Periconceptional Diet and Medication Use in Human Subfertility

With emphasis on **polycystic ovary syndrome** and **semen parameters**

Nicole Antoinette Huijgen

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Periconceptional Diet and Medication Use in Human Subfertility

With emphasis on **polycystic ovary syndrome** and **semen parameters**

Het periconceptionele dieet en geneesmiddelgebruik in relatie tot subfertiliteit bij de mens

In het bijzonder het polycysteus ovarieel syndroom en semen parameters.

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Chapter 1

Introduction

RATIONALE

Subfertility

Worldwide 70-80 million couples experience difficulties in conceiving [1]. In about 10-15% of these couples subfertility is present defined as; "A disease of the reproductive system characterized by the failure to achieve a clinical pregnancy within 12 months or more of regular unprotected sexual intercourse" [2]. A routine fertility investigation has to be performed to investigate whether causes can be attributed to a female factor, a male factor or a combination of both and to design an individual treatment plan [3]. Several techniques can improve pregnancy rates such as artificial insemination, ovulation induction, or *in-vitro* fertilization (IVF), and new insights and enhanced techniques arise. Unfortunately, a substantial part of the subfertile couples will experience mild to severe physical complications and psychological and emotional constrains (such as medicalization on the relationship, sexual well-being) due to the fertility treatment [4]. Since major health care and societal costs are involved in fertility treatment, subfertility has economic and societal consequences as well. For example the mean costs per live birth with IVF treatment are 87,748 euro [5].

Diet and medication

The chances of a successful fertility treatment are mediated by complex interactions between non-modifiable risk factors, for example age and ethnicity, and modifiable factors, such as obesity, smoking or an inadequate diet. When improving in particular these modifiable risk factors, patients can contribute to their own health and thereby chances of pregnancy for example by changing inadequate lifestyles [6].

Our group has shown before that a healthy diet in women improves the ovarian response, embryo morphology and pregnancy chances [6-8]. Moreover, in men an adequate diet is associated with a better semen quality by decreasing the sperm DNA fragmentation index and increasing the semen concentration [9]. The absorption of nutrients can be inhibited by specific types of medication, resulting in a drug-induced malabsorption which leads to nutritional deficiencies especially during chronic medication use. Gastric acid secretion is necessary for the absorption of nutrients and the acid-suppressant drugs histamine H2 receptor antagonists (H2RA) and proton pump inhibitors (PPI) are associated with zinc and B12 vitamin deficiencies [10-12]. For example B vitamin deficiencies are not only observed in users of acid-suppressant drugs, but also in users of antiepileptic drugs, metformin, colchicine, antibiotics, methotrexate and cholestyramine [13-19]. Thus, we hypothesize that chronic use of specific medication might impact on male as well as on female fertility as a consequence of secondary nutrient deficiencies.

One-carbon metabolism

The involvement of micro- and macronutrients in metabolic pathways is complex and numerous, however the one-carbon pathway (1-C) is of interest in human reproduction and in particular during the periconception period [20]. Subfertility and most reproductive failures originate in the periconception period and can arise as a partial consequence of a derangement in 1-C metabolism. A healthy diet provides substrates (folate, methionine) and cofactors (cobalamin, vitamin B6 and B2) that are necessary for a proper functioning of the 1-C metabolism (Figure 1). Therefore, a low serum folate and cobalamin and an elevated plasma tHcy are indicators of a deranged 1-C metabolism [20].

Nutrigenomics is a new field of research with a focus on gene-nutrient interactions of which 1-C metabolism is a nice example [21]. This metabolism is essential for the development of oocytes and spermatocytes, since it is involved in protein and DNA synthesis and DNA methylation.

Methylation is essential for the regulation of gene expression, the supply of methyl groups through 1-C metabolism is essential for the programming of gene transcription through the binding of methyl groups to CpG islands. Therefore, deficiencies of methyl groups easily lead to hypomethylation and as such can result in derangements in the programming of genes involved in disease (Figure 1).

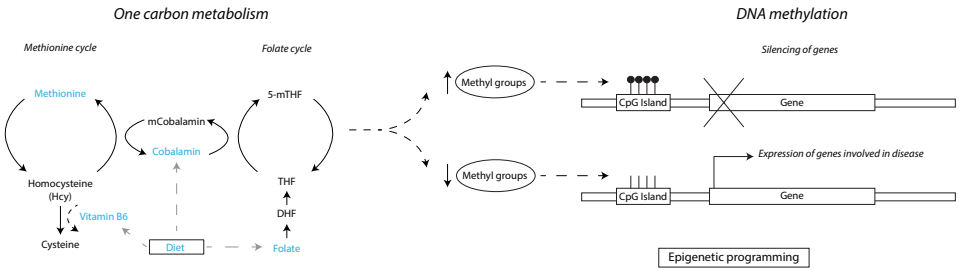

Figure 1 | Summary of the 1-C metabolism and the role in epigenetic programming.

Abbreviations; 5-mTHF = 5-methyl-tetrahydrofolate, THF = tetrahydrofolate, DHF = dihydrofolate. Adapted from Twigt et al. (2010)

An optimal 1-C metabolism is necessary for the most active phase of ovarian follicular development which starts 14 weeks before conception and therefore in women we have defined this moment as the start of the periconception period. During this window it is important to have an optimal health status and to use an adequate diet for epigenetic programming. The complete spermatogenic cycle in men covers a time span of 10 weeks, which means that the preconception period in men is only slightly shorter than in women [20]. From this background it is important to emphasize that preconception care and interventions should start at least three months before conception.

FEMALE SUBFERTILITY; POLYCYSTIC OVARY SYNDROME

PCOS

Polycystic ovary syndrome (PCOS) is the most common cause of anovulatory subfertility with an estimated prevalence of 5-15% [22,23]. This syndrome is characterized by ovarian dysfunction (OD), hyperandrogenism (HA) and/or polycystic ovarian morphology (PCOM) [24]. The pathogenesis of PCOS is not yet fully understood but considered a complex disorder, in which genetic variants together with environmental factors contribute to the diversity of the clinical spectrum of PCOS.

Patients can be categorized into four phenotypes according to the presence of the following features; (1) HA, OD and PCOM, (2) HA and OD, (3) HA and PCOM, (4) OD and PCOM. The first three phenotypes comprise the hyperandrogenic PCOS patients, which might have a higher prevalence of cardiovascular and metabolic riskfactors in comparison to the non hyperandrogenic PCOS phenotypes [25, 26].

In PCOS an elevated LH secretion is one of the findings which occurs due to abnormal gonadotropin dynamics (Figure 2) [27]. Although the aetiology is unknown, it is reported that an increased GnRH pulse frequency is induced by an impaired sensitivity of the hypothalamic GnRH pulse generator to feedback inhibition of progesterone [28]. Elevated levels of LH result in hyperandrogenism, due to the stimulation of theca cells in the ovary. A relative FSH deficiency together with the elevated levels of androgens might result in the arrest of follicular maturation [28]. Without the maturation of follicles and the selection of one dominant follicle anovulation occurs. The accumulated small antral follicles produce elevated levels of AMH, often used as marker for ovarian function [29]. Recently, an interesting new theory emerged due to the fact that AMH type II Receptors were detected in the hypothalamus. Subsequent research suggest that AMH, which is elevated in most PCOS patients, might reach through the blood-brain barrier GnRH neurons and might in this way alter GnRH pulsatility leading to LH hypersecretion and relative FSH shortage [30].

The role of homocysteine as marker of 1-C metabolism has been extensively investigated in PCOS [31]. Moreover it has been shown that a healthy diet improves the clinical course of PCOS [32, 33]. Because overweight and obesity are features of a poor diet and sedentary lifestyle and often present in PCOS, it is not surprising that dietary interventions promoting weight loss are important in the treatment of PCOS [34]. Diet can influence hyperandrogenism, cycle regularity, metabolic and psychiatric outcome and therefore the general recommendation is to achieve weight loss using an adequate diet [32,33,35-40]. Furthermore in subfertile women, it was shown that biomarkers of 1-C metabolism in blood reflected the concentration in follicular fluid and are associated with the number of oocytes, the follicle diameter and with embryo quality [41-43]. In the same study population it was also observed that the adherence to an adequate dietary pattern reduced the estradiol concentration and improved the chance of pregnancy after conventional ovarian hyperstimulation treatment [44].

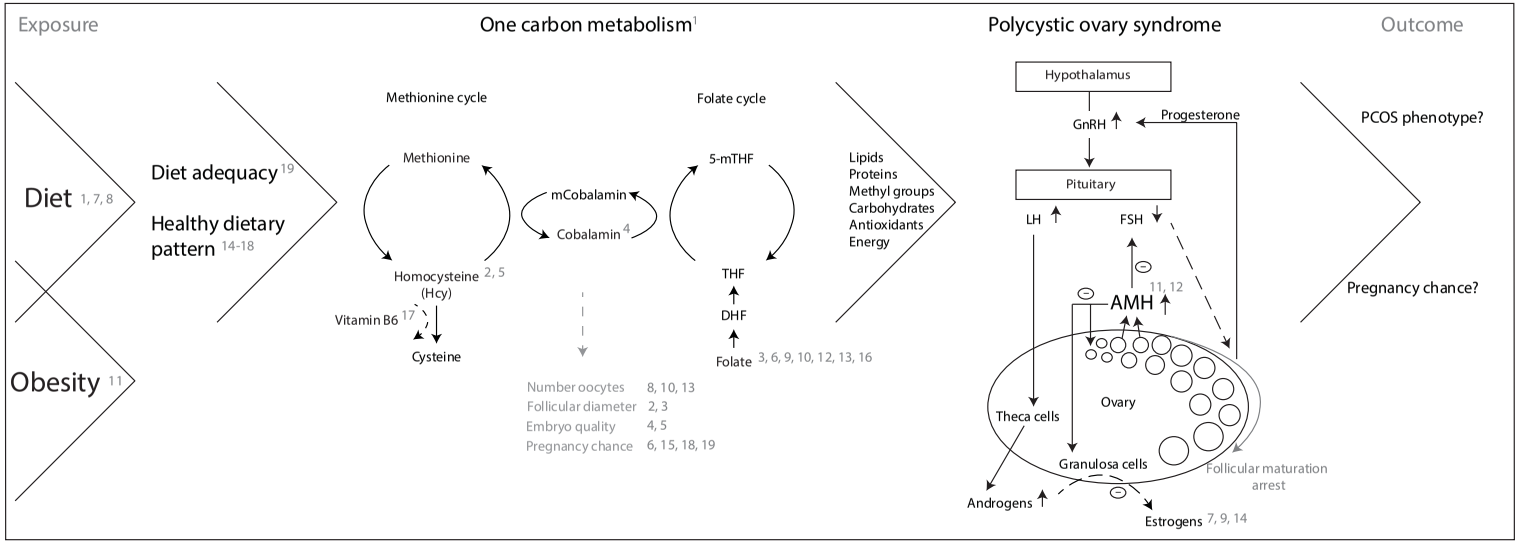

Figure 2 | Hypothesis of the role of the diet in the pathophysiology of PCOS.

Abbreviations; GnRH = Gonadotropin Releasing Hormone, LH = Luteinizing Hormone, FSH = Follicle Stimulating Hormone, AMH = Anti-Müllerian hormone, tHcy = total Homocysteine.
[1] Verkleij et al. 2007, [2-3] Boxmeer et al. 2008, [4-6] Boxmeer et al. 2009, [7-10] Hammiche et al. 2011, [11-12] Hammiche et al. submitted, [13] Twigt et al. 2011, [14-15] Twigt et al. submitted, [16-18] Vuijkovic et al. 2010, [19] Twigt et al. 2012

MALE SUBFERTILITY

Male factor subfertility can be caused by several factors, such as testicular insufficiency, genetic abnormalities, endocrine disturbances, obstructive diseases, infections or due to stress, medication use or sexual disturbances. Previous research demonstrated the important contribution of 1-C metabolism in spermatogenesis (Figure 3) [45,46]. Therefore, the further investigation of determinants affecting the substrates and cofactors of 1-C metabolism as well as the determination of its biomarkers (cobalamin, folate, homocysteine) can be of interest.

AIMS OF THE THESIS

Worldwide, subfertility is still a rather common and complex problem. Since the pathogenesis is in most cases unknown, we aimed to investigate periconceptional modifiable determinants of 1-C metabolism in association with human subfertility. The main aims are to investigate;

(1) the Preconception Dietary Risk score (PDR score) and the mHealth coaching program Smarter Pregnancy (www.slimmerzwanger.nl) as tool to assess and change diet adequacy and empower health care professionals and patients. (Part 1)
(2) the impact of the diet in the development and treatment of the clinical spectrum of PCOS. (Part 2)
(3) the influence of medication use on semen parameters. (Part 3)

METHODOLOGY

For this study we used data of the 'Achieving a healthy pregnancy' study, the 'COLA database' (screeningsprogram for patients with irregular menstrual cycles; cycle disturbances, oligo- or amenorrhea), the Dutch Integrated Primary Care Information (IPCI) database, and of the Slimmer Zwanger survey.

Achieving a healthy pregnancy study

Between 2007 and 2012 all couples planning a pregnancy and visiting the outpatient clinic of the division of Reproductive Medicine of the department of Obstetrics and Gynaecology of the Erasmus Medical Center in Rotterdam, received preconception counselling at the 'Achieving a Healthy Pregnancy' clinic. Both women and men completed a questionnaire to obtain information on general characteristics and six additional questions comprising habitual diet inadequacy, which were based on the Dutch food based dietary guidelines and from which a summary PDR score was calculated. Venous blood samples were obtained for the determination of the biomarkers of 1-C metabolism: serum and red blood cell (RBC) folate, serum cobalamin and plasma total homocysteine concentrations (tHcy). Anthropometrics were performed by trained counsellors. Semen parameters were extracted from medical records. Additionally, the couples were invited to participate in the Rotterdam Periconception cohort (Predict study), a periconception cohort study with follow up until one year after pregnancy for which they received a Food Frequency Questionnaire to be completed at home and returned by mail for the detailed assessment of the habitual dietary patterns.

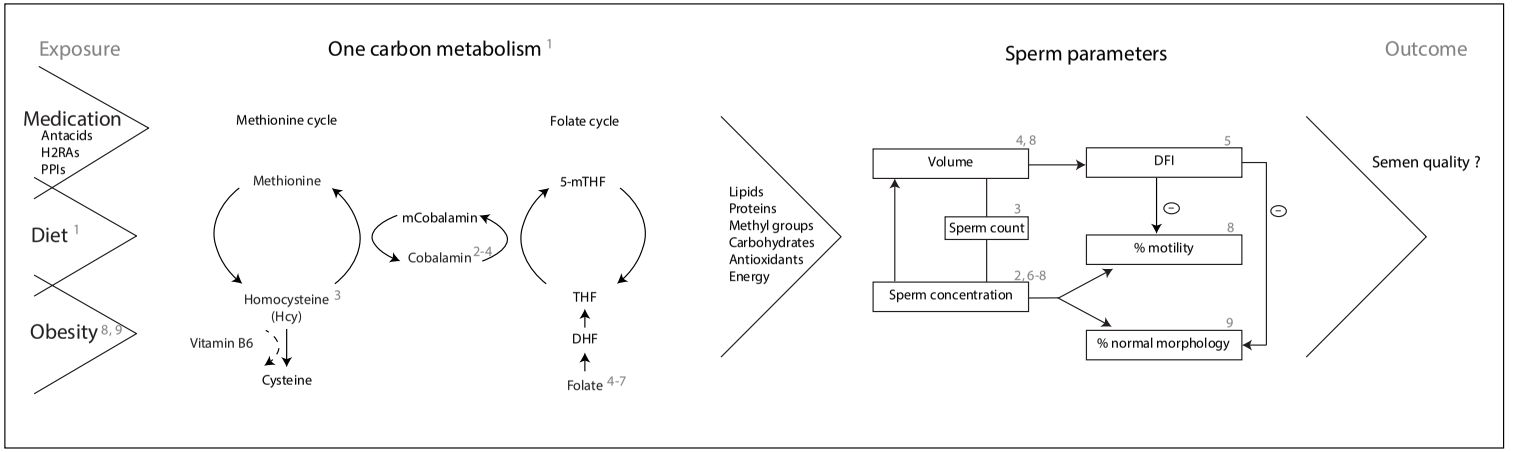

Figure 3 | Hypothesis of the role of the diet and medication use on semen parameters.

Abbreviations; tHCY = total Homocystein, DFI = DNA fragmentation index.

[1] Verkleij et al. 2007, [2] Boxmeer et al. 2007, [3-5] Boxmeer et al. 2009, [6-7] Ebisch et al. 2006, [8] Hammiche et al. 2012, [9] Sermondade et al. 2013

The COLA database

The women with oligo- or anovulation were also screened for PCOS at the outpatient clinic for cycle disturbances ('COLA'; cycle disturbances, oligo- or amenorrhea) during which a standardized physical examination and an extensive endocrine evaluation were performed by trained professionals. PCOS was diagnosed according to the Rotterdam criteria [24] and two of the following symptoms needed to be present: oligo- or anovulation, hyperandrogenism, and/ or polycystic ovaries.

The Dutch Integrated Primary Care Information

The IPCI database consists of medical records of 1.5 million patients from 720 general practitioners spread across the Netherlands. We selected a cohort of men planning pregnancy and with available semen analysis data from all patients visiting the general practitioner between 1996 and 2013. Additional information on general characteristics was extracted automatically for the period of 12 months preceding the semen analysis using search codes and text strings.

Smarter Pregnancy survey

www.slimmerzwanger.nl is a Dutch personal web based lifestyle coaching program on the smartphone, identifying inadequate diet and lifestyle in couples contemplating pregnancy or being pregnant. After a baseline online identification of risk factors, a 6 months coaching program is generated. The content of the individual coaching consists of the baseline screening and follow up screening at 6, 12, 18 and 24 weeks of the program as well as a maximum of 3 interventions per week comprising of SMS and push-messages, as well as e-mail messages containing tips, recommendations, additional questions addressing behavior, pregnancy status, Body Mass Index (BMI), adequacy of the diet, and seasonal recipes and vouchers. A personal page of the website represents a couples results and provides access to additional modules supporting physical activity, an agenda improving the compliance of hospital appointments and intake of medication, and a module to monitor the safety of prescribed medication. For the participation in the survey women and men visiting the outpatient clinic of the Department of Obstetrics and Gynaecology of the Erasmus Medical Center were offered a free subscription of this coaching program between January 2012 and December 2013.

OUTLINE OF THE THESIS

In Part 1, **chapter 2,** we describe the validity of the use of the PDR score as a tool to assess the inadequacy of the habitual diet in clinical practice.
In **chapter 3** the first results of the personal online lifestyle coaching program Smarter Pregnancy are presented.

In Part 2, **chapter 4**, we report on associations between the PDR score and PCOS severity.
In **chapter 5** we identify dietary patterns in association with PCOS severity of phenotypes and the chance of an ongoing pregnancy.

In Part 3, **chapter 6**, we present the investigations of the use of medication for gastric acid-related symptoms and the impact on pH dependent B vitamins and semen parameters in men visiting our tertiary hospital.
In **chapter 7** we describe the associations between the use of proton pump inhibitors and semen quality in a population based cohort of men visiting the general practitioner as a validation of the previous study.

Chapter 8 covers the general discussion of the methodological considerations, the main findings and the suggestions for future research. In **chapter 9** the summary is provided.

PART 1

New screening tools and mHealth intervention

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Chapter 2

The Preconception Dietary Risk score; a simple tool to assess an inadequate habitual diet for clinical practice

ABSTRACT

Background and aims

Worldwide unhealthy dietary behaviors in women and men in reproductive age are an increasing problem with adverse effects on reproduction. This emphasizes the need for a simple tool to assess the habitual diet in clinical practice. The aim of this study was to evaluate the use of the Preconception Dietary Risk score as a tool to determine the inadequacy of the habitual diet.

Methods

We investigated 139 patients (68 women, 71 men) planning pregnancy at the outpatient clinic. A summary Preconception Dietary Risk score was calculated from seven questions to assess the inadequacy of the diet using the six Dutch guidelines for the consumption of bread, oils/fats, vegetables, fruit, meat and fish. The Preconception Dietary Risk score was used to predict the nutrient intakes derived from the Food Frequency Questionnaire and validated with the biomarkers of one-carbon metabolism in blood.

Results

The Preconception Dietary Risk score assessed an inadequate habitual diet in 55.4% of women and 54.2% in men and revealed a sensitivity of more than 80% for an inadequate intake of bread, vegetables, fruit, meat and fish. ANOVA revealed significantly positive linear trends of the Preconception Dietary Risk score for saturated fat intake, and significantly negative trends for the intake of protein, EPA, DHA, fiber, folate and vitamin B6, B12 and C intake. Furthermore, a significant inverse correlation was observed between the Preconception Dietary Risk score and serum vitamin B12 (Spearman's $\rho = -0.172$, $p = 0.046$).

Conclusions

The Preconception Dietary Risk score is a sensitive, quick and simple tool to assess an inadequate habitual diet in clinical practice.

INTRODUCTION

Worldwide healthy dietary behaviors are deteriorating [6,47,48]. Dutch men and women in reproductive age, showed an insufficient consumption of more than 90% for fruit and vegetables, more than 70% for fish and 100% for fiber [49]. In addition, excessive intakes of saturated fats, animal proteins and carbohydrates are observed [49]. These qualitative poor diets result in relative deficiencies of essential vitamins and minerals, and as such contribute not only to the epidemic of obesity and ageing diseases, but also to reproductive failures [50,51].

The composition of the diet of women and men during the preconception period is considered increasingly important, because during this time frame of 14 weeks before conception female and male germ cells develop in order to be fertilized [20]. During this period, reproductive tissues of especially women also have to be replenished with essential micronutrients to cover the nutritional needs of the embryo as well as the placenta during the first trimester of mainly histiotrophic nourishment. The amount of evidence addressing the detrimental influences of a qualitative poor diet on reproduction with long-term consequences for parental- and offspring health is overwhelming [52-54]. Moreover, maternal but also paternal obesity, a resulting phenotype of a poorly composed diet and a more sedentary lifestyle, is in a similar manner associated with increased risks of reproductive failures and adverse pregnancy outcome [55-57]. Furthermore, it has been observed that poor maternal adherence to a healthy Mediterranean dietary pattern deteriorates chances of pregnancy and increases the risk of spina bifida and fetal growth restriction in offspring [8,52,58].

A change towards a healthier lifestyle can be achieved in a relatively short time and the preconception and pregnancy period is considered as 'the window of opportunity' to change poor dietary habits and to achieve lifestyle changes [59]. Therefore, all couples planning pregnancy should be personally counselled on the inadequacy of the diet during a preconception visit.

Previous research has shown that a self-administered questionnaire prior to the preconception visit is a valid method to identify risk factors [60]. To determine the inadequacy of the habitual diet, we created the Preconception Dietary Risk score (PDR), as summary score to assess the inadequate intake of six food groups, derived from a self-administered questionnaire and based on the six dietary guidelines of the Netherlands Nutrition Center [61].

The current study aims to evaluate the PDR score as a clinical screening tool to assess the inadequacy of the habitual diet in couples planning pregnancy. We compared the PDR score with nutrient intakes from a validated semi-quantitative Food Frequency Questionnaire (FFQ) [62] and biomarkers of one-carbon metabolism, important in reproduction and significantly influenced by the diet [49,62].

MATERIALS AND METHODS

Study population

Between November 2010 and October 2011, couples visiting the preconception outpatient clinic "Achieving a Healthy Pregnancy" [59] of the department of Obstetrics and Gynaecology of the Erasmus MC, University Medical Center in Rotterdam, the Netherlands were invited to participate in the Rotterdam Predict study [63], a preconception cohort study with follow up during pregnancy. Couples were eligible when they were living near Rotterdam and had an adequate understanding of the Dutch language in reading and writing. In the preparation of the preconception visit, all couples completed a Preconception Questionnaire (PQ) at home to obtain data on age, ethnicity, education, indication for referral, the use of medication, folic acid supplement, tobacco, drugs, beverages containing caffeine or alcohol, physical exercise, and diet inadequacy. The questions about habitual diet inadequacy were based on the Dutch food based dietary guidelines on average consumptions of bread, oils/fats, vegetables, fruit, meat and fish from which a summary PDR score was calculated (Table 1) [61]. Ethnicity and education level were classified according to the definition of Statistics Netherlands [47].

During the study period a total of 860 patients (women and men) received preconception counselling of which 428 patients were eligible because of an adequate understanding of the Dutch language in speaking and reading and living in the Rotterdam area. A subgroup of 139 patients participating in the Predict study were available for analysis (68 women and 71 men) after exclusion of patients not responding the FFQ [62] (n= 173; 40%), patients completing the FFQ [62] r >1 month after the PQ (n= 89; 21%) and when patients reported substantial changes in diet between the moment of completing the PQ and the FFQ [62] (n= 27; 6%) (vegetarian, energy-restricted diet). During the preconception visit, a trained counsellor screened and advised couples by using the PDR score from the PQ. Anthropometric measurements were performed during the visit, i.e. height, weight, waist- and hip circumference, systolic and diastolic blood pressure and body mass index (BMI) was calculated. Non-fasting blood samples were obtained by venipuncture for the determination of biomarkers of one-carbon metabolism i.e. serum and red blood cell (RBC) folate, serum total vitamin B12 and plasma total homocysteine concentrations (tHcy). The participating patients received a FFQ [62] to be completed at home and returned by mail. All questionnaires and materials were anonymously processed. This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving patients were approved by the Medical Ethical and Institutional Review Board of the Erasmus University Medical Center in the Netherlands. Written informed consent was obtained from all participants.

