2.5 and 3.0, type I error of 0.05 and a power of 80%. It revealed that the sample sizes needed are between 47 to 68 cases and 75 to 108 controls. This indicates that the available 50 cases and 81 controls provided our study with sufficient power to detect significant effects of a low adherence to the Mediterranean diet.

General Conclusion

The identification of the Mediterranean dietary pattern and its association with SB risk is valuable in understanding how the overall diet can be linked to biomarkers, metabolic pathways and risk estimates of disease. In future, this may lead to opportunities for more natural and flavoursome strategies for the target group in addition to currently implemented pharmacological interventions. Encouraging women to adhere to a Mediterranean diet will not only protect against cardiovascular diseases but may be beneficial for reproductive outcome as well.

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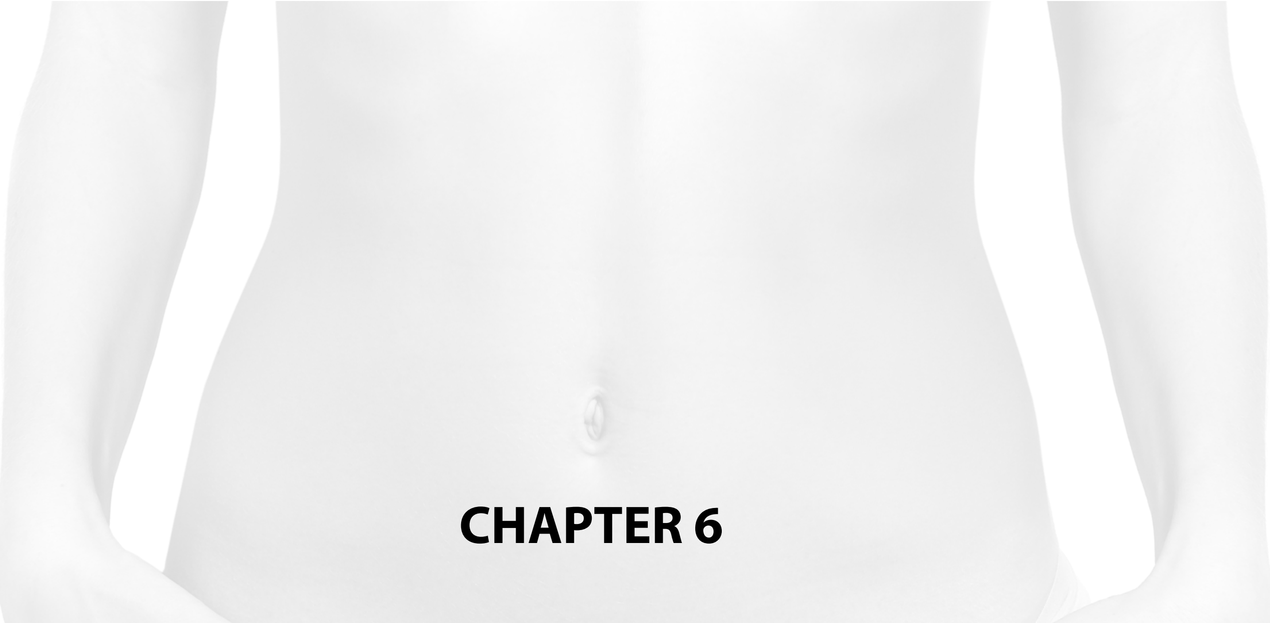

CHAPTER 6

A maternal dietary pattern characterized by fish and seafood is associated with a reduced risk of congenital heart defects in the offspring

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ABSTRACT

Objective

Derangements in the maternal one carbon pathway increase the risk of congenital heart defects (CHD) in the offspring and may be linked to the diet. This study aims to identify dietary patterns related to biomarkers of methylation of this pathway and to investigate associations with CHD risk in the offspring.

Methods

As part of a case-control study in the western part of the Netherlands, we investigated 179 mothers of a child with CHD and 231 control mothers of a non-malformed child. We identified dietary patterns related to the plasma biomarkers S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) by the reduced rank regression (RRR) method. The dietary patterns were validated with individual nutrient intakes and the biomarkers in blood of the B-vitamins and homocysteine by linear and logistic regression models. Risks for CHD were assessed with relation to the estimated dietary patterns.

Results

A dietary pattern comprising of high intakes of snacks and sugar-rich products and non-alcoholic drinks was positively associated with SAH (β 0.92, $p \leq 0.001$). Strong adherence to this One Carbon Poor diet slightly increased homocysteine ($p \leq 0.10$) without affecting CHD risk (OR 0.8, 95%CI 0.5; 1.4). A dietary pattern with high intakes of fish and nuts was positively associated with SAM (β 0.44, $p \leq 0.001$) and inversely with SAH (β -0.08, $p \leq 0.001$). Mothers who strongly adhered to this One Carbon Rich dietary pattern showed increased serum ($p \leq 0.05$) and red blood cell ($p \leq 0.01$) folate and a reduced risk of CHD offspring (OR 0.3, 95%CI 0.2; 0.6).

Conclusion

A One Carbon Rich dietary pattern characterized by high maternal fish intakes associated with a reduction of CHD risk comparable to the effects of periconceptional folic acid use.

INTRODUCTION

Worldwide each year around 8 million children are born with a congenital malformation, of which the congenital heart defects (CHD) is the largest group.[1] Its complex aetiology is characterized by an interplay between genetic, environmental, and lifestyle factors. The importance of adequate nutrition in the periconception period is well established.[2] It is shown that folic acid fortification of foods significantly reduces the prevalence of not only neural tube defects but also of CHD.[3, 4] The B-vitamin folate is a substrate and vitamin B2, B3, B6 and B12 serve as cofactors in the one carbon pathway. Together with choline these B-vitamins are involved in the remethylation of homocysteine into methionine. The methyl groups provided by folate, methionine and choline are ultimately important for the transmethylation of S-adenosylmethionine (SAM) into S-adenosyl-homocysteine (SAH) after which homocysteine is formed. Evidence from animal and first human studies shows that methyl groups play a role in the epigenetic programming of DNA during the periconception period and embryogenesis.[5-7] Methyl groups are needed for the methylation of lipids, proteins, chromatin, and DNA. This is supported by our recent study showing that the maternal use of a low dose of folic acid in the periconception period significantly increases the methylation of IGF2 gene in the child.[8]

In humans SAM and SAH plasma concentrations and their ratio are frequently used markers of global DNA methylation potential.[9] Low SAM concentrations and/or high SAH concentrations may result in cellular hypomethylation, which is associated with chromosomal instability and abnormal chromosomal segregation.[10] This is relevant with respect to our earlier finding that a maternal state of global hypomethylation, i.e., low SAM/SAH ratio and high SAH concentration, is associated with an increased risk of CHD and Down syndrome offspring.[11]

From this background we aimed to 1) identify dietary patterns related to the methylation biomarkers of the one carbon pathway, 2) to validate the identified dietary patterns with nutrient intakes and other biomarkers of the one carbon pathway, and 3) to investigate associations between adherence to the identified maternal dietary patterns and the risk of CHD in the offspring.

METHODS

Study Population

The subjects are enrolled in the HAVEN study, a Dutch acronym of the ongoing case-control family study designed to investigate determinants in the pathogenesis and prevention of CHD. The study has been conducted in the western part of the Netherlands and has been described in detail previously.[12] From June 2003, this study has included cases from 4 University Medical Centres and controls in collaboration with the child health centres of 'Thuiszorg Nieuwe Waterweg Noord' in the Rotterdam area. These child health centres are part of the Dutch Health Care system, where all newborns are regularly checked on for health, growth, and development by physicians specialized in child health care. The domain population comprised both case children and control children living in the Western part of the Netherlands. All children were between 11 and 18 months of age, and there was no familial relationship between cases and controls. The selection of the included CHD phenotypes was mainly based on experimental and epidemiological studies that showed that derangements in the one carbon pathway are involved in the etiology.[12-15] The CHD was diagnosed in the first year of life by two paediatric cardiologists trained at the same University Medical Centre in Leiden, The Netherlands, using ultrasound and/or cardiac catheterization and/or surgery. Case and control children were eligible for inclusion if they were singletons, and their parents were familiar with the Dutch language in reading and writing. Only biological parents could participate.

all triads
818 triads: cases 352 / controls 466

complete data (missings in FFQ and biomarkers excluded)
813 triads: cases 349 / controls 464

ethnicity (non-European excluded)
659 triads: cases 295 / controls 364

diet (difference in diet excluded)
598 triads: cases 269 / controls 329

breastfeeding (current breastfeeding excluded)
582 triads: cases 259 / controls 323

pregnancy (current pregnancy excluded)
531 triads: cases 233 / controls 298

vitamin use (current B-vitamin use excluded)
410 triads: cases 179 / controls 231

Figure 1 | Flowchart of the selection criteria of the study population

Between June 2003 and August 2008, 352 case-triads (child, mother, and father) and 466 control triads were included. The study protocol was approved by the Central Committee on Research involving Human Subjects and the Institutional Review Boards of all participating hospitals. Informed consent was obtained from all parents.

Food Frequency Questionnaire

At the standardized study moment of approximately 16 months after the index-pregnancy, mothers filled out a validated food frequency questionnaire (FFQ) at home that covered the food intake of the 4 weeks before the study moment. This FFQ was developed by the division of Human Nutrition, Wageningen University, Wageningen, the Netherlands, and validated for intake of energy, B-vitamins, and fatty acids.[16, 17] Moreover, a self-administered general questionnaire was also filled out by the mother at home from which data were obtained on educational level and ethnicity, alcohol, tobacco, medication, and B-vitamin use at the study moment and during the periconception period of the index-pregnancy. The periconception period was defined as 4 weeks before conception until 8 weeks after conception. The use of folic acid in the periconception period was defined as the daily use of at least

400μg of folic acid, either in a multivitamin preparation or as a single tablet during the whole period. Mothers who did not use a folic acid preparation, or only during a part of the periconception period, were classified as non-users. We defined medication use as any use of medication during the periconception period. Educational level and ethnicity were classified according to the definition of Statistics Netherlands.[18]

During the hospital visit, the FFQ and general questionnaire were checked in a standardized manner for completeness and consistency by the researcher. Furthermore, standardized anthropometric measurements were performed, including maternal height and weight (anthropometric rod and weighing scale; SECA, Hamburg, Germany). Body mass index (BMI) was calculated by weight divided by the squared height. Fasting venous blood samples were drawn for measurement of the biomarkers of methylation in plasma: SAM, SAH, and plasma total homocysteine (tHcy), red blood cell (RBC) folate, and serum folate and vitamin B12 concentrations.

Biomarkers

Immediately after blood sampling, an EDTA tube was put on ice and a serum separator tube was kept at room temperature. Both tubes were centrifuged at 4,000 x g for ten minutes at 4°C and separated within one hour. All samples were stored at -80°C and measured anonymously in batches within five months of collection. To determine SAM and SAH, we used liquid chromatography tandem mass spectrometry (LC-MS/MS; Waters acquity UPLC premier XE, Milford, MA, US) as previously described.[11] tHcy was also determined using LC-MS/MS. Serum folate and vitamin B12 were routinely determined by immuno-electrochemiluminescence immunoassay (ECLIA) on the Roche Modular E170 (Roche Diagnostics GmbH, Mannheim, Germany). RBC folate was measured in the hemolysate of whole-blood with ascorbic acid for stabilization. The RBC folate concentration was calculated according to the following formula: (nM haemolysate folate x 10/haematocrit) – (nM serum folate x [1 – hematocrit] / hematocrit) = nM RBC folate. The inter-assay coefficients of variation (CV) for SAM were 4.4% at 70.8 nmol/L, 4.4% at 100.8 nmol/L and 4.8% at 143.2 nmol/L, for SAH 4.2% at 24.2 nmol/L, for tHcy 5.9% at 15.3 μmol/L and 3.4% at 39.3 μmol/L, for folate 9.5% at 8.3 nmol/L and 3.2% at 20.2 nmol/L, and for vitamin B12 5.1% at 125 pmol/L and 2.9% at 753 pmol/L.

Statistics

Due to the case-control character of the study, we first performed the dietary pattern analysis on the FFQ data filled out by the controls (n 231) by reduced rank regression (RRR), because the group of control mothers best represent the general population of women in reproductive ages. All 200 food items in the FFQ filled out by all mothers were classified into 22 predefined food groups similar in nutrient content and use, and comparable with the grouping schemes reported in literature.[19] The RRR method was applied with CANOCO (Canonical Community Ordination, version 4.5, Microcomputer Power, Ithaca, NY, US) software for Windows to identify dietary patterns from the energy adjusted food groups among the control mothers that explain the largest proportion of variation in the biomarkers of methylation SAM and SAH.[20, 21]

Dietary patterns were identified by extracting linear combinations of the predicting variables, i.e., the energy adjusted food groups, that explain as much variation in response variables as possible, i.e., SAM and SAH. All food groups were entered and scaled before RRR analysis.[22] The linear combinations identified with RRR are represented by β estimates in Table 2. The overall p for the explained variance of the dietary pattern analysis was tested using the permutation method. RRR identified two factors, e.g. dietary patterns, as the number of patterns to be identified equals the number of selected response variables.

Table 1 | Baseline characteristics of mothers with CHD in the offspring and controls

	cases (n 179)	*controls (n 231)*	*p*
at conception			
age[1,2]	33.1 (4.3)	32.4 (4.3)	0.14
BMI[1,3]	24.9 (6.5)	24.1 (4.9)	0.03
pregnancy length[4,5]	39.4 (2.4)	40.0 (2.1)	≤0.01
at study moment			
energy intake[1,6]	8856 (2023)	9041 (2349)	0.39
high education[7]	127 (29.1)	64 (27.7)	0.22
medication[7]	33 (18.4)	44 (19.0)	0.90
smoking[7]	32 (17.9)	44 (19.0)	0.80
alcohol[7]	100 (55.9)	143 (61.9)	0.23
biomarkers[5]			
SAM[8,9]	76.8 (13.9)	80 (15.4)	0.03
SAH[8,9]	14.3 (4.0)	14.2 (4.1)	0.80
homocysteine[8,10]	10.9 (4.2)	10.3 (3.9)	0.10
vitamin B12[11,12]	271.0 (158.5)	244.5 (135.3)	0.14
folate[9,11]	14.2 (5.9)	13.4 (5.7)	0.50
folate[9,13]	617.5 (246.5)	607.1 (226.5)	0.89
Periconception			
medication[7]	46 (25.7)	33 (14.3)	≤0.01
use of B-vitamins[7]	98 (54.7)	133 (57.6)	0.62
smoking[7]	35 (19.6)	41 (17.7)	0.70
alcohol[7]	70 (39.1)	81 (35.1)	0.47

Data are displayed by [1]mean (se), [2]years, [3]kg/m², [4]weeks, [5]median (iqr), [6]kJoule/day, [7]n (%), [8]plasma, [9]nmol/L, [10]µmol/L, [11]serum, [12]pmol/L, [13]red blood cells.