Table 1 | The summary PDR score, calculated by the intake of six foods based on Dutch guidelines.[a]

PDR item	Preconception Questionnaire	Food Frequency Questionnaire
1	1) How many slices of bread do you eat daily? A: None, less than one, 1, 2-3, 4-5, 6-7, 7/> 2) What type of bread do you eat? A: White, brown, variable **PDR score 1;** White or variable 0-7 slices, or brown <4-5 slices.	1) How often did you eat bread in the previous month? A: None, 1 day/month, 2-3 days/month, 1 day/wk, 2-3 days/wk, 4-5 days/wk, 6-7 days/wk 2) When eating bread, how many slices of bread did you eat? A: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 3) What type of bread did you eat in the previous month? A: White, whole-wheat, brown, other
2	What type of cooking oils/fats do you use and how often do you use them weekly? A: Butter, margarine, liquid butter, vegetable oils, other. (Frequency; 0, 1, 2, 3, 4, 5, 6, 7) **PDR score 1;** Usage of butter, margarine.	4) What type of cooking fat did you usual use to cook meat, fish, vegetables, fried eggs, potatoes or meat replacers? (Answered per item separately) A: Butter, margarine, diet margarine, liquid margarine, olive oil, sunflower oil, other
3	How often do you eat 200 grams of vegetables weekly (cooked or raw)? A: 0, 1, 2, 3, 4, 5, 6, 7 **PDR score 1;** <7 days a week.	5) How often did you eat vegetables (cooked/raw) in the previous month? A: None, 1 day/month, 2-3 days/month, 1 day/wk, 2-3 days/wk, 4-5 days/wk, 6-7 days/wk 6) How many servings (1 serving= 50 grams) did you usually eat? A: 1-2, 3-4, 5-6, 7-8
4	How many pieces of fruit do you eat daily? A: none, 1 or less, 1-2, 2 or more **PDR score 1;** <2 pieces daily	7) How often did you eat fruits in the previous month? A: None, 1 day/month, 2-3 days/month, 1 day/wk, 2-3 days/wk, 4-5 days/wk, 6-7 days/wk 8) When eating fruit, how many pieces of fruit did you eat daily? A: 1, 2, 3, 4, 5, 6
5	How often do you eat meat weekly? A: 0, 1, 2, 3, 4, 5, 6, 7 **PDR score 1;** <3-4 times a week	9) How often did you eat meat in the previous month? A: None, 1 day/month, 2-3 days/month, 1 day/wk, 2-3 days/wk, 4-5 days/wk, 6-7 days/wk 10) When eating meat, how many servings did you eat on average? A: 0.5, 1, 1.5, 2/>
6	How often do you eat fish weekly? A: 0, 1, 2, 3, 4, 5, 6, 7 **PDR score 1;** <2 times a week	11) How often did you eat fish in the previous month? A: None, 1 day/month, 2-3 days/month, 1 day/wk, 2-3 days/wk, 4-5 days/wk, 6-7 days/wk 12) When eating fish, how many servings of fish did you eat on average? A: 0.5, 1, 1.5, 2/>

[a] Based on the dietary guidelines of the Netherlands Nutrition Center [61].

Dietary assessment

Based on the dietary guidelines of The Netherlands Nutrition Center regarding six food groups, the PDR score was calculated using seven questions from the PQ which reflects the habitual diet without a specific time frame (Table 1**)** [61]**.** When patients did not meet one of the dietary guidelines, one point was administered, resulting in a summary PDR score. Since six guidelines were evaluated, the PDR score had a maximum of 6 points and a minimum of 0 points. The higher the score, the more inadequate the food intake. In addition, couples completed a validated, semi-quantitative FFQ [62], developed by the division of Human Nutrition, Wageningen University, The Netherlands to estimate habitual food intake over the previous four weeks [62,64]. The FFQ [62] consists of 196 food items and comprises questions on consumption frequency, preparation methods and portion sizes according to Dutch household measures [62]. The intake of an individual nutrient was calculated by multiplying nutrient density with the consumed amounts of the food products containing the nutrient of interest. Nutrient densities as reported in the Dutch Food Composition Database of 2006 were used for all calculations and standard portion sizes were verified by use of the portion size table [65].

Laboratory analyses

For the determination of serum folate and vitamin B12, venous blood samples were drawn into dry vacutainer tubes and allowed to clot. After centrifugation at 2,000 xg, serum was collected and the samples were analyzed during routine laboratory procedures using an immune electro chemo luminescence assay (E170; Roche Diagnostics GmbH, Mannheim, Germany). For the determination of RBC folate and plasma tHcy, venous blood samples were drawn into ethylenediamine tetraacetate (EDTA)-containing vacutainer tubes. The EDTA-blood samples were kept on ice and plasma was separated by centrifugation within one hour for determination of tHcy. Directly after blood sampling, 0.1 ml EDTA tube was hemolysed 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 xg after which the folate concentration was measured in the hemolysate. RBC folate was calculated using the following formula: nM RBC folate = (nM hemolysate folate x 10/hematrocit) – (nM serum folate x [1 – hematocrit] /hematocrit). tHcy in EDTA plasma was determined using high-performance liquid chromatography with reversed phase separation and fluorescence detection [66]. Inter-assay coefficients of variation for serum folate were 4.5% at 13 nmol/L and 5.7% at 23 nmol/L, for serum cobalamine 3.6% at 258 pmol/L and 2.2% at 832 pmol/L and for plasma tHcy were 4.8% at 14.6 µmol/L and 3.3% at 34.2 µmol/L. The detection limit for serum folate was 1.36 nmol/L, for serum cobalamine 22 pmol/L and for plasma tHcy 4 µmol/L.

Statistical analyses

In order to classify individuals according to meeting the six Dutch dietary guidelines, data of the PQ and FFQ [62] were compared and the sensitivity and specificity of the PQ was calculated using FFQ [62] as reference. Considering that there is no general agreement about the acceptable level of sensitivity for this assessment, values of more than 80% were considered acceptable. Because portion sizes are not assessed with the PQ, standard portion sizes as reported in the portion size table were used [65]. We considered several potential confounders; gender, ethnicity, educational level, medication and folic acid use, smoking, alcohol or drug use and BMI. Chi-Square and Mann Whitney U tests were performed to test differences between women and men in the study group. Chi square tests and Kruskal Wallis tests were performed in women and men separately between the study group, the excluded group and the non-eligible group. Significant differences were subsequently tested pairwise between groups. Spearman's correlation coefficients were calculated between the PDR score and biomarkers. Multivariable linear regression analysis was used to predict with the PQ the consumption per food group in grams per day derived from the FFQ [62]. The nutrient intakes derived from the FFQ [62] were energy-adjusted using the nutrient density method according to Willett and were normalized after log-transformation. ANOVA was used to assess differences in nutrient intake according to the PDR scores (p value for linear trend) and ANCOVA was used to adjust for gender, ethnicity, education level and energy intake. All statistical analyses were performed using SPSS for Windows (version 17.0.2, SPSS, Inc., Chicago, IL, USA). The level of significance was set to $p < 0.05$ for all analyses.

RESULTS

The general characteristics of the 139 participants (68 women and 71 men) are shown in table 2. Women were younger and less likely to use drugs, caffeine and alcohol than men. Furthermore, women showed higher levels of folate (RBC and serum) and lower levels of tHcy than men, in line with their frequent use of folic acid containing supplements. The dietary inadequacies assessed with the PDR score were comparable between men and women (Table 2). The maximum PDR score of six, did not occur in our study group. In both women and men, we observed some differences in general characteristics between the excluded group, the non eligible group and the study group. In women and men the proportion of Dutch participants was significantly higher in the study group (women 76.5%, men 78.9%) than in the non-eligible group (women 47.2%, men 53.3%; both $p < 0.01$). In men the study group showed a higher frequency of intermediate educational level compared to the non-eligible group (46.5% vs. 32.7%; $p < 0.05$). In women, the study group used more often alcohol (51.5% vs. 35.8%; $p < 0.05$) and folic acid supplements (66.2% vs. 51.2%; $p < 0.05$) than the non-eligible group.

Table 2 | Characteristics of the study group.

	Women (n= 68)	**Men** (n= 71)
Age (y)	31(21-42) **	35 (21-60)
Ethnicity		
Dutch	52 (76.5%)	56 (78.9%)
Non-Dutch	16 (23.5%)	15 (21.1%)
Educational level		
Low	9 (13.2%)	11 (15.5%)
Intermediate	30 (44.1%)	33 (46.5%)
High	28 (41.2%)	26 (36.6%)
Indication for referral		
Subfertility	62 (91.2%)	–
Recurrent miscarriages	3 (4.4%)	–
BMI (kg/m^2)	25.4 (16.7-45.9)	26.1 (19.2-40.5)
25-30(kg/m^2);	20 (29.4%)	31 (43.7%)
≥30 (kg/m^2);	14 (20.6%)	12 (16.9%)
Waist-hip ratio (cm)	0.78 (0.63-1.34)**	0.87 (0.74-1.05)
Systolic blood pressure (mmHg)	110 (62-170)**	124 (90-160)
Diastolic blood pressure (mmHg)	72 (40-120)**	80 (58-110)
Exposure		
Medication use 'yes'	32 (47.1%)	23 (32.4%)
Folic acid use 'no'	23 (33.8%) **	68 (95.8%)
Supplement use 'no'	40 (58.8%) **	58 (81.7%)
Lifestyle		
No physical exercise	32 (47.1%)	30 (42.3%)
Smoking	18 (26.5%)	24 (33.8%)
Drugs	1 (1.5%) **	10 (14.1%)
Caffeine [a]	2 (2.9%) *	9 (12.7%)
Alcohol	35 (51.5%) *	49 (69.0%)
Biomarkers		
Folate, RBC(nmol/L)	1255 (713-1928)**	1054 (640-1999)
Folate, serum(nmol/L)	31.6 (10-45) **	18.3 (9-40)
Vit B12, serum(pmol/L)	309.5 (145-1218)	301 (138-839)
tHcy, plasma(µmol/L)	8.5 (5-44)**	11.3 (7-60)
PDR score [b]		
Mean	55.4%	54.2%
Median	3 (0-5)	3 (0-4)

Table 2 | (*Continued*)

	Women (n= 68)	**Men** (n= 71)
PDR per item		
Bread	47 (69.1%)	40 (56.3%)
Oils/fats	34 (50%)	31 (43.7%)
Vegetables	44 (64.7%)	48 (67.6%)
Fruit	45 (66.2%)	55 (77.5%)
Meat	8 (11.8%)	7 (9.9%)
Fish	48 (70.6%)	50 (70.4%)

Data are either presented as median (range) or as number of subjects (%). Pearson Chi-Square test or Mann-Whitney U test between women and men (* P value <0.05,** P value <0.01). [a] Caffeine use was defined as 6 or more cups/day. [b] Number (percentage) of individuals with inadequate dietary intake according to cut-off points used for calculating the PDR score.

When we compared the PDR score items, both women and men from the study group showed less inadequacy in consuming meat than the non-eligible group (women 11.8% vs. 29.8%; p <0.01 and in men 9.9% vs. 22.9% p <0.05). Table 3 shows the sensitivity and specificity of the PDR score to classify the inadequate intakes per food group in the total study group and stratified by gender using the FFQ [62] as reference. In the total study group the sensitivity was lowest for oils/fats (63.1%) and highest for fruit (99.0%) and the specificity was lowest for fish (36.6%) and highest for meat (83.9%). In women compared with men, we observed higher sensitivities for almost all food groups, except for meat and fish. The specificity in women was also higher for fish and vegetable consumption.

Table 3 | Differences in the sensitivity and specificity of the PDR score for all subjects and stratified for gender.

	Study group (n= 139)		**Women** (n= 68)		**Men** (n= 71)	
	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
Bread [a]	95.4%	39.2%	100%	9.5%	90.0%	60.0%
Oils/fats [b]	63.1%	76.7%	70.6%	76.5%	54.8%	76.9%
Vegetables	81.5%	55.3%	84.1%	62.5%	79.2%	47.8%
Fruit	99.0%	30.8%	100%	26.0%	98.2%	37.5%
Meat	93.3%	83.9%	87.5%	83.3%	100%	84.4%
Fish	93.9%	36.6%	93.8%	50.0%	94%	23.8%

[a] One missing in the bread PDR item (n= 70 men). [b] One missing in the oils/fats PDR item (n= 70 men).

In women and men the lowest sensitivity was shown for oils/fats and the highest for bread in women and meat in men. The specificity was lowest for bread in women and fish in men and highest for meat in both women and men. The multivariable linear regression analyses

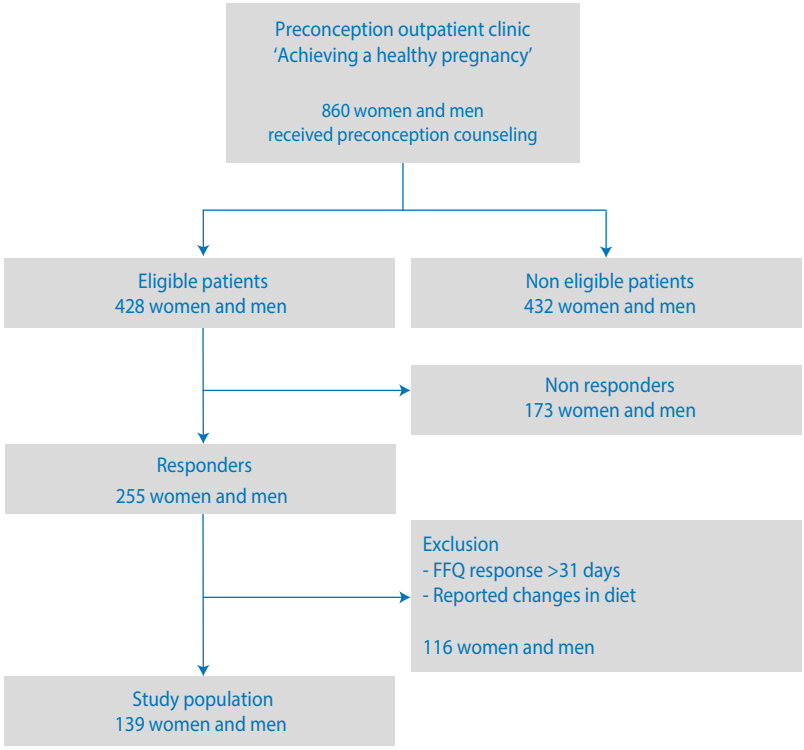

Figure 1 | Flow diagram of the study population selection

Table 4 | Prediction of the consumption per food group (g/day) of the FFQ by the preconception questionnaire (n= 139).

	Unadjusted Model				Adjusted Model [a]			Equal consumption in PQ and FFQ (g/day) [b]
	Constant	β	R^2	P value	β [a]	R^2	P value	
Bread [c]	11.6	0.853*	0.426	<0.001	0.657*	0.578	<0.001	78.9
Vegetables	18.1	0.908 *	0.126	<0.001	0.764 *	0.250	<0.001	196.7
Fruit	26.8	0.776 *	0.434	<0.001	0.752 *	0.518	<0.001	119.6
Meat	36.1	0.775*	0.142	<0.001	0.424*	0.384	0.008	160.0
Fish	2.8	0.664*	0.381	<0.001	0.571*	0.365	<0.001	8.3

[a] Multivariable linear regression analysis adjusted for gender, ethnicity, education level, folic acid supplement use, BMI, smoking, alcohol and energy intake. [b] The level of equal consumption according to the PQ and the FFQ was calculated by substituting y with x in the regression formula (y = βx + constant) derived from the unadjusted model. [c] One missing in the bread PDR item (n= 138).

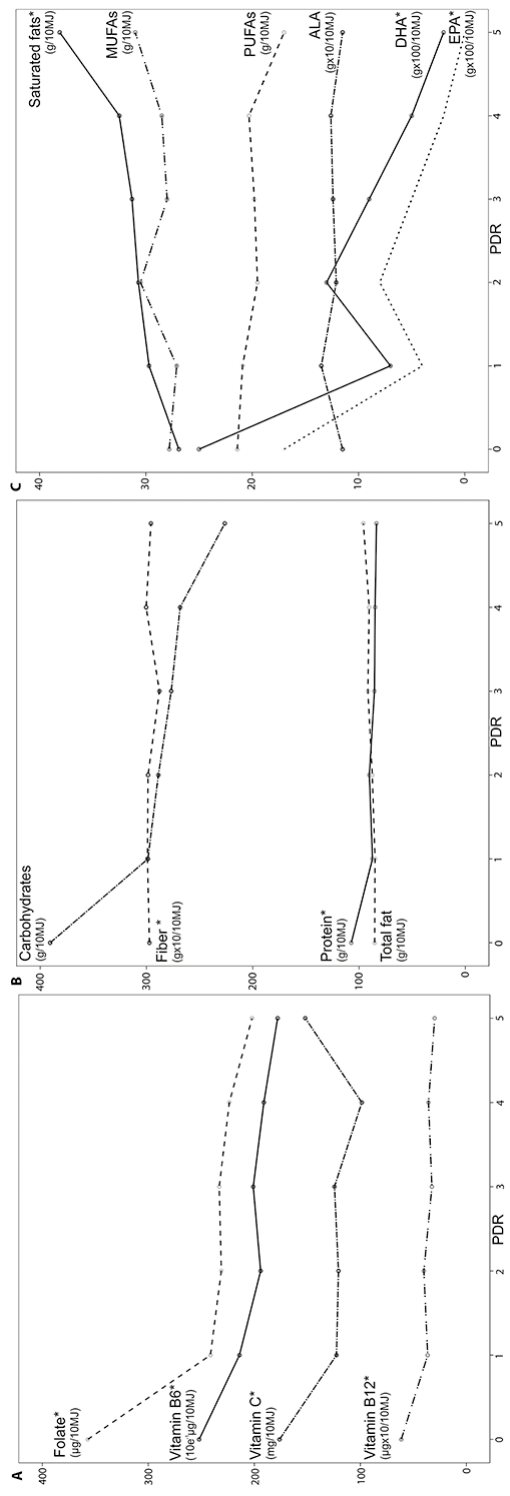

Figure 2 | The PDR score and energy-adjusted nutrient levels based on the FFQ. A. Energy-adjusted folate (p= 0.002), vitamin B6 (p= 0.001), vitamin C (p= 0.004) and vitamin B12 (p= 0.013). B. Energy-adjusted carbohydrates (p= 0.871), fiber (p= <0.001), total fat (p= 0.254) and protein (p= 0.002). C. Energy-adjusted saturated fats (p= 0.019), MUFAs (p= 0.901), PUFAs (p= 0.931), ALA (p= 0.758), DHA (p= <0.001) and EPA (p= <0.001).

The y scale varies for each nutrient. *ANOVA testing for trend in energy adjusted intake level (P value, weighted linear term)

for the consumption of each food group in grams per day from the PQ and FFQ [62] and adjusted for gender, ethnicity and educational level and energy intake revealed significantly positive associations (Table 4). The analysis of the prediction of the energy-adjusted nutrient intakes of the FFQ [62] by the PDR score, using ANOVA, showed significant linear trends for the intake of protein, saturated fats, EPA, DHA, fiber, vitamin B6, B12, folate and vitamin C (Figure 2, Supplement Table 1). After adjustment for gender, ethnicity, educational level and BMI, significances remained except for total fat (p= 0.36, data not shown). The correlation between the PDR score and the biomarkers of the one-carbon metabolism showed an inverse correlation with vitamin B12 only (Spearman's ρ= -0.172, p= 0.046, data not shown).

DISCUSSION

In couples visiting a preconception outpatient clinic, the prevalence of an inadequate habitual diet defined by the PDR score and based on the Dutch guidelines, was 55%. The PDR score demonstrated a more than 80% sensitivity for inadequate intake of bread, vegetables, fruit, meat and fish. The PDR score specificity varied from 30.8% to 83.9%. From these data we conclude that the PDR score is a sensitive and simple tool for clinical practice to assess especially inadequacies of the habitual diet.

An important finding was that on nutrient level a higher PDR score was independent for gender significantly associated with a higher consumption of saturated fats and a lower consumption of protein, EPA, DHA, fiber, vitamin B6, vitamin B12, folate and vitamin C, suggesting adherence to a Western dietary pattern [67]. The importance for this validation study is emphasized by the rising figures of qualitative malnutrition and obesity in the reproductive population. The high frequency of overweight and obesity (40.2%) in the Dutch men and women in reproductive age [47] substantiate the 55% diet inadequacy in our subfertile study population. Furthermore, Hammiche et al. previously reported the high frequency of unhealthy dietary behavior in men and women attending this preconception clinic [59]. In addition, Inskip et al. showed similar high percentages of non-compliance to the nutrition- and lifestyle recommendations in women attempting to achieve pregnancy [48]. The further need of a valid dietary assessment tool is supported by the recent findings of Twigt et al. showing that in couples undergoing IVF/ICSI treatment, even one point improvement in the PDR score is associated with a 65% increased chance of ongoing pregnancy [6]. We observed a small but inverse significant correlation between the PDR score and serum vitamin B12. This is an interesting finding considering the associations between a poor vitamin B12 status and fertility parameters [43,46]. It is very likely that due to folic acid supplement use in women and men, no significant correlations were observed with folate and tHcy. This is supported by Verkleij-Hagoort et al. showing significant correlations of folate and vitamin B12 intake as estimated by the same FFQ [62] with serum and RBC folate and serum vitamin B12 in a population of the same age not using vitamin supplements [64]. However, it must be mentioned that the correlation between biomarkers and diet is always hampered due to the

complexity of nutrition as composite determinant influenced by several conditions such as age, smoking, and alcohol consumption [68-70].

The strengths and limitations in the interpretation of the findings have to be addressed. The response rate of the eligible study group determined by the return of the FFQs [62] was 60%, which is an average response rate compared to others varying between 46% and 76% [71,72]. The sensitivity analysis showed general comparability of the study group, the excluded group and the non-eligible group, thereby excluding selection bias. With the exception of ethnicity, educational level, alcohol use, and folic acid use in the non-eligible group. Adjustment for ethnicity did not significantly affect the association between the PDR score and nutrient intake levels. We recommend for future research however that the generalizability of the PDR score in non-Dutch populations should be further evaluated. Strength of the PDR score is that it is a very simple and practical tool consisting of seven questions only, to be filled out by a couple in a matter of minutes, justifying an immediate individual result and conclusion on the inadequacy of the habitual diet. On the contrary, the FFQ [62] – is an extensive and time consuming list of 196 questions and is not used for giving advice on an individual level. Other dietary scores have been developed, however they are based on nutrient intake levels derived from a 24-hrs recall records and they are therefore time consuming in calculating a summary score [73,74]. Another strength of this study is that both questionnaires comprised the same time period and showed significant associations on nutrient intake levels. The estimation of the validity of the data of women and men separately and as a couple, is both interesting and important, because their dietary intake is often correlated. For all six food groups the multivariable linear regression models significantly predicted the nutrient intake in grams per day estimated with the FFQ [62]. Overestimation of meat, bread and fish intake, slightly occurred using the PDR score to predict the consumption in grams per day. However, it must be emphasized that the PDR score is designed to judge the inadequacy of the intake of the food groups and not to estimate consumption level. For the purpose of estimating the exact level of consumption the FFQ remains the Gold standard. A limitation of the study is that the PQ and FFQ [62] have correlated errors, since both questionnaires are retrospective and estimate usual consumption as opposed to recording actual consumed foods in detail [75]. Furthermore, it is known that individuals, with overweight or obesity, underreport food intake in the FFQ [62,75]. On the other hand, in the general population, the intake of healthy foods is often over reported in the FFQ [62,76]. For that reason we adjusted the analysis for BMI and total energy intake. The aim of the PDR sore is to estimate the inadequacy of the habitual diet and not to estimate unhealthy overconsumption. Therefore, the PDR score is not intended to classify the total diet as a more or less healthy diet. For example the patients with a PDR score of 1 reported the highest total daily energy intakes and showed the highest BMI. To improve the PDR score the addition of questions on fast-food, snacks and sweets can be considered to estimate unhealthy overconsumption as well. Another limitation of the PDR score is that vegetarians are classified as having an inadequate intake of meat, while it is possible to reach

an adequate nutrient intake when replacing meat with other foods. Therefore, such questions can be added for this subgroup in particular.

In conclusion, diet is a composite determinant and increasingly associated with health and reproductive outcome. This study shows the validity of the PDR score in assessing the inadequacy of the habitual diet and quality of the nutrient composition in a simple and efficient manner.

Supplemental Table 1 | Intake levels of energy and some main nutrients for men and women, calculated using the FFQ, stratified for PDR score.[a]

	0 (n= 6)	**1** (n= 14)	**2** (n= 39)	**3** (n=46)	**4** (n= 30)	**5** (n= 2)	**P value**[b]
Energy (kJ/day)	8703.5 7258.1-13064.3	11660.4 4907.6-20789.7	8735.1 4648.2-15502.7	8055.3 4213.4-17062.2	8306.2 4915.8-14435.0	9945.5 8723.3-11167.6	0.042
Carbs (g/day)	246.7 213.6-378.0	350.0 119.1- 734.2	242.7 123.9-442.0	236.8 88.0-475.9	256.8 138.8-445.2	296.2 243.0-349.5	0.062
Carbs (g/10MJ)	297.2 266.4-330.1	299.1 235.0-353.1	298.5 226.2-373.4	287.8 208.8-372.6	300.3 236.0-381.6	295.8 278.6-313.0	0.871
Protein (g/day)	96.8 66.5-130.9	102.7 61.6-174.9	71.4 47.0 - 144.4	69.9 32.8-154.3	68.9 43.7-97.9	82.3 79.4-85.2	<0.001
Protein (g/10MJ)	107.3 83.0-133.7	87.4 74.1-132.5	90.3 58.5-130.1	85.5 58.7-117.0	84.9 51.9-112.1	83.6 76.3-91.0	0.002
Total fat (g/day)	65.2 59.5-121.1	103.0 45.4-145.1	78.4 34.7-172.0	68.8 33.4-207.2	79.2 38.7-151.7	94.8 87.6-102.0	0.233
Total fat(g/10MJ)	85.3 62.9-92.7	85.0 68.1-114.3	87.6 58.2-114.6	91.7 54.5-130.5	90.5 58.8-117.5	95.9 91.4-100.4	0.254
SFAs (g/day)	24.2 18.7-35.8	36.5 16.0-55.1	25.8 10.8-67.6	24.3 10.5-76.8	27.5 12.0-57.4	37.5 36.0-39.1	0.706
SFAs(g/10MJ)	26.9 24.4-31.4	29.7 21.6-37.2	30.7 18.6-46.6	31.3 15.6-53.1	32.5 19.8-48.9	38.1 35.0-41.2	0.019
MUFAs (g/day)	20.3 18.3-40.9	33.9 15.5-51.5	26.3 11.8-55.2	22.4 11.2-64.2	24.4 11.5-52.4	30.8 27.3-34.3	0.078
MUFAs (g/10MJ)	27.8 19.2-31.3	27.1 21.5-39.5	30.5 20.5-41.7	28.0 16.6-46.7	28.5 13.8-40.4	31.0 30.8-31.3	0.901
PUFAs (g/day)	15.6 12.5 - 33.8	23.6 9.4 - 37.4	17.5 8.0 - 41.7	16.2 5.4 - 47.3	17.8 7.7 - 30.2	16.6 16.5 - 16.7	0.103
PUFAs (g/10MJ)	21.4 11.9 - 25.9	20.9 13.2 - 29.9	19.5 9.1 - 30.7	19.8 10.5 - 43.6	20.3 13.0 - 27.4	17.0 14.8 - 19.2	0.931
ALA (g/day)	0.99 0.84 - 1.21	1.43 0.65 - 3.19	1.13 0.42 - 2.79	0.93 0.34 - 2.49	1.09 0.41 - 3.49	1.15 0.98 - 1.33	0.230

Supplemental Table 1 | *(Continued)*

	0 (n= 6)	**1** (n= 14)	**2** (n= 39)	**3** (n=46)	**4** (n= 30)	**5** (n= 2)	**P value**[b]
ALA (g/10MJ)	1.15 0.84 - 1.43	1.35 0.79 - 2.43	1.21 0.40 - 2.54	1.24 0.67 - 2.67	1.26 0.55 - 2.69	1.15 1.12 - 1.19	0.758
EPA (g/day)	0.12 0.03 - 0.48	0.06 0.01 - 0.27	0.06 0.00 - 0.25	0.05 0.00 - 0.27	0.03 0.00 - 0.16	0.00 0.00 - 0.00	<0.001
EPA (g/10MJ)	0.17 0.04 - 0.49	0.04 0.01 - 0.34	0.08 0.00 - 0.45	0.05 0.00 - 0.37	0.02 0.00 - 0.21	0.00 0.00 - 0.00	<0.001
DHA (g/day)	0.18 0.07 - 0.73	0.10 0.02-0.41	0.11 0.01-0.40	0.09 0.01 - 0.41	0.05 0.01 - 0.27	0.02 0.01 - 0.02	<0.001
DHA (g/10MJ)	0.25 0.10 - 0.73	0.07 0.02 - 0.52	0.13 0.01 - 0.71	0.09 0.01 - 0.57	0.05 0.01 - 0.32	0.TG HYE3 02 0.01 - 0.02	<0.001
Fiber (g/day)	36.6 24.7 - 46.6	32.8 18.8 - 80.1	22.7 8.9 - 52.7	21.5 3.8 - 42.2	21.6 8.0 - 34.3	22.2 21.7 - 22.8	<0.001
Fiber (g/10MJ)	39.1 33.9 - 45.5	29.9 26.6 - 44.1	28.9 8.4 - 53.5	27.7 8.9 - 50.8	26.8 12.9 - 39.9	22.6 20.4 - 24.8	<0.001
B6 (mg/day)	2.37 1.2 - 2.8	2.40 1.4 - 4.8	1.72 1.1 - 2.9	1.64 0.5 - 3.2	1.56 0.6 - 2.4	1.78 1.5 - 2.1	<0.001
B6 (mg/10MJ)	2.52 1.7 - 3.1	2.14 1.6 - 2.9	1.94 1.4 - 3.3	2.01 1.1 - 3.2	1.91 1.2 - 2.7	1.78 1.7 - 1.9	0.001
B12 (µg/day)	5.21 2.2 - 13.6	4.21 2.8 - 7.7	3.57 1.2 - 12.0	2.76 1.3 - 8.1	2.92 0.4 - 8.6	2.98 2.6 - 3.4	<0.001
B12 (µg/10MJ)	6.14 3.1-13.9	3.65 1.9-11.7	4.01 1.7-8.8	3.25 1.6-13.2	3.57 0.5-11.0	2.99 2.99-3.0	0.013
Folate (µg/day)	348.9 186.7-383.3	294.9 154.7-660.1	203.5 99.0-429.6	194.1 52.8-422.8	186.9 110.0-328.0	207.3 131.4-283.3	<0.001
Folate (µg/10MJ)	357.1 256.4-443.1	241.4 193.1-534.5	231.1 106.3-453.2	233.1 125.2-399.5	223.5 103.3-386.4	202.1 150.6-253.7	0.002
C (mg/day)	173.8 58.0-226.7	151.1 72.2-345.9	106.0 38.0-186.9	93.1 9.7-201.4	75.7 33.9-179.4	163.4 44.6-282.1	<0.001
C (mg/10MJ)	176.2 79.7-282.6	122.5 83.3-325.2	120.6 40.8-307.7	124.4 23.1-198.8	98.6 48.4-250.3	151.9 51.1-252.6	0.004

Values are depicted as median (range). [a] Two missings in the PDR items bread and oils/fats. [b] ANOVA testing for trend in energy adjusted, log transformed intake level (P value, weighted linear term).

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Chapter

3

Impact of an mHealth Platform for pregnancy on nutrition and lifestyle of the reproductive population: a survey

ABSTRACT

Background

Poor nutrition and lifestyle behaviors exert detrimental effects on reproduction and health during the life course. Therefore, lifestyle interventions during the periconceptional period can improve fertility, pregnancy outcome, and health of subsequent generations.

Objective

This survey investigates the compliance, usability, and initial effectiveness of the Web-based mHealth platform, Smarter Pregnancy.

Methods

A free subscription to the mHealth platform, Smarter Pregnancy, was provided to couples contemplating pregnancy (n= 1275) or already pregnant (n= 603). After baseline identification of inadequate nutrition and lifestyle behaviors, a personal online coaching program of 6 months was generated. Using multiple imputation and the generalized estimating equation model with independent correlations, we estimated the changes from inadequate to adequate nutrition and lifestyle behaviors over time. Subgroup analyses were performed for (1) overweight and obese women (body mass index [BMI] ≥25 kg/m^2), (2) pregnant women at the start of the program, and (3) couples.

Results

A 64.86% (1218/1878) compliance rate was observed and 54.7% (range 39.2-73.4%) of participants rated the program usability as positive or very positive. Adequate nutrition and lifestyle behaviors at baseline were 21.57% (405/1878) for vegetable intake, 52.61% (988/1878) for fruit intake, 85.44% (1303/1525) for folic acid use, 86.79% (1630/1878) for no tobacco use, and 64.43% (1210/1878) for no alcohol consumption. After 6 months of coaching, these lifestyle behaviors improved by 26.3% (95% CI 23.0 to 29.9) for vegetable intake, 38.4% (95% CI 34.5 to 42.5) for fruit intake, 56.3% (95% CI 48.8 to 63.6) for folic acid use, 35.1% (95% CI 29.1 to 41.6) for no tobacco use, and 41.9% (95% CI 35.2 to 48.9) for no alcohol consumption. The program showed the strongest effectiveness for participating couples.

Conclusions

This novel Web-based mHealth platform shows high compliance and usability, and users demonstrate improvements in nutrition and lifestyle behaviors. The next step will be further validation in randomized controlled trials and implementation.

INTRODUCTION

Worldwide, more than 45 million couples are contemplating pregnancy, of which around 22 million remain involuntarily childless. Moreover, of the more than 360 million pregnancies worldwide per year, at least 90 million end in miscarriage, 18 million result in congenital malformation, and 40 million result in children small for their gestational age. These reproductive and pregnancy failures largely originate in the periconceptional period, during which development and function of gametes, embryonic organs, and the placenta are programmed [20]. Poor periconceptional nutrition and lifestyle not only affect fertility and pregnancy outcome, but can also derange epigenetic programming with long-lasting health consequences [77]. Therefore, effective nutrition and lifestyle interventions in particular during this window of time will be an investment in healthy pregnancy and the health of current and future generations. Currently, the most effective preconceptional interventions comprise weight loss, improvement of nutrition, use of folic acid supplements, and lowering the use of tobacco [78,79].

Unfortunately, women and men contemplating pregnancy or pregnant couples, as well as health care professionals, are often not aware of the detrimental effects of poor lifestyle behaviors [59,80,81].

These behaviors often accumulate not only in an individual, but also in couples, in particular among those with a low socioeconomic status, increasing the risk of a poor pregnancy outcome [82,83].

Therefore, it should be the responsibility of both health care professionals and patients to improve inadequate nutrition and lifestyle. To this aim, we previously developed and implemented a specific preconception outpatient clinic tailored to improve nutrition and lifestyle, which showed a 30% reduction of inadequate nutrition and lifestyle and a 65% increased chance of ongoing pregnancy after in vitro fertilization (IVF) treatment [6,59].

Obstacles of lifestyle counseling as part of periconceptional (clinical) care, however, require special expertise and time without reimbursement of costs. Mobile health (mHealth) has the potential to transform health care delivery and to overcome obstacles by providing individual, tailored, and repeated information. Evidence is accumulating that mobile technology can effectively improve inadequate nutrition, lifestyle, and medication adherence [84]. Therefore, we developed the online, device-independent, Web-based coaching platform, *Smarter Pregnancy* [85]. This platform was based on scientific evidence of effective nutrition and lifestyle interventions, prevention and educational programs for noncommunicable diseases [86,87], and behavioral models, as well as our experience from the preconception outpatient clinic [59,88]. This mHealth platform aims to empower women, men, and health care professionals to improve inadequate nutrition and lifestyle. It also demonstrates the need for easily accessible, evidence-based interventions to improve the quality and effectiveness of periconceptional (clinical) care, the success of reproduction and pregnancy outcomes, as well as the prevention of disease during the life course [89,90].

Here we investigate the compliance, usability, and initial effectiveness of the Dutch version of this Web-based mHealth platform on changing inadequate nutrition and lifestyle behaviors in prepregnant women and their partners.

METHODS

Study population

In 2012 and 2013, women and men contemplating pregnancy or pregnant couples living in Rotterdam, the Netherlands, visiting the Erasmus Medical Center (MC), University Medical Center, or midwifery practices in Rotterdam, were recruited to the study. Recruits were invited to sign up for a free subscription to the Web-based Smarter Pregnancy platform [85]. This included 6 months of coaching on the most prevalent inadequate nutrition and lifestyle behaviors (ie, vegetable, fruit, and alcohol intake) or the most strongly demonstrated associations of behaviors with fertility and pregnancy course and outcome (ie, tobacco and folic acid supplement use).

Adequate daily intakes are defined as at least 200 grams of vegetables and at least two pieces of fruit, a folic acid supplement of 400 µg, and no tobacco or alcohol use [91]. Men were screened on the same behaviors, except for folic acid supplement use. Evaluation of the results of the baseline survey and the four follow-up screening surveys are shown on each participant's personal page as lifestyle risk scores in graphs and text, accompanied by personal advice according to preconceptional recommendations and Dutch guidelines [91]. If a participant completes the final screening survey at 6 months, we consider this as maximum compliance. More details are described in the next paragraph.

Smarter Pregnancy

The coaching model developed for the Smarter Pregnancy platform is based on our research and expertise from the last 25 years on the impact of nutrition and lifestyle on reproduction as well as on pregnancy course and outcome [6,48,59,88,92]. In addition, we incorporated the following into the platform: results from the literature, Prochaska and Diclemente's transtheoretical model with a focus on the readiness for behavioral change, Bandura's social cognitive theory for self-efficacy, and Fogg's behavior model to include triggers to motivate and increase the ability to change [93-95]. Features of the attitude, social influence, and self-efficacy (ASE) model for coaching are applied; the ASE model has been frequently used for developing health education and prevention. Elements of this model comprise individual attitude, social influence, and self-efficacy aimed at the understanding and motives of people to engage in specific behavior [96].

The content of the individual coaching consisted of the baseline screening and follow-up screening at 6, 12, 18, and 24 weeks of the program. Coaching also included a maximum of three interventions per week comprised of short message service (SMS) text messaging and email messages containing tips, recommendations, vouchers, seasonal recipes, and additional

questions addressing behavior, pregnancy status, body mass index (BMI), and adequacy of the diet. Every 6 weeks, participants were invited to complete a short, online, follow-up screening survey to monitor the change in their inadequate nutrition and lifestyle behaviors. Results from the screening session compared to the previous screening sessions were shown on their personal page (see Figure 1). This page also provided access to additional modules (ie, applications) to support physical activity, an agenda to improve the compliance of hospital appointments and intake of medication, and a module to monitor the safety of prescribed medication. A summary of all individual results were available to be obtained at any point by the participant, and to be handed over or sent by email to the health care professional for further evaluation and support of preconceptional and antenatal care.

This mHealth platform complied with the highest rules of legislation for medical devices in Europe; therefore, it received the Conformité Européenne, classe 1 (CE-1), classification (2013) and can be used to improve the quality of medical care.

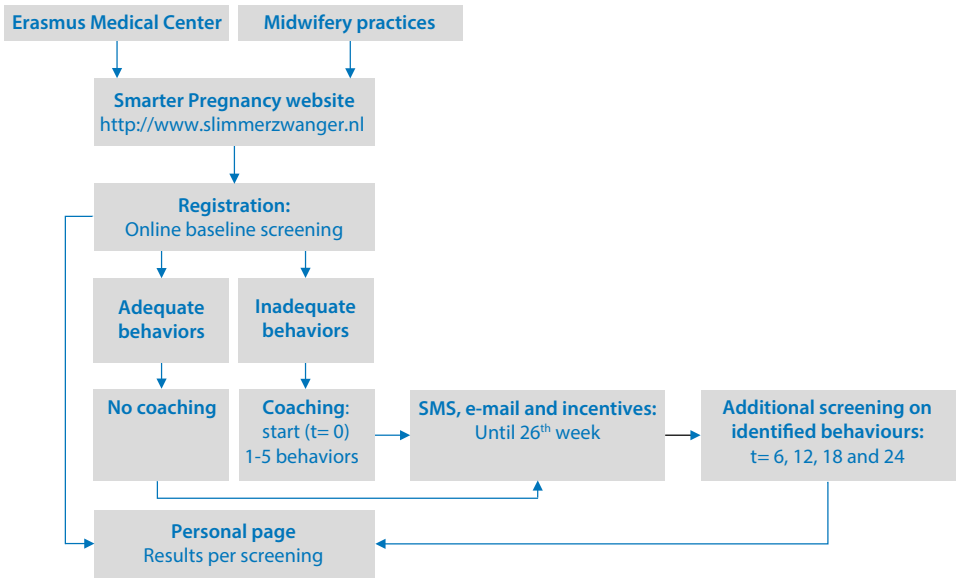


Figure 1 | Overview of the web-based Smarter Pregnancy program: registration, identification of inadequate nutrition and lifestyle behaviors and coaching. SMS: short message service.

Statistical analysis

We analyzed all participants who completed or prematurely resigned from the platform. Compliance was defined by the percentage of participants who completed the 6-month program. Usability was assessed using a digital evaluation form containing 26 questions whose answers were scored using a 4-point Likert scale; the ratings were *negative*, *neutral*, *positive*, and *very positive*. This was used to report on participants' satisfaction with the

platform, which was subdivided into three categories: (1) design and interface, (2) content and coaching, and (3) perception and personal benefit. General characteristics and lifestyle behaviors were compared using chi-square tests for proportions, and *t* tests and Mann Whitney U tests for continuous variables. Using a generalized estimating equation (GEE) model with an independent working correlation matrix, we modeled the fraction that scored *adequate* at each of the follow-up time points. In order to minimize selection bias, we used multiple imputation models to handle missing data of the participants who prematurely resigned. Therefore, a separate model was built for each of the five lifestyle behaviors of interest using all available information on each of the time points, as well as the subgroup indicators to impute the missing values. For each nutrition and lifestyle behavior, we examined those individuals that scored inadequate at baseline.

Subgroup analyses were performed between (1) normal weight and overweight or obese women defined as having a BMI of <25.0 and ≥25.0 kg/m^2, respectively, (2) nonpregnant and pregnant women at the start of the program, and (3) women-only participants and couples, who were defined as the woman and her male partner who followed his own personal coaching program at the same time, which was also dependent on pregnancy status. To create the area under the curve (AUC) of the linear predictor as an overall measure of effectiveness of the program, we calculated the average of the log odds ratio at the specific time points. For each subgroup, this average was compared with that of its complement (eg, obese versus nonobese, pregnant versus nonpregnant, and couples versus women without a participating male partner). SPSS version 21.0 (IBM Corp, Armonk, NY) software package was used and the level of significance was set to 0.05 for all analyses.

Ethical approval

All data were anonymously processed. This survey was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving patients were approved by the Medical Ethical and Institutional Review Board of the Erasmus MC, University Medical Center, Rotterdam, the Netherlands. Digital informed consent was obtained from all participants, allowing us to use the data for analysis.

RESULTS

Compliance and usability

Study compliance was 64.86% (1218/1878) among all participants who activated the program. Additional digital evaluation forms sent every 4 months to new participating women were received from 357 women out of 1878 (19.01%), of which 69.2% (247/357) were highly educated. The usability of the program was judged as positive or very positive by 54.7% of participants, and ranged from 39.2% (content and coaching) to 73.4% (design and interface) (see Figure 2).

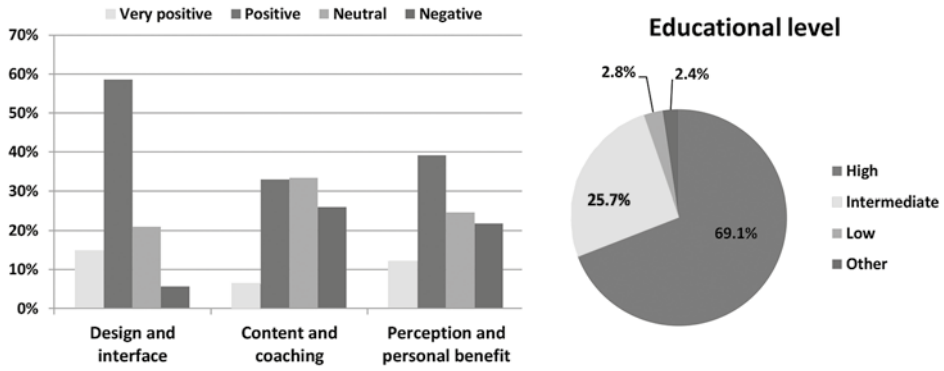


Figure 2 | Results of the evaluation of usability based on 357 evaluation forms. Usability of the Smarter Pregnancy program was subdivided into three program characteristics (left) and by participant educational levels (right).

Baseline characteristics

We evaluated 1878 out of 2003 (93.76%) participants after exclusion of 125 (6.24%); these participants were excluded because of nonactivation due to incomplete registration or no data entry after subscribing to the application (see Figure 3). The baseline characteristics of the cohort (n= 1878) who completed or prematurely resigned from the platform are depicted in Table 1. They are classified according to gender and further subdivided into groups that (1) completed the last screening and (2) resigned prematurely from the platform. No significant differences were observed in women and men that completed or resigned prematurely from the platform with regard to age, height, BMI, percentage of overweight and obesity, mean vegetable and fruit intake, percentage of inadequate folic acid supplement, and tobacco and alcohol use. The woman-to-man ratio of the participants was 4.3 to 1. Of the total group of 1525 registered women, 603 (39.54%) reported to be pregnant at baseline, of which 416 (69.0%) completed the program and 187 (31.0%) prematurely resigned (p= 0.04).

Baseline nutrition and lifestyle behaviors

Adequate nutrition and lifestyle behaviors at baseline were 21.57% (405/1878) for vegetable intake, 52.61% (988/1878) for fruit intake, 85.44% (1303/1525) for folic acid use, 86.79% (1630/1878) for no tobacco use, and 64.43% (1210/1878) for no alcohol consumption. The most prevalent inadequate behavior among both women and men was vegetable intake, which was 78.75% (1201/1525) and 77.1% (272/353), respectively. Inadequate fruit intake was observed in 43.21% (659/1525) of the women and 65.4% (231/353) of the men, whereas only 14.56% (222/1525) of the women reported no folic acid supplement use. Tobacco use was reported for 11.34% (173/1525) and 21.2% (75/353) of the women and men, respectively. Alcohol consumption was reported in 27.73% (423/1525) of all women and 69.4% (245/353)

of all men. Women who resigned from the platform prematurely showed a significantly higher percentage of alcohol consumption of 31.6% (165/522) versus 25.72% (258/1003) (p= 0.02).

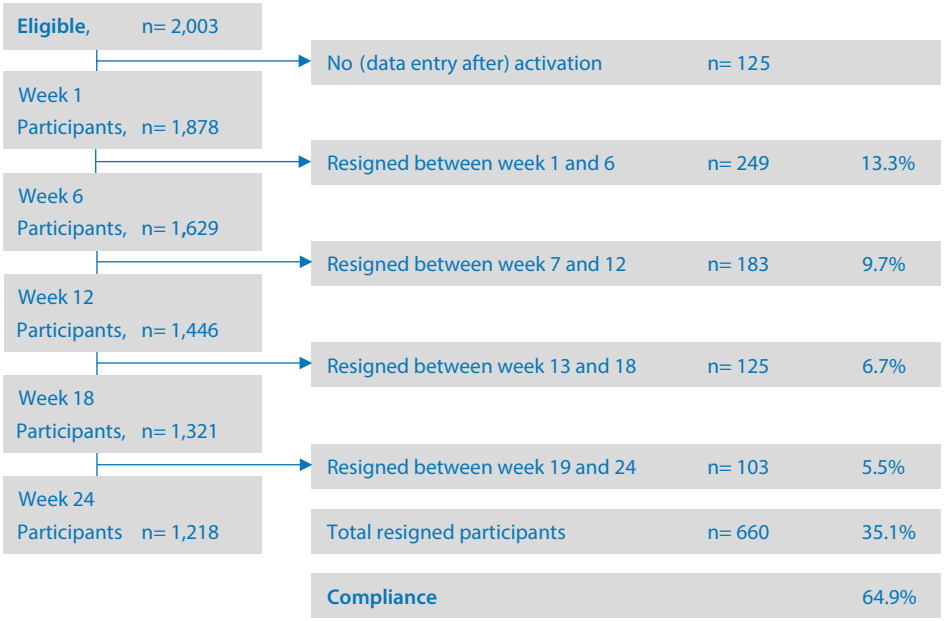


Figure 3 | Flowchart of the Smarter Pregnancy survey. Percentages are based on the total participants (n= 1878) in week 1.

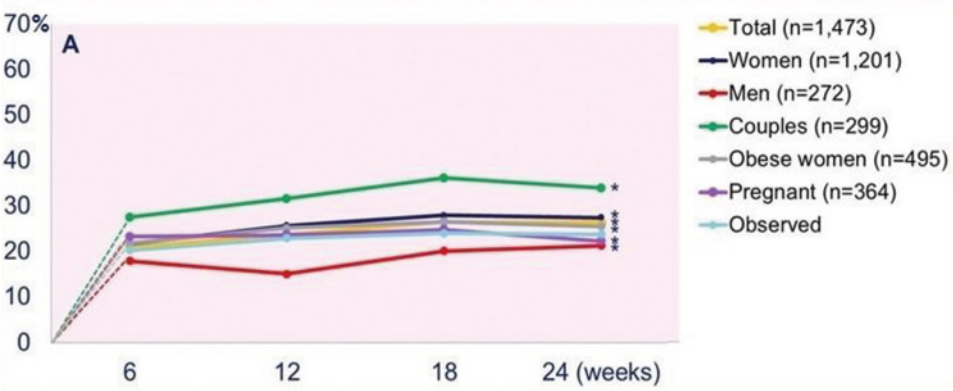
Effectiveness

Figure 4 depicts the changes in nutrition and lifestyle behaviors of the total and specific subgroups. Results at every follow-up screening point have been compared to baseline values. At baseline, vegetable intake was inadequate in 1473 out of 1878 participants (78.43%). An improvement of 20.9% (95% CI 18.5 to 23.5) was observed after 6 weeks and persisted to an increase up to 26.3% (95% CI 23.0 to 29.9) at 6 months (see Figure 4, A). Inadequate fruit intake was observed in 890 out of 1878 participants (47.39%) at baseline and improved by 36.1% (95% CI 33.0 to 39.3) and 38.4% (95% CI 34.5 to 42.5) at 6 weeks and 6 months, respectively (see Figure 4, B). The figures for inadequate folic acid supplement use observed in 222 out of 1525 women (14.56%) showed a decrease of 53.6% (95% CI 46.8 to 60.3) and 56.3% (95% CI 48.8 to 63.6) at 6 weeks and 6 months, respectively (Figure 4, C). At baseline, the prevalence of tobacco and alcohol use was 248 out of 1878 (13.21%) and 668 out of 1878 (35.57%), respectively. Tobacco and alcohol use were further reduced by 23.8% (95% CI 16.8 to 32.6) and 27.0% (95% CI 22.4 to 32.1) at 6 weeks and 35.1% (95% CI 29.1 to 41.6) and 41.9% (95% CI 35.2 to 48.9) at 6 months, respectively (Figure 4, D and E). All percentages are depicted in Multimedia Appendix 1.

Table 1 | Baseline characteristics of participants.