All case and control mothers were assigned scores on the two identified dietary patterns, calculated as the product of the food group value and its factor loading derived from the control group and summed across foods. The case and control mothers were classified into tertiles according to the magnitude of their factor score, i.e., the degree to which the individual nutritional intakes agreed to the dietary pattern extracted from the control group. The association between the dietary patterns and the concentrations of tHcy, folate and vitamin B12 in blood was examined with linear regression analysis (Table 3).

Table 2 | Association between food groups and One Carbon Poor and One Carbon Rich dietary patterns performed with RRR with food groups as predictor variables for SAM and SAH in 231 control mothers

food group	*One Carbon Poor diet*	*p*	*One Carbon Rich diet*	*p*
alcohol	– 0.01 (0.01)	0.38	0.03 (0.02)	0.22
cereals	0.08 (0.08)	0.31	– 0.05 (0.13)	0.67
butter	1.19 (1.08)	0.27	0.25 (1.70)	0.89
dairy	– 0.01 (0.04)	0.80	0.11 (0.06)	0.08
eggs	– 0.03 (0.07)	0.65	– 0.03 (0.10)	0.81
fish	0.12 (0.21)	0.58	0.96 (0.33)	≤0.01
fruit	0.00 (0.02)	0.89	– 0.02 (0.03)	0.52
legumes	0.09 (0.09)	0.28	0.11 (0.13)	0.43
margarine	1.09 (0.77)	0.16	1.28 (1.20)	0.29
mayonnaise	0.07 (0.21)	0.74	0.20 (0.33)	0.54
meat	0.06 (0.16)	0.68	0.17 (0.25)	0.48
non-alcoholic drinks	0.01 (0.01)	0.03	0.01 (0.01)	0.54
nuts	0.22 (0.11)	0.05	0.35 (0.18)	0.05
refined grains	0.06 (0.05)	0.27	– 0.05 (0.09)	0.55
potatoes	0.01 (0.03)	0.60	0.02 (0.04)	0.65
sauces	0.00 (0.19)	0.99	0.17 (0.29)	0.55
snacks	0.16 (0.06)	≤0.01	0.04 (0.09)	0.65
soup	0.01 (0.01)	0.13	0.01 (0.01)	0.37
sweets	0.27 (0.12)	0.02	0.01 (0.18)	0.94
vegetable oils	0.76 (1.30)	0.55	0.73 (2.00)	0.71
vegetables	0.00 (0.03)	0.99	– 0.05 (0.05)	0.31
whole grains	0.03 (0.04)	0.43	0.01 (0.06)	0.86

Data are displayed by β estimates and standard error in parentheses.

To assess the risk of CHD offspring, we analyzed whether the degree of adherence to the identified dietary pattern differed between case and control mothers, as shown in Table 4. We calculated tertiles of the dietary patterns to estimate CHD risks and present them by odds ratios (OR) and 95% confidence intervals (95%CI). The distributions of case and control mothers with intermediate and high adherence to the respective dietary pattern were compared with the reference group of mothers with low adherence to the respective dietary pattern. In a logistic regression model the estimates were adjusted for potential confounders, such as maternal age, BMI, educational level, periconception folic acid/multivitamin use, smoking, alcohol and medication use.

Table 3 | Pearson r coefficients between the energy adjusted macro- and micronutrient intakes and dietary patterns in 231 control mothers

nutrients	*One Carbon Poor diet*	*p*	*One Carbon Rich diet*	*p*
total fat[1]	−0.064	0.33	−0.077	0.24
saturated fats[1]	−0.041	0.54	−0.103	0.12
MUFA[1]	−0.032	0.63	−0.072	0.28
PUFA[1]	−0.087	0.19	0.003	0.97
LA[1]	−0.036	0.58	0.037	0.57
ALA[1]	0.044	0.50	0.033	0.62
EPA[1]	0.109	0.10	0.131	0.05
DHA[1]	0.085	0.20	0.131	0.05
cholesterol[2]	0.013	0.85	0.046	0.48
total protein[1]	0.055	0.40	0.164	0.01
carbohydrates[1]	0.078	0.24	−0.017	0.80
fiber[1]	0.042	0.53	0.065	0.33
alcohol[1]	−0.103	0.12	0.046	0.48
vitamin B6[2]	−0.016	0.81	0.138	0.04
vitamin B12[3]	0.125	0.06	0.183	≤0.01
folate[3]	0.053	0.43	0.129	0.05
zinc[2]	0.071	0.29	0.145	0.03
retinol[3]	0.060	0.36	0.045	0.49
thiamin[2]	0.090	0.17	0.130	0.05
riboflavin[2]	0.025	0.70	0.175	0.01
nicotinamide[2]	0.109	0.10	0.183	≤0.01
vitamin C[2]	−0.012	0.85	0.065	0.33
vitamin E[2]	−0.124	0.06	−0.024	0.71

Data are presented by correlation coefficients in [1]g/day, [2]mg/day, µg/day.

Differences in general characteristics between case and control mothers were tested with student's t-test for age and energy intake, Mann-Whitney U test for the remaining continuous variables, and chi-square test for the categorical variables. Maternal age and log-transformed energy intake are presented as mean with range (p25-p75). BMI at the study moment, and duration of pregnancy showed skewed distributions even after log-transformation and are presented as median with inter-quartile range (p25-p75). Kruskall Wallis U-test was used to test differences in BMI and duration of pregnancy. The chi-square test for linear association was used to investigate differences in educational level, medication use, smoking, alcohol use and B-vitamin intake (Table 1).

RESULTS

We excluded 170 out of 349 case and 233 out of 464 control mothers because of the following reasons: non-European ethnicity, dietary differences at the study moment compared with the periconception period, breastfeeding, pregnancy, and/or the use of folic acid at the study moment. This resulted in 179 case and 231 control mothers for analysis (Figure 1). The CHD phenotypes (n 179) included were tetralogy of Fallot (n 25), transposition of the great arteries (n 37), atrioventricular septal defect (n 16), peri-membranous ventricular septal defect (n 41), coarctation of the aorta (n 21), aortic valve stenosis (n 6), pulmonary valve stenosis (n 26) and hypoplastic left heart syndrome (n 7). Table 1 presents the general characteristics which reveal that when compared to controls, BMI (p 0.03) in case mothers was higher and gestational age shorter (p ≤0.01), Moreover, case mothers reported more use of medication (p ≤0.01)periconception, and showed higher SAM concentration (p 0.03).

Table 4 | The correlations between the biomarkers and the dietary pattern in 231 control mothers[1]

	One Carbon Poor diet			
	low	*intermediate*	*high*	
biomarker	*(n 74)*	*(n 79)*	*(n 78)*	*p[8]*
SAM[2,3]	74.7 (12.2)	82.1 (12.2)	83.8 (16.5)	≤0.001
SAH[2,3]	11.5 (1.4)	14.3 (1.3)	17.3 (2.9)	≤0.001
tHcy[4,5]	10.0 (2.8)	10.5 (4.0)	10.9 (4.7)	0.10
vitamin B12[4,6]	260 (131)	237 (139)	246 (143)	0.72
folate[3,4]	13.0 (6.2)	13.6 (5.6)	13.5 (5.7)	0.62
folate[3,7]	627 (243)	588 (222)	593 (233)	0.93
	One Carbon Rich diet			
	low	*intermediate*	*high*	
biomarker	*(n 68)*	*(n 78)*	*(n 85)*	*p[8]*
SAM[2,3]	69.4 (4.2)	78.7 (5.0)	90.4 (8.8)	≤0.001
SAH[2,3]	12.8 (5.2)	13.9 (3.3)	15.2 (3.9)	≤0.001
tHcy[4,5]	10.3 (3)	10.7 (3.9)	10.4 (4.3)	0.11
vitamin B12[4,6]	240 (128)	259 (157)	240 (122)	0.79
folate[3,4]	13.5 (4.1)	13.0 (6.3)	14.3 (7.0)	0.03
folate[3,7]	592 (191)	599 (214)	637 (291)	≤0.01

Data are presented by median (IQR), [2]plasma, [3]nmol/L, [4]serum, [5]µmol/L, [6]pmol/L, [7]red blood cell. [8]P values have been calculated in a linear regression model on log-transformed continuous biomarker variables.

The RRR analysis of the control mothers revealed a dietary pattern explaining 10.6% of the variance in SAH (p ≤0.001) and a dietary pattern related to SAM explaining 4.2% of the variance (p ≤0.001). The strong positive association between the first factor and SAH (β 0.92, p ≤0.001) reflects a diet poor in methyl-donors and was therefore labelled as the One Carbon Poor diet. This diet was also positively, albeit marginally, associated with SAM (β 0.03, p ≤0.001). This One Carbon Poor dietary pattern contained in particular high intakes of snacks and sugar-rich products and non-alcoholic drinks (Table 2), which were not significantly correlated with nutrient intakes (Table 3). A high adherence to this dietary pattern was associated with elevated concentrations of tHcy (p 0.02) across the tertiles (Table 4).

The second dietary pattern showed a positive association with SAM (β 0.44, p ≤0.001). This diet was inversely, albeit marginally, association with SAH (β -0.08, p ≤0.001). This One Carbon Rich dietary pattern contained especially high intakes of fish and other seafood (Table 2). Adherence to this dietary pattern was significantly correlated with high intakes of total protein, B-vitamins, i.e., vitamin B1, B2, B3, B6, and B12 and omega-3 fatty acids, i.e., eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), and zinc (Table 3). A high adherence to this One Carbon Rich dietary pattern was associated with higher levels of serum folate (p ≤0.01)and RBC folate (p 0.005) (Table 4).

Only BMI was associated with both the adherence to the One Carbon Poor (p ≤0.01) and One Carbon Rich dietary pattern (p ≤0.001). In contrast to the One Carbon Poor dietary pattern, strong adherence to the One Carbon Rich dietary pattern significantly reduced CHD risk (crude OR 0.59, 95%CI 0.37; 0.96, Table 5). After adjustment for maternal age, BMI, educational level, periconception use of folic acid, alcohol, medication and smoking, the risk estimate became even more prominent (OR 0.32, 95%CI 0.18; 0.59).

DISCUSSION

In this study we identified a maternal One Carbon Poor and One Carbon Rich dietary pattern reflecting the bioavailability of methyl-donors. The One Carbon Rich dietary pattern characterized by high intake of fish and seafood provided in particular total protein, vitamin B1, B2, B3, B6, and B12, zinc, EPA and DHA. The adherence to this dietary pattern was reflected in higher RBC and serum folate levels. A very important finding is that strong adherence to the One Carbon Rich dietary pattern is also associated with a 70% reduced risk of CHD offspring. This estimate is comparable to the preventive effect of the periconceptional use of a low dose of synthetic folic acid.[14, 23]

The One Carbon Poor dietary pattern, characterized by high intakes of snacks, sugar-rich foods, and non-alcoholic drinks, resembles a Western dietary pattern, and did not affect the other biomarkers levels and CHD risk. It has been shown before that low SAM and high SAH are often accompanied by hyperhomocysteinemia, which is also a risk factor for CHD and neural tube defects.[5, 11, 12, 15, 24] In line with Shoob et al. showing that a methionine-rich diet is associated with a reduced risk of neural tube defects, we demonstrated an association between adherence to the One Carbon Rich diet and a reduction in CHD risk.[25] This finding substantiates our earlier finding that adherence to a Mediterranean dietary pattern, also rich in one carbon groups and related to high serum and RBC folate levels, is associated with a reduction in the risk of spina bifida in the offspring.[26]

The underlying mechanisms by which derangements in the one carbon pathway induced by compromised dietary intakes of its substrates and cofactors, contribute to the prevention of birth defects is largely unknown. Nutrition plays a key role in epigenetic programming of embryonic growth and development, in which one carbon groups, in particular methyl groups, play a significant role in DNA methylation and histone modification.[7, 27] Animal studies show that dietary availability of methyl groups affects gene methylation. Supplementation of mice with methyl donors before or in early pregnancy increases the level of DNA methylation, and changes the phenotype of the offspring.[6, 28] A study in rats showed that maternal dietary protein restriction during pregnancy, including low methionine, leads to a persistent decrease in methylation of several genes. This pattern of hypomethylation was not present in rats supplemented with folic acid.[29] Furthermore, Sinclair et al.[30] demonstrated in sheep that deficiencies of B-vitamins and methionine in the preconception diet leads to both hypomethylation and hypermethylation of the offspring's genome accompanied by an unhealthy phenotype. These findings underline the importance of periconceptional adherence to the One Carbon Rich dietary pattern as determinant of future health and disease risk in the offspring thereby substantiating the developmental origin hypothesis of health and disease.[27]

Table 5 | The association between adherence to the dietary patterns and the risk of having offspring with CHD

	One Carbon Poor diet			
	cases	*controls*	*crude OR*	*adjusted OR[1]*
adherence	*(n 179)*	*(n 231)*	*(95% CI)*	*(95% CI)*
low	63 (35.2%)	74 (32.0%)	1.00 (Ref)	1.00 (Ref)
intermediate	58 (32.4%)	79 (34.2%)	0.86 (0.54 - 1.39)	0.96 (0.56 - 1.64)
high	58 (32.4%)	78 (33.8%)	0.87 (0.54 - 1.41)	0.81 (0.47- 1.40)
	One Carbon Rich diet			
	cases	*controls*	*crude OR*	*adjusted OR[1]*
adherence	*(n 179)*	*(n 231)*	*(95% CI)*	*(95% CI)*
low	69 (38.5%)	68 (29.4%)	1.00 (Ref)	1.00 (Ref)
intermediate	59 (33.0%)	78 (33.8%)	0.75 (0.46 - 1.20)	0.64 (0.37 - 1.11)
high	51 (28.5%)	85 (36.8%)	0.59 (0.37 - 0.96)	0.32 (0.18 - 0.59)

[1]Adjusted for maternal age at index-pregnancy, BMI, educational level, periconception folic acid/multivitamin use, smoking, alcohol intake, and medication use.