	Women (n= 1525)		P value	Men (n= 353)		P value
	Completed (n= 1003)	Stopped (n= 522)		Completed (n= 215)	Stopped (n= 138)	
General						
Age (years), median (IQR[a])	31.2 (27.7-34.6)	31.5 (27.9-35.2)	.81 [b]	33.7 (30.1-37.0)	34.6 (30.4-38.1)	.64 [b]
Height (cm)	169.0 (164.0-174.0)	170.0 (165.0-175.0)	.53 [b]	183.0 (179.0-190.0)	185.0 (181.0-188.0)	.16 [b]
Pregnant (yes), n (%)	416 (41.5)	187 (35.9)	.04 [c]	– [d]	–	–
BMI (kg/m^2)						
Total group BMI	24.0 (21.3-27.6)	24.0 (21.7-27.0)	.53 [b]	25.2 (23.7-27.8)	25.3 (23.2-27.5)	.30 [b]
Overweight (BMI 25-30)	27.1 (25.8-28.4)	26.7 (25.9-28.1)	.25 [b]	26.6 (25.5-28.1)	27.2 (25.9-28.2)	.48 [b]
Overweight, n (%)	266 (26.5)	139 (26.7)		96 (44.7)	62 (45.0)	
Obese (BMI 30–60)	32.9 (31.3-35.8)	32.7 (31.2-36.1)	.52 [b]	31.3 (30.8-35.1)	31.7 (30.3-35.1)	.42 [b]
Obese, n (%)	141 (14.1)	68 (13.0)		22 (10.2)	10 (7.2)	
Nutrition						
Total group vegetable intake (g/day)	135.7 (96.4-185.7)	142.9 (100.0-185.7)	.90 [b]	142.9 (100.0-192.9)	150.0 (107.1-185.7)	.88 [b]
Inadequate vegetable intake (<200)	785 (78.3)	416 (79.9)	.23 [c]	162 (75.3)	79.7 (110)	.19 [c]
Total group fruit intake (pieces/day)	2.3 (1.3-3.4)	2.1 (1.3-3.3)	.32 [e]	1.4 (0.7-2.3)	1.4 (0.5-2.2)	.46 [e]
Inadequate fruit intake (<2)	427 (42.6)	232 (44.6)	.23 [c]	139 (64.7)	92 (66.7)	.29 [c]
Lifestyle						
Folic acid (no), n (%)	150 (15.0)	72 (13.8)	.59 [c]	–	–	–
Smoking (yes), n (%)	119 (11.9)	54 (10.3)	.40 [c]	48 (22.3)	27 (19.6)	.60 [c]
Alcohol (yes), n (%)	258 (25.7)	165 (31.7)	.02 [c]	151 (70.2)	94 (68.1)	.72 [c]

Data are presented as median (Interquartile range) and number(percentages) unless stated otherwise. [a]IQR: interquartile range. [b]Independent t test. [c]Pearson chi-square test. [d]N/A: not applicable. [e]Mann Whitney U test.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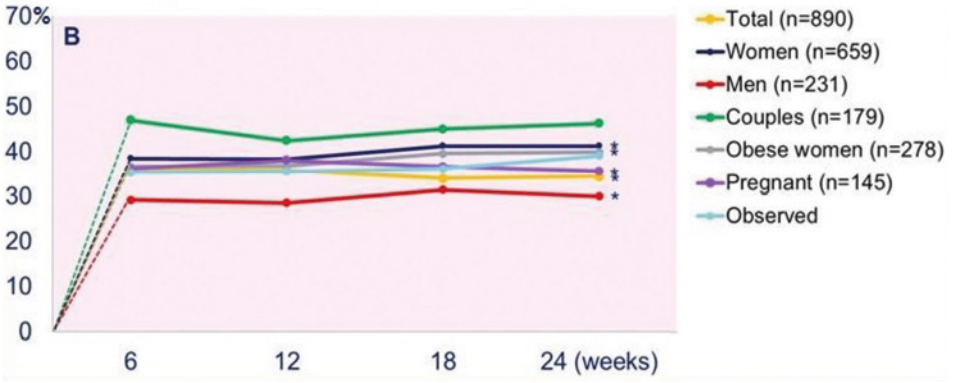
24 (weeks)
Total (n=1,473)
Women (n=1,201)
Men (n=272)
Couples (n=299)
Obese women (n=495)
Pregnant (n=364)
Observed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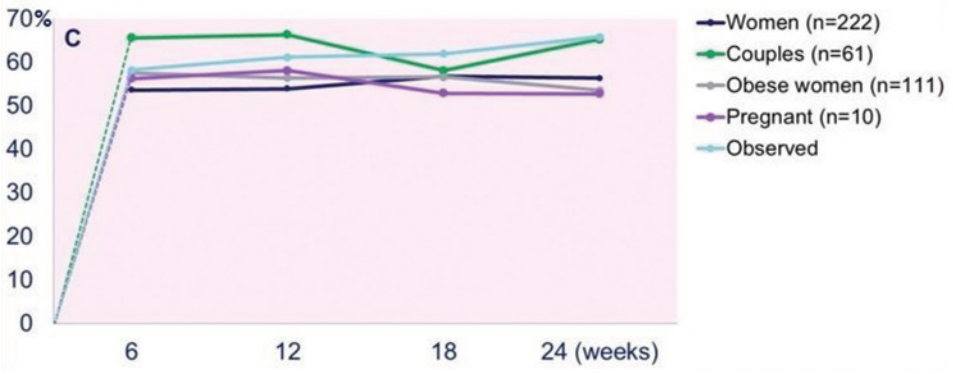
24 (weeks)
Total (n=890)
Women (n=659)
Men (n=231)
Couples (n=179)
Obese women (n=278)
Pregnant (n=145)
Observed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Women (n=222)
Couples (n=61)
Obese women (n=111)
Pregnant (n=10)
Observed

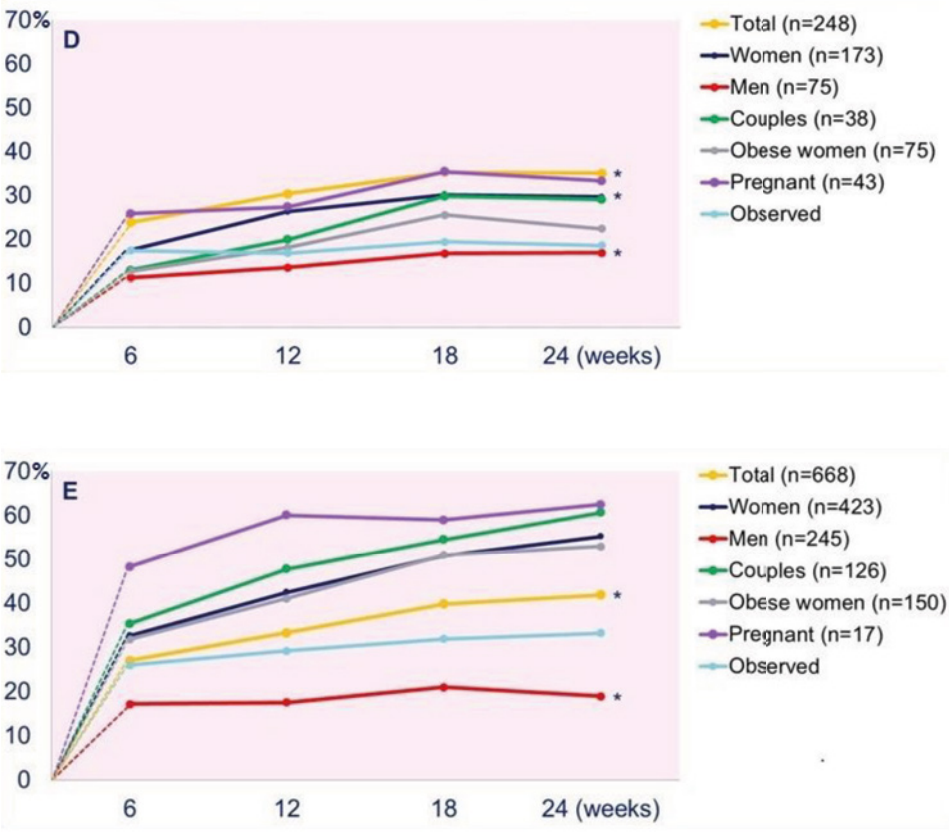

Figure 4 | Vegetable intake (A), fruit intake (B), folic acid use (C), tobacco use (D), and alcohol consumption (E) by participants. Improvement of behavior from inadequate at baseline to adequate at every screening point is shown as the percentage (y-axis) of the total group or subgroup. The dotted lines representing the change in relation to baseline are included to improve the interpretation of the graphs. *p <0.05 at all screening points. All percentages (per screening point) and areas under the curve, including P values, are included in Multimedia Appendix 1.

Subgroup: overweighed and obese women

Baseline screening revealed 614 out of 1525 (40.26%) and 190 out of 353 (53.8%) overweight and obese women and men, respectively. Subgroup analysis showed patterns of inadequate nutrition and lifestyle behaviors in these women and men comparable to the total group (see Figure 4). The AUCs of the five inadequate lifestyle behaviors were comparable in overweight and obese (BMI ≥25 kg/m^2) and nonobese (BMI <25 kg/m^2) women and men (see Multimedia Appendix 1).

Subgroup: women pregnant at entry

A trend of comparable improvement of vegetable, fruit, and folic acid intake was shown in pregnant and nonpregnant women. Cessation of tobacco and alcohol use was higher in

pregnant women although the groups were small (n= 10 and n= 17, respectively). The AUCs did not differ significantly (see Multimedia Appendix 1).

Subgroup: couples

A total of 353 couples were coached, of which 215 (60.9%) completed the 6 months of coaching. The program was most effective on changing inadequate nutrition and lifestyle behaviors, except for tobacco use, when both the women and men used the program compared to the group of women only (see Figure 4).

DISCUSSION

Smarter Pregnancy is the first CE-1-certified, Web-based, personal mHealth platform tailored to convert inadequate to adequate nutrition and lifestyle behaviors in couples during the prepregnancy and pregnancy periods. This survey highlights the very high prevalence of inadequate intake of vegetables, fruit, and folic acid supplements, as well as tobacco and alcohol use in both women and men in the prepregnancy and pregnancy periods. Previous research by Hammiche et al. and Vujkovic et al targeting the same period showed comparable results for inadequate vegetable and fruit intake (32.7-80.6%), inadequate folic acid supplement use (18.9-37.9%), tobacco use (11.3-31.0%), and alcohol use (35.5-66.0%) [9,59]. Screening tools and programs, such as *ZwangerWijzer* [97] and *Healthy Pregnancy 4 All*, have been developed and are being implemented [98,99]. However, routine preconceptional care is still only scarcely available. There is some evidence from other groups substantiating that eHealth and mHealth can support and enhance preventive preconceptional health care interventions.

The strengths of this survey are the high number of participants (n= 1878), the high compliance of 64.86% (1218/1878) of participants to complete the 6 months of coaching, the positive feedback of the usability, participation of couples, and the analysis in which selection bias was limited by multiple imputation. The high appreciation of usability and initial effectiveness of this program on improving lifestyle behaviors suggests increased awareness and strong adherence to the given insights and recommendations. A possible explanation for these results is the multifunctional, interactive, and individual character of the coaching, which is distinctive compared to most eHealth and mHealth tools providing information only without taking individual conditions into account. Other strengths are the prospective and automatic data collection, as well as the subgroup analyses addressing the influence of pregnancy status, overweight and obesity, gender, and the participation of individuals or couples.

Our previous research has shown that a short self-administered risk score is a valid method to identify adequate or inadequate vegetable and fruit intake on both food group and nutrient levels [88].

Moreover, the percentages of these inadequate nutrition and lifestyle behaviors are in line with our data from the preconceptional outpatient clinic [6,59].

Limitations of this survey are the absence of validation by biomarkers and, inherent to the design of a survey, the absence of a control group. Moreover, using the Internet and a website in the Dutch language excludes groups using other languages and those having less access to the Internet. In general, the endless opportunities of mHealth tools and knowing how to access them can be of unprecedented importance, especially with regard to health care. The rise of mobile technology by mobile phones, with more than one billion users worldwide, and other handheld devices also contributes to accessibility regarding online information and recommendations concerning healthy nutrition and lifestyle behaviors during the preconceptional period [99,100].

Couples contemplating pregnancy are often unaware of the availability and importance of these recommendations [48,59,81,101]. Unfortunately, health care professionals are often unfamiliar with up-to-date, evidence-based preconception care; it should be their responsibility to educate and increase patient awareness concerning healthy lifestyle behaviors in order to improve their chances to conceive and ensure a healthy prenatal environment for all couples [81]. Our findings contribute to previous research suggesting that both women and men should be involved in preconceptional care [102].

We demonstrated that the support of the partner by utilizing the same platform increases the effect of this intervention. It is known that changing inadequate nutrition and lifestyle behaviors and maintaining healthy behavior is hard to accomplish, especially when there is a possibility that the goal to become pregnant will not be reached. Currently, only a small group of women that will not conceive spontaneously and those with a previous complicated pregnancy may receive preconceptional counseling by a health care professional (eg, general practitioner or gynecologist). Because the Smarter Pregnancy program has the potential as an mHealth platform to reach and educate a much larger population, including men, its use and implementation in health care is of interest to patients, health care professionals, and health care insurance companies to reduce health care costs in the future. The initial results of this survey were encouraging; this opens up the opportunity of implementation and conducting randomized controlled trials to further substantiate the findings on changing nutrition and lifestyle behaviors, and to further demonstrate the clinical effectiveness and cost-effectiveness of this mHealth platform in several target groups.

In conclusion, Smarter Pregnancy is a mHealth Web-based coaching platform that has the potential to improve and maintain healthy nutrition and lifestyle behaviors in women as well as men and, in particular, couples in the prepregnancy and pregnancy periods. These findings are important for further improvement of the quality and accessibility of preconceptional and pregnancy care, fertility, pregnancy course and outcome, and ultimately health from the earliest moment and throughout the life course.

Multimedia Appendix 1

	N	T= 6	T= 12	T= 18	T= 24	AUC-sub	AUC –compl	Diff.	P-value
Vegetable intake, % (95% CI)									
Observed	–	20.2	22.8	23.9	23.7	N/A	N/A	N/A	N/A
Total	1,473	20.9 (18.5-23.5)	23.6 (21.2-26.2)	26.5 (23.0-30.2)	26.3 (23.0-29.9)	-1.15	N/A	N/A	N/A
Women	1,201	21.6 (19.0-24.4)	25.6 (22.8-28.6)	27.9 (24.3-31.9)	27.4 (23.8-31.4)	-1.07	-1.56	0.49	.004
Men	272	17.8 (12.8-24.2)	15.0 (10.2-21.3)	20.0 (14.5-26.8)	21.2 (15.5-28.3)	-1.56	-1.07	-0.49	.004
Pregnant	364	23.2 (17.8-29.6)	23.6 (18.6-29.4)	24.7 (18.9-31.7)	22.2 (16.7-29.0)	-1.21	-1.02	0.19	.177
Overweight and obese	495	21.7 (18.1-25.7)	25.1 (20.7-30.0)	26.5 (21.4-32.3)	25.5 (20.0-31.9)	-1.12	-1.04	-0.08	.519
Couples	299	27.4 (22.4-33.0)	31.6 (25.5-38.3)	36.1 (29.5-43.3)	33.9 (27.8-40.5)	-0.74	-1.20	0.46	.0009
Fruit intake, % (95% CI)									
Observed	–	35.4	35.6	36.2	39.1	N/A	N/A	N/A	N/A
Total	890	36.1 (33.0-39.3)	35.8 (31.9-40.0)	38.7 (34.0-43.8)	38.4 (34.5-42.5)	-0.53	N/A	N/A	N/A
Women	659	38.5 (34.6-42.6)	38.4 (33.1-43.9)	41.3 (35.4-47.6)	41.3 (36.1-46.7)	-0.41	-0.88	0.47	.005
Men	231	29.2 (23.3-35.8)	28.6 (22.3-35.7)	31.5 (25.2-38.6)	30.0 (21.4-40.2)	-0.88	-0.41	-0.47	.005
Pregnant	145	36.3 (28.2-45.2)	38.1 (30.1-46.8)	36.7 (26.7-47.9)	35.7 (24.2-49.0)	-0.54	-0.38	0.17	.355
Overweight and obese	278	36.7 (29.9-44.0)	36.8 (28.5-46.0)	39.6 (30.6-49.3)	39.9 (30.0-50.7)	-0.48	-0.37	0.11	.482
Couples	179	47.0 (38.2-56.0)	42.5 (34.2-51.1)	45.0 (36.0-54.4)	46.3 (37.1-55.6)	-0.22	-0.49	0.27	.087
Folic acid supp. use, % (95% CI)									
Observed	–	58.2	61.1	61.9	65.8	N/A	N/A	N/A	N/A
Women	222	53.6 (46.8-60.3)	53.9 (46.5-61.1)	56.8 (48.7-64.5)	56.3 (48.8-63.6)	0.18	N/A	N/A	N/A
Pregnant	10	56.2 (2.3-98.6)	58.0 (3.0-98.4)	52.9 (8.9-92.8)	52.7 (7.6-93.8)	-0.72	0.21	-0.93	.577
Overweight and obese	111	57.5 (47.1-67.2)	56.4 (46.9-65.5)	56.6 (45.8-66.8)	53.5 (42.8-64.0)	0.27	0.09	0.18	.47
Couples	61	65.6 (52.0-77.0)	66.3 (50.1-79.5)	58.1 (43.9-71.0)	65.3 (52.5-76.2)	0.55	0.04	0.51	.099

Multimedia Appendix 1 | *(Continued)*

	N	T= 6	T= 12	T= 18	T= 24	AUC-sub	AUC –compl	Diff.	P-value
Smoking, % (95% CI)									
Observed	–	17.4	16.8	19.3	18.6	N/A	N/A	N/A	N/A
Total	248	23.8 (16.8-32.6)	30.4 (24.4-37.2)	35.3 (28.5-42.8)	35.1 (29.1-41.6)	-0.85	N/A	N/A	N/A
Women	173	25.4 (17.4-35.3)	34.1 (26.2-42.9)	38.7 (30.1-48.0)	38.1 (29.7-47.4)	-0.72	-1.18	0.46	.110
Men	75	20.2 (11.3-33.4)	21.8 (13.5-33.3)	27.4 (16.7-41.5)	27.9 (16.9-42.5)	-1.18	-0.72	-0.46	.110
Pregnant	43	25.8 (12.2-46.6)	27.4 (15.1-44.4)	35.5 (15.5-62.1)	33.3 (16.9-55.0)	-0.86	-0.68	-0.18	.617
Overweight and obese	75	22.0 (12.7-35.5)	29.0 (18.0-43.1)	39.7 (25.5-55.8)	35.4 (22.4-51.0)	-0.84	-0.65	-0.19	.552
Couples	38	27.2 (12.9-48.4)	34.7 (19.9-53.1)	45.3 (29.8-61.7)	44.7 (29.0-61.6)	-0.63	-0.75	0.12	.736
Alcohol consumption, % (95% CI)									
Observed	–	25.3	29.2	31.9	33.3	N/A	N/A	N/A	N/A
Total	668	27.0 (22.4-32.1)	33.3 (29.8-37.1)	39.8 (34.3-45.6)	41.9 (35.2-48.9)	0.63	N/A	N/A	N/A
Women	423	32.7 (27.2-38.6)	42.5 (37.0-48.0)	50.7 (44.3-57.1)	55.2 (46.1-63.9)	-0.22	-1.49	1.27	.031
Men	245	17.2 (12.3-23.5)	17.5 (12.7-23.7)	21.0 (14.6-29.2)	18.9 (13.7-25.5)	-1.49	-0.22	-1.27	.031
Pregnant	17	48.2 (25.9-71.3)	60.0 (35.5-80.4)	58.9 (31.8-81.5)	62.4 (35.5-83.3)	0.22	-0.24	0.46	.325
Overweight and obese	150	31.9 (24.1-40.8)	41.0 (33.3-49.3)	50.9 (42.0-59.8)	52.9 (37.6-67.7)	-0.25	-0.20	-0.05	.788
Couples	126	35.4 (26.4-45.5)	47.8 (36.0-59.8)	54.5 (41.1-67.2)	60.7 (50.1-70.3)	-0.04	-0.30	0.26	.207

Data are presented per risk factor per screening point as the percentage of improvement from inadequate to adequate behavior of the total group or subgroup, including the 95% confidence interval. Area under the curve (AUC) is presented as log odds ratio: AUC subgroup versus AUC complement, difference, and corresponding P value.

PART 2

Periconceptional diet and PCOS

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Chapter

4

Are dieting and dietary inadequacy a second hit in the association with polycystic ovary syndrome severity?

ABSTRACT

Background

The composition of the diet is of increasing importance for the development and maturation of the ovarian follicles. In Polycystic Ovary Syndrome (PCOS) healthy dietary interventions improve the clinical spectrum. We hypothesized that dieting and diet inadequacy in the reproductive life course is associated with impaired programming of ovarian follicles and contributes to the severity of the PCOS phenotype.

Methods and Findings

To determine associations between the use of a self-initiated diet and diet inadequacy and the severity of the PCOS phenotype, we performed an explorative nested case control study embedded in a periconception cohort of 1,251 patients visiting the preconception outpatient clinic.

218 patients with PCOS and 799 subfertile controls were selected from the cohort and self-administered questionnaires, anthropometric measurements and blood samples were obtained. The Preconception Dietary Risk Score (PDR score), based on the Dutch dietary guidelines, was used to determine diet inadequacy in all women. The PDR score was negatively associated to cobalamin, serum and red blood cell folate and positively to tHcy. PCOS patients (19.9%), in particular the hyperandrogenic (HA) phenotype (22.5%) reported more often the use of a self-initiated diet than controls (13.1%; p= 0.023). The use of an inadequate diet was also significantly higher in PCOS than in controls (PDR score 3.7 vs. 3.5; p= 0.017) and every point increase was associated with a more than 1.3 fold higher risk of the HA phenotype (adjusted OR 1.351, 95% CI 1.09-1.68). Diet inadequacy was independently associated with the anti-Müllerian Hormone (AMH) concentration (β 0.084; p= 0.044; 95% CI 0.002-0.165) and free androgen index (β 0.128; p= 0.013; 95% CI 0.028-0.229).

Conclusions

The use of a self-initiated diet and diet inadequacy is associated with PCOS, in particular with the severe HA phenotype. This novel finding substantiated by the association between diet inadequacy and AMH needs further investigation.

INTRODUCTION

Polycystic Ovary Syndrome (PCOS) is the most common endocrine disorder affecting young women in reproductive age, resulting in subfertility and increased risks of cardiovascular related disease in later life [103]. The estimated prevalence of PCOS varies between 5-15% [22,23]. PCOS is characterized by oligo- or anovulation (ovulatory dysfunction; OD), clinical and/or biochemical signs of hyperandrogenism (HA) and/or polycystic ovaries (Polycystic ovarian morphology; PCOM), and gene-environment interactions play a significant role in the variability within PCOS phenotypes [24,104]. The clinical spectrum and severity in PCOS is heterogeneous, including reproductive, metabolic and psychological features. Because of the profound cardiovascular and metabolic disturbances in hyperandrogenic PCOS patients, it is recommended to distinguish two subtypes of PCOS according to the severity of disease (HA-PCOS and non HA-PCOS) [25]. Personal behaviors, such as dieting, smoking, alcohol- and drug use, stress and exercise are considered environmental factors that are important for reproductive functioning [20,81]. The composition of the preconception diet is of increasing interest, because over- and undernutrition derange metabolic and endocrine pathways, especially the B vitamin dependent one-carbon (1-C) pathway [20,42,105]. During the development of the germ cells and the maturation of the ovarian follicles, the methyl groups mainly derived from B vitamins, methionine and choline are used for DNA synthesis and phospholipid and protein biosynthesis. Deficiencies of these nutrients are inversely associated with ovarian follicle development, the number of oocytes retrieved for IVF treatment, embryo quality and pregnancy outcome [20,42,105]. Worrisome is that in more than 50% of subfertile couples planning pregnancy the preconception diet is inadequate [59]. However it is promising that improvement of the diet can increase the chance of ongoing pregnancy up to 65% [6]. The prevalence of obesity and PCOS is increasing and although obesity is not the cause of PCOS it contributes to the clinical spectrum [106]. Moreover, weight loss can improve the clinical spectrum including menstrual regularity, insulin resistance and quality of life [33] and dietary interventions are therefore part of the clinical treatment of PCOS. In the polycystic ovary, an arrest of follicular maturation results in the accumulation of small antral follicles that produce elevated levels of Anti-Müllerian Hormone (AMH) [29]. AMH concentration is therefore a marker of PCOS severity which is inversely associated with a healthy diet, irrespective of weight loss [107]. Psychological co-morbidities such as depression, eating and anxiety disorders, which can affect the nutritional state are also more prevalent in PCOS [108]. Another potential driver for differences in dieting could be obesity although previous research showed that dieting behavior is independent of current BMI, but strongly associated with negative emotions and problematic behaviors [109]. Dietary behavior is acquired during childhood and remains rather stabile over time only varying during episodes of illnesses, dieting and increased needs [110-113]. Dieting habits can be transferred unknowingly since the practices mothers adopt predict the children's diet quality [114] and because parents contribute to adolescents' motivation to diet [115].

Here we hypothesize that regular self-initiated dieting and using an inadequate diet is associated with alterations in the 1-C pathway and with impaired functioning of ovarian follicles which contributes to the clinical spectrum of PCOS. We performed a case-cohort study embedded in a prospective periconception cohort study of couples referred for subfertility, to investigate whether the use of a self-initiated diet and dietary inadequacy are associated with PCOS severity and phenotype.

MATERIALS AND METHODS

Study design

Between October 2007 and March 2011, all couples planning pregnancy and visiting the outpatient clinic of the Department of Obstetrics and Gynaecology at the Erasmus MC, University Medical Center Rotterdam, a tertiary hospital in the Netherlands, were offered nutrition and lifestyle counselling at the preconception outpatient clinic 'Achieving a Healthy Pregnancy' [6,59].

In this study women completed a questionnaire from which we extracted general characteristics, such as age, ethnicity (Dutch or Non-Dutch), educational level (low, intermediate or high), the use of a diet (energy restricted, vegetarian, macrobiotic, vegan, other) and of six main food groups (Preconception Dietary Risk score), folic acid supplements (yes/no), vitamin supplements (yes/no), medication (yes/no), alcohol (yes/no), and smoking (yes/no), physical exercise (yes/no), and the experience of stress (yes/no). Ethnicity and education level were classified according to the definition of Statistics Netherlands (www.cbs.nl; 2012). The validated summary PDR score, based on the Dutch food based dietary guidelines was used to assess diet inadequacy in the outpatient clinical setting [6,88]. When patients did not meet the dietary guidelines for the food group, one point was administered. Since six guidelines were evaluated, the PDR score had a maximum of 6 points and a minimum of 0 points. Thus, the higher the score the more inadequate the intake according to the guideline. The adequate intake per food group was defined by the following guidelines: 4-5 slices of whole wheat bread daily, the use of monounsaturated or polyunsaturated oils/fats, 200 grams of vegetables daily, 2 pieces of fruit daily, 3-4 servings of meat weekly and 2 servings of fish weekly [61]. During the visit at the outpatient clinic the questionnaires were verified by a trained counsellor.

Subsequently non-fasting blood samples were obtained by venipuncture and anthropometrics (i.e. height, weight and circumferences) were measured. Patients with oligo- or anovulation were screened for ovulatory dysfunction by trained professionals according to a standardized protocol.

The diagnose of PCOS was based on the Rotterdam criteria [24] including the presence of oligo- or anovulation (i.e. menstrual bleeding interval between 35-182 days or absence of menstrual bleeding for more than 182 days), hyperandrogenism (Ferriman-Gallwey score ≥9 and/or a Free Androgen Index >4.5), and/ or polycystic ovaries (volume exceeding 10 cm^3 and/ or the follicle count was ≥12).

Study population

During the study period 1,348 couples visited the preconception outpatient clinic of which 1,251 participated in the study and provided a written informed consent. Two hundred and fifty one patients were diagnosed with PCOS and 33 patients were excluded because of an uncertain diagnosis or the period between enrolment and PCOS diagnosis comprised more than one year. From the remaining 1,000 patients we excluded patients with an incomplete fertility medical record (n= 80), patients with ovulatory disorders such as imminent ovarian failure, WHO 1 or WHO 2 disorders (n= 107) and fertile patients (n= 14) who were referred because of a complicated obstetrical history. This resulted in a control group of 799 subfertile patients (Figure 1). According to phenotype, we divided the PCOS group (n= 218) in the HA (n= 112) and non HA (n= 106) phenotype.

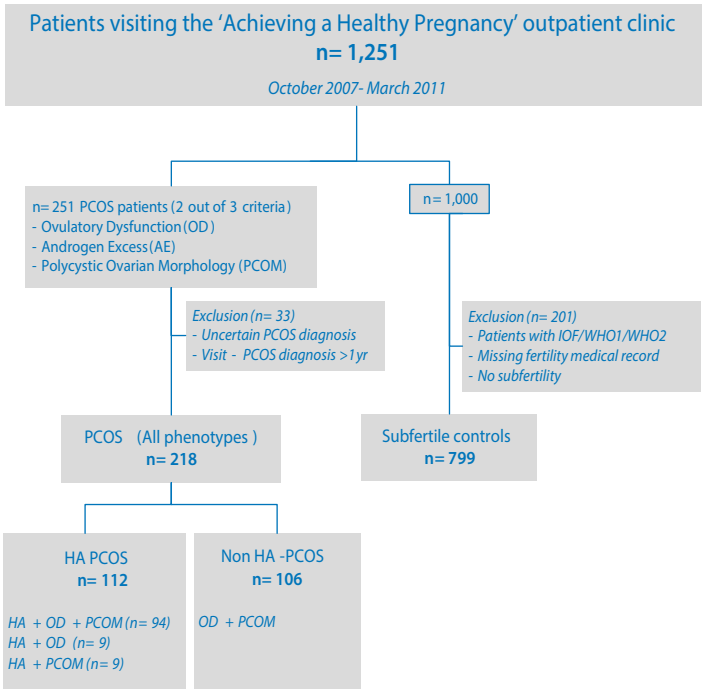

Figure 1 | Flowchart of the selection of the study population.
Abbbreviations; IOF = imminent ovarian failure; WHO1 = hypogonadotropic hypogonadal anovulation; WHO2 = normogonadotropic normoestrogenic anovulation; HA = hyperandrogenic.

Biomarkers

On the day of the visit at the outpatient clinic blood samples were drawn and processed within 2 hours after withdrawal. Serum was stored at -20°C until assayed. In blood the biomarkers of 1-C metabolism serum and red blood cell (RBC) folate, serum cobalamin, and plasma total homocysteine (tHcy) were determined in all study participants. tHcy was measured using

HPLC-TandemMS. For the determination of serum folate and cobalamin an immune electro chemoluminescence assay was used (E170; Roche Diagnostics GmbH, Mannheim, Germany) and for the determination of RBC folate the ADVIA 120 Hematology Analyzer was used (Bayer Diagnostics, Leverkusen, Germany).

For clinical purposes the patients with PCOS were additionally screened on hormonal and metabolic parameters. Testosterone was measured using radioimmunoassay (RIA) (Siemens DPC, Los Angeles, CA) and glucose levels were measured by using a Hitachi 917 analyzer (Roche Diagnostics, Almere, The Netherlands). The Free Androgen Index (FAI) was calculated as follows; 100 x [T (nmol/L) / Sex hormone-binding globulin (SHBG) (nmol/L)]. An immunoluminometric assay (Immulite® platform, Siemens Diagnostic Product Corporation, Los Angeles, CA) was used to measure LH, FSH, SHBG, androstenedione, insulin and DHEAS. Serum AMH (Anti-Müllerian Hormone) levels were measured by using an in-house double-antibody ELISA (GenII; Beckman Coulter) as previously described [116]. Intra- and interassay coefficients of variation were, respectively, <5% and <15% for LH, <3% and <18% for FSH, <3% and <5% for T, <8% and <11% for AD, <4% and <5% for SHBG, <9% and <11% for DHEAS, <5% and <8% for AMH, and <6% and <8% for insulin.

Statistical analyses

Continuous variables are presented as median with interquartile range. Dichotomous and categorical variables are presented as count and proportions. Chi Square tests with comparison of column proportions were performed with a Bonferroni correction to compare the distribution of categorical variables between PCOS phenotypes and subfertile controls. Mann Whitney U Tests were performed to compare the total group of PCOS with the subfertile controls. Kruskal Wallis tests with pairwise comparisons adjusted for the number of pairwise tests were performed for comparison of controls, HA-PCOS patients and non HA-PCOS patients. Simple linear regression analysis were conducted to establish associations between the PDR score and the biomarkers of 1-C metabolism. To achieve normality a natural log transformation was performed for cobalamin, RBC Folate and tHcy and a square root transformation for serum folate. Multivariable logistic regression analysis was applied to calculate the risk of PCOS after one point increase of the PDR score for each phenotype separately with adjustment for the potential confounders body mass index (BMI), age, educational level, ethnicity, medication use and vitamin supplement use. These covariates were selected because of the significant association with the PDR score or when significant differences revealed between PCOS and controls. Multivariable linear regression analysis were performed to investigate associations between the PDR score and hormonal parameters in PCOS only with adjustments. Statistical analyses were performed using SPSS software for Windows (version 21.0, IBM SPSS, Statistics for Windows, Armonk, NY: IBM Corp). The level of significance was set to 0.05 for all analyses.

Ethical approval

All questionnaire data and materials were processed anonymously. This study was conducted according to the guidelines of the Declaration of Helsinki, and all procedures involving patients were approved by the Medical Ethical and Institutional Review Board at the Erasmus MC, University Medical Center in Rotterdam, the Netherlands. Written informed consent was obtained from all patients.

RESULTS

To evaluate selection bias we present the general characteristics of the included and excluded patients in Supplemental Table 1. The general characteristics were comparable in both groups except the slightly younger age, higher percentage of smoking and alcohol use and slightly smaller waist circumference and waist-hip ratio in the study group.

PCOS study population

In Table 1 the general characteristics of PCOS patients (n= 218) and subfertile control patients (n= 799) are shown. The PCOS group was significantly younger and used less often alcohol, folic acid and vitamin supplements than subfertile controls, which is validated with the lower concentrations of serum and RBC folate observed in the PCOS group. They also used more often a self-initiated diet and were more frequently obese (BMI ≥30).

PCOS phenotypes

In Table 1 we depict the PCOS group divided into the HA (n= 112) and non HA phenotype (n= 106) and compare the general characteristics with subfertile controls (n= 799). The HA and non HA phenotype patients were both significantly younger than subfertile controls. Patients with the HA phenotype exercised less than non HA-PCOS patients and they used less often alcohol than non HA-PCOS patients or subfertile controls. Patients with the HA phenotype were also more likely to use a self-initiated diet and used less often folic acid supplements than subfertile controls and showed a significantly higher BMI and waist circumference compared to the non HA phenotype and subfertile controls. Moreover, the waist hip ratio, used as indicator of central obesity, was significantly lower in the non HA phenotype, compared to the HA phenotype and subfertile controls. Patients with the HA phenotype showed lower levels of serum and RBC folate than the non HA phenotype and subfertile controls.

Dietary inadequacy assessed with the PDR score

A higher mean PDR score was observed in the PCOS group than in the subfertile controls (3.7 vs. 3.5; p= 0.017). Patients with the HA phenotype also showed a higher mean PDR score (3.9; p <0.001) than the non HA phenotypes (3.5) and the subfertile controls (3.5). The PDR score per food group was comparable between the groups, except for a higher percentage of inadequate meat and fish intake in the HA phenotype (Figure 2).

Table 1 | Preconception general characteristics.

	Total PCOS (n= 218) (Group A)	HA-PCOS (n= 112) (Group A1)	non HA-PCOS (n= 106) (Group A2)	Subfertile controls (n= 799) (Group B)	P value (A, B)	P value (A1, A2, B)
Age (years)	28.5 (25.5-31.3)	27.7 (24.5-30.0)[a]	29.0 (27.2-32.2)[a]	33.1 (29.6-36.4)[b]	<0.01	<0.01
Ethnicity						
Dutch	122 (56.0%)	54 (48.2%)	68 (64.2%)	445 (56.0%)	1.00	0.06
Other	96 (44.0%)	58 (51.8%)	38 (35.8%)	350 (44.0%)	–	–
Educational level						
Low	30 (14.6%)	20 (18.7%)	10 (10.1%)	120 (15.8%)	0.75	0.09
Intermediate	97 (47.1%)	55 (51.4%)	42 (42.4%)	336 (44.2%)	–	–
High	79 (38.3%)	32 (29.9%)	47 (47.5%)	304 (40.0%)	–	–
Lifestyle Parameters						
Diet (Yes)	43 (19.9%)	25 (22.5%)[b]	18 (17.1%)[a, b]	105 (13.1%)[a]	0.01	0.02
PDR score (mean; sd)	3.7 (1.14)	3.9 (1.12)[a]	3.5 (1.13)[b]	3.5 (1.13)[b]	0.02	<0.01
Folic acid supplement (No)	92 (42.4%)	55 (49.1%)[b]	37 (35.2%)[a, b]	268 (33.5%)[a]	0.02	<0.01
Vitamin supplement (No)	145 (67.4%)	78 (70.3%)	67 (64.4%)	471 (59.5%)	0.03	0.07
Medication use (Yes)	75 (34.6%)	41 (36.6%)	34 (32.4%)	255 (32.0%)	0.48	0.63
Alcohol (Yes)	101 (46.3%)	39 (34.8%)[b]	62 (58.5%)[a]	439 (54.9%)[a]	0.02	<0.01
Smoking (Yes)	51 (23.5%)	29 (26.1%)	22 (20.8%)	175 (22.3%)	0.70	0.59
Physical exercise (No)	98 (51.0%)	56 (60.2%)[a]	42 (42.4%)[b]	366 (49.4%)[a, b]	0.68	0.04
Stress (Yes)	64 (34.4%)	30 (33.7%)	34 (35.1%)	245 (33.6%)	0.83	0.96
Measurements						
BMI (kg/m^2)	25.6 (22.0-31.2)	29.2 (24.9-33.2)[a]	23.1 (20.6-26.6)[b]	24.5 (22.0-28.3)[c]	0.03	<0.01
BMI categories						
<20	21 (9.6%)	3 (2.7%)[a]	18 (17.0%)[b]	54 (6.8%)[a]	<0.01	<0.01
≥20 <25	82 (37.6%)	27 (24.1%)[b]	55 (51.9%)[a]	373 (47.0%)[a]	–	–
≥25 <30	52 (23.9%)	31 (27.7%)[a]	21 (19.8%)[a]	228 (28.8%)[a]	–	–
≥30	63 (28.9%)	51 (45.5%)[b]	12 (11.3%)[a]	138 (17.4%)[a]	–	–

Table 1 | *(Continued)*

	Total PCOS (n= 218) (Group A)	HA-PCOS (n= 112) (Group A1)	non HA-PCOS (n= 106) (Group A2)	Subfertile controls (n= 799) (Group B)	P value (A, B)	P value (A1, A2, B)
Waist circumference (cm)	85 (74-97)	93 (83-105)[a]	77 (71-89)[b]	84 (75-93)[c]	0.28	<0.01
Waist hip ratio	0.81 (0.75-0.87)	0.84 (0.80-0.91)[a]	0.78 (0.74-0.83)[b]	0.81 (0.75-0.87)[c]	0.66	<0.01
Biochemical Parameters						
Cobalamin (pmol/L)	305 (232-392)	282 (215-371)	322 (254-416)	310 (237-411)	0.51	0.09
RBC Folate (nmol/L)	960 (769-1182)	861 (718-1067)[a]	1059 (869-1380)[b]	1037 (831-1328)[b]	<0.01	<0.01
Folate (nmol/L)	25.1 (15.6-36.1)	21.3 (13.4-30.9)[a]	29.1 (17.8-43.4)[b]	30.1 (19.0-41.3)[b]	<0.01	<0.01
tHcy (µmol/L)	8.8 (7.4-10.2)	8.5 (7.3-10.2)	9.1 (7.7-10.2)	8.4 (7.1-9.9)	0.08	0.12

Note: Values are expressed as median (interquartile range) or number (%). HA = Hyperandrogenic; PDR score = Preconception Dietary Risk score; BMI = Body Mass Index; RBC Folate = Red Blood Cell Folate; tHcy = homocysteine. Normal range biochemical parameters; Folate ≥8 nmol/L, RBC folate ≥500 nmol/L, Cobalamin ≥145 pmol/L, tHcy <15 µmol/L. For pairwise comparisons; each subscript letter denotes a subset of categories whose column proportions do not differ significantly from each other.

Table 2 | Multivariable logistic regression analyses; The risk of PCOS after one point increase in the Preconception Dietary Risk score compared to subfertile controls.

	HA-PCOS			non HA-PCOS		
	OR	P value	95% CI	OR	P value	95% CI
Unadjusted:	1.427	0.000	1.183-1.721	0.978	0.805	0.817-1.170
Model I: Adjusted for BMI, ethnicity, education, vitamin supplement use and medication use	1.520	0.000	1.240-1.863	0.937	0.520	0.767-1.143
Model II: Adjusted for age, BMI, ethnicity, education, vitamin supplement use and medication use.	1.351	0.007	1.087-1.679	0.866	0.176	0.703-1.067

Abbreviations; HA = Hyperandrogenic; OR = Odds Ratio; CI = Confidence Interval; BMI = Body Mass Index.

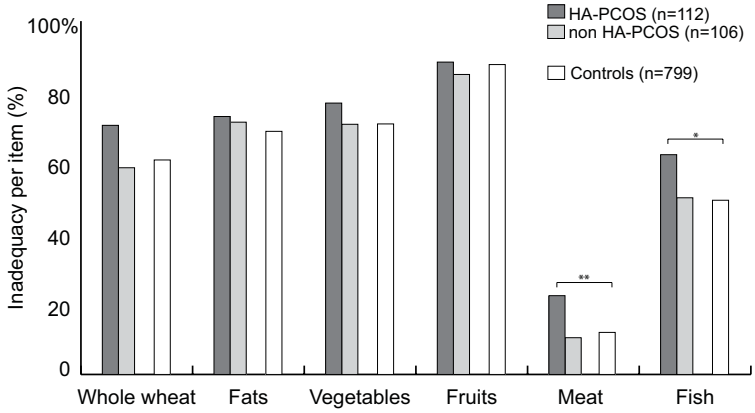

Figure 2 | The items of the Preconception Dietary risk score. Depicted are the percentages of inadequate dietary intake for each food group. Meat* and fish** intake differed significantly between the controls, HA and non HA phenotype (respectively p= 0.006 and p= 0.039)

Table 2 shows that one point increase in the PDR score was associated with a 1.4 fold higher risk of the HA phenotype (unadjusted OR 1.427, 95% CI 1.183 to 1.721). The PDR score was inversely associated with age (β -0.037, 95% CI -0.051 to -0.023, p <0.001), educational level (category high β -0.311, 95% CI -0.522 to -0.100, p= 0.004) and the use of vitamin supplements (β -0.192, 95% CI -0.336 to -0.048, p= 0.009) and therefore these covariates were selected for adjustment of the analyses. Age was a confounder and inversely associated with the severity of the PCOS phenotype and positively associated with the PDR score. After adjustment in model 1 the risk of the HA phenotype slightly increased (OR 1.520, 95% CI 1.240 to 1.863) and attenuated in model 2 (OR 1.351, 95% CI 1.087 to 1.679), but remained significant. The PDR score was not associated with the risk of the non HA phenotype.

The PDR score and biomarkers of 1-C metabolism

To investigate the association between dietary inadequacy and the markers of 1-C metabolism, we subsequently performed linear regression analyses in the PCOS patients and subfertile controls with the separate biomarkers cobalamin, RBC folate, serum folate and tHcy. A negative linear association was established between the PDR score and cobalamin (unadjusted β -0.052; p <0.001; 95% CI -0.074 to -0.030), RBC folate (unadjusted β -0.045; p <0.001; 95% CI -0.067 to -0.023) and serum folate (unadjusted β -0.193; p <0.001; 95% CI -0.295 to -0.091). A positive linear association was observed between the PDR score and tHcy (unadjusted β 0.030; p <0.001; 95% CI 0.014 to 0.045). Additional adjustment for age, BMI, ethnicity, education, vitamin supplement use and medication use slightly attenuated the effect estimates of the four biomarkers and remained significant.

Diet and hormonal and metabolic parameters in PCOS patients

In Table 3 the serum characteristics of the PCOS phenotypes are depicted. The PDR score was positively associated with AMH and FAI. A positive linear association was established between the PDR score and AMH (unadjusted β 0.084; p= 0.044; 95% CI 0.002 to 0.165), however after adjustment for age and BMI this association was no longer significant.

This association was stronger when repeated only in the HA+OD+PCOM and OD+PCOM PCOS patients (unadjusted β 0.109; p= 0.011, 95% CI 0.025 to 0.193) and remained significant after adjustment for age (β 0.100; p= 0.022, 95% CI 0.015 to 0.185) and BMI (β 0.102; p= 0.018, 95% CI 0.018 to 0.186).

The PDR score was also positively associated with FAI (unadjusted β 0.128; p= 0.013; 95% CI 0.028 to 0.229), but lost significance after adjustment for age and BMI.

Table 3 | Serum characteristics according to PCOS phenotype.

	HA-PCOS (n= 112)	non HA-PCOS (n= 106)
Hormonal Parameters		
AMH (µg/L)	10.3 (6.7-14.8)	7.6 (4.8-11.2)**
FSH (U/l)	6.1 (5.2-7.2)	5.9 (3.9-7.8)
LH (U/l)	9.8 (6.7-14.6)	6.6 (4.4-11.7)**
Progesterone (nmol/l)	1.2 (0.9-1.9)	1.5 (0.9-10.2)*
17-hydroxyprogesterone (nmol/l)	2.9 (2.2-4.4)	2.7 (1.9-5.8)
Estradiol (pmol/L)	225.5 (181.0-300.5)	234.0 (143.0-363.0)
SHBG (nmol/l)	27.0 (21.4-39.7)	56.4 (45.3-81.6)**
Testosterone (nmol/l)	2.0 (1.6-2.8)	1.2 (0.9-1.6)**
FAI	7.0 (5.5-10.7)	2.2 (1.4-3.0)**
Androstenedione (nmol/l)	11.8 (9.7-17.4)	8.6 (6.4-10.4)**
DHEAS(µmol/L)	5.7 (3.9-7.4)	4.8 (3.3-6.1)**
Metabolic Parameters		
Insulin (pmol/l)	67.0 (40.0-118.0)	34.0 (17.0-57.0)**
Glucose (mmol/l)	4.9 (4.6-5.3)	4.7 (4.5-5.0)**
TG (mmol/L)	1.0 (0.8-1.6)	0.9 (0.7-1.2)*
Total–C (nmol/L)	5.4 (4.6-6.7)	5.2 (4.5-6.7)
HDL-C (nmol/L)	1.4 (1.1-1.8)	1.7 (1.4-2.2)**
LDL-C (nmol/L)	3.8 (3.1-4.6)	3.4 (2.8-4.4)*
Apolipoprotein A1	209.6 (183.3-256.7)	239.4 (198.0-281.8)**
Apolipoprotein B	124.1 (103.6-148.8)	115.2 (84.1-136.1)**

Note: Values are expressed as median (interquartile range), * = p <0.05; ** = p <0.01. *Abbreviations;* HA = Hyperandrogenic; FSH = Follicle Stimulating Hormone; LH = Luteinizing Hormone; SHBG = Sex Hormone Binding Globulin; FAI = Free Androgen Index; DHEAS = dehydroepiandrosterone sulfate; TG = Triglycerides; Total-C = total Cholesterol; HDL-C = HDL-cholesterol; LDL-C = LDL-cholesterol. Mann Whitney U Tests were performed.

DISCUSSION

This explorative study demonstrates that patients with PCOS more often use a self-initiated diet and an inadequate diet than subfertile controls. This is substantiated by the use of a more inadequate diet in patients with the more severe HA phenotype and the positive association between diet inadequacy and the AMH concentration in PCOS patients.

The strength of the study is the large sample size and case-cohort design to reduce selection and information bias. Furthermore, standardized anthropometric measurements were performed by trained counsellors who verified all questionnaire data individually during the preconception visit at the outpatient clinic. Several adjustments were made including BMI to minimize the presence of underreporting and overreporting of dietary intake. The PDR score was previously validated in the same study population and is shown to be a sensitive and simple tool to assess diet inadequacy [88]. Another strength of the study is that biomarkers were measured to validate the PDR score and that all laboratory determinations were measured in the same hospital. The PDR score estimates dietary inadequacy and not unhealthy overconsumption which is a limitation [88]. We are aware that although the PDR score covers the six main food groups, it does not reflect the total nutrient and energy intake. The control group consisted of patients referred for subfertility which reduces the external validity of the study. We were able to use clinical and laboratory data for PCOS diagnosis, which were not available for subfertile controls. Despite numerous efforts, genetic research does not elucidate the developmental origin of PCOS. We suggest that this can be due to a more significant role of gene-nutrient interactions affecting epigenetic mechanisms of multiple pathways regulating ovarian function during the course of life. Previous research demonstrated that genes are prenatally programmed by androgen exposure in utero, resulting in disturbed ovarian and adrenal steroidogenesis [117], impaired oocyte development [118] and PCOM [119]. The diet is important in epigenetics by supplying substrates and methyl groups that are important for gene methylation, programming and subsequent expression and silencing of the genome. Dietary inadequacies can therefore lead amongst other pathways to derangements in the 1-C pathway, resulting in hypomethylation and altered gene expression [20,120]. As a first hit the nutritional environment in-utero can affect reproductive function in the offspring through impaired gonadal organogenesis before birth [120-122]. In rabbits, Leveille et al. showed prenatal and postnatal effects of the diet on ovarian function and morphology by the number of atretic follicles [123]. Not only during early life but also postnatally through puberty and adolescence, diet can affect gene expression as second or third hit influencing disease occurrence and severity. Adolescents suffering from overweight and psychological problems are at risk to develop nutritional inadequacies [108,124]. Therefore these data support our hypothesis that the use of a self-initiated diet and dietary inadequacies can contribute to a clinical heterogeneous spectrum of PCOS phenotype development. This study showed an association between the use of a more inadequate diet in PCOS patients and disease severity which is further underlined by a lower serum and RBC folate. Homocysteine concentrations

were higher as well, although this did not reach statistical significance. The study of Rodrigues et al supports this finding showing a poor diet quality in PCOS patients (56% inadequacy according to the Brazilian healthy eating index) [125]. Studies comparing diet quality in PCOS to controls are contradictory. Comparable dietary compositions in PCOS and controls are reported [126-128], whereas the group of Moran et al observed a higher diet quality in PCOS [129]. A high prevalence of folate deficiency and increased levels of homocysteine is previously observed in PCOS substantiating our results and the association with the involvement of the one-carbon pathway [130,131]. An explanation for the different findings in other studies is that in all other studies the assessment of diet inadequacy was performed always after diagnosing PCOS. Moreover, due to the exclusion of dieting, non-stabled weighted or normal weighted PCOS patients in other studies, selection bias could explain the different results. Information bias due to the awareness of PCOS and the intention to treat can also explain the differences with other studies. Finally, in contrast to other studies, we also investigated the association with the severity of the PCOS phenotypes and compared them with subfertile controls.

Our results are in line with studies showing the importance of dietary treatment in PCOS for reproductive and metabolic outcome [107,132,133]. Furthermore, in overweighed PCOS patients it is observed that folate supplement use had beneficial effects on metabolic profiles [134], which indicates the involvement of an impaired 1-C pathway in PCOS.

The positive linear association between the PDR score and AMH concentration, and PCOS severity very much supports the function of AMH as marker of the spectrum [135]. This is supported by Nybacka et al showing a decrease in AMH in PCOS patients following a 4 month calorie restricted, well-balanced diet [107]. Another observation is that PCOS gradually disappears during ageing [136,137]. We suggest that the known improvement of the diet during the course of life also contributes to its disappearance which is supported by the gradual decrease of AMH during ageing [138]. FAI as marker for androgen excess was also positively associated with diet inadequacy, which is in line with the findings of others [132]. According to the biological gradient criteria of Hill, the associations of AMH and FAI with the PDR score strengthen the relationship between diet inadequacy and PCOS severity. This is substantiated by a stronger linear association between the PDR score and AMH after exclusion of the subpopulation without PCOM (HA+OD) or without OD (HA+PCOM). After adjustment for age and BMI this association remained significant, demonstrating the heterogeneity of the hyperandrogenic phenotype.

To conclude, this explorative study shows for the first time that the use of a self-initiated diet and diet inadequacy are associated with PCOS as well as PCOS phenotype. These findings emphasize the need for prospective research from the reproductive life course onwards with a focus on the role of the inadequacy of the diet in the developmental origins of PCOS. Moreover, in PCOS treatment more attention should be given to the adherence of an adequate diet.

Supplemental Table 1 | Sensitivity analysis.