The One Carbon Rich dietary pattern provides in particular B-vitamins. This supports our earlier finding that a high intake of these nutrients reduced CHD risk in offspring.[31, 32] Furthermore, this dietary pattern was also positively correlated with zinc, being a cofactor of methionine synthase implicated in the conversion of homocysteine into methionine.[33] The positive correlation with the omega-3 fatty acids EPA and DHA intakes may be explained by their homocysteine reducing effect, and contribute to the discussion of the beneficial effects of fish intake on pregnancy outcome.[34, 35]

Previously we investigated associations between lifestyle factors and the biomarkers of methylation SAM and SAH in the same HAVEN-study among the control mothers.[36] It revealed that BMI is a strong determinant of SAM, and to a lesser extent of SAH. Our current analysis in the larger study group confirms this finding by showing that a high intake of fish and seafood is also associated with an increase in BMI. A high maternal BMI, however, is a known risk factor for CHD in the offspring[37] and is in our study independently associated with an increased risk of CHD in the offspring (BMI ≥30 vs. BMI ≤30, OR 2.5, 95%CI 1.4; 4.6).

Methionine, choline and betaine are also important donors of methyl groups and their uptake is associated with a significant decrease in plasma SAH and an increase in SAM:SAH, methionine, and glutathione:GSSG. It has been suggested that foods rich in these compounds may prevent birth defects as well.[38, 39] Unfortunately, the FFQ used in our study is not validated for precise assessment of the intakes of these nutrients. Therefore, it would be interesting to study in future the effects of these nutrients in association with CHD risk.

The case-mothers had a higher BMI, and reported a lower energy-intake which introduces the concern of differential underreporting between obese and non-obese women. It has been shown that nutritional underreporting increases together with the increase of BMI.[40] We tested this potential confounder by estimating the mean basal metabolic rate (BMR) using the new Oxford equation for women aged 30-60 years: BMR (mJoule/day) = 0.0407 x weight (kg) + 2.90. The physical activity level (PAL) was calculated by dividing the mean reported energy intake (EI) by the mean BMR.[41] A PAL cut-off value of 1.35 was used to evaluate under-reporting. In case mothers the PAL was 1.39 and 1.44 in controls. Additional trend analysis, revealed a significant trend for a decrease in PAL with an increase in BMI. The PAL for case and control mothers with BMI >25, 1.31 and 1.27, respectively, and may suggest underreporting. This association, however, is independent of the case or control status and thus non-differential and thereby not disturbing the validity of our results.

The main advantage of RRR is that all food groups are used in predicting the global methylation status by SAM and SAH biomarkers. This sophisticated method includes all dietary information in which no foods have been omitted. Nevertheless, restricting the model to predicting methylation biomarker levels, other pathways between diet and disease that also may be involved are disregarded a priori.

Some strengths and limitations of this study have to be addressed. We used a standardized study moment of approximately 16 months after the index-pregnancy, which we consider as one of the main methodological strengths compared to case control studies on congenital malformations by others.[26, 32] The study moment is within 2 years after pregnancy thereby minimizing recall bias regarding periconception lifestyle behaviours. Furthermore, misclassification of cases and controls is prevented, since most CHD are diagnosed during the first year of life. If misclassification of controls has occurred, this will only have lead to underestimation of the observed associations.The validated FFQ was filled out at the study moment and covered the intake of the previous 4 weeks whereby day to day variability of food intake is minimized. The study moment is 2 years after conception of the index-pregnancy in the same season as the periconception period. Thus, the seasonal influences on food-intake are comparable between these periods. In addition, we have showed earlier that the maternal nutritional status assessed more than 1 year after delivery can be used as a valid estimate of the preconception maternal nutritional status.[42] This is also supported by others showing that every individual adheres to a habitual dietary pattern that is only influenced by periods of illnesses, dieting, pregnancy, breastfeeding and growth spurts. After adjustment of the analysis for those factors as we did, dietary patterns are very stable during life.[43-45] An advantage of our study population was that it was very homogeneous due to the exclusion of non-European mothers.

Methodological limitations in the case–control study concern the degree in which the measured maternal biomarkers and BMI at study moment truly reflect the periconceptional status. Because differences are not to be thought to be differential between cases and controls, they do not pose a threat to the validity of our findings.

General Conclusion

Maternal adherence to a One Carbon Rich dietary pattern mainly reflected by high intake of fish and seafood is associated with a reduced CHD risk in the offspring. This finding could be a first link between periconception maternal nutrition and epigenetic regulation of embryonic development and growth.

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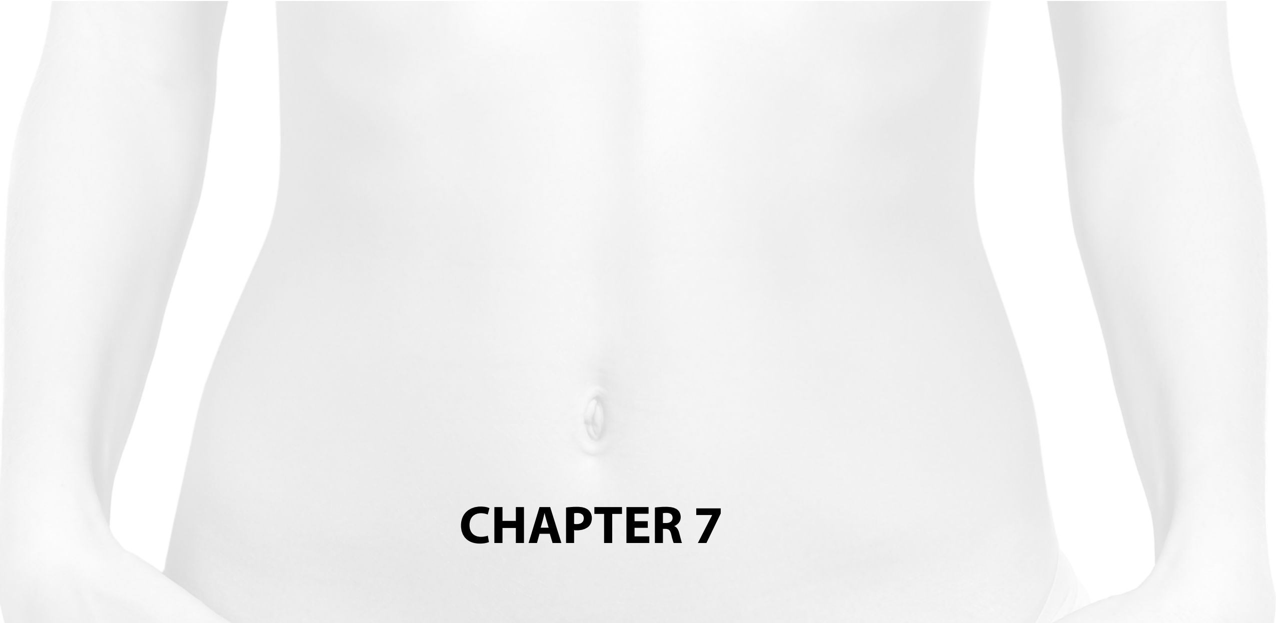

CHAPTER 7

Maternal Western dietary patterns and the risk of developing a cleft lip with or without cleft palate

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ABSTRACT

Objective

To identify maternal dietary patterns in association with a cleft lip or cleft palate or both in the offspring.

Methods

In a case–control study of 203 mothers of a child with a cleft lip or cleft palate and 178 mothers with non-malformed offspring, maternal nutritional intakes were assessed 14 months after the birth of the index child to estimate the preconception intake. We measured serum and red blood cell folate, serum vitamin B12, whole blood vitamin B6, and total plasma homocysteine as biomarkers. Dietary patterns were analyzed by principal component factor analysis. Univariate and multivariate analyses were performed and odds ratios (OR) with 95% confidence intervals (95%CI) calculated.

Results

Two major dietary patterns were identified. The Western dietary pattern, e.g., high in meat, pizza, legumes, and potatoes, and low in fruit, was associated with a higher risk of a cleft lip or cleft palate, OR 1.9 (95%CI 1.2; 3.1). This risk remained significant after adjustment for potential confounders of maternal education and smoking at the study moment, and periconception vitamin use. This dietary pattern was associated with lower red blood cell folate (p 0.02), vitamin B6 (p ≤0.001), vitamin B12 (p 0.02), and higher homocysteine (p 0.05) concentrations. The use of the Prudent pattern, e.g., high intakes of fish, garlic, nuts, vegetables, increased vitamin B12 (p ≤0.001) and serum folate (p 0.05) levels, was not associated with cleft lip or cleft palate risk.

Conclusion

The use of the maternal Western diet increases the risk of offspring with a cleft lip or cleft palate approximately two fold. Therefore, dietary and lifestyle profiles should be included in preconception screening programs.

INTRODUCTION

Cleft lip with or without a cleft palate (CLP) is a serious birth defect, with varying birth prevalence rates among populations, gender, and geographic regions.[1] These children have to undergo several treatments by various specialists, which have an enormous effect on their lives. Therefore, there is a strong imperative toward a better understanding of the cause of CLP, in which, in particular, maternal periconception exposures are of interest. Because harmful exposures can be avoided or treated, the preconception identification of risk factors may contribute to future primary prevention programs. Evidence on the importance of maternal nutrition, lifestyle, and subtle genetic variants in the pathogenesis of CLP is increasing.[2] So far research on the association between maternal nutrition and CLP has mainly been focused on single nutrients, such as folate, vitamin B6, vitamin A, or zinc.[3-5] Observational and intervention studies suggest a beneficial effect of periconception multivitamin supplementation on the occurrence of CLP.[3,5-8] The identification of dietary patterns has recently become of considerable interest and has been related to cardiovascular disease, type 2 diabetes, and cancer.[9-11] So far, data on maternal dietary patterns and pregnancy outcome, in particularly birth defects, are lacking.

Therefore, we investigated whether maternal dietary patterns are associated with 1) the concentrations of folate, vitamin B12, vitamin B6, and homocysteine in blood and 2) the risk of having a child with CLP. Because it is not yet feasible to investigate these aims in a prospective cohort study, we have chosen as the best alternative conducting a case–control study at a fixed study moment approximately 14 months after the index-pregnancy. At that study moment, the nutritional status of the mother is rather comparable with the periconception period, because nutritional intake is stable during life except for periods of dieting, breastfeeding, and extreme growth.

METHODS

Study Population

A case–control study was conducted comprising of 225 case mothers of a child with a CLP and 217 control mothers of a non-malformed child, as described previously.[5] All mothers were Dutch Europeans and studied at the standardized study moment of approximately 14 months after delivery of the index child. Exclusion criteria were pregnancy, breastfeeding, folic acid containing supplement use at the study moment, a different diet at the study moment than in the periconception period, and extreme nausea starting after the first week of pregnancy. The institutional review boards of the participating hospitals approved the study protocol, and written informed consent was obtained from every participant.

General Questionnaire

We extracted the following data from the general questionnaires: age, body weight, height, education, smoking, vitamin intake and other lifestyle factors. Body mass index (BMI) was calculated as the ratio weight (kg) per height (m^2). Education was categorized into low (primary, lower vocational, intermediate secondary, or intermediate vocational), intermediate (higher secondary), and high education (higher vocational or university). Mothers were considered smokers when any smoking (cigarettes, cigars, or pipe) was reported. Data on folic acid only or multivitamin intake composed of content, dosage, and frequency.

Food Frequency Questionnaire

The nutritional intake was assessed using a validated food frequency questionnaire (FFQ).[12,13] We standardly collected the nutritional intake at approximately 14 months after the index pregnancy. The rationale was that nutritional habits in general are rather constant, with the exception of periods of dieting, pregnancy, and breastfeeding.[14,15] The validity of the data is strengthened by the time at which the FFQ was filled out, corresponding with 24 months after the preconception period and in the same season as the preconception period.

Table 1a | Baseline characteristics of case and control mothers in the Western diet

		Western diet		
	low	*intermediate*	*high*	
characteristic	*(n 127)*	*(n 127)*	*(n 127)*	*p*
age[1,2]	32.5 (27.3 - 38.3)	32.2 (27.4 - 39.1)	31.7 (24.9 - 39.6)	0.15
BMI[1,3]	23.3 (18.8 - 30.9)	23.9 (20.1 - 30.5)	25.9 (20.7 - 39.6)	0.01
high education[4]	67 (58.3)	37 (31.4)	26 (21.3)	≤0.001
folic acid[4,5]	60 (47.6)	40 (31.7)	48 (37.8)	0.27
smoking[4,5]	17 (13.6)	25 (19.8)	46 (36.2)	≤0.001
alcohol[4,5]	88 (69.8)	69 (54.8)	54 (42.5)	≤0.001
vitamin use[4,5]	27 (21.3)	26 (20.5)	24 (18.9)	0.81
energy Intake[1, 6, 7]	8.7 (5.9 - 12.3)	9.2 (5.8 - 13.5)	8.8 (5.9 - 12.5)	0.92
nutrient intakes[1,6]				
total protein[8]	82.8 (64.2 - 99.8)	81.3 (66.9 - 98.3)	78.1 (61.7 - 99.1)	0.01
carbohydrates[8]	266 (224 - 34)	260(223 - 295)	252(208 - 305)	≤0.001
saturated fats[8]	34.9 (27.6 - 43.5)	36.9 (29.3 - 45.5)	38.2 (30.3 - 48.1)	≤0.001
MUFA[8]	31.6 (25.2 - 38.7)	33.4 (27.8 - 41.4)	35.8 (27.4 - 44.4)	≤0.001
PUFA[8]	15.3 (9.2 - 23.7)	16.5 (4.7 – 25.0)	17.0 (12.2 - 24.1)	0.001
cholesterol[9]	199 (146 - 269)	207 (137 - 282)	24 (140 - 306)	0.01
fiber[8]	25.0 (19.0 - 31.7)	24.1 (17.6 - 31.2)	21.5 (15.6 - 28.9)	≤0.001
beta-carotene[10]	1642 (960 - 2514)	1509 (836 - 2514)	1268 (565 - 2188)	≤0.001
ascorbic acid[9]	124 (80 - 203)	104 (61 - 192)	80 (45 - 132)	≤0.001
thiamin[9]	1.13 (0.90 - 1.44)	1.4 (0.85 - 1.44)	1.05 (0.82 - 1.40)	0.001
riboflavin[9]	1.76 (1.24 - 2.35)	1.69 (1.14 - 2.51)	1.54 (0.87 - 2.37)	≤0.001
pydridoxine[9]	1.71 (1.38 - 2.40)	1.67 (1.32 - 2.05)	1.60 (1.25 - 2.07)	≤0.001

Data presented by [1]median (min-max), [2]years,[3]kg/m², [4]periconception, [5]n (%), [6]study moment, [7]mJoule/day, [8]g/day, [9]mg/day, [10]µg/day.

Moreover, most birth defects are diagnosed during the first postnatal year, which is important to reduce misclassification of diagnosis in the control group. The FFQs were mailed to the mothers and filled out at home. In the FFQ the answers can be given in frequency per day, week, month, year, or never. For several food items, additional questions were asked about the frequency of consumption and preparation methods, including the addition of condiments. The amounts consumed were estimated by using household measures or coloured photographs of foods showing different portion sizes. The researcher verified the completeness and consistency of the FFQ in a standardized way. Mean daily nutrient intake was estimated by multiplying the frequency of consumption of the food items by the portion size and the nutrient content per gram. Total energy intake, the intake of macro-nutrients, vitamins, minerals, were calculated by using the computerized version of the 1996 Dutch food composition table.[16]

Table 1b | Baseline characteristics of case and control mothers in the Prudent diet

		Prudent diet		
	low	*intermediate*	*high*	
characteristic	*(n 127)*	*(n 127)*	*(n 127)*	*p*
age[1,2]	31.7 (26.9 - 35.2)	32.2 (26.8 - 38.6)	32.5 (25.7 - 39.9)	0.08
BMI[1,3]	24.0 (20.1 - 35.9)	25.0 (17.8 - 37.2)	23.8 (19.6 - 30.21)	0.75
high education[4]	33 (28.4)	47 (39.8)	50 (41.3)	0.05
folic acid[4,5]	49 (38.9)	48 (38.1)	51 (40.2)	0.23
smoking[4,5]	33 (26.2)	26 (20.6)	29 (23.0)	0.90
alcohol[4,5]	68 (54.0)	72 (57.1)	71 (55.9)	0.52
vitamin use[4,5]	20 (15.7)	28 (22.0)	29 (22.8)	0.26
energy intake[1, 6, 7]	8.7 (5.8 - 12.5)	9.2 (6.3 - 12.3)	9.0 (5.6, 13.1)	0.02
nutrient intakes[1,6]				
total protein[8]	80.3 (61.9 - 98.4)	81.7 (64.8 - 99.8)	80.2 (63.8 - 98.6)	0.93
carbohydrates[8]	262 (213 - 304)	260 (221 - 302)	256 (216 - 303)	0.06
saturated fats[8]	36.8 (29.7 - 48.1)	36.7 (29.5 - 45.9)	36.4 (28.3 - 44.3)	0.60
MUFA[8]	33.4 (26.6 - 44.1)	33.3 (27.3 - 41.3)	34.1 (25.6 - 42.8)	0.23
PUFA[8]	15.5 (10.5 - 23)	15.9 (4.5 - 22.5)	17.3 (12.4 - 24.8)	≤0.001
cholesterol[9]	209 (137 - 298)	207 (148 - 297)	202 (139 - 271)	0.20
fiber[8]	22.6 (16.3 - 31)	23.3 (16.4 - 29.9)	24.5 (17.9 - 31.1)	≤0.001
beta-carotene[10]	1314 (767 - 2275)	1499 (912 - 2462)	1595 (978 - 2524)	≤0.001
ascorbic acid[9]	99.4 (53.4 - 179.7)	100.6 (60.0 - 198.8)	102.7 (59.8 - 199.8)	0.45
thiamin[9]	1.09 (0.86 - 1.41)	1.10 (0.85 - 1.43)	1.40 (0.85 - 1.44)	0.27
riboflavin[9]	1.67 (0.99 - 2.44)	1.67 (1.15 - 2.37)	1.64 (1.05 - 2.31)	0.27
pydridoxine[9]	1.66 (1.27 - 2.19)	1.65 (1.27 - 2.01)	1.67 (1.36 - 2.08)	0.58

Data presented by [1]median (min-max), [2]years, [3]kg/m², [4]periconception, [5]n (%), [6]study moment, [7]mJoule/day, [8]g/day, [9]mg/day, [10]μg/day.

The relative validity was assessed by comparing the data collected from this questionnaire with data drawn from 24-hour recalls, which has been repeated 12 times. The study demonstrated that the reproducibility and validity of food groups and nutrients was acceptable and comparable to other

FFQs.[13] Therefore, in our study periconception period was defined as daily intake from 4 weeks before through 8 weeks after conception. Periconception vitamin intake and mild nausea or vomiting were included in the data set for analysis. Nausea was characterized by duration, period, and severity. Severe nausea or vomiting was defined as starting after the first week of pregnancy and resulting in a change or decrease in food intake.

Table 2 | Maternal dietary patterns from low to high adherence and the association with the risk of cleft lip and or palate in the child[1]

Western diet	*cases (n 203)*	*controls (n 178)*	*OR (95% CI) crude*	*OR (95% CI) adjusted[2]*	*OR (95% CI) adjusted [4]*
low (n 127)	58 (28.6)	69 (38.8)	1.0 (Ref)	1.0 (Ref)	1.0 (Ref)
intermediate (n 127)	67 (33.0)	60 (33.7)	1.3 (0.8 - 2.2)	1.2 (0.7 - 2.1)	1.2 (0.8 - 2.1)
high (n 127)	78 (38.4)	49 (27.5)	1.9 (1.2 - 3.1)	1.7 (1.0 - 3.0)	1.8 (1.0 - 2.9)
Prudent diet	*cases[5]*	*controls*	*OR (95% CI)*	*OR (95% CI)[3]*	*OR (95% CI)[4]*
low (n 127)	68 (33.5)	59 (33.1)	1.0 (Ref)	1.0 (Ref)	1.0 (Ref)
intermediate (n 127)	64 (31.5)	63 (35.4)	0.9 (0.5 - 1.4)	0.8 (0.5 - 1.4)	0.7 (0.5 - 1.2)
high (n 127)	71 (35.0)	56 (31.5)	1.1 (0.7 - 1.8)	1.3 (0.8 - 1.8)	1.0 (0.6 - 1.7)

[1]Adherence groups were calculated by summing of intake food groups weighted by their factor loadings. Adjusted for [2]maternal education, smoking and alcohol consumption at the study moment, [3]maternal education, [4]periconceptional maternal folic acid intake and/or multivitamin intake.

Biomarker Assay

From 170 mothers a fasting venous blood sample was obtained to measure serum and RBC folate, serum vitamin B12, whole blood vitamin B6 as pyridoxal-'5-phosphate, and total plasma homocysteine.[16-18] All laboratory analyses were performed blinded. Failure in blood sampling or laboratory testing resulted in approximately 5% missing values.

Statistics

The procedure for calculating dietary patterns by using food consumption data from the FFQ has been described, validated, and reproduced in detail elsewhere.[20] To reduce the number of dimensions and to maintain the essence of the nutritional value of the foods, we reduced the number of food items from 102 into 36 predefined food groups. The food groups were comparable to those reported by others.[21,22] Food items that did not fit into any of the groups were considered as food groups on their own, e.g., pizza, tea, and beer. After pooling of the case and control mothers, we performed principal factor analysis on all food groups. The Kaiser Meyer Olkin measure of sampling adequacy was used to exclude variables from the model due to multicollinearity.[23] The number of factors to preserve was determined by factor interpretability and Eigenvalues greater than 1. Bartlett's test of sphericity was used to test the equality of the eigenvalues.[24] Pearson r correlation coefficients were analyzed by principal component factor analysis with subsequent varimax rotation to reveal the nutritional characteristics of each factor.

The factor score for each pattern was calculated by adding up the intakes of the food groups weighted by the factor loadings. Each mother received a score for each factor solution to describe the similarity of her diet to the respective dietary pattern. The factor model was confirmed with reliability analysis. First, the set of 381 mothers was divided randomly into two groups. Principal components factor analysis was performed on one group, and followed by the other group to confirm the model. The exploratory factor and reliability test were performed with SPSS 11.0.1 software (SPSS Inc., Chicago, IL, US).

Maternal age at the time of the study, BMI, and energy intake are presented as medians with 5^{th} and 95^{th} percentiles. Tertiles of the dietary patterns were calculated based on factor scores. Differences between tertiles were evaluated by analysis of variance and tested for linearity. For each dietary pattern the differences in baseline characteristics were tested using the chi-square test. The tertiles of each dietary pattern of the total group of mothers and 85^{th} percentile (n 58), 90^{th} percentile (n 39), and 95^{th} percentile (n 19) were used for risk estimation by odds ratios and 95% confidence intervals (95%CIs). Linear regression analysis was used to find associations between dietary patterns and nutrient intakes adjusted for energy intake.[25] The nutrient residual method was used to adjust for total energy intake on micronutrient and macronutrient intakes.[24] The distributions of the energy-adjusted nutrient intakes and maternal biomarker concentrations were positively skewed. Therefore, log-transformation was performed, and the data are presented as geometric means with 5^{th} and 95^{th} percentiles. Differences between tertiles per dietary pattern were evaluated in an unconditional linear regression model. A multivariate model was used to analyze the effect of potential confounders, such as maternal education, smoking status, and alcohol consumption at the study moment.

RESULTS

After exclusion of 22 case mother and 39 control mothers, we evaluated the data of 203 case mothers and 178 control mothers. The CLP group comprised 171 mothers of a child with a CLP and 32 mothers of a child with a cleft palate only. Five factors were selected from factor analysis. The first three factors represented typically Western dietary habits. Therefore, they were combined into one dietary pattern and called the Western dietary pattern. Factor 4 and factor 5 were combined and called the Prudent dietary pattern. The reliability test was positive and substantiates the validity of the data. The first dietary pattern, the Western diet, consisted of high intakes of organ meat, red meat, processed meat, pizza, legumes, potatoes, French fries, condiments, and mayonnaise, but low intakes of fruit. The Western diet explained 28.2% of the total variance cumulatively.

The Prudent dietary pattern explained 7.1% of total variance and contained high intakes of fish, garlic, nuts, and vegetables and was characterized by a higher frequency of warm meals per day. A high adherence to the Western dietary pattern was associated with an increased BMI ($p \leq 0.01$), decreased maternal educational level ($p \leq 0.05$), more smoking ($p \leq 0.001$) and decreased alcohol consumption ($p \leq 0.001$) (Table 1). The Western dietary pattern is also characterized by higher intakes of saturated fats, mono-unsaturated and poly-unsaturated fatty acids, and cholesterol and lower intakes of total proteins, carbohydrates, fibre, β-carotene, ascorbic acid, thiamine, riboflavin, and pyridoxine.

Table 3 | Concentration of vitamins and homocysteine per level of maternal adherence to the Western and Prudent dietary patterns[1]

Western diet	*folate[2,3]*	*folate[2,4]*	*vitamin B12[2,5]*	*vitamin B6[3,6]*	*homocysteine[7,8]*
low	15.9	718	279	65.5	4.0
(*n 71)*	(5.9, 71.0)	(357, 1394)	(102, 593)	(41.8, 128)	(7.3, 22.1)
intermediate	13.6	618	260	55.7	4.6
(n 49)	(6.8, 29.7)	(286, 1364)	(130, 530)	(36.5, 98.5)	(7.6, 16.4)
high	13.5	598	222	54.1	12.3
(*n 50)*	(5.5, 53.9)	(332, 1229)	(82, 483)	(31.8, 105.7)	(7.8, 19.7)
P	0.40	0.02	0.02	≤0.001	0.05

Prudent diet	*folate[2,3]*	*folate[2,4]*	*vitamin B12[2,5]*	*vitamin B6[3,6]*	*homocysteine[7,8]*
low	12.5	623	206	60.4	4.9
(*n 51)*	(5.3, 49.7)	(314, 1365)	(71, 472)	(36.0, 120.0)	(8.3, 22.1)
intermediate	15.2	675	261	59.7	4.5
(*n 55)*	(5.8, 62.7)	(360, 1387)	(48, 506)	(35.0, 143.0)	(7.2, 19.1)
high	15.6	654	298	57.5	4.2
(*n 64)*	(7.0, 45.8)	(300, 1320)	(154, 614)	(35.4, 93.6)	(7.7, 18.7)
p	0.05	0.59	≤0.001	0.42	0.27

Data are presented by [1]geometric mean (p5,p95), [2]serum, [3] nmol/L, [4]red blood cell, [5]pmol/L, [6]whole blood, [7]plasma, [8]μmol/L.

Mothers with a high adherence to the Prudent diet were generally more highly educated ($p \leq 0.05$) and showed higher intakes of poly-unsaturated fats, fibre, and β-carotene. Average concentrations of vitamin B12 and folate increased with maternal adherence to the Prudent diet (Table 3). We stratified the dietary patterns for CLP and cleft palate only offspring and no significant differences were revealed, p 0.65 and p 0.26, respectively. No significant differences were observed between the general characteristics of mothers of a CLP and a cleft palate only-child. The cleft palate only sample size was too small for further analysis. On average, RBC folate, vitamin B12, and B6 concentrations decreased and total homocysteine levels increased with a higher adherence to the Western dietary pattern (as shown in Table 3).

Mothers with a high adherence to the Western dietary pattern demonstrated a significantly increased CLP risk, odds ratio 1.9 (95%CI 1.2; 3.1). After adjustment for maternal education, smoking and alcohol use at the study moment the increased risk for CLP remained significant. After adjusting for periconception folic acid or multivitamin intake, the significantly increased risk for CLP remained. When considering the highest percentiles of the Western diet, a positive trend for CLP risk was determined ranging from an odds ratio of 2.0 (95%CI 1.1; 3.6) for the 85^{th} percentile to 3.5 (95%CI 1.1; 10.7) for the 95^{th} percentile with a p 0.024 for trend (data not shown). The Prudent dietary pattern was not associated with increased CLP risk (Table 2).

DISCUSSION

Two major maternal dietary patterns have been identified in association with the risk of CLP offspring. These patterns have been labelled as the Western and Prudent dietary pattern, which is in line with others.[9-11] In contrast to the Prudent diet, mothers with a high adherence to the Western diet showed an approximately two-fold higher risk of having a child with a CLP, which was independent of periconception folic acid supplementation. Mothers with a high adherence to the Western diet were of a lower educational level and also demonstrated characteristics of an unhealthy lifestyle, such as a high BMI and smoking. These associations are in line with studies of others.[9,11,21,22]

The relationship between diet and lifestyle is very interesting and may contribute to the future identification of specific risk profiles in the preconception counselling of mothers-to-be. Maternal hyperhomocysteinemia is a risk factor for adverse outcome, such as a CLP.[8] Studies on associations between dietary patterns and concentrations of B-vitamins and homocysteine are limited.[21] Therefore, of interest are the associations between a high adherence to the Western pattern and high plasma total homocysteine, low RBC folate, low vitamin B12, and low pyridoxal-'5-phosphate concentrations that fit with a high intake of meat and low intakes of fruit and vegetables. The Prudent diet was positively associated with vitamin B12 and serum folate, very likely due to the high fish and vegetable consumption.