	Study population (n= 1017)	Excluded population (n= 234)
Age (years)	32.1 (28.4 -35.8)	33.1 (29.6-37.2)**
Ethnicity		
Dutch	567 (56.0%)	122 (53.5%)
Other	446 (44.0%)	106 (46.5%)
Educational level		
Low	150 (15.5%)	31 (14.2%)
Intermediate	433 (44.8%)	103 (47.2%)
High	383 (39.6%)	84 (38.5%)
Lifestyle Parameters		
Diet (Yes)	148 (14.6%)	38 (16.5%)
PDR score (mean; sd)	3.54 (1.14)	3.47 (1.20)
Folic acid supplement use (No)	360 (35.4%)	89 (38.0%)
Vitamin supplement use (No)	616 (61.2%)	129 (55.6%)
Medication use (Yes)	330 (32.6%)	78 (33.9%)
Alcohol (Yes)	540 (53.1%)	100 (42.7%)**
Smoking (Yes)	226 (22.5%)	35 (15.2%)*
Physical exercise (No)	464 (49.7%)	110 (50.5%)
Stress (Yes)	309 (33.7%)	87 (40.7%)
Measurements		
BMI (kg/m^2)	24.8 (22.0-28.9)	24.8 (22.5-28.8)
BMI in categories (kg/m^2)		
<20	75 (7.4%)	17 (7.3%)
≥20 <25	455 (45.0%)	109 (46.6%)
≥25 <30	280 (27.7%)	57 (24.4%)
≥30	201 (19.9%)	51 (21.8%)
Waist circumference (cm)	84 (75-94)	85 (77-97)*
Waist hip ratio	0.81 (0.75-0.87)	0.82 (0.77-0.90)*
Biochemical Parameters		
Cobalamin (pmol/L)	309.0 (236-409)	313.5 (246-431)
RBC Folate (nmol/L)	1022.0 (817-1303)	994.5 (815-1268.5)
Folate (nmol/L)	28.5 (18.0 -40.3)	25.6 (17.8-37.4)*
tHcy (µmol/L)	8.50 (7.1-10.0)	8.5 (7.1-10.2)

Note:Values are expressed as median (interquartile range) or number (%),* = p <0.05; ** = p <0.01. Abbreviations; PDR score = Preconception Dietary Risk score; BMI = Body Mass Index; RBC Folate = Red Blood Cell Folate; tHcy = homocysteine. Normal range biochemical parameters; Folate ≥8 nmol/L, RBC folate ≥500 nmol/L, Cobalamin ≥145 pmol/L, tHcy <15 µmol/L. Chi Square tests and Mann Whitney U Tests were performed.

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Chapter 5

Dietary patterns and the phenotype of polycystic ovary syndrome: the chance of ongoing pregnancy

ABSTRACT

Polycystic ovary syndrome (PCOS) is generally considered a complex disorder caused by interactions between genetic and environmental factors. In a sub-cohort of women with PCOS visiting the preconception outpatient clinic of a tertiary hospital with follow-up in a periconception cohort, we identified specific dietary patterns and adherence in patients with PCOS with and without hyperandrogenism and the chance of ongoing pregnancy.

Food frequency questionnaires were available from 55 patients diagnosed with PCOS during follow-up in routine clinical practice, including 25 with hyperandrogenism and 30 without hyperandrogenism. Strong adherence to the healthy dietary pattern was inversely associated with the hyperandrogenic PCOS phenotype (Adjusted OR 0.27; 95% CI 0.07 to 0.99).

In women with PCOS overall, a strong adherence to the healthy dietary pattern showed a three-fold higher chance of ongoing pregnancy (adjusted OR 3.38; 95% CI 1.01 to 11.36) and an association with anti-Müllerian hormone concentration (β -0.569 µg/L; 95% CI -0.97 to -0.17). The effect of this dietary pattern on the chance of ongoing pregnancy and AMH suggests causality, which needs further investigation in prospective studies in the general population.

INTRODUCTION

Polycystic ovary syndrome (PCOS) is an endocrine disorder with a prevalence of 5-10% and characterized by oligo- or anovulation, hyperandrogenism (HA) and/or polycystic ovaries [22,24,139]. Nowadays, PCOS is generally considered a complex genetic disorder with interactions between environmental factors, such as diet, obesity, and genetic susceptibility caused by single nucleotide polymorphisms [140,141]. These nutrient-gene interactions, in particular, influence gene expression, resulting in a variety of diseases even with transgenerational effects [21]. The one-carbon (1-C) pathway plays an essential role in epigenetics, in which methyl groups derived from nutrients such as B vitamins are used for DNA synthesis and phospholipid and protein biosynthesis [20]. Previous studies have shown that dietary patterns rich in these B vitamins are associated with increased chances of pregnancy and decreased chances of adverse pregnancy outcome, confirming the involvement of the 1-C pathway [7,8,52,58,59,67,142]. Dietary intervention studies of specific nutrients have shown various improvements in the features of PCOS, substantiating the hypothesis that nutrition affects biological processes involved in the clinical spectrum of PCOS phenotypes [32,35-40,143]. Moreover, we recently showed that the use of a self-initiated diet and diet inadequacy are associated with PCOS and with the severity of the PCOS phenotype, which is further substantiated by a positive association between diet inadequacy and the Anti-Müllerian Hormone concentration [144]. In this continuous search for the best treatment for PCOS, to the best of our knowledge no observational studies have assessed dietary patterns in relation to the heterogeneity of the PCOS phenotype. Therefore, we explored which dietary patterns are distinctive for women with PCOS and whether these dietary patterns are associated with the more severe hyperandrogenic PCOS phenotype or the mild non-hyperandrogenic PCOS phenotype [25,144]. Additionally, we investigated whether the established dietary patterns are associated with the chance of ongoing pregnancy.

MATERIALS AND METHODS

Design

Couples referred to our hospital because of subfertility visited the preconception tailored nutrition and lifestyle counselling clinic during their first visit at the Department of Obstetrics and Gynaecology at the Erasmus MC, University Medical Center Rotterdam, a tertiary hospital in the Netherlands, between October 2010 and October 2012. They were additionally invited to participate in the ongoing Rotterdam. Periconceptional Cohort, the Predict study [59,145]. Women with oligoovulation or anovulation were screened for ovulatory dysfunction. Trained professionals diagnosed PCOS according to a standardized protocol, based on the Rotterdam criteria [24] including the presence of oligoovulation or anovulation, hyperandrogenism (Ferriman-Gallweyscore ≥9, a Free Androgen Index >4.5, or both), polycystic ovaries, or both. Two subtypes of PCOS were distinguished according to the severity of the phenotype: hyperandrogenic PCOS and non-hyperandrogenic PCOS [25,144]. The AMH was also used as

marker of PCOS severity, as elevated levels are the result of an arrest of the selection of the dominant follicle with a concomitant increase in the number of small (pre)-antral follicles in the polycystic ovary. During the visit at the outpatient clinic, standardized anthropometric measurements, i.e. height, weight and circumferences, and non-fasting blood samples, were obtained. Participants completed a general questionnaire and a food frequency questionnaire (FFQ) at baseline, which were used for counselling during the visit. Couples were counselled twice with a fixed time interval of 3 months [59]. From the general questionnaire, data were obtained on age, ethnicity, educational level, the use of a diet, the use of folic acid or vitamin supplements, medication, alcohol or tobacco and physical exercise. The validated semi-quantitative FFQ consisting of 196 food items was developed by the division of Human Nutrition, Wageningen University, The Netherlands, and was used to estimate habitual food intake over the previous 4 weeks before the preconception visit [62,64,88]. To calculate the individual micro- and macro-nutrient intakes, the nutrient densities as reported in the Dutch Food Composition Database of 2011 were multiplied by the consumed amounts of foods with consideration of preparation methods and portion sizes [146]. Twenty-three food groups were classified, to reduce the 196 food items by the summation of food items with a similar nutrient content (Supplementary Table 1) [8,147]. Dietary patterns were extracted from the women with PCOS only, because of the heterogeneity of the subfertile control group comprising women with ovulatory disorders (such as imminent ovarian failure, WHO 1), tubal infertility or unexplained subfertility. Clinical and fertility outcome parameters, such as type of fertility treatment, ongoing pregnancy (intrauterine pregnancy with positive heart action confirmed by ultrasound at 12 weeks gestation; yes/no), and time to pregnancy were extracted from medical records and follow-up stopped in January 2015.

Biomarkers

Venous blood samples were collected and the biomarkers of 1-C metabolism were determined; serum and red blood cell (RBC) folate, serum cobalamin and plasma total homocysteine (tHcy). At the preconception visit, venous blood samples were drawn in a vacutainer ethylenediamine tetraacetate (EDTA) tube and in a dry vacutainer tube (BD Diagnostics, Plymouth, UK). The dry vacutainer tubes were centrifuged at 2000 g for 15 min. The serum was collected and analyzed using an immunoelectro-chemoluminescence assay for the measurement of serum folate and cobalamin (E170; Roche Diagnostics GmbH, Mannheim, Germany). EDTA-blood was kept on ice and, immediately after blood sampling, 0.1 ml EDTA blood was haemolysed with 0.9 ml freshly prepared 1.0% ascorbic acid. The ADVIA 120 Hematology.

Analyzer (Bayer Diagnostics, Leverkusen, Germany) was used to determine the haematocrit. Within 1 h, the haemolysate was centrifuged for 5 min at 1000 g after which folate was determined on a Sysmex XE-2100 (Goffin Meyvis, Etten-Leur, The Netherlands). The RBC folate was calculated using the following formula: (nM haemolysate folate × 10/haematocrit) – [nM serum folate × (1- haematocrit) /haematocrit] = nM RBC folate. Plasma tHcy was determined

using a sensitive liquid chromatography tandem mass spectrum method (HPLC-Tandem MS, Waters Micromass Quattro Premier XE Mass Spectrometer with Acquity UPLC system, Milford, Massachusetts, USA). Inter-assay coefficients of variation for serum folate were 4.5% at 13 nmol/L and 5.7% at 23 nmol/L, for serum cobalamine 3.6% at 258 pmol/L and 2.2% at 832 pmol/L; for plasma tHcy were 4.8% at 14.6 µmol/L and 3.3% at 34.2 µmol/L. Serum AMH levels were measured by using a commercially available ELISA assay (AMH Gen II ELISA, A79765, Beckman Coulter; Inc., USA) [148]. Radioimmunoassay was used to measure testosterone (Siemens DPC, Los Angeles, CA) and the Hitachi 917 analyser was used to measure glucose levels (Roche Diagnostics, Almere, The Netherlands). The Free Androgen Index (FAI) was calculated as follows; [100 × T (nmol/L) / Sex hormone-binding globulin (SHBG) (nmol/L)]. LH, FSH, SHBG, androstenedione, insulin and dehydroepiandrosterone sulphate were determined by immunoluminometric assay (Immulite® XPi, Siemens, Los Angeles, CA). Intra- and interassay coefficients of variation were as follows, respectively: <5% and <8% for AMH, <3% and <18% for FSH, <5% and <15% for LH, <4% and <5% for sex hormone binding globulin, <3% and <5% for testosterone, <8% and <11% for androstenedione, <9% and <11% for dehydroepiandrosterone sulphate and <6% and <8% for insulin. Homeostatic model assessment-insulin resistance was calculated using the following formula (insulin (pmol/l) × glycaemia (mmol/l) /135).

Statistical analyses

Principal component factor analysis was used to summarize dietary patterns by unrotated extraction in PCOS patients. The number of identified dietary patterns was based on an eigen value over 1 and the break point in the scree plot (depicting the eigen values of each pattern; the components plotted on the steep slope contribute highly to the patterns) [149]. After examining the contents of all identified dietary patterns, one and three were interpreted as, respectively, an unhealthy and healthy dietary pattern (Supplementary Table 1 and Figure 1). Patterns two and four were not distinctively healthy or unhealthy and were, therefore, not further considered. Each participant was assigned personalized scores for the two patterns, which represents the comparability of the individual's diet to the two dietary patterns. All participants were subsequently classified based on ranking into a poor (lower half) or strong (upper half) adherence to the healthy and the unhealthy dietary pattern. The adherence to both patterns are uncorrelated, although a participant with a high score of adherence to the healthy dietary pattern will very likely have a low score of adherence to the unhealthy dietary pattern. When a participant has a moderate diet not consisting of very healthy or unhealthy items, the degree of adherence for both patterns would be low and the participant will be scored in both analyses in the group 'poor adherence'. The physical activity level (PAL), an estimate for underreporting, was calculated using the Schofield formula for basal metabolic rate [150]. Chi-squared tests were carried out to compare categorical variables and Mann-Whitney U Tests to compare continuous variables. Multivariable logistic regression analysis

was applied to calculate the risk of the more severe hyperandrogenic PCOS phenotype in case of a strong adherence to the healthy pattern and unhealthy pattern, adjusted for total energy intake (respectively model 1 and 3). In model 2, several confounders were considered and age and body mass index (BMI) were added to the logistic regression analyses. Ethnicity and educational level showed no significant correlation with the dietary patterns, and were therefore not added to the adjusted model. Multivariable logistic regression analysis was also used to predict the chance of ongoing pregnancy in case of strong adherence to the healthy pattern and unhealthy pattern. Multivariable linear regression analyses was applied to investigate the association between natural logtransformed AMH and the two patterns. SPSS software for Windows (version 21.0, IBM SPSS, Statistics for Windows, Armonk, NY: IBM Corp) was used for statistical analyses. The level of significance was set to $P \leq 0.05$ for all analyses.

Ethical approval

All data and materials were processed anonymously. Written informed consent was obtained from all patients to participate in both studies. This study was conducted according to the guidelines of the Declaration of Helsinki, and all procedures involving patients were approved by the Medical Ethical and Institutional Review Board at the Erasmus MC, University Medical Center in Rotterdam, the Netherlands on 6 August, 2013; reference number MEC-2013-378.

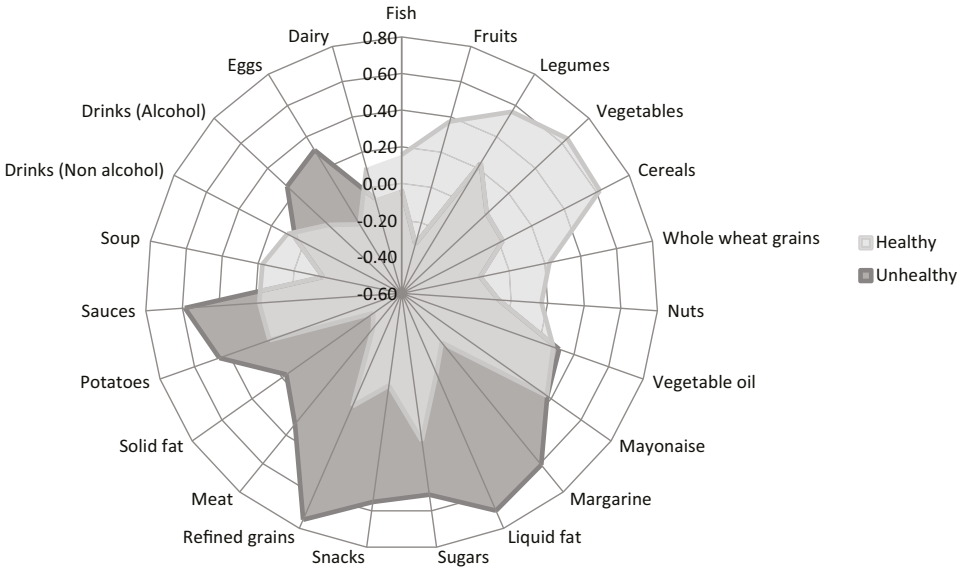

Figure 1 | Food group factor loadings for an unhealthy (dark grey) and healthy (light grey) dietary pattern; extracted from food frequency questionnaires of 55 women with PCOS. The overlap between the two patterns is coloured the middle gradient of grey. Factor loadings over 0.20 can be considered as foods highly related to the dietary pattern. Food group factor loadings range from -1 (no correspondence) to 1 (highly corresponding).

RESULTS

A total of 1017 sub-fertile women visited the preconception outpatient clinic between 2010 and 2012, of which follow-up of 453 (44.5%) women was ensured in the Predict Study. In 164 out of 1017 women (16.1%), a diagnosis of PCOS was made, of which 81 (49.4%) participated in the Predict study. In 55 women with PCOS, a completed FFQ was available for analysis, revealing a response rate of 67.9%. To evaluate selection bias, we present the general characteristics of the included and excluded patients with or without PCOS in the Predict study in (Supplementary Table 2). The general characteristics were comparable except for a younger age, higher BMI, higher waist circumference and waist-hip ratio in the included group of women with PCOS. The women with PCOS without a FFQ were slightly younger than the women with PCOS in the study group. The non-PCOS study participants showed the oldest median age and the lowest BMI, waist circumference and waist-hip ratio compared with the PCOS participants without a FFQ and the included women with PCOS. Dietary patterns in women with PCOS were identified with principal component factor analysis and the first component or 'dietary pattern' (14.3% variance explained; the highest proportion of variance in total food consumption with the least loss of information per dietary pattern) represented a pattern high in refined grains, liquid fat, margarine, sauces, snacks and sugars, and low in fruits, whole grain, soup, diary, nuts and fish and was therefore labelled 'unhealthy'.

The third component (9.3% variance explained) represented a pattern high in vegetables, cereals and legumes and low in fat (solid, margarine and liquid), meat, snacks, eggs and alcohol and was labelled 'healthy'. We did not further investigate the less distinctive second and fourth dietary pattern, comprising both healthy and unhealthy items within one pattern.

In Table 1 we presented the general characteristics of 55 women with PCOS stratified into 25 patients with the hyperandrogenic PCOS phenotype and 30 patients with the non-hyperandrogenic PCOS phenotype. In contrast to non-hyperandrogenic PCOS, women with hyperandrogenic PCOS were more often of non-Dutch origin, had a lower level of education, were more frequently obese and showed a higher median BMI, waist circumference and waist-hip ratio. All lifestyle parameters were comparable in both groups except a stronger adherence to the healthy dietary pattern in women with non-hyperandrogenic PCOS compared with women with hyperandrogenic PCOS. In addition, we observed a lower median serum and RBC folate concentration in women with hyperandrogenic PCOS. A similar PAL was observed for women with hyperandrogenic PCOS (1.08) and for women with non-hyperandrogenic PCOS (1.29).

Dietary patterns and the risk of the hyperandrogenic phenotype

The results of the multivariable logistic regression analyses are presented in Table 2. Strong adherence to the healthy dietary pattern was inversely associated with the hyperandrogenic PCOS phenotype only (β -1.26; OR 0.28; p= 0.03; 95% CI 0.09 to 0.88, adjusted for total energy intake), which remained significant after additional adjustment for BMI and age (β -1.31; OR

0.27; p= 0.047; 95% CI 0.07 to 0.99). The adherence to the unhealthy dietary pattern, however, was not associated with the hyperandrogenic or non-hyperandrogenic PCOS phenotype.

Table 1 | Preconception characteristics of women with hyperandrogenic and non-hyperandrogenic polycystic ovary syndrome

	HA-PCOS (n=25)	non HA-PCOS (n=30)	
Age (years)	29 (28-31)	29 (27-33)	NS
Ethnicity, n (%)			
Dutch	11 (44.0%)	25 (83.3%)	0.002
Other	14 (56.0%)	5 (16.7%)	
Educational level, n (%)			
Low	3 (12.5%)	1 (3.3%)	0.04
Intermediate	14 (58.3%)	10 (33.3%)	
High	7 (29.2%)	19 (63.3%)	
Healthy dietary pattern, n (%)			
Strong adherence	8 (32.0%)	19 (63.3%)	0.02
Poor adherence	17 (68.0%)	11 (36.7%)	
Unhealthy dietary pattern, n (%)			
Strong adherence	13 (52.0%)	14(46.7%)	NS
Poor adherence	12 (48.0%)	16 (53.3%)	
Diet (weight reducing; Yes), n (%)	1 (0.04%)	6 (20.7%)	NS
Energy intake (kJ/day)	7935 (6053–8758)	8028 (6791–9881)	NS
Folic acid supplement use (No) , n (%)	12 (48.0%)	5 (16.7%)	NS
Vitamin supplement use (Yes), n (%)	7 (28.0%)	13 (43.3%)	NS
Medication use (Yes), n (%)	11 (44.0%)	14 (46.7%)	NS
Alcohol (Yes), n (%)	16 (64.0%)	14 (46.7%)	NS
Smoking (Yes), n (%)	5 (20.0%)	7 (23.3%)	NS
Physical exercise (No), n (%)	13 (52.0%)	10 (34.5%) [a]	NS
Measurements			
BMI (kg/m^2)	31.0 (28.9-34.5)	24.6 (21.2-28.7)	0.000
<20, n (%)	0 (0.0%)	2 (6.7%)	0.004
≥20 <25, n (%)	2 (8.0%)	13 (43.3%)	
≥25 <30, n (%)	7 (28.0%)	8 (26.7%)	
≥30, n (%)	16 (64.0%)	7 (23.3%)	
Waist circumference (cm)	93 (86-106)	83 (74-94)	0.001
Waist hip ratio	0.84 (0.82-0.86)	0.78 (0.73-0.82)	0.001

Table 1 | *(Continued)*

	HA-PCOS (n=25)	non HA-PCOS (n=30)	
Biochemical Parameters			
Cobalamin (pmol/L)	312.0 (246-419)	333.5 (260-460)	NS
RBC Folate (nmol/L)	1108 (956-1223)	1238 (1082-1417)	0.03
Folate (nmol/L)	22.6 (14.8-36.1)	32.1 (26.0-45.40)	0.02
tHcy (µmol/L)	8.5 (7.3-9.9)	8.4 (7.3-9.0)	NS

Poor (lower half) or strong (upper half) adherence based on ranking. Chi-square tests and Mann-Whitney U Tests were conducted to compare groups. Normal biochemical parameters; folate ≥8 nmol/L, red blood cell folate ≥500 nmol/L, cobalamin ≥145 pmol/L, homocysteine <15 µmol /L. PCOS, polycystic ovary syndrome; NS, not statistically significant.[a] One missing in physical exercise item; total n= 29.

Table 2 | Multivariable logistic regression analyses for the risk of hyperandrogenic polycystic ovary syndrome.

	B values	OR	95% CI	P value
Healthy dietary pattern				
Model 1				
Strong adherence (>p50)[a]	-1.26	0.28	(0.09 to 0.88)	0.03
Energy intake (kJ/day)	0.00	1.00	(1.00 to 1.00)	NS
Model 2				
Strong adherence (>p50)	-1.31	0.27	(0.07 to 0.99)	0.047
Energy intake (kJ/day)	0.00	1.00	(1.00 to 1.00)	NS
Age (years)	-0.10	0.91	(0.75 to 1.11)	NS
Body Mass Index	0.20	1.22	(1.07 to 1.39)	0.003
Unhealthy dietary pattern				
Model 1				
Strong adherence (>p50)	0.87	2.39	(0.56 to 10.26)	NS
Energy intake (kJ/day)	0.00	1.00	(1.00 to 1.00)	NS
Model 2				
Strong adherence (>p50)	1.10	3.01	(0.59 to 15.40)	NS
Energy intake (kJ/day)	0.00	1.00	(1.00 to 1.00)	NS
Age (years)	-0.08	0.92	(0.77 to 1.12)	NS
Body Mass Index	0.20	1.23	(1.08 to 1.40)	0.002

NS, not statistically significant. [a] Strong adherence is based on ranking (upper half).

General characteristics of patients with poor or strong adherence to the healthy dietary pattern

The PCOS characteristics, stratified according to a poor or strong adherence to the healthy dietary pattern are presented in Supplementary Table 3. Women with poor adherence to the healthy pattern more often had hyperandrogenic PCOS than those with a strong adherence.

Table 3 | Patient characteristics according to the occurrence of ongoing pregnancy.[a]

	Ongoing Pregnancy (n= 24)	No Pregnancy (n= 26)	P value
PCOS phenotype			
HA PCOS, n (%)	8 (33.3%)	15 (57.7%)	NS
Non-HA PCOS, n (%)	16 (66.7%)	11 (42.3%)	
Healthy dietary pattern			
Strong adherence, n (%)	15 (62.5%)	9 (34.6%)	0.049
Poor adherence, n (%)	9 (37.5%)	17 (65.4%)	
Unhealthy dietary pattern			
Strong adherence, n (%)	10 (41.7%)	15 (57.7%)	NS
Poor adherence, n (%)	14 (58.3%)	11 (42.3%)	
Fertility treatment			
No	5 (20.8%)	4 (15.4%)	NS
Yes	19 (79.2%)	22 (84.6%)	
Weight reduction only	4	14	
IUI	2	–	
Ovulation induction	9	2	
IVF	4	5	
Vaso-vasostomy	–	1	
Time to pregnancy (days)			
Fertility treatment	415 (93-925)	–	
No fertility treatment	229 (63-797)	–	
Female Subfertility			
Primary	20 (83.3%)	17 (65.4%)	NS
Secondary	4 (16.7%)	9 (34.6%)	
Male subfertility (VCM <10) [b]			
Yes	5 (20.8%)	3 (14.3%)	NS
No	19 (79.2%)	18 (85.7%)	

Values are expressed as mean (range). Poor (lower half) or strong (upper half) adherence based on ranking. Chi-squared tests were conducted to compare groups. Dashes mean zero/not applicable. IUI, intrauterine insemination; NS, not statistically significant; PCOS, polylcystic ovary syndrome; VCM, volume × concentration × percentage progressively moving spermatozoa.

[a] Five patients were excluded; one unkown pregnancy outcome and four miscarriages [b] Five semen analyses were missing.

The anthropometric measurements, hormonal and metabolic assessments were comparable between the two groups except for AMH. The median AMH concentration was significantly higher in patients with a poor adherence to a healthy dietary pattern. Subsequently, multivariable linear regression analyses showed a significant inverse association between the adherence to the healthy dietary pattern and AMH concentration (β -0.57 g/L; p= 0.006;

95% CI -0.97 to -0.17), with adjustment for total energy intake. Additional adjustment for age and BMI, did not change this association (β -0.56; p= 0.008; 95% CI -0.97 to -0.15).

Clinical outcome parameters

Women with PCOS (hyperandrogenic and non-hyperandrogenic) and an ongoing pregnancy, more often strongly adhered to the healthy dietary pattern compared with non-pregnant women with PCOS (Table 3). When we applied multivariable logistic regression analysis, we observed a three-fold higher chance of ongoing pregnancy in patients with a strong adherence to the healthy pattern (OR 3.38; p= 0.049; 95% CI 1.01 to 11.36), which attenuated after adjustment for age and BMI (OR 3.09; p= 0.08; 95% CI 0.86 to 11.05). No association was found between the unhealthy pattern and the chance of ongoing pregnancy. In women with PCOS with an ongoing pregnancy, the number receiving fertility treatment for primary or secondary subfertility or male subfertility was comparable with nonpregnant women with PCOS. The androgenic profile of women with PCOS with hyperandrogenism and without hyperandrogenism are presented in Supplementary Table 4.