Our findings may suggest a significant effect of the maternal adherence to the Western dietary pattern on the risk of CLP offspring and herewith extend our previous findings from the multivariate analysis of food frequency questionnaire data.[3,5,8] Those studies revealed adequate or slightly increased intakes of macronutrients and marginal intakes of several micronutrients in mothers of a CLP child compared with controls. The use of dietary patterns for identifying associations with lifestyle factors in combination with biomarkers and CLP risk is substantiated by these new findings and may improve the identification of a specific risk profile. Moreover, dietary patterns show several advantages above focusing on individual nutrients and foods, because synergistic effects between nutrients or foods may be more easily detected and the results seem to be more easily translated into practical advice to mothers-to-be.

Strength of the study is that we tested the data for validity and reproducibility by a reliability analysis. We divided the data set randomly into two halves, the principal component factor analysis was performed on each half, and the results were compared.

The first factor loading matrix showed the same findings as the full data set. In the second factor loading matrix there was only one food group, i.e., alcohol-free beer that did not match. This food group did not significantly affect the dietary patterns. Thus, the two analyses represent a study and a replication with valid results that support the validity of our findings. The results reveal that the adherence to the Western dietary pattern during the preconception period may increase the risk of a child with a CLP. A limitation of our study is that we have not measured the nutritional intakes during the preconception period. This would require a very large preconception cohort study which is unfortunately not yet feasible. Therefore, we have chosen for the best alternative, namely, to study maternal exposures by conducting case–control studies at a fixed study moment at approximately 14 months after the index pregnancy to estimate the exposures in the preconception and periconception period.[26-28] At that study moment the nutritional status of the mother is very likely comparable to the preconception and periconception period after excluding pregnant and breastfeeding women and those who have used a different diet at that moment compared with the preconception period. The rationale is that nutritional intake is rather stable during life except for periods of dieting, breastfeeding, and extreme growth. This is further supported by others.[14,15]

Our study shows that the concentrations of homocysteine, folate, vitamin B12, and B6 are significantly associated with the dietary patterns. Moreover, the validity is supported by a study on cardiovascular disease in the general Dutch population showing similar dietary patterns as in the mothers of our study. We have used other studies in nutritional epidemiology as guideline for our analysis to reduce possible bias.[29] Nevertheless, the observed associations should be evaluated with respect to the results from food analysis. Bias in reported energy intake is associated with variable bias in estimated macronutrient intake. Moreover, environmental factors and genetic variations also affect embryogenesis and make it always difficult to detect association between CLP and dietary patterns. More studies, however, should be performed in the reproductive population to substantiate our data.

General Conclusion

Current guidelines of preconceptional care emphasize that nutrition and certain lifestyle factors play an important role in early pregnancy. The identification in our study of the Western dietary pattern validated by some biomarkers and accompanied by unhealthy lifestyle factors may therefore be a first step in the profiling of preconceptional risks of mothers-to-be.

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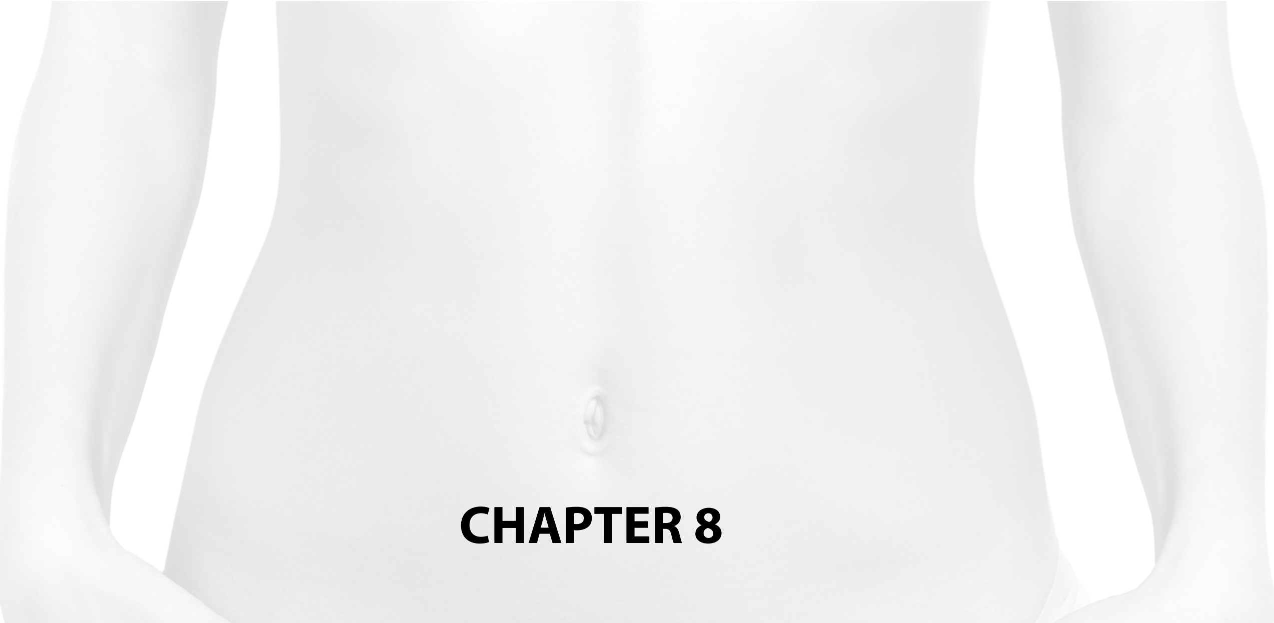

CHAPTER 8

General Discussion

'Behold, I have given you every seed-bearing herb, which is upon the surface of the entire earth, and every tree that has seed-bearing fruit; it will be yours for food.'

Genesis 1:29

BACKGROUND

All living organisms rely on food sources for reproduction and survival. After consumption, digestion, and absorption of foods, nutrients (e.g. carbohydrates, fats, proteins, vitamins and minerals) are made available for growth, repair, maintenance, and proper functioning of the genome, cells and tissues. Our daily needs can be met by a diverse range of foods. Food requirements are increased during several periods of life, in particular during pregnancy, to ensure an appropriate growth of the placenta, fetus, and maternal tissues. Ideally, women should enter pregnancy in an optimal health and nutritional status to enhance the chance of a successful pregnancy course and outcome.[1, 2]

In 2002, the ´Nutrition Today Matters Tomorrow´ report of the *March of Dimes Task Force* acknowledged the importance of the maternal preconception nutritional status, and urgently recommended the establishment of evaluation methods to determine the impact of dietary patterns with regard to its contributions to pregnancy outcome.[3] This report specifically stated that the *´efforts to improve diet quality should focus on the diet as a whole, not on single vitamins or minerals´*. Considering the need for evidence-based knowledge on the impact of the whole diet on reproductive performance and pregnancy outcome, this thesis aimed the following:

1. to identify patterns in food intake in the Dutch reproductive population;
2. to validate the dietary patterns with biomarkers of the homocysteine pathway;
3. to assess the relationship between preconceptional dietary patterns and reproductive performance in men and women;
4. to study the effects of periconceptional maternal dietary patterns on the risk of several congenital malformations in the offspring.

KEY FINDINGS

Throughout the studies in this thesis two general dietary patterns were recurrently observed: 1) the joint intakes of fruit, vegetables, vegetable oil, and whole grains, reflecting Healthy foods that have been part of the diet of our local ancestors in post-agricultural period (e.g. Mediterranean, Health Conscious, and Traditional Dutch diet) and 2) joint intakes of snacks, meat and sweets, corresponding with the dietary habits of Western post-industrial societies. The Healthy dietary patterns increased the biomarker concentrations of folate, vitamin B6 and B12 and decreased homocysteine. A high adherence to a Western dietary pattern has opposite effects on the biomarkers, i.e., increased homocysteine and decreased B-vitamin concentrations.

In general, the adherence to a High Healthy – Low Western diet was associated with better reproductive performance and pregnancy outcome, such as: 1) improved sperm quality, 2) increased chance of conception after fertility treatment, and 3) a reduction in the birth prevalence of spina bifida, congenital heart malformations, and orofacial clefts.

Beneficial effects of maternal dietary patterns on maternal dietary patterns on maternal conditions and pregnancy course have been substantiated in the Generation R study (Table 1). This large urban birth cohort study in Rotterdam, the Netherlands demonstrated inverse associations between the Mediterranean dietary pattern and blood pressure during pregnancy as well as intrauterine fetal growth restriction (Table 1). In the following section of the discussion we will elaborate on the determinants of dietary intake, and discuss the possible causal pathways of the association between dietary patterns and reproductive performance and pregnancy outcome.

Table 1 | Associations between dietary patterns and biomarkers of the homocysteine pathway, together with reproductive outcomes across studies performed in the Dutch reproductive population in the Netherlands between 1999 and 2009

dietary pattern	*foods*	*biomarkers*	*reproductive outcomes*
Health Conscious	fruit, vegetables, fish, whole grains	folate, vitamin B6	sperm quality[20]
Mediterranean	fruit, vegetables, legumes, vegetable oil, fish, whole grains	folate, vitamin B12, vitamin B6, homocysteine, SAM	spina bifida,[21] IVF treatment outcome,[22] fetal growth,[23] heart malformations,[24] pregnancy hypertension[25]
Traditional Dutch	potatoes, whole grains, meat, margarine	folate	sperm quality[20]
Western	snacks, sweets, meat, potatoes, mayonnaise	folate, vitamin B12, vitamin B6, SAH, homocysteine	cleft lip and/or palate,[26] congenital heart malformations[24]

Based on the following studies: [20]Vujkovic et al. 2009, [21]Vujkovic et al. 2009, [22]Vujkovic et al. 2010, [23]Timmermans et al. 2010, [24]Borst-Obermann/Vujkovic et al. 2010, [25]Timmermans et al. 2010, and [26]Vujkovic et al. 2007.

General Determinants of Dietary Intake

With the abundance of so many types of foods in our wealthy industrialized society, one might question why so many similarities are observed regarding patterns in food intake across the different studies with so many consistent influences on reproductive performance and pregnancy outcome.

It is known that several generic factors can affect food intake. A major factor of food choice is sensory perception.[4] Since caloric availability was scarce in our ancient habitat, human species have adapted neurophysiological mechanisms to prefer fatty and sweet tasting products that naturally contain many calories, through which chances of survival are maximized during famines. These foods taste very good, are relatively cheap and are abundantly available all over the world (e.g. meat, snacks and sweets). However, the content of vitamins and nutrients in these foods are generally low. Studies show that a high adherence to a Western diet increases the risk of chronic conditions, such as cardiovascular diseases, hypertension, type 2 diabetes, stroke, certain cancers, muscular-skeletal disorders, and mental health conditions.[5-11] We observed that a maternal preference for Western foods in the periconception period negatively affects IVF/ICSI treatment outcome, and increases the probability of spina bifida and orofacial cleft in the offspring **(Chapter 3, 5, and 7)**. Other studies showed that intrauterine exposure to a hyper-caloric but nutrient-poor diet predisposes the offspring to chronic conditions later in life, such as cardiovascular disease trough excessive maternal lipid intake, and metabolic disorders through excessive maternal carbohydrate intake.[12,13] Although the underlying mechanisms are unknown, it is hypothesized that maternal nutrition contributes to the programming of the fetal cardiovascular system and hypothalamic–pituitary–adrenal (HPA) axis.[14,15] When postnatal nutrition differs from exposure in utero, it is hypothesized that the formed organs and tissues are not optimally able to adapt to new environmental stimuli, which in the long-term has harmful consequences for health and increases disease risk, e.g., type 2 diabetes,[16] hypertension,[17] obesity,[18] and cardiovascular disease.[19]

Another behavioural regulator of food choice is income. This was also observed across our studies by the consistent positive associations between educational level and the Healthy dietary patterns. A study among US pregnant women showed that maternal dietary quality is especially influenced by age, parity, and BMI and indicates that cultural, demographic, attitudinal, and social factors influence food choice.[27] These factors may influence the awareness, the means, discipline, and self-control of individuals to regulate their dietary intake by choosing and buying Healthy foods. This may be a plausible explanation for the observed trend that higher educated women more often having healthier offspring than poor educated women.[28]

B-Vitamins and the Homocysteine Pathway

Fruit, vegetables, and grains have been the dominant foods of the human diet throughout evolution. These foods provide B-vitamins, such as folate, which is, amongst others, responsible for the supply of methyl donors (CH3 molecules) through the homocysteine pathway. Methyl donors are important for the proper programming of DNA methylation patterns in the periconception period.[29] Inadequate establishment of DNA methylation patterns in offspring through maternal nutritional deficiency may contribute to congenital malformations, growth retardation, and chronic diseases in later life.[30] The increasing interest in the epigenetic properties of foods challenged us to investigate which dietary patterns were most predictive of methyl group bioavailability and the risk of congenital heart defects in the offspring *(Chapter 6)*. We showed that the intakes of fish and nuts seemed to be associated with the methyl content of the diet. Further research is needed to replicate these findings, and elucidate the underlying mechanisms.

An important finding of the current thesis is that couples seeking fertility treatment may benefit from vitamin B6. Previous research showed that an improved vitamin B6 status in women with fertility problems increased reproductive performance, i.e. a 40% higher chance of conception and a 30% lower risk of early miscarriage.[31] Findings from our FOLFO study performed in an IVF/ICSI population are herewith in line as the vitamin B6 concentration in blood were increased by a high adherence to Healthy dietary patterns. These Healthy dietary patterns were also associated with an improvement in sperm quality (Chapter 2) and an increased chance of conception after fertility treatment **(Chapter 3)**.

Vitamin B6 may be involved in one of the underlying mechanisms between diet and reproductive health, representing a good candidate to test in a clinical setting.

Poly-Unsaturated Fatty Acids

The Healthy dietary patterns (Health Conscious and Mediterranean) are also characterized by high contents of vegetable oils and fish. These foods are the predominant sources of poly-unsaturated fatty acids (PUFAs), which are involved in essential metabolic processes of inflammation, and implicated in cellular and DNA signalling as transcription factors. As precursors of different prostaglandins they are important for the initiation of the menstrual cycle, estradiol response, follicle development and growth, embryo morphology, endometrial receptivity, implantation, and maintenance of pregnancy. Adequate intakes of fish, nuts, vegetable oils and other dietary sources of PUFAs may therefore be beneficial in women and men during their reproductive years and in particular in those undergoing IVF/ ICSI treatment.[32,33] Furthermore, studies recently observed that low maternal periconceptional fish intake was associated with increased risk of suboptimal neurodevelopment in childhood.[34,35] More studies, however, are needed to accurately assess and replicate the possible benefits to a child's development. Given that currently only 9% of the Dutch reproductive population adheres to the general recommendations of eating fish twice a week, campaigns are needed to improve fish intake in the reproductive population especially.[36]

METHODOLOGICAL REFLECTIONS

Before any conclusive statements about causality in the associations between dietary patterns and human reproduction can be drawn, a number of methodological issues have to be considered, such as study design, selection of the study population, the reliability and time window of dietary exposure assessment, the identification of covarying components, and generalizability of the findings.

Selection and Covariates

In the prospective preconception FOLFO study in couples with fertility problems the compliance of participation was 74%. The case-control triad study populations, in which the congenital malformations as outcome have been studies, showed a lower compliance. Primary reasons for participation refusal was the burden to travel to the hospital, to provide blood samples, especially in children, and filling out of questionnaires with no financial reward. The question is whether this introduced a bias in selection. To quantify the potential degree of selection bias we compared the general characteristics between case and control mothers in the case-control studies.[37] We observed that general characteristics were overall comparable (e.g. BMI, folic acid use, smoking, and alcohol consumption). Differences were observed in maternal age and periconception use of medication in the study on congenital heart defects. Furthermore, the controls in the spina bifida and orofacial cleft study were more often higher educated. These data indicate that selection bias was minimal, and cases and controls are representative of the same study base. To increase maximum accuracy of effect estimates in our studies, we included these variables as covariates in all analyses to eliminate their confounding effects.

To test the external validity, i.e., extrapolation to the general population, we compared the general characteristics of the control mothers in the case-control studies with those of the mothers of the population-based cohort Generation R. Again, most characteristics were comparable, i.e., maternal age, BMI, folic acid use, smoking, and alcohol consumption. Only ethnicity was significantly different between the study groups, which can be explained by the difference in study locations, i.e., Generation R and FOLFO study have been conducted in the multi-ethnic population of Rotterdam and the case-control triad studies on spina bifida and orofacial cleft all over the Netherlands. The control mothers in the study on congenital heart malformations were lower educated, and reported more frequent use of periconception folic acid supplements than the cohort of mothers in Generation R. This can be explained by the high frequency of non-Dutch ethnicities in this birth cohort. Therefore, due to minimal differences in general characteristics, the results may be generalized with a reasonable degree of certainty to the overall reproductive population.

Assessment of Dietary Intake

Food frequency questionnaires are useful tools to measure dietary intake, but its self-administrative nature may lead to information bias, especially underreporting of nutritional intake, because in general individuals have a natural tendency to report behaviour that adheres more closely to social norms and public health recommendations. This is especially a sensitive issue for obese people. We solved this problem by including BMI as a covariate in the analyses.

To ensure that dietary intake at the moment of inquiry is comparable to the periconception period, a study moment of 15 months after the birth of the index-child was chosen. This period corresponds with 24 months post conception, minimizing within-person exposure misclassification due to seasonal variation.

Short-term changes in dietary intake and recall bias complicate the assessment of nutritional assessment by food frequency questionnaires, and therefore we assessed dietary intake to cover a the period of the previous three months (spina bifida and orofacial cleft) and one month (FOLFO and HAVEN study), a period in time which is least susceptible to inaccurate memory recall.[38] To maximize the correspondence between study moment and periconceptional period we excluded mothers who reported dietary restriction, illness, nausea, pregnancy, and lactation. We validated this hypothesis by comparing the preconception dietary intake and dietary intake after birth women in a subgroup of the FOLFO study. It revealed that in general dietary intake was comparable between these two moments in time.[39] This further substantiates the validity of the assessment of nutritional intake at 15 months postpartum as a proxy of the periconception period.

Nutrients or Dietary Patterns?

Human nutrition can be classified in various ways. When classified in nutrient content it has the advantages that identified beneficial nutrients can be targeted to the relevant audience in the form of a nutrient supplement, of which the advice of taking folic acid containing supplements to women with a pregnancy wish is a well-known success story worldwide. However, because nutrients are highly correlated, it is often difficult to identify the individual nutrients responsible for a certain health outcome. Recently, classifying nutrition in terms of dietary patterns has become popular for the following reasons: 1) foods and dietary patterns reflect real-world eating behaviour, 2) dietary patterns have a relevant cumulative effect on health, and 3) beneficial foods can be effectively translated into food-based dietary guidelines.[40] It should be kept in mind that with dietary pattern analysis it is more difficult to identify one biological pathway leading to health and disease risk. However, the human diet contains a wide range of nutrients that act in concert on many biological pathways. Therefore, the scientific tradition of nutrient-based analysis and the novel dietary pattern driven techniques should not be seen as polar opposites, but as complementary approaches which together can strengthen causality in the diet-disease relationship and ensure that no relevant information is missed.

Biochemical Markers of the Diet

Biomarkers are objective estimates of the diet, of which those in the homocysteine pathway are frequently used in nutritional epidemiologic studies. We have recently shown that these biomarkers determined at 15 months post partum after the birth of the index-child are reasonably good estimates of the periconception nutritional status, which further substantiates the validity of the findings in our studies.[39]

PUBLIC HEALTH AND POLICY IMPLICATIONS

Scientific research has shown that maternal nutrition in the preconception period has a great impact on pregnancy outcome, but also on health and disease in later life. Malnutrition during pregnancy has a great impact not only on the health of the current but also of the future generations. Worrisome is therefore that at present a large part of the Dutch reproductive population shows an inadequate nutritional status. The Dutch National Food Consumption Survey in 2003 showed that only 10% of the population met the dietary guidelines for vegetables, fruit and saturated fat intake.[36] Only 9% of the population satisfies the current recommendations of eating fish twice a week. These worrying statistics raise the question whether there are interventions possible which will change dietary patterns in order to be healthy and produce healthy offspring?

Currently, many countries are becoming aware of the consequences of malnutrition which has given rise to the initiation of nutrition campaigns. The Netherlands Nutrition Centre is a powerful organization that promotes healthy, tasty and safe food in the Dutch society. Their noble but difficult task is to stimulate dietary changes of the general population. The difficulty lies in the fact that lifestyle changes in general – and nutritional changes in particular – may be more effective when recommended recurrently and on an individual level than by general population campaigns.

Individual responsibility for lifestyle and nutritional behaviour can only be taken if the person is aware, willing and able to make rational decisions. Therefore, there is a need for nutrition education programmes as well as access to clear non-misleading information on foods. A nice example is the project 'Hidden Fat', an initiative of the Netherlands Nutrition Centre to educate consumers about food, and specifically different types of 'good' and 'bad' fats by means of a FatGuide.

Another example is that of the EU regulation on food labelling (e.g. clarity, relevancy, and health claims) to empower individuals to get relevant information on nutrient content from food packages and make balanced dietary choices.[41]

An individual's dietary choice is heavily influenced and shaped by culture and the media. Furthermore, availability and affordability of foods are also eminent determinants of food intake. Although the Netherlands has one of the highest living standards in the world, it seems that a substantial part of the population is not able to eat adequately, especially in socio-economically deprived neighbourhoods. As the global food prices keep rising and the economic market has not stabilized, the group of food-insecure people is expected to grow. Food aid and food distribution programmes are therefore important initiatives to support the unemployed and most deprived of our society by relocating food surpluses via charity organizations and local social services. Currently, food aids consist mostly of energy dense products (e.g. bread, sugar, and meat) but the range of foods provided should preferably be expanded with fruit, vegetables, and/or vitamin supplements.

Businesses can also contribute to the burden of malnutrition by offering healthy foods at the work-place. Simple and cost-effective measures can be implemented together with employee organisations to improve the quality of food offered during lunch. The accessibility of food is also major determinant of its consumption, and therefore efforts in the workplace could include: 1) supply cafeteria with free or low-cost fruit and vegetables, 2) offer whole grain bread only, 3) reintroduce the traditional Friday fish day, 3) eliminate fatty dressings at the salad bar, 4) and reduce the food-product range of snacks and sweets. The Erasmus Medical Centre University in Rotterdam serves as a good role model as an organization that takes responsibility by stimulating a healthy lifestyle and nutrition.[42,43] One example is preconceptional lifestyle and nutrition counselling program to employees who want to become pregnant. The department of Public Health offers free fruit to their employees, and this attitude is worthy of being followed by more departments, organizations, and companies.

These initiatives should be supported especially in low socio-economic populations and deprived regions by the Government, where the highest health benefits can be achieved. It is important to note that its success on long term might be determined by the degree of preservation of cultural habits and tradition in intervention program, as compliance to imposed changes in behaviour is often of short length in time. By respecting ethnic backround and identity, individual will, dedication and self-control may be improved.

FUTURE RESEARCH DIRECTIONS

It is important to keep gaining knowledge and understanding the relationship between human nutrition and reproductive health. Future aetiological research should consider using the same methods employed in this thesis, such as principal component factor analysis and reduced rank regression to investigate the impact of the overall diet in addition to traditional single nutrient analysis. Furthermore, it is well known that differences exist in dietary patterns between ethnic groups, which should further investigated in these populations to identify culture-specific foods that are most likely to be most effective when implemented in food-based dietary guidelines in countries other than West Europe and North America.

The trend of obesity in both developed and developing countries are of global concern. The nutritional status and sedentary lifestyle of Dutch adolescents at present justifies the raised concerns regarding major health problems that are expected in future. Future research should therefore emphasize the effects of hyper-caloric nutrition on physiological functions, mental performance, reproductive health and pregnancy outcome. Because of the importance of physical activity on the regulation of nutritional status and body weight, nutritional epidemiological studies would benefit including measures of physical activity.[44]

The dietary patterns observed in this thesis have provided us with new insights in the foods that should be stimulated as well as those that should be omitted in order to contribute to successful reproductive performance and pregnancy outcome. Given that a substantial part of the Dutch population has an inadequate nutritional status, national efforts to improve lifestyle, nutrition and physical activity are crucial to ensure a healthy Dutch generation in future.[42,43] The Netherlands Nutrition Centre provides valuable instruments and means to educate consumers on nutrition and it's consequences on health. The identification of a high Healthy – low Western diet from this thesis, as a reproductive health promoting diet, consisting of high intakes of fruit, vegetable, fish, vegetable oils, and whole grains, and low intakes of sweets, snacks, refined grains, and meat should be replicated in other studies. A large observational cohort study, such as Generation R, provides the necessary resources and means to substantiate these findings with regard to pregnancy outcome, physical functioning, and mental development in childhood. Ultimately, molecular biological studies should provide insights about the specific mechanisms through which dietary patterns modify epigenetic mechanisms involved in fetal developmental processes.

Dietary Patterns and Human Reproduction

This thesis aimed to identify the various pieces in the complex puzzle known as the human diet, and delineate pieces specifically involved for reproductive performance and pregnancy outcome. Like in arts, music, and literature, true beauty hides in a proper balance among all individual elements. And it appears that human reproduction is prosperous when Healthy foods provided by nature are chosen over Western foods manufactured by modern man. The identification of the High Healthy – Low Western diet has enriched our understanding of the relationship between diet and reproductive processes and offers opportunity for implementation through food-based dietary guidelines in preconceptional care initiatives in the Netherlands. First experimental trials of nutrition intervention in pregnant women show promising and exciting results.[45]

Preconceptional care and nutrition education programs are an indispensible part of the National Health System to reduce fertility disorders and perinatal morbidity, and improve the health of future children, providing equal chances for optimal growth and survival from the beginning of the lifespan for all.

The future anticipates new discoveries elucidating the relationship between dietary patterns and human reproduction, and it will be the quest of future molecular biologists, clinicians, and epidemiologists to keep contributing to the understanding of the underlying mechanisms.

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APPENDICES

Summary | Samenvatting | List of Abbreviations

Authors and Affiliations | Publications and Awards

About the Author | Phd Portfolio | Word of Thanks

Summary

Not only is the human diet very complex, it also has a vast impact on reproductive performance and pregnancy outcome. Research so far successfully allocated adverse pregnancy outcomes to maternal nutritional deficiencies. Considering the need for more understanding of the impact of the whole diet on reproductive performance and pregnancy outcome, this thesis aimed to identify patterns in food intake of the reproductive population, and investigated its effects on various reproductive outcomes. In the introduction of this thesis the importance of dietary intake around the period of conception is emphasized with regard to its public health potential.

The objectives of this thesis were investigated in different studies. These studies have been described in two parts. In **Part I**, dietary patterns were identified in couples undergoing IVF/ICSI fertility treatment, and studied in association with the quantity and quality of several fertility outcome parameters, e.g. sperm, follicles, oocytes, embryo, and chances of conception. The focus in **Part II** was the influence of maternal dietary patterns and risk of congenital malformations in the offspring. Three common congenital malformations were investigated: spina bifida, heart defects, and orofacial clefts.

Part I

Results from the studies described in **Part I** are derived from the prospective FOod, Lifestyle and Fertility Outcome-study (FOLFO-study). This study was set up to investigate the influence of preconceptional nutrition and lifestyle on fertility outcome parameters. Between 2004 and 2007, subfertile couples undergoing IVF/ICSI treatment at the Erasmus University Medical Centre, Rotterdam, the Netherlands, were invited to participate. After exclusion of couples with a known cause of subfertility and ethnic background, 161 couples were included in the analyses. General and food frequency questionnaires were provided on the day of oocyte retrieval or semen sample collection, filled out by all participants, and returned on the day of embryo transfer.

In **Chapter 2** we investigated the role of dietary patterns on biomarkers of the homocysteine pathway and on sperm quality in 161 male participants of the FOLFO-study. During their visit at the andrology outpatient clinic blood and semen samples were collected. Folate, vitamin B6, vitamin B12 and homocysteine were determined in blood. Semen analysis included sperm concentration, motility, and morphology, and DNA integrity according to WHO criteria. Two major dietary patterns were identified. The Health Conscious dietary pattern showed high intakes of fruit, vegetables, fish and whole grains. The Traditional Dutch dietary pattern was characterized by high intakes of meat, potatoes, and whole grains, and low intakes of non-alcoholic drinks and sweets. Concentrations of homocysteine in blood and seminal plasma were significantly lower and vitamin B6 in blood was increased in males with a high adherence to the Health Conscious diet. An inverse association was demonstrated between the Health Conscious diet and loss of DNA integrity of the sperm cells. The Traditional Dutch diet was positively associated with red blood cell folate and sperm concentrations.

In **Chapter 3** and **Chapter 4** the fertility outcome parameters of 161 female participants of the FOLFO-study were examined with regard to dietary patterns and fatty acid intake. Data was used from blood samples collected on cycle day 2 and the day of hCG administration. The two leading follicles with corresponding monofollicular fluids were collected for each participant. Folate, vitamin B6, vitamin B12 and homocysteine were determined in all samples. Fertility outcome parameters after IVF/ICSI treatment consisted of: estradiol concentrations, number of follicles, fertilization rate, embryo quality and outcome of the pregnancy test.