DISCUSSION

In this periconceptional cohort study, we observed that strong adherence to a healthy dietary pattern, comprising of fruits, vegetables, fish and whole grains, was associated with the non-hyperandrogenic PCOS phenotype and a lower AMH concentration. Moreover, hyperandrogenic and non-hyperandrogenic PCOS patients with strong adherence to a healthy dietary pattern also demonstrated a three-fold higher chance of ongoing pregnancy. These findings are in line with our recent study, in which we observed that dieting and diet inadequacy was associated with alterations in the 1-C pathway, contributing to the clinical spectrum of PCOS [144]. Patients with hyperandrogenic PCOS more often reported the use of a self-initiated diet than controls, and the use of an inadequate diet was associated with a higher risk of the hyperandrogenic phenotype. Diet inadequacy was also independently associated with the AMH concentration, suggesting a more significant role of gene-nutrient interactions affecting epigenetic mechanisms of multiple pathways regulating ovarian function during the course of life [20].

In a study estimating dietary intake with a 7-day food diary, Barr et al. observed a higher percentage energy from fat in PCOS patients compared with data of women participating in a national dietary survey [151]. Another study using the combination of a FFQ and a 4-day food diary did not observe differences in nutrient intake in women with PCOS compared with healthy control women. The intake of only high glycaemic foods, however, was assessed separately, and the PCOS group consumed significantly more white bread than controls [127]. Graff et al. also observed that, in PCOS, a high dietary glycemic index was associated with a less favourable anthropometric and metabolic profile [143]. No differences in the total energy intake were observed between HA and non-HA PCOS patients, although an overall increase

in energy intake with a higher diet quality in PCOS patients is previously reported [129]. In these observational studies, nutrient contents were compared instead of food groups. Food group analyses are easier to interpret and the derived dietary patterns are more suitable in communication with patients. More recently Moran et al. showed that women with PCOS were more likely to consume a Mediterranean dietary pattern compared with women without PCOS. This finding is supposed to be the effect of adopting a healthy dietary pattern after being diagnosed with PCOS [152]. Unfortunately PCOS phenotypes were not determined in this study and, to our knowledge, the present study is the first to observe associations between dietary patterns and the severity of the PCOS phenotype. Numerous studies have investigated the composition of the ideal PCOS weight loss diet to improve PCOS features, such as hyperandrogenism, cycle regularity, metabolic and psychiatric outcome, but so far the results are rather conflicting. These diets vary from high to low in carbohydrates, proteins, and fat or combinations of the previous [32,35-40].

Therefore, weight loss should always be accomplished by adequate intakes of healthy foods [33]. Our study showed alterations in biomarkers of 1-C metabolism in women with hyperandrogenism and PCOS, more specifically lower serum and red blood cell folate levels, whereas Hcy and cobalamin levels were comparable to women with non-hyperandrogenic PCOS. Other studies reporting lower Hcy levels in women with PCOS when folic acid supplements were used also observed an improvement in metabolic profile and biomarkers for oxidative stress [134,153,154]. This substantiates the involvement of 1-C metabolism, in which an inadequate intake of nutrients interferes with the metabolic profile of the PCOS phenotype. Moreover, we observed that a healthy diet, rich in B vitamins, may be a crucial factor associated with the hyperandrogenic phenotype; in fact it was recently shown that this phenotype constitutes the most severe metabolic phenotype in women with PCOS. The beneficial effect of a healthy diet on hyperandrogenism is supported by studies observing a decrease in FAI and testosterone [35,40,155]. Although we found comparable FAI and testosterone levels between the groups, others observed significant differences [32,37,38]. The association between dietary composition and an improvement in AMH has been described previously [107]. The latter observation might indicate a similar improvement in follicle dynamics. Women in this study who adhered more to the healthy diet showed an increased chance of becoming pregnant, which is in line with previous research showing beneficial effects of supplementation of B vitamins on levels of Hcy and consequently on pregnancy rate in women with PCOS undergoing infertility treatment [154].

This is also substantiated by previous research in subfertile women undergoing IVF–ICSI, in which a 'Mediterranean' diet containing high intakes of not only fruits and vegetables, but also vegetable oils increased vitamin B6 in blood and in follicular fluid and improved the chance of pregnancy after IVF–ICSI [8]. Nutrition and lifestyle counselling of couples planning pregnancy leads to an improved diet adequacy and also increases the chance of ongoing pregnancy [7, 59].

This emphasizes the importance of an adequate dietary intake in women planning pregnancy, but especially in those with PCOS. Although we have to keep in mind that increasing age and obesity might be stronger factors influencing pregnancy chance, as the chance of ongoing pregnancy was attenuated after adjustment of the multivariable logistic regression analysis. The present study has some limitations. Although all FFQs were checked for completeness, the FFQ estimates the consumption of the previous 4 weeks instead of recording the actual consumed foods during the periconceptional period of 14 weeks before until 10 weeks after conception [20].

An underestimation of dietary intake can occur in obese or overweighed patients [62], and the study population of women with PCOS were more obese than the excluded participants who did not have PCOS. All analyses were adjusted for BMI and total energy intake, although the intake could have been underestimated more in the hyperandrogenic phenotypes. In both groups, under-reporting occurred; however, we have to bear in mind that the minimum PAL of 1.35 is only plausible for individuals who have a stable bodyweight [150]. Furthermore, significant differences were found between the groups in ethnicity and educational level, although no significant correlation was found with the two dietary patterns. Further research is, therefore, necessary to investigate the effect of social and cultural factors. We could not use a control group of healthy individuals, which limits the generalizability of our results to other populations. Distinctive differences in food groups in the subfertile women with ovulatory disorders, tubal infertility or unexplained subfertility makes these women less appropriate to pool as control group for dietary pattern analyses. Overcorrection of the estimates would very likely occur when adjusting for all covariates caused by the limited sample size; therefore, we choose for adjustment of energy intake, BMI and age as most important covariates and are aware that residual confounding cannot be excluded.

Finally, all lifestyle factors were assessed at baseline and lifestyle changes during follow-up could have influenced pregnancy chance as well. Some strengths of the study are the preconceptional recruitment and prospective design in one tertiary hospital setting, the sensitivity analysis showing no selection bias and the high response rate. The standardized diagnosis of PCOS according to the Rotterdam criteria limited the occurrence of measurement errors; similarly, all blood sampling and analysis were carried out in one laboratory following a standardized protocol, which further increased the validity of the data. Finally, the biomarkers of 1-C metabolism were used as extra validation of the FFQ data.

In conclusion we show that, in women with PCOS, strong adherence to a healthy dietary pattern is related to the non-hyperandrogenic phenotype and to an increased chance of ongoing pregnancy. Therefore, information on the importance of the diet and offering of validated interventions should be part of the initial assessment and treatment of women with PCOS. Future prospective studies in the general population should be conducted to further validate our findings and to investigate intergenerational effects of the diet in the developmental origin of PCOS in offspring.

Supplementary Table 1 | Food group loadings for eligible dietary patterns.

	First dietary pattern	Second dietary pattern	Third dietary pattern	Fourth dietary pattern
Variance explained [a] (%)	14.3%	10.3%	9.3%	9.1%
Initial Eigen value	3.290	2.374	2.135	2.087
Alcoholic Drinks	0.255	-0.228	-0.053	-0.190
Cereals	0.023	0.263	0.619	-0.229
Dairy	-0.063	0.601	0.112	0.157
Eggs	0.315	-0.492	-0.160	-0.126
Fish	-0.033	-0.138	0.156	-0.329
Fruits	-0.320	-0.192	0.374	0.422
Legumes	0.233	0.091	0.560	-0.426
Mayonnaise	0.374	0.270	0.371	-0.337
Meat	0.322	0.317	-0.353	0.272
Non Alcoholic Drinks	0.054	0.323	0.103	0.324
Nuts	-0.068	0.055	0.166	0.347
Potatoes	0.456	0.559	0.165	0.082
Sauces	0.586	-0.498	0.179	0.177
Snacks	0.551	0.390	-0.091	0.057
Soup	-0.161	-0.242	0.176	0.277
Sugars	0.511	0.335	0.213	-0.015
Vegetables	0.037	-0.269	0.638	0.344
Solid fat	0.170	-0.098	-0.411	-0.197
Margarine	0.609	0.053	-0.252	0.424
Liquid fat	0.695	-0.102	-0.122	0.452
Vegetable Oils	0.314	-0.447	0.280	0.135
Refined Grains	0.749	-0.342	0.073	-0.345
Whole grains	-0.161	-0.099	0.225	0.529

Extraction Method: Principal Component Analysis. [a]The highest proportion of variance in total food consumption with the least loss of information per dietary pattern.

Supplementary Table 2 | Participants in the Predict study.

	Included PCOS study participants with FFQ (n= 55)	Excluded; PCOS study participants without FFQ (n= 26)	Excluded; non-PCOS study participants (n= 372)	P value
Age (years)	29 (27-32)	27.5 (26-31)	32.0 (29.0-36.0)	<0.001
Ethnicity, n (%)				
Dutch	36 (65.5)	15 (57.7)	225 (60.6)	NS
Other	19 (34.5)	11 (42.3)	146 (39.4)	
Educational level, n (%)				
Low	4 (7.4)	3 (12.0)	53 (14.6)	NS
Intermediate	24 (44.4)	14 (56.0)	166 (45.6)	
High	26 (48.1)	8 (32.0)	145 (39.8)	
Diet (weight reducing; yes), n (%)	7 (13.0)[a]	5 (19.2%)	43 (11.7%)[b]	NS
Folic acid supplement use (no), n (%)	17 (30.9)	12 (46.2)	129 (34.7)	NS
Medication use (yes), n (%)	25 (45.5)	14 (53.8)	192 (51.6)	NS
Alcohol (yes), n (%)	30 (54.5)	8 (30.8)	171 (46.0)	NS
Smoking (yes), n (%	12 (21.8)	10 (38.5)	84 (22.6)	NS
Physical exercise (no), n (%)	23 (42.6)[c]	13 (52.0)[d]	162 (44.0)[e]	NS
Measurements				
Body mass index (kg/m^2)	28.6 (22.9-33.3)	26.3 (22.6-34.2)	24.6 (21.8-28.3)	0.001
<20, n (%)	2 (3.6)	1 (3.8)	34 (9.2)	0.003
≥20 <25, n (%)	15 (27.3)	9 (34.6)	161 (43.5)	
≥25 <30, n (%)	15 (27.3)	6 (23.1)	104 (28.1)	
≥30, n (%)	23 (41.8)	10 (38.5)	71 (19.2)	
Waist circumference (cm)	87 (77-99)	86 (78-98)	81 (73-90)	0.001
Waist-hip ratio	0.81 (0.76-0.85)	0.82 (0.77-0.88)	0.78 (.74-0.84)	0.025
Biochemical parameters				
Cobalamin (pmol/L)	333 (249-449)	356 (290-514)	328 (250-437)	NS
RBC folate (nmol/L)	1158 (1027-1353)	1235 (954-1454)	1213 (955-1427)	NS
Folate (nmol/L)	31.0 (20.6-45.4)	24.8 (15.9-36.0)	31.2 (18.6-42.7)	NS
Homocysteine (µmol/L)	8.5 (7.3-9.4)	8.0 (7.1-9.4)	8.2 (6.9-9.7)	NS

Values are expressed as median (interquartile range) or number (%). Chi-squared and Kruskal-Wallis tests were conducted to compare groups. One[a] and three[b] items missing for diet, and one[c], one[d] and four[e] items missing for physical exercise. Normal biochemical parameters: folate ≥8 nmol/L, RBC folate ≥500 nmol/L, cobalamin ≥145 pmol/L, homocysteine <15 µmol/L. NS, not statistically significant; PCOS, polycystic ovary synrome; RBC, red blood cell.

Supplementary Table 3 | Characteristics of PCOS patients with a poor or strong adherence to the healthy dietary pattern

	Poor adherence to a healthy dietary pattern (n= 28)	Strong adherence to a healthy dietary pattern (n= 27)	P value
PCOS phenotype, n(%)			
HA PCOS	17 (60.7%)	8 (29.6%)	0.02
Non-HA PCOS	11 (39.3%)	19 (70.4%)	
Hyperandrogenism , n(%)	17 (60.7%)	8 (29.6%)	0.02
Ovulatory dysfunction, n(%)	28 (100%)	26 (96.3%)	NS
Polycystic ovarian morphology, n(%)	26 (92.9%)	27 (100%)	NS
Measurements			NS
BMI (kg/m^2)	29.0 (24.3-34.0)	27.7 (22.6.7-32.9)	NS
Waist circumference (cm)	86 (83-100)	88 (74-98)	NS
Waist-Hip ratio	0.82 (0.78-0.85)	0.80 (0.74-0.85)	NS
RR Systolic	110 (107-122)	112 (109-125)	NS
RR Diastolic	71 (70-80)	75 (70-82)	NS
Hormonal Parameters			
AMH (µg/L)	8.30 (6.20-13.59)	5.90 (3.30-7.30)	0.008
FSH (U/l)	5.45 (4.10-7.60)	5.30 (4.30-6.60)	NS
LH (U/l)	6.95 (5.15-10.25)	5.80 (4.30-13.70)	NS
Progesterone (nmol/l)	1.10 (0.65-2.45)	1.00 (0.60-4.90)	NS
17- hydroxyprogesterone (nmol/l)	2.35 (1.50-3.40)	2.00 (1.50-5.70)	NS
Estradiol (pmol/L)	198.50 (170.50-261.50)	166.00 (110.00-390.00)	NS
SHBG (nmol/l)	39.55 (21.90-61.45)	41.10 (33.70-70.40)	NS
Testosterone (nmol/l)	1.46 (1.20-2.00)	1.50 (1.00-1.90)	NS
FAI	5.15 (2.32-6.47)	3.70 (1.78-4.61)	NS
Androstenedione (nmol/l)	11.20 (7.49-14.35)	8.44 (5.64-12.10)	NS
DHEAS(µmol/L)	4.93 (3.26-6.20)	4.26 (3.31-5.54)	NS
Metabolic Parameters			
Insulin (pmol/l)	41.50 (19.00-89.50)	43.00 (18.00-86.00)	NS
Glucose (mmol/l)	4.90 (4.65-5.20)	4.80 (4.60-5.20)	NS
HOMA-IR	1.50 (0.66-3.31)	1.37 (0.61-3.10)	NS

Note: Values are expressed as median (interquartile range). Poor (lower half) or strong (upper half) adherence based on ranking. Mann Whitney U Tests were conducted. NS, not statistically significant.
Abbreviations; HA = Hyperandrogenic; FSH = Follicle Stimulating Hormone; LH = Luteinizing Hormone; SHBG = Sex Hormone Binding Globulin; FAI = Free Androgen Index; DHEAS = dehydroepiandrosterone sulfate; HOMA-IR = homeostatic model assessment – insulin resistance.

Supplementary Table 4 | The androgenic profile of the women enrolled.

	Hyperandrogenic PCOS (n= 25)	Non-hyperandrogenic PCOS (n= 30)
Hormonal parameters		
SHBG (nmol/l)	24.20 (19.10-38.80)	62.50 (40.30-77.60)
Testosterone (nmol/l)	1.70 (1.30-2.20)	1.25 (1.00-1.70)
FAI	6.22 (5.26-7.38)	2.32 (1.70-3.49)
Androstenedione (nmol/l)	11.30 (8.96-14.90)	8.09 (5.74-11.90)
DHEAS (µmol/L)	5.40 (3.94-6.51)	4.09 (3.01-5.18)
Definition of hyperandrogenism		
Ferriman–Gallwey score ≥9	8	–
FAI >4.5	23	–

Values are expressed as median (interquartile range) or number. *Abbreviations;* DHEAS = dehydroepiandrosterone sulphate; FAI = Free Androgen Index; PCOS = polycystic ovary syndrome; SHBG = sex hormone binding globulin.

PART 3

Periconceptional medication use and semen parameters

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Chapter

6

Effect of medications for gastric acid-related symptoms on total motile sperm count and concentration: a case-control study in men of subfertile couples from the Netherlands

ABSTRACT

Introduction

Gastric acid-related symptoms are highly prevalent in the general population (21-40%) and more than 11% of individuals use medication for the treatment of these symptoms. The uptake of micronutrients is dependent on the gastrointestinal potential of hydrogen (pH).

Objective

We hypothesized that medication affecting the gastrointestinal pH reduces the availability of B vitamins thereby deranging 1-C metabolism and detrimentally affecting spermatogenesis.

Methods

This explorative nested case-control study in men of subfertile couples investigated associations between medication used for gastric acid-related symptoms and semen parameters. We included 40 men using medication for gastric acid-related symptoms and 843 men not using medication. Semen analyses were performed between 70 days before and 21 days after the visit.

Results

The use of medication was associated with a twofold higher risk of a low total motile sperm count (TMSC $<1x10^6$, OR 2.090, p= 0.049) and negatively with sperm concentration (β -0.320, p= 0.028). Red blood cell folate was positively associated with TMSC (β 0.257, p= 0.026), sperm count (β 1.679, p= 0.013) and ejaculate volume (β 0.120, p= 0.023) and total homocysteine was negatively associated with sperm count (β -0.077, p= 0.021).

Conclusion

Here we delineate associations between the use of medication for gastric acid-related symptoms and poor semen quality in men of subfertile couples. The use of medication for gastric acid-related symptoms is associated with a twofold higher risk of a low TMSC and a decreased sperm concentration. Although these findings warrant further research on causality, the associations between folate, tHcy and semen quality emphasize the importance of preconception counselling in male subfertility.

INTRODUCTION

Around 15% of couples are subfertile -pregnancy not achieved after 1 year of unprotected intercourse- of which half remain involuntarily childless [3]. In 45% the cause can be attributed to a male factor [156]. To contribute to future treatment and prevention of male subfertility it is of great importance to investigate causes and the underlying pathophysiology.

In 20-40% of the general population gastric acid-related symptoms are present, and in about 11% medication is used [157,158]. However, its impact on semen parameters has scarcely been studied. Antacids, proton-pump inhibitors (PPIs) and H2-receptor antagonists (H2RAs) induce a prolonged increase in gastric pH, impairing the gastro intestinal absorption of nutrients, including folate and cobalamin, these being essential for spermatogenesis [12,159,160].

B vitamin shortages derange 1-C metabolism and induce hyper-homocysteinemia, which causes excessive oxidative stress and interferes with biological processes and DNA structures [20].

Seminal plasma folate, cobalamin and total homocysteine (tHcy) are associated with the sperm DNA fragmentation index and sperm concentration [45,46]. This is supported by the detrimental effects of poor nutrition and beneficial effects of a healthy diet, on sperm parameters [9,45,161]. In addition, folic acid and zinc supplements increase sperm count, concentration and seminal plasma folate concentration [45,50,162].

Therefore this explorative study aims to investigate whether the use of medication for gastric acid-related symptoms is associated with semen parameters and biomarkers of 1-C metabolism in men of subfertile couples.

MATERIALS AND METHODS

Study design

Between 2007 and 2012, couples contemplating pregnancy and visiting the outpatient clinic of the department of Obstetrics and Gynaecology at the Erasmus MC, University Medical Center in Rotterdam, The Netherlands, were offered preconception counselling.

These couples were referred because of subfertility, i.e. when pregnancy is not achieved after 1 year of unprotected intercourse [3]. Men were invited to participate in the study and completed a self-administered questionnaire to obtain data on medication use (type, dosage and frequency), smoking (yes/no), use of alcohol (yes/no), recreational drugs (yes/no) and folic acid supplements (yes/no). At the visit the questionnaires were verified by a trained counselor, and height and weight were measured to calculate body mass index (BMI kg/m^2). The information on nutrition and lifestyle was screened for risk factors, and individual advice on inadequacies was given during the visit. Men who provided written informed consent and a semen analysis between 70 days before and 21 days after the visit were included in the study. This window was defined because it covers 3 months of spermatogenesis during which medication use was assessed and reduces confounding by counselling. We excluded men with semen samples provided by microsurgical epididymal sperm aspiration (MESA), percutaneous

epididymal sperm aspiration (PESA) or retrograde ejaculation. Men with incomplete data and men using other medication for gastric acid-related symptoms were excluded as well.

Semen sample collection and analysis

Semen analyses were performed on the clinical indication of subfertility and these data were obtained from medical records. The examination and processing were performed before 2010 according to the fourth edition of the World Health Organization (WHO) laboratory manual and afterwards according to the fifth edition [163,164]. Men received a leaflet with instructions on how to provide a semen sample. The obligatory abstinence period for producing a semen sample was 3-5 days. The sample had to be collected in a non-toxic plastic container provided by the laboratory and had to be delivered within 1 h of production at the outpatient clinic of the Division of Reproductive Medicine at the Erasmus MC, University Medical Center, Rotterdam, The Netherlands. To avoid large changes in temperature, the semen sample had to be kept at ambient temperature during transport [165].

After liquefaction the following sperm parameters were assessed by using a Zeiss microscope (Carl Zeiss, Oberkochen, Germany): ejaculate volume (normal range: >2.0 mL); sperm concentration (normal range: $>20x10^6$ sperm/mL); and percentage of motile and immotile spermatozoa (normal range: >50% with grade A+B motility). Sperm concentration was determined with an improved Neubauer Hemocytometer counting chamber. Total sperm count was calculated as the product of ejaculate volume and sperm concentration. Sperm motility parameters type A (rapid progressive motility); type B (slow or sluggish progressive motility); type C (local motility); and type D (immotility) were assessed at 37°C. Total Motile Sperm count (TMSC) was calculated as the product of ejaculate volume, sperm concentration and grade A+B motility. A total motile sperm count $<1x10^6$ was defined as low because it represents severe oligoasthenoteratozoospermia [166,167]. All semen analyses were performed in one center and laboratory which participates in the external quality control scheme of the Dutch Foundation for Quality Assessment in Clinical Laboratories (SKML). The staff performing these analyses are trained and tested annually according to the certified ESHRE course for performing semen analyses.

The laboratory of the Erasmus Medical Center received the certification of the International Organization for Standardization (ISO9001), the international standard for quality measurement. The between technician variation for sperm concentration was 1.6% (Intra class correlation coefficient (ICC) 0.992, 95 % CI 0.978 to 0.997). The between technician variation for sperm motility was 2.4% (ICC 0.988, 95% CI 0.972 to 0.996).

Biomarkers

At the preconception visit, venous blood samples were drawn in a vacutainer ethylenediamine tetraacetate (EDTA) tube for the determination of red blood cell (RBC) folate and plasma tHcy; for serum folate and cobalamin, a dry vacutainer tube (BD diagnostics, Plymouth,

UK) was used. EDTA-blood was kept on ice and plasma was separated by centrifugation within one hour for determination of tHcy. Immediately after blood sampling, 0.1 ml EDTA blood was hemolysed with 0.9 ml freshly prepared 1.0% ascorbic acid. The haematocrit in EDTA blood was determined on an ADVIA 120 Hematology Analyzer (Bayer Diagnostics, Leverkusen, Germany). The hemolysate was centrifuged for 5 minutes at 1000 xg after which folate was measured in the hemolysate. RBC folate was calculated with the following formula: (nM haemolysate folate × 10/haematocrit) – [nM serum folate × (1- haematocrit) / haematocrit] = nM RBC folate. tHcy was determined using a sensitive liquid chromatography tandem mass spectrum method (HPLC-Tandem MS, Waters Micromass Quattro Premier XE Mass Spectrometer with Acquity UPLC system, Milford, Massachusetts, United States). Blood obtained in dry vacutainer tubes was centrifuged at 2,000 xg, serum was collected and during routine laboratory procedures analyzed using an immunoelectro-chemoluminescence assay (E170; Roche Diagnostics GmbH, Mannheim, Germany). Inter-assay coefficients of variation for serum folate were 4.5% at 13 nmol/L and 5.7% at 23 nmol/L, for serum cobalamine 3.6% at 258 pmol/L and 2.2% at 832 pmol/L and for plasma tHcy were 4.8% at 14.6 µmol/L and 3.3% at 34.2 µmol/L.

Statistical analyses

Chi-Squared tests and Mann-Whitney U tests were performed to evaluate differences between the groups. To perform linear and logistic regression analyses, a normal distribution of the sperm parameters was achieved with root transformation of ejaculate volume and sperm count, and fourth root transformation of TMSC and sperm concentration. Univariable and multivariable linear and logistic regression models were applied to study associations between medication use and sperm parameters. For the univariable linear regression analyses of sperm parameters, RBC folate was assessed in pmol/L for better interpretation of the estimate (pmol/L= nmol/Lx10^3). Age, ethnicity, smoking and alcohol use were selected as potential confounders based on associations with semen parameters. The cut off concentration for serum folate of ≥22.5 nmol/L was used to confirm folic acid supplement use [168]. Statistical analyses were performed using SPSS software for Windows (version 21.0, IBM SPSS, Statistics for Windows, Armonk, NY: IBM Corp) with a level of significance of 0.05.

RESULTS

A total of 2255 men visiting the preconception outpatient clinic were eligible for participation, from whom we excluded 137 who did not provide written informed consent and 846 who did not provide a semen analysis between 70 days before and 21 days after the visit. From the remaining 1272 men, we excluded 23 with MESA, PESA or retrograde ejaculation samples, ten with incomplete data, 329 using any other medication and 27 without information on medication use. This resulted in the analysis of 883 men, of whom 40 used medication for gastric acidrelated symptoms (PPIs n= 34, antacids n= 3, and H2RAs n= 3), and 843 controls

not using any medication. Of the 40 medication users, the use was daily in 29 men, weekly in two men and when needed in seven men. This data was missing in two men. General characteristics of included (n= 883) and excluded (n= 1235) men of subfertile couples are presented in Supplemental Table 1. All characteristics were comparable between the groups except a slightly lower age (34 y vs. 35 y, p= 0.003) and higher percentage of no medication use in the study population (95.5% and 52.6% non-users, p <0.001) inherent to the selection. Table 1 shows the general characteristics of medication users (n= 40) and controls (n= 843). In medication users, age was significantly higher (38 and 34 years, p= 0.002) and alcohol and drug use was lower than in controls (55% and 71%, p= 0.031 and 0.0% and 7.7%, p= 0.029 respectively). Serum and RBC folate tended to be higher and cobalamin and tHcy tended to be lower in medication users, albeit not significantly. The abstinence period and time to semen analysis were comparable between the groups. All sperm parameter values were slightly but not significantly lower in medication users except for a higher ejaculate volume (3.4 and 2.8 mL, p= 0.036). Figure 1 depicts the median values and interquartile ranges of sperm concentration, TMSC and sperm motility.

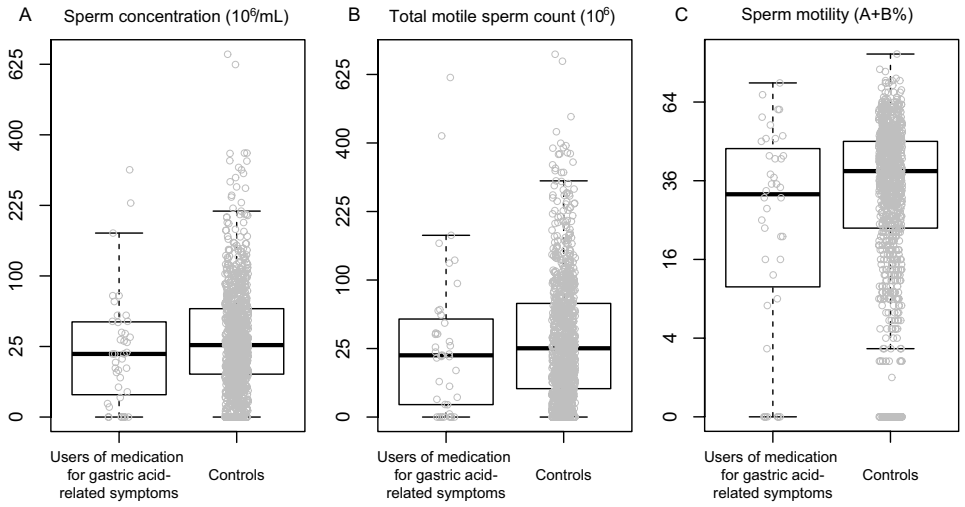

Figure 1 | **A** Sperm concentration (p= 0.090), **B** total motile sperm count (p= 0.209) and **C** motility (A+B%) (p= 0.095) in men using medication for gastric acid-related symptoms (n= 40) and controls (n= 843).

The Box plots display the 25th and 75th percentile, the horizontal line marks the median value and the whiskers extend to the minimum and maximum observations that are within 1.5 times the interquartile range

Table 1 | General characteristics of the study population of men of subfertile couples visiting the preconception outpatient clinic.

	Users of medication for gastric acid-related symptoms (n= 40)	Controls (n= 843)
Age (years)	38 (34-42)**	34 (30-39)
BMI (kg/m^2)	25.9 (23.4-29.3)	26.1 (23.9-28.6)
Ethnicity (Dutch)	24 (60.0%)	533 (63.5%)
Previous child (Yes)	9 (23.7%)	157 (19.4%)
Stress (Yes)	5 (15.6%)	99 (13.1%)
Smoking (Yes)	17 (42.5%)	281 (33.4%)
Alcohol (Yes)	22 (55.0%)*	597 (71.0%)
Recreational drugs (Yes)	0 (0.0%)*	65 (7.7%)
Folic acid supplement use (Yes)	11 (28.9%)	175 (22.6%)
Biochemical Parameters		
Folate (nmol/L)	17.6 (12.6-25.0)	17.2 (13.4-21.8)
RBC Folate (nmol/L)	987 (828-1109)	952 (779-1126)
Cobalamin (pmol/L)	279 (240-399)	299 (239-381)
tHcy (µmol/L)	11.0 (9.7-13.5)	11.5 (9.7-13.5)
Sperm Parameters		
Sperm count (10^6/ejaculate)	58.6 (8.4-119.6)	66.3 (20.7-147.6)
Ejaculate volume (mL)	3.4 (2.4-4.2)*	2.8 (1.8-3.9)
Sperm concentration (10^6/mL)	20 (3-46)	26 (9-59)
Sperm motility (A+B%)	32 (11-47)	39 (23-49)
Total motile sperm count (10^6/ejaculate)	20.3 (0.8-51.3)	25.2 (4.2-68.9)
Abstinence period (days)	4 (3-5)	4 (3-6)
Time to semen analysis (min)	40 (25-55)	45 (30-60)

Values are expressed as median (IQR) or number (%); Chi Square and Mann Whitney Tests were performed: * = p <0.05 ** = p <0.01;
Abbreviations; BMI = Body Mass Index; RBC Folate = Red Blood Cell Folate; tHcy = homocysteine. Normal range biochemical parameters; Folate ≥8 nmol/L, RBC folate ≥500 nmol/L, Cobalamin ≥145 pmol/L, tHcy <15 µmol/L.

Being a Dutch native was associated with a lower sperm concentration (β -0.128, 95% CI -0.23 to -0.02, p= 0.016) and a higher ejaculate volume (β 0.126, 95% CI 0.07 to 0.18, p <0.001). Smoking was negatively associated with TMSC (β -0.173, 95% CI -0.30 to -0.05, p= 0.008), sperm count (β -1.138, 95% CI -1.88 to -0.40, p= 0.003) and ejaculate volume (β -0.093, 95% CI -0.15 to -0.04, p= 0.001). Alcohol use was positively associated with sperm motility (β 2.506, 95% CI 0.26 to 4.75, p= 0.029), TMSC (β 0.130, 95% CI 0.001 to 0.26, p= 0.048) and ejaculate volume (β 0.069, 95% CI 0.01 to 0.13, p= 0.020). Age, BMI, folic acid supplement use and drug use were not significantly associated with sperm parameters.

Table 2 | Uni- and multivariable linear regression analyses in men of subfertile couples with a semen analysis performed within an interval of 70 days before and 21 days after the visit (n= 883).

Predictor	Ejaculate volume √(mL) β (s.e.)	Sperm concentration $\sqrt[4]{}$(10^6/mL) β (s.e.)	Sperm count √(10^6/ejaculate) β (s.e.)	Total motile sperm count $\sqrt[4]{}$(10^6/mL) β (s.e.)	Sperm motility (A+B%) β (s.e.)
Use of medication for gastric acid-related symptoms (unadjusted)	0.132 (0.076)	-0.311 (0.144)*[a]	-0.624 (1.009)	-0.248 (0.171)	-5.403 (2.963)
Use of medication for gastric acid-related symptoms (Model 1)	0.142 (0.075)	-0.307 (0.145)*	-0.522 (1.008)	-0.234 (0.170)	-5.301 (2.964)
Use of medication for gastric acid-related symptoms (Model 2)	0.161 (0.076)*	-0.320 (0.145)*	-0.504 (1.012)	-0.217 (0.172)	-4.561 (2.982)

Data depicted as β and standard error (s.e.),* = p <0.05. The regression coefficient (β) indicates the increase or decrease (-) change per unit of the sperm parameter. [a] Backward transformation to the original scale results in a decrease of 10.5 10^6/mL in the sperm concentration when medication for gastric acid-related symptoms was used. Model 1: adjusted for smoking, Model 2: adjusted for smoking, alcohol use, age and ethnicity.

Table 2 presents associations between medication for gastric acid-related symptoms and a decreased sperm concentration (β -0.311, SE 0.144). Backwards transformation to the original scale, resulted in a decrease in sperm concentration of $10.5x10^6$/mL in medication users. Moreover a two-fold higher risk of low total motile sperm count was observed in medication users (unadjusted OR 2.158, 95% CI 1.05 to 4.43, p= 0.036). After adjustment for smoking this risk slightly increased (OR 2.161, 95% CI 1.05 to 4.44, p= 0.036). Additional adjustment for alcohol use, age and ethnicity slightly attenuated the risk estimate (OR 2.090, 95% CI 1.00 to 4.35, p= 0.049). Adjustment of the latter model for the WHO manual edition did not affect the results (OR 2.148, 95% CI 1.02 to 4.48, p= 0.042). The multivariable linear regression analysis showed the same (β -0.323, SE 0.146, p= 0.027).

Univariable linear regression analyses revealed positive associations between RBC folate (pmol/L) and TMSC (β 0.257, 95% CI 0.03 to 0.48, p= 0.026), sperm count (β 1.679, 95%CI 0.35 to 3.01, p= 0.013) and ejaculate volume (β 0.120, 95%CI 0.02 to 0.22, p= 0.023). tHcy was negatively associated with sperm count (β -0.077, 95% CI -0.14 to -0.01, p= 0.021). Serum folate and cobalamin were not associated with any sperm parameter.

DISCUSSION

This explorative study shows that use of antacids, PPIs and H2RAs is associated with impaired semen parameters in men of subfertile couples visiting a tertiary hospital in the Netherlands. These medication users showed a decreased sperm concentration and a two-fold higher risk of low TMSC.

The strengths of the study are the standardized semen sample collection and semen analyses in a single and qualified laboratory center, thereby limiting risks of methodological and measurements errors. Protocols were used by trained counselors for data collection, verification of medication use in a defined window, and measurements. To exclude selection bias we also showed that general characteristics were largely comparable in included and excluded men. Weaknesses are, firstly, that due to the small sample sizes, the individual medicines (PPIs, H2RAs and antacids) could not be evaluated, and, secondly, confounding by indication, because in all medication users gastro intestinal symptoms were present. Although no abnormal semen parameters were shown in men with digestive diseases, these issues have to be addressed in future research [169]. Under-reporting of medication use is an issue to be considered, because previous research reported that 11% of Dutch men and women used medication for gastric acid-related symptoms, which is much higher than the 5% in our study [157]. Because these medicines are also available over-the-counter, a validation through pharmacy databases was not possible. In addition, frequency of medication use, but not duration of use, was taken into account, which is a limitation but compensates the reliability of current use. Therefore this study can also not assess the -although very unlikely- harmful pharmacological effects of a single dose on semen quality.

One semen sample of each man was available which does not account for intra-individual variation of semen parameters. Residual confounding by a sedentary lifestyle, sauna visits, nutritional behavior or incompleteness of the sample collection cannot be excluded as well and has to be addressed in future [170,171]. Confounding however was reduced by selecting semen samples only in a strictly defined window which was independent of exposures and bias due to the exclusion of conditions affecting semen parameters, i.e. varicoceles or cryptorchidism. Our findings are supported by Hayashi et al who reported that quitting or switching H2RAs improved semen quality [172]. Others have shown a reduction in sperm viability in a dose- and time-dependent manner, when liquefied semen samples were exposed to H2RAs [173]. Both Ranitidine and Cimetidine were considered to be acting as anti-androgens, but only the use of cimetidine impaired hypothalamic-pituitary-gonadal function [174]. Ranitidine, the only H2RA reported in our study, did not bind to androgen receptors or influence basal serum concentrations of testosterone, luteinizing hormone, follicle stimulating hormone or prolactin in fertile men. The mean sperm count decreased during Ranitidine treatment, although this difference was not significant [175]. The higher ejaculate volume in medication users is new and so far cannot be explained by a pharmacological mechanism of action. Our previous study investigating the effect of the duration of PPIs only on semen quality substantiates the potential harmful effects observed in this study. A three-fold higher risk of a low TMSC was observed in men visiting the general practitioner when PPIs were used between 12 and 6 months preceding semen analysis, suggesting that a long-term increase in gastric pH results in a decline of sperm quality [176].

Detrimental effects of BMI, age or alcohol consumption on semen quality were not observed. This may be due to the small variation of these conditions and effect estimates relative to the large variations in sperm parameters. Our studies and that of others reported that obesity or central adiposity are associated with impaired sperm motility, ejaculate volume, sperm concentration, TMSC [57,177] and azoospermia or oligozoospermia [178]. The latter study also reported no differences in sperm concentration across BMI categories. Age above 60 has been associated with a raised sperm DNA fragmentation index, but similar to our results, was not significantly associated with other sperm parameters [179]. The association between alcohol use and semen parameters is not substantiated by the rather conflicting literature and is complicated because the majority of men in this study used alcohol [180-184]. In addition, smoking negatively influenced TMSC, ejaculate volume and sperm count, which is in line with studies of others [185,186]. From these data we conclude that differences in general characteristics of the study populations, sample sizes, precision of exposure assessment, and the effects at the extremes of conditions only (BMI >30, excessive alcohol consumption) might explain the differences in associations between BMI, age, alcohol use and sperm parameters.

Associations between the biomarkers of 1-C metabolism RBC folate, tHcy and semen quality are in line with our studies and that of others [45,46,187]. Moreover, these observations are supported by studies demonstrating associations between folate intake by supplements or

food and semen quality [161,188]. A traditional Dutch diet is positively associated with RBC folate and with sperm concentration, and a diet rich in fruits and vegetables is negatively associated with seminal plasma tHcy and sperm DNA fragmentation index (DFI) [9,45]. In our study, the slightly lower cobalamin level in medication users did not differ significantly from non-users. This may be explained by a higher percentage of vitamin supplement users in medication users resulting in higher serum and RBC folate, albeit not significantly, and the small sample size. The use of folic acid supplements was not associated with semen quality and therefore not included in the multivariable analyses [45,50,161,162]. This is very likely due to the limited folic acid supplement use in our study.

CONCLUSION

The use of medication for gastric acid-related symptoms is associated with impaired semen parameters in men of subfertile couples contemplating pregnancy. These first findings have to be interpreted with caution and emphasize the need for further research on causality and awareness of potential detrimental effects of medication for gastric acid-related symptoms on semen quality.

Supplemental Table 1 | General characteristics of the study population and the excluded population.

	Study population (n= 883)	Excluded population (n= 1235)
Age (years)	34 (30-39)	35 (30-40)**
BMI (kg/m^2)	26.1 (23.9-28.6)	26.3 (23.9-29.0)
Ethnicity (Dutch)	557 (63.4%)	735 (60.9%)
Smoking (Yes)	298 (33.8%)	378 (31.1%)
Alcohol (Yes)	619 (70.3%)	842 (69.2%)
Recreational drugs (Yes)	65 (7.4%)	101 (8.3%)
Folic acid supplement use (Yes)	186 (22.9%)	252 (23.6%)
Medication use		
No	843 (95.5%)	631 (52.6%)**
Medication for gastric acid-related symptoms	40 (4.5%)	27 (2.3%)
Other	–	542 (45.2%)
Biochemical Parameters		
Folate (nmol/L)	17.2 (13.4-21.8)	17.4 (13.7-22.1)
RBC Folate (nmol/L)	956 (783-1124)	962 (807-1134)
Cobalamin (pmol/L)	298 (239-383)	301 (238-390)
tHcy (µmol/L)	11.5 (9.7-13.5)	11.3 (9.6-13.6)

Values are expressed as median (IQR) or number (%); Chi Square and Mann Whitney Tests were performed: ** = p <0.01; *Abbreviations;* BMI = Body Mass Index; RBC Folate = Red Blood Cell Folate; tHcy = homocysteine. Normal range biochemical parameters; Folate ≥8 nmol/L, RBC folate ≥500 nmol/L, Cobalamin ≥145 pmol/L, tHcy <15 µmol/L.

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Chapter 7

Are proton-pump inhibitors harmful for the semen quality of men in couples who are planning pregnancy?

ABSTRACT

Objective

To determine associations between proton-pump inhibitor (PPI) use and semen parameters in young men of couples who are planning pregnancy.

Design

Case-control study of a population-based registry. General practitioner patients comprising 2,473 men from couples planning pregnancy with a recorded semen analysis: 241 with a low total motile sperm count (TMSC ≤1) and 714 with TMSC >1 as matched controls.

Main Outcome Measure(s)

Exposure to PPI; PPI dosage.

Result(s)

The study of data from between 1996 and 2013 from the Integrated Primary Care Information database in the Netherlands, which incorporates the medical records of 1.5 million patients from 720 general practitioners, found that the use of PPIs in the period between 12 and 6 months before semen analysis was associated with a three-fold higher risk of low TMSC (odds ratio 2.96; 95% confidence interval 1.26-6.97) adjusted for age and other medication. Use of PPIs during the 6 months immediately before the semen analysis was not statistically significantly associated with low TMSC.

Conclusion(s)

The use of PPIs in the period 12 to 6 months preceding semen analysis is associated with a three-fold higher risk of low TMSC, which suggests that a long-term increase in gastric pH results in a decline of sperm quality. This finding emphasizes the need for more preconceptional research and counseling on the potential effects of medication use on semen quality.

INTRODUCTION

In one out of ten couples, pregnancy is not achieved after one year of unprotected intercourse [3,189]. Subfertility is a major burden affecting 48.5 million people worldwide and in a considerable number a male factor is involved [156]. A method for quantifying the severity of male factor subfertility is the assessment of sperm parameters, among which total motile sperm count (TMSC) is a reliable parameter for predicting the chance of a spontaneous ongoing pregnancy [190]. The observed continuous decline in sperm quality over the last decades has been worrisome, as it seems to be related to increased exposure to endocrine disruptors, unhealthy lifestyles, obesity, and increased age [179,191,192]. Health status declines accompany aging, which goes together with an increase in medication use. In the Netherlands the use of prescribed medication in men between 20 and 25 years of age is 18.7%, and rises to 52.9% by the ages of 55 to 65 years [193].

Proton-pump inhibitors (PPIs) are the most commonly used medication for gastro-oesophageal reflux disease and peptic ulcer disease. Its prevalence is 26.9% in men and women with gastrointestinal symptoms and 5% in those without gastrointestinal symptoms [157]. In comparison with H2-receptor antagonists (H2RAs) and antacids, PPIs are most effective in decreasing gastric acidity [194]. A strong disulfide bond, binding irreversibly to the hydrogen-potassium ATPase pump, leads to a specific inactivation of the enzyme, which results in a long-lasting impairment of gastric acid secretion [195].

The increase in pH that accompanies PPI use reduces the uptake of B vitamins, which are essential for biologic processes involved in DNA synthesis and cellular growth and development such as spermatogenesis [12,45,46]. Thus, patients on long-term PPI treatment should be periodically screened for nutrient deficiencies [12,160]. The other suggested adverse effects of PPIs are direct gonadotoxic effects and interactions with the hypothalamic-pituitary-gonadal axis. To explore these effects further, we examined whether the use of PPIs is associated with impaired semen quality in a population based study of men in couples who are planning pregnancy and have been visiting a general practitioner.

MATERIALS AND METHODS

Design

Our case-control study was conducted using the Dutch Integrated Primary Care Information (IPCI) database. The IPCI database, which consists of medical records of 1.5 million patients from 720 general practitioners (GPs) spread across the Netherlands, is validated to be used for pharmacoepidemiologic research [196]. It complies with European Union guidelines on the use of data for medical research. More details concerning the IPCI-database have been provided elsewhere [197].

Patients who visited a GP between 1996 and 2013 are registered in the IPCI database with anonymous longitudinal information (coded and in free text) on age, symptoms, test results, disease outcome, referrals, and prescription data. The database comprises all men with at

least 12 months of data registered before the study period (1996 and 2013). We extracted additional information on obesity, weight, smoking, and the use of alcohol in the period of 15 months preceding the visit until the day of the semen analysis by using search codes and text strings. From the prescription files we extracted data on dosage, frequency, and duration of medication use in the same time window based on the Anatomical Therapeutic Chemical (ATC) classification system. The Scientific Advisory Board of the IPCI Project approved the study protocol and use of IPCI data (Project number: 01/13).

Study population

A study cohort was selected of men aged ≥18 years during the study period and found via one of the following search codes; 'A97.02' (wish to become parents), 'Y10' (male sub/infertility), 'W15' (female sub/infertility), or the additional text strings 'sperm', 'semen', 'ferti', 'androl', and 'YS' (the text code for semen analysis). Records including the text 'vasectomy', 'sterilisation', 'spermatocele', 'hematospermia', or 'epididymitis' in the same notation were automatically excluded. Every medical record was manually validated and the semen analysis data were extracted. The manual review excluded the false hits such as patients without semen analysis data or without the wish to become parents. In the study population we only included men in couples who were planning a pregnancy and with a recorded semen analysis at the first visit (Table 1). We excluded men with a history of testicular surgery, chemotherapy or radiotherapy, congenital absence of the vas deferens, varicocele, or who had an incomplete or unreliable semen analysis (for example, due to fever). Subsequently, we excluded men without any recorded prescription data or with an incomplete medication history (Figure 1).

Cases and controls

Cases were defined as men with a low TMSC ≤1 which is the clinical diagnosis of severe oligoasthenoteratozoospermia [167]. All men with a TMSC >1 were selected as controls and were matched with cases managed by the same GP.

Proton-pump inhibitor exposure

Proton-pump inhibitor exposure (ATC code A02BC) was assessed for the period 12 to 6 months preceding the semen analysis (period 1) and in the period 6 months immediately before the semen analysis (period 2). Men without PPI exposure in the corresponding period were defined as non-users. Exposure to other ATC-coded drugs in the period 12 to 0 months before the semen analysis was defined as "Medication use other." We expressed PPI dosage as the defined daily dosage (DDD) – the assumed average maintenance dose per day for a drug used for its main indication in adults – to account for differences in the standard dosing regimen between the types of PPIs. The cumulative PPI dose was calculated by multiplying the DDD and the duration of use. If the duration of use was missing, we imputed a value calculated by dividing the prescribed number of units by the dosing regimen.

Table 1 | General characteristics of cases and controls in sperm analysis and proton-pump inhibitor use analysis based on the Integrated Primary Care Information database in the Netherlands.

	Cases (TMSC ≤1) (n= 241)	Controls (TMSC >1) (n= 714)
Age (years)	34 (30-39)	34 (31-38)
Body mass index >25 (kg/m^2)		
Yes	21 (77.8%)	43 (72.9%)
No	6 (22.2%)	16 (27.1%)
Missing	214	655
Smoking		
Yes	38 (63.3%)	71 (46.7%)
No	22 (36.7%)	81 (53.3%)
Missing	181	562
Alcohol use		
Yes	16 (59.3%)	65 (77.4%)
No	11 (40.7%)	19 (22.6%)
Missing	214	630
Sperm Parameters:		
pH	8.0 (7.7-8.1)	8.0 (7.7-8.0)
Sperm count (10^6/ejaculate)	2.6 (1.1-9.2) *	103.7 (48.0-198.0)
Ejaculate volume (mL)	2.5 (1.5-3.5) *	3.0 (2.0-4.0)
Sperm concentration (10^6/mL)	2.1 (0.3-9.0) *	43.0 (21.0-81.0)
Sperm motility (A+B%)	0.0% (0.0%-5.0%) *	43.6% (31.3%-56.0%)
Total motile sperm count (10^6/ejaculate)	0.0 (0.0-0.3) *	47.2 (16.6-96.8)
Abstinence period (days)	3 (2-5)	3 (2.5-5)
Time to semen analysis (min)	0:40 (0:30-1:00)	0:40 (0:30-0:55)

Values are expressed as median (IQR) or as number (%); TMSC = total motile sperm count. * p <0.01

Semen sample data

The following semen sample data were extracted; ejaculate volume (mL); sperm concentration (10^6/mL); sperm motility parameters type A (rapid progressive motility, %) and type B (slow or sluggish progressive motility, %); total sperm count (10^6/ejaculate); and total motile sperm count (TMSC, 10^6/ejaculate) calculated as the product of ejaculate volume, sperm concentration and grade A+B motility. Additional information was collected on the abstinence period, time between semen collection and analysis, pH, and completeness of the sample.

Semen analyses were performed in hospital-affiliated laboratories participating in the external quality control scheme of the Dutch foundation for quality assessment in clinical laboratories (www.SKML.nl). The staff performing these analyses has trained and tested annually according to the certified European Society of Human Reproduction and Embryology (ESHRE) course for performing semen analyses. Both clinical and nonclinical laboratories have to be ISO15189

certified (the worldwide standard for medical laboratories) and are controlled by the Dutch institute for accreditation of laboratories (www.cckl.nl).

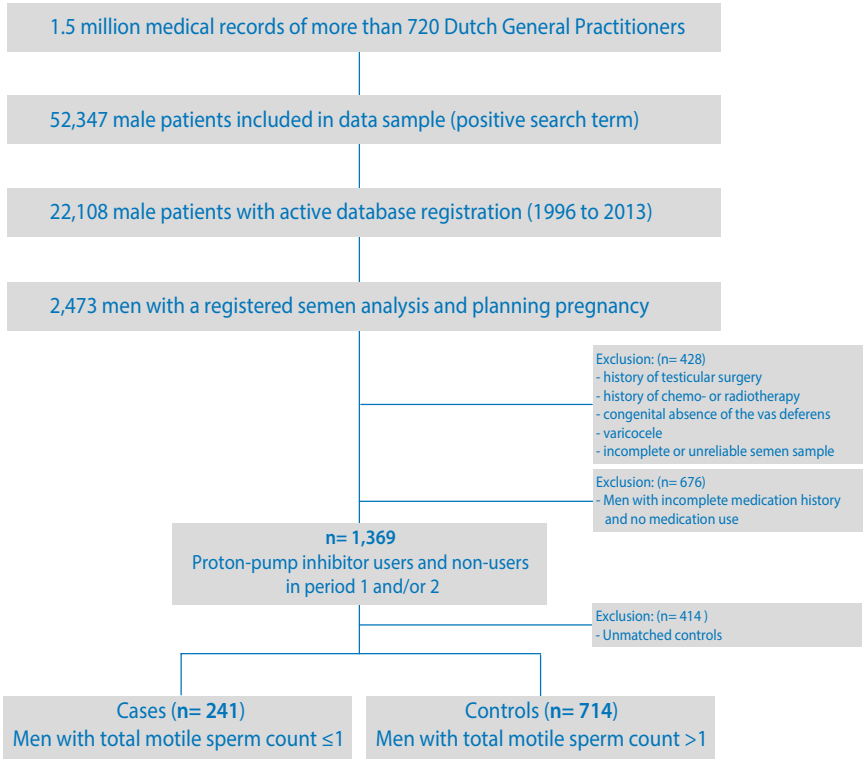


Figure 1 | Flowchart of data acquisition for model 1 of the conditional logistic regression analysis.

Statistical analyses

Chi-square tests and Mann-Whitney U tests were used to test differences between cases and controls, and Chi-square tests and Kruskal Wallis tests in case of comparisons of more than two groups. In PPI users and non-users, the effects of PPI use in two consecutive periods of 6 months preceding semen analysis were analyzed for cases matched to controls by GP using conditional logistic regression with adjustment for age and other medication