Two dietary patterns were identified among the female participants in this study. The first dietary pattern was labelled as Health Conscious – Low Western, consisting of high intakes of fruit, vegetables, fish and whole grains and low intakes of snacks, meats and mayonnaise. The second diet, the Mediterranean diet was characterized by high intakes of vegetable oil, vegetables, fish, and legumes, and low intakes of snacks. These healthy foods and drinks are traditionally consumed by people living in countries near the Mediterranean Sea. A high adherence to the Health Conscious – Low Western diet resulted in increased red blood cell folate concentrations, whereas a high adherence to the Mediterranean diet increased both red blood cell folate as well as vitamin B6 concentrations in blood. Vitamin B6 levels were also elevated in follicular fluid. Furthermore, a high adherence by the couple to the Mediterranean diet increased the probability of pregnancy with 40%. The Mediterranean diet is well-known for its bioavailability of B-vitamins from vegetables and grains, as well as unsaturated fatty acids from vegetable oils and fish. Subsequently, we demonstrate in **Chapter 4** that omega-3 polyunsaturated fatty acids intake has estradiol level regulating properties during IVF/ICSI fertility treatment, and improves embryo morphology.

In conclusion, **Part I** acknowledges the importance of dietary patterns for male spermatogenesis, embryo morphology and chances of pregnancy in couples undergoing IVF/ICSI treatment. A Healthy dietary pattern consisting of high intakes of fruit, vegetable, whole grains, vegetable oils, nuts and legumes seems to be beneficial for the reproductive health of the Dutch population. Fish and vegetable oils are good dietary sources of omega fatty acids and should be encouraged in women during their reproductive years and in particular in those undergoing IVF/ICSI treatment. A promising candidate for reproductive health is vitamin B6. It seems to have health-promoting effects on both sperm quality and probability of conception. Future studies however are needed for additional insights in the possible mechanisms.

Part II

The objective of **Part II** was to investigate whether maternal dietary patterns during pregnancy affect biomarkers of the homocysteine metabolism and the risk of congenital malformations in the offspring. **Part II** is based on case-control triad studies that were conducted in the Netherlands. All participants in all three studies were of Dutch Caucasian origin. The investigations comprised the collection of data by general and food frequency questionnaires. Blood samples were taken at a standardized study moment of approximately 14 months after birth of the index-child to determine the biochemical determinants folate, vitamin B6, vitamin B12 and homocysteine in the mother. This moment reflects 24 months after the periconceptional period of the index-pregnancy and is assumed a reliable proxy of the preconceptional maternal nutrient intake.

In **Chapter 5** the results of the case-control triad carried out in collaboration with spina bifida centres in the Netherlands between 1999 and 2001 are described. In this study, 50 families were included with a child suffering from a nonsyndromic spina bifida recruited in collaboration with Spina Bifida teams. Eighty one control families, with children without malformations, were recruited either by a case family or by nurseries and infant welfare centres in the city of Nijmegen and surrounding areas. Principal component factor analysis and reduced rank regression were used to identify most prevalent food patterns in this population, and the food groups predictive of maternal biomarkers of the homocysteine pathway. The Mediterranean dietary pattern was most prominently prevalent in this study, characterised by high intakes of fruit, vegetables, vegetable oil, fish, legumes and cereals, moderate alcohol intake, and low intakes of potatoes and sweets. The Mediterranean dietary pattern was positively associated with folate and vitamin B12 and inversely associated with homocysteine concentrations.

The increase in the bioavailability of folate through food intake was comparable to the use of vitamin supplements. Furthermore, we observed that women with a high adherence to a Mediterranean diet were approximately 70% less likely to give birth to a child with spina bifida.

In **Chapter 6** the results are presented from the ongoing HAVEN study, conducted at the Erasmus University Medical Centre in Rotterdam, the Netherlands. From 2004 onwards, case children with parents participated in collaboration with the departments of Paediatric Cardiology of the Rotterdam Erasmus University Medical Centre, Leiden University Medical Centre, and the Amsterdam University Medical Centre. The control children and their parents were invited in collaboration with the child health centres of 'Thuiszorg Nieuwe Waterweg Noord' in the surroundings of Rotterdam. In our study sample comprising 179 cases and 231 control mothers' we related dietary patterns to the plasma biomarkers of methylation S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) with reduced rank regression method. Two dietary patterns were identified, the One Carbon Poor dietary pattern increased SAH concentrations and was characterized by a dietary intake comprising high intakes of snacks, sweets, nuts and non-alcoholic drinks. The One Carbon Rich dietary pattern substantially increased both SAM and SAH levels, and consisted of high intakes of fish and nuts. We observed that a high adherence to the One Carbon Rich dietary pattern reduced the risk of CHD offspring with 70%. This effect is surprisingly comparable to the effect of the maternal Mediterranean diet in the prevention of spina bifida in offspring.

In **Chapter 7** we described a study for which 203 children with a non-syndrome orofacial cleft were included in collaboration with cleft palate teams through the Netherlands between 1998 and 2000. The 178 control children had no malformations and the control triads were recruited either by a case family or by using posters and leaflets at nurseries and infant welfare centres in the city of Nijmegen and surrounding areas. Two major dietary patterns were identified in this study. The Western dietary pattern, e.g., high in meat, pizza, legumes, and potatoes, and low in fruit, was associated with an approximately two fold increased of cleft lip or cleft palate in the offspring, a risk estimate that remained significant after adjustment for potential confounders. The Western dietary pattern was associated with lower folate, vitamin B6, vitamin B12, and higher homocysteine concentrations. The use of the Prudent pattern, e.g., high intakes of fish, garlic, nuts, vegetables, increased vitamin B12 and folate levels, was not associated with risk of orofacial cleft.

Concluding from **Part II**, it seems that a strong maternal adherence to Healthy dietary patterns (e.g. Mediterranean and One Carbon Rich) reduces the risks of congenital malformations; risk reductions which are comparable with the use of folic acid containing supplements in the periconception period. In contrast, a strong adherence to a Western diet shows an increased risk of orofacial cleft in the offspring.

Chapter 8 provides a general reflection on the main findings and places them in a broader context of scientific knowledge and public health initiatives. We have observed throughout all studies that dietary patterns affect biomarkers of the homocysteine pathway, and show comparable preventive effects on the occurrence of poor reproductive outcome comparable as folic acid containing supplements. Therefore, it is not unlikely that these findings will be implemented in food-based dietary guidelines to address the Dutch population on reproductive healthFood-based dietary guidelines de facto provide easy, safe and natural ways to improve nutritional status. The underlying mechanisms on reproductive performance and pregnancy outcome – as observed in this thesis – remain unknown and warrant further investigation and clinical trials are needed to investigate the true efficacy of these findings.

Dietary Patterns and Human Reproduction

This thesis aimed to identify various pieces of the complex puzzle known as the human diet, and delineate which components are beneficial for reproductive performance and pregnancy outcome. Like in arts, music, and literature, true beauty hides in a proper balance among all individual elements. The discovery that a High Healthy – Low Western diet is benefical for human reproduction has enriched our understanding and offers opportunity for implementation through food-based dietary guidelines in preconceptional care initiatives in the Netherlands. It will be the quest of future molecular biologists, clinicians, and epidemiologists to keep contributing to the understanding of the underlying mechanisms.

Samenvatting

De voedingsinname van de mens in de moderne samenleving is complex en heeft een grote invloed op de voortplanting. Onderzoek tot nu toe heeft aangetoond dat specifieke voedingstekorten vóór en tijdens de zwangerschap bij kunnen dragen aan zwangerschapscomplicaties, zoals aangeboren afwijkingen. Het doel van dit proefschrift was om de meest voorkomende voedingspatronen van vrouwen en mannen in de reproductieve leeftijd te identificeren en te onderzoeken welke invloed deze uitoefenen op de fertiliteit en zwangerschapsuitkomst. In **Hoofdstuk 1** wordt het belang van de voeding rondom de bevruchting en in de eerste zwangerschapsweken beschreven. De vraagstellingen en mogelijke implicaties van de resultaten beschreven in dit proefschrift worden duidelijk toegelicht. De vraagstellingen zijn onderzocht in verschillende studies zijn opgesplitst in twee gedeelten. In **Deel I** bestaat de onderzoekspopulatie uit vrouwen en hun partner die een fertiliteitbehandeling ondergaan, waarvan de voedinginname bij beiden in de periode vóór de bevruchting zijn geëvalueerd. Hun voedingspatronen zijn bestudeerd in relatie tot de kwantiteit en kwaliteit van verschillende fertiliteitsparameters, zoals de zaadkwaliteit, het aantal follikels en eicellen, de morfologie van het embryo, en de kans op zwangerschap. In **Deel II** worden voedingspatronen in de periode rondom de bevruchting én in de eerste weken van de zwangerschap bestudeerd. De mate van het gebruik van deze voedingspatronen van de moeder zijn vervolgens gerelateerd aan het risico op het voorkomen van aangeboren afwijkingen bij het kind. Het gaat hier om de aangeboren afwijkingen spina bifida, hartafwijkingen, en lip en/of gehemelte spleet.

Deel I

De resultaten van de studies beschreven in **Deel I** zijn afkomstig van de FOLFO-studie (Food, Lifestyle en Fertility Outcome). Deze prospectieve studie is opgezet om de invloed van de preconceptionele voeding en leefstijl te onderzoeken op fertiliteitsparameters bij vrouwen en mannen die een fertiliteitbehandeling ondergaan. Tussen 2004 en 2007 zijn hiervoor subfertiele paren met een zwangerschapswens geïncludeerd op de polikliniek Voortplantingsgeneeskunde van het Erasmus MC te Rotterdam. Vrouwen en mannen met een bekende oorzaak voor de subfertiliteit en een niet-Kaukasische etnische achtergrond konden niet deelnemen. Dit resulteerde in de analyse van 161 vrouwen en hun partner. Algemene- en voedingsvragenlijsten werden uitgedeeld op de dag van de eicelpunctie of op de dag dat het zaadmonster werd ingeleverd. De ingevulde vragenlijsten werden ingeleverd op de dag van de terugplaatsing van het embryo in de baarmoeder.

In **Hoofdstuk 2** is de invloed van de voedingspatronen onderzocht op biomarkers van de foliumzuur-homocysteine stofwisseling in het bloed en in de zaadvloeistof, en op de zaadkwaliteit in 161 mannen. Tijdens het bezoek aan de polikliniek werd bloed en een zaadmonster verzameld waarin foliumzuur, vitamine B6, vitamine B12 en homocysteine werden bepaald. Het zaadonderzoek bestaat uit het bepalen van de concentratie, het volume, de progressieve beweeglijkheid, en de morfologie en DNA integriteit. De twee meest voorkomende voedingspatronen in deze studie waren: 1) het Gezonde voedingspatroon, gekenmerkt door een hoge inname van groente, fruit, vis en volkoren granen, en 2) het Traditioneel Hollandse voedingspatroon, gekarakteriseerd door de innamen van vlees, aardappelen, groente en volkoren granen, en weinig niet-alcoholische dranken en zoetigheden. Het strikte gebruik van het Gezonde voedingspatroon ging gepaard met een lager homocysteine gehalte in het bloed en in de zaadvloeistof, en een hoger vitamine B6 gehalte in het bloed. Bovendien was de afbraak van het DNA in de zaadcellen door het strikte naleven van een Gezonde voedingspatroon minder. Het Traditioneel Hollandse voedingspatroon verhoogde het foliumzuur gehalte in de rode bloedcellen en het aantal zaadcellen.

In **Hoofdstuk 3** en **Hoofdstuk 4** zijn de effecten van voedingspatronen en onverzadigde vetzuren op verschillende fertiliteitparameters bestudeerd in 161 vrouwen. De bloedmonsters werden afgenomen op cyclusdag 2 en de dag van de hCG toediening. Ook werden van elke vrouw monofolliculaire vloeistoffen uit de twee grootste voorliggende follikels verzameld. Foliumzuur, vitamine B6, vitamine B12 en homocysteine werden bepaald in bloed en monofolliculaire vloeistof. De fertiliteitparameters bestonden uit het oestradiol gehalte, het aantal follikels, het percentage bevruchte eicellen, de morfologie van het embryo en de uitslag van de biochemische zwangerschapstest.

Twee voedingspatronen bij deze vrouwen geïdentificeerd. Het eerste Gezonde voedingspatroon bestond uit vooral een hoge inname van groente, fruit, vis en volkoren granen en een lage inname van snacks, vlees en mayonaise. Het tweede Mediterrane voedingspatroon was gekenmerkt door de inname van vooral plantaardige oliën, groente, vis en peulvruchten, en weinig snacks. De naam voor dit voedingspatroon is afkomstig van de voedingsmiddelen die van oudsher in landen rondom de Middellandse Zee worden gebruikt. Afhankelijk van de mate waarin het Gezonde voedingspatroon werd geconsumeerd was ook het foliumzuur gehalte in de rode bloedcellen hoger. Het strikte gebruik van een Mediterraan voedingspatroon ging gepaard met een hoog foliumzuur en vitamine B6 gehalte in het bloed, en vitamine B6 gehalte in de monofollicullaire vloeistof. Bovendien was het strikte gebruik van het Mediterrane voedingspatroon door de vrouw en haar partner geassocieerd met een 40% verhoogde kans op zwangerschap.

In **Hoofdstuk 4** tonen we aan dat de omega-3 meervoudig onverzadigde vetzuren – kenmerkend van een Mediterraan voedingspatroon – het oestradiol gehalte in het bloed tijdens de ovariele stimulatie behandeling beïnvloeden, en de morfologie van het embryo verbeteren.

Deel II

Het doel was om in **Deel II** van het proefschrift de invloed van maternale periconceptionele voedingspatronen te bestuderen in relatie met de foliumzuur-homocysteinestofwisseling en het risico op het krijgen van een kind met aangeboren afwijking. **Deel II** is gebaseerd op een drietal Nederlandse patiënt-controle familie studies (kind, moeder, en vader). Alle moeders waren van Kaukasische en Nederlandse afkomst en hebben een algemene- en voedingsvragenlijst ingevuld. Bloedmonsters zijn bij alle moeders afgenomen om de gehaltes van het foliumzuur, vitamine B6, vitamine B12 en homocysteine te bepalen. Alle gegevens en metingen zijn op een gestandaardiseerd studiemoment uitgevoerd, omstreeks 14 maanden na de geboorte van het index-kind dat overeen komt met ongeveer 24 maanden (2 jaar) na de conceptie. Van dit moment wordt aangenomen dat de bepalingen een betrouwbare schatting geven van de preconceptionele status.

In **Hoofdstuk 5** worden de resultaten van het patiënt-controle familie onderzoek beschreven, uitgevoerd in samenwerking met spina bifida centra in Nederland tussen 1999 en 2001. In totaal zijn 50 families met een kind met een niet syndromale spina bifida geïncludeerd. Één-en-tachtig (81) controle families werden geworven via de spina bifida families of via posters en foldertjes bij kinderdagverblijven in Nijmegen en omgeving. De controle kinderen hadden geen aangeboren afwijkingen. Het Mediterrane voedingspatroon was het meest voorkomende patroon in deze studie, gekenmerkt door hoge innamen van fruit, groenten, plantaardige oliën, vis, peulvruchten en volkoren granen, en een matig alcoholgebruik en lage inname van aardappelen en zoetigheden. Naarmate de moeder het Mediterrane voedingspatroon strikter gebuikte waren de foliumzuur en vitamine B12 gehalten hoger en het homocysteine gehalte in het bloed lager.

Een strikt gebuik van het Mediterrane voedingspatroon van de zwangere vrouw in de periconceptionele was geassocieerd met een 70% lager risico op een kind met een spina bifida, een effect dat vergelijkbaar is met de inname van een foliumzuurhoudend supplement.

In **Hoofdstuk 6** worden de resultaten gepresenteerd van de HAVEN-studie (afgekort voor Hart Afwijkingen, Vasculaire status, Erfelijkheid en Nutriënten) uitgevoerd vanuit het Erasmus MC te Rotterdam in Nederland. Vanaf 2004 zijn 179 families geïncludeerd in samenwerking met de afdelingen Kindercardiologie van het Erasmus MC, het Leids Universitair Medisch Centrum, en het VU Medisch Centrum en Academisch Medisch Centrum in Amsterdam. Twee-honderd-één-en-dertig controle families zijn geworven in samenwerking met consultatiebureaus van 'Thuiszorg Nieuwe Waterweg Noord' in de regio Rotterdam. In de controle groep zijn twee voedingspatronen geïdentificeerd die gecorreleerd waren aan de S-Adenosylmethionine (SAM) en S-Adenosylhomocysteine (SAH) gehalten van de foliumzuur-homocysteinestofwisseling. Het eerste voedingspatroon – geaccocieerd met een hoog SAH gehalte – bevatte vooral hoge innamen van snacks, zoetigheden, en niet-alcoholische drankjes. Het tweede voedingspatroon – gekenmerkt door hoge SAM gehalten – wordt gekarakteriseerd door een hoge inname van vis en zeevruchten. Wij hebben kunnen aantonen dat een strikt gebruik van het SAM voedingspatroon van de moeder in de periconceptionele periode het risico op aangeboren hart afwijkingen met 70% vermindert. Verrassend genoeg is de sterkte van dit effect wederom vergelijkbaar met het preventieve effect van het Mediterraan voedingspatroon of een foliumzuur houdend supplement op het voorkomen van spina bifida bij het kind **(Hoofdstuk 5)**.

In **Hoofdstuk 7** beschrijven we een patiënt-controle familie studie waarin 203 kinderen met een niet syndromale lip en/of gehemeltespleet (schisis) en de beide ouders zijn geincludeerd in samenwerking met alle 9 schisis teams in Nederlands tussen 1998 en 2000. Een controle groep van 178 kinderen zonder aangeboren afwijkingen en de beide ouders werd geworven via posters en folders op kinderdagverblijven en consultatiebureaus in Nijmegen en omgeving. De twee meest voorkomende voedingspatronen in deze onderzoekspopulatie waren: 1) het Westerse voedingspatroon, met hoge innamen van bewerkt vlees, pizza, peulvruchten en aardappelen, en weinig groente, en 2) het Gezonde voedingspatroon, gekenmerkt door de innamen van vis, knoflook, noten en groenten. Het strikte gebruik van het Westerse voedingspatroon verhoogde het homocysteine gehalte en verlaagde foliumzuur, vitamine B6, en vitamine B12 gehalten in het bloed. Het Gezonde voedingspatroon verhoogde met name de vitamine B12 en foliumzuur gehaltes in het bloed.

Het strikte gebruik van het Westerse voedingspatroon van de moeder in de periconceptionele periode was geassocieerd met een 2 maal verhoogt risico op een kind met een lip en/of gehemelte spleet. Er werd geen verband aangetoond tussen het Gezonde voedingspatroon en deze aangeboren afwijking.

De resultaten beschreven in **Deel II** van dit proefschrift suggereren dat een strikt gebruik van gezonde voedingspatronen (Mediterraan en SAM) beschermen tegen het risico op het krijgen van een kind met een aangeboren afwijking. De grootte van de gezondheids-effecten zijn merkwaardig genoeg vergelijkbaar zijn met het gebruik van een foliumzuur-houdend supplement in de periconceptionele periode.

In **Hoofdstuk 8** worden de belangrijkste bevindingen van dit proefschrift bediscussieëerd. Er wordt uitvoerig ingegaan op de toegepaste onderzoeksmethoden en de klinische relevantie en implicaties van de resultaten voor de gezondheidszorg. Samenvattend zijn voedingspatronen geïdentificeerd die de foliumzuur-homocysteine stofwisseling beïnvloeden en als zodanig een aantal fertiliteitsparameters en het verloop en de uitkomst van de zwangerschap. De grootte van de gevonden associaties bleken consistent te zijn en vergelijkbaar met de inname van een foliumzuurhoudend supplement. Deze kennis moet dan ook worden geimplementeerd in de preconceptie zorg bestemd voor vrouwen en hun partner voor en tijdens de zwangerschap. Meer onderzoek is nodig om onze resultaten te bevestigen.

Voedingspatronen en de Menselijke Voortplanting

Het doel van dit proefschrift was om componenten van het natuurlijke menselijke eetgedrag te identificeren die een invloed hebben op verschillende processen van de menselijke voortplanting. Net als in kunst, muziek en literatuur, bevindt ware schoonheid zich in de harmonie der individuele elementen. Zo hebben we aangetoond dat een gezond voedingspatroon, gekenmerkt door hoge innamen van groenten, fruit en vis, maar lage innamen van bewerkt vlees en snacks, de fertiliteit en zwangerschapsuitkomst doen verbeteren. Het is belangrijk dat deze resultaten in de toekomst worden gecommuniceerd naar aanstaande ouders zodat ook zij een eigen bijdrage kunnen leveren aan hun individuele gezondheid en die van hun kinderen.

List of Abbreviations

ALA	alpha-linolenic acid
BMI	body mass index
BMR	basal metabolic rate
CHD	congenital heart defects
CI	confidence interval
CLP	cleft lip with/without palate
CV	coefficients of variation
DFI	DNA fragmentation index
DHA	docosahexaenoic acid
DRI	dietary reference intake
EPA	eicosapentaenoic acid
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
OR	odds ratio
p	probability
PAL	physical activity level
PCA	principal component factor analysis
PLP	pyridoxal-'5-phosphate
MUFA	mono-unsaturated fatty acid
PUFA	poly-unsaturated fatty acid
r	correlation coefficient
RBC	red blood cell
rFSH	recombinant follicle stimulating hormone
RRR	reduced rank regression
SAH	s-adenosylhomocysteine
SAM	s-adenosylmethionine
SB	spina bifida
sd	standard deviation
se	standard error
SHBG	sex hormone binding globulin
tHcy	total plasma homocysteine
β	unstandardized regression coefficient

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Ocké MC from the National Institute for Public Health and the Environment, Bilthoven, the Netherlands.

Publications and Awards

A maternal dietary pattern characterized by fish and seafood is associated with a reduced risk of congenital heart defects in the offspring. Obermann-Borst SA / **Vujkovic M,** de Vries JH, Wildhagen MF, Looman CW, de Jonge R, Steegers EA, Steegers-Theunissen RP (submitted)

The Mediterranean Diet during early pregnancy is significantly associated with fetal growth and the risk of intrauterine growth restriction. Timmermans S, Steegers-Theunissen RP, **Vujkovic M,** den Breeijen H, Russcher H, Lindemans J Hofman A, Jaddoe VW, Steegers EA (submitted)

Increased preconceptional omega-3 polyunsaturated fatty acid intake improves embryo morphology. Hammiche F, **Vujkovic M,** Wijburg W, de Vries JH, Laven JS, Steegers-Theunissen RP (submitted)

Major patterns of food consumption and maternal blood pressure during pregnancy in the Generation R Study. Timmermans S, Steegers-Theunissen RP, **Vujkovic M,** den Breeijen H, Hofman A, Jaddoe VW, Steegers EA (in review)

The preconceptional Mediterranean dietary pattern in couples undergoing in vitro fertilization / intracytoplasmic sperm injection treatment increases the chance of pregnancy. **Vujkovic M,** de Vries JH, Lindemans J, Macklon NS, van der Spek PJ, Steegers EA, Steegers-Theunissen RP. Fertil Steril 2010 (Feb 18th, epub ahead of print)

The maternal homocysteine pathway is influenced by riboflavin intake and MTHFR polymorphisms without affecting the risk of orofacial clefts in the offspring. **Vujkovic M,** Steegers EA, van Meurs J, Yazdanpanah N, van Rooij IA, Uitterlinden AG, Steegers-Theunissen RP. Eur J Clin Nutr 2010;64:266-73

Associations between dietary patterns and semen quality in men undergoing IVF/ICSI treatment. **Vujkovic M,** Steegers EA, de Vries JG, Dohle GR, Lindemans J, van der Spek PJ, Macklon NS, Bonsel GJ, Steegers-Theunissen RP. Hum Reprod 2009;24:1304-12

The maternal Mediterranean dietary pattern is associated with a reduced risk of spina bifida in the offspring. **Vujkovic M,** Steegers EA, Looman CW, Ocké MC, van der Spek PJ, Steegers-Theunissen RP. BJOG 2009;116:408-15

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** Jury Wladimiroff Research Award, RGOC Rotterdam, 2006*

About the Author

Marijana Vujković is the firstborn daughter of Momčilo Vujković and Biserka Krstevska. She was born on a Friday, April 18^{th} 1980 at the Erasmus University Medical Centre in Rotterdam. She has a younger sister, Dragana Vujković.

Marijana passed secondary school at the Libanon Lyceum in Rotterdam (1992 – 1999). She completed a Bachelor degree in Computer Science with a specialization in Bioinformatics at the Rotterdam University (HRO) in Rotterdam (2000 – 2005).

In 2005, she was appointed as PhD student at the department of Obstetrics and Gynaecology of the Erasmus University Medical Centre under the supervision of prof.dr. R.P.M. Steegers-Theunissen, prof.dr. E.A.P. Steegers, and prof.dr. P.J. van der Spek. Her thesis emphasizes the impact of dietary patterns on reproductive performance and pregnancy outcome.

During her PhD project she completed an MSc program in Health Science, with a specialization in Epidemiology at the Netherlands Institute for Health Sciences (NIHES) of the Erasmus University Rotterdam (2008 – 2010).

All research findings have been published in peer-review medical journals, and her research has been acknowledged with the annual 'Jury Wladimiroff Researcher Award' by the Rotterdam Gynaecologists Education Cluster (RGOC) in 2006. Marijana has presented her findings on numerous of (inter)national conferences and meetings.

In the future she hopes to pursue her ambition to becoming a scientist who combines research with teaching activities. In this respect she will continue working as a post-doctoral researcher in the department of Obstetrics and Gynaecology at the Erasmus University Medical Centre (prof.dr. E.A.P. Steegers).

PhD Portfolio

student	Marijana Vujković
department	Obstetrics and Gynaecology Erasmus University Medical Centre Rotterdam, The Netherlands
research school	Netherlands Institute for Health Sciences (NIHES)
PhD period	Aug 2005 – Sept 2010
promotors	prof.dr. R.P.M. Steegers-Theunissen prof.dr. E.A.P. Steegers prof.dr. P.J. van der Spek

YEAR	ECTS	TRAINING
		general academic skills
2010	0.3	Methodologie van Patiëntgebonden Onderzoek en Voorbereiding van Subsidieaanvragen, Erasmus MC
2008	0.3	PhD introduction day, Erasmus MC
		research skills
2009	0.7	Partek Training Course, MOLMED
		in-depth courses
2007	6.0	Molecular and Cell Biology – B, MGC
2006	0.7	SNP's and human diseases. MOLMED/NIHES
		presentations
2007	0.3	TNO Quality of Life Spring research meeting. Zeist
2006	0.7	Sophia Children's hospital Circle of life symposium. Rotterdam
2005	0.3	MOLMED Bioinformatics Bridge meeting. Rotterdam
		(inter)national conferences
2010	1.4	11th Royan congress. Tehran, Iran
2010	1.4	57th annual meeting of the SGI. Orlando, Florida, US
2009	1.4	NVOG Gynaecongres. Utrecht
2009	1.4	56th annual meeting of the SGI. Glasgow, Scotland, UK
2007	0.3	Symposium Generation R Fetal Growth and Development. Rotterdam
2007	1.4	54th annual meeting of the SGI. Reno, Nevada, US
2006	0.7	Sanger Institute Congress on Bioinformatics Applications. Cambridge, UK
2006	1.4	53rd annual meeting of the SGI. Toronto, Ontario, Canada
2005	1.4	52nd annual meeting of the SGI. Los Angeles, California, US

YEAR	ECTS	TRAINING
		seminars and workshops
2007	1.4	14th MGCGraduate Student Symposium. Maastricht
2007	0.3	MOLMED Molecular Medicine Day 2007. Rotterdam
2006	0.7	Spotfire European User Seminar. Amsterdam
2006	0.3	MOLMED Molecular Medicine Day 2006. Rotterdam
2005	0.7	Spotfire European User Seminar. Cologne, Germany

YEAR	ECTS	GRANT APPLICATIONS, REVIEWING PAPERS
2010	0.7	Scholarship: ERACOL Academic Exchange Program for Panama City, Panama
2010	0.3	Travelgrant: Royan Institute
2010	0.3	Travelgrant: Erasmus Trustfonds
2009	0.7	Review Paper: Birth Defects Research Part A
2009	0.3	Travelgrant: Erasmus Trustfonds
2007	0.3	Travelgrant: Erasmus Trustfonds
2006	0.3	Travelgrant: Erasmus Trustfonds

YEAR	ECTS	TEACHING
		lecturing
2010	4.0	Introduction to SPSS: for MOLMED affiliated PhD students
2009	2.0	Statistics: for gynaecologists, students and midwives, Breda Hospital
2009	2.0	Statistics: for 4th year Medical students, Erasmus MC
		supervising practicals and excursions
2007	1.0	Organization of the annual excursion 'Labday', Erasmus MC
		supervising students and colleagues
2010	2.0	Marieke Both, PhD student, Erasmus MC
2009	2.0	Sylvia Obermann-Borst, PhD student, Erasmus MC
2009	2.0	Sarah Timmermans, PhD student, Erasmus MC
2008	2.0	Fatima Hammiche, PhD student, Erasmus MC

ERASMUS UNIVERSITEIT ROTTERDAM

Word of Thanks

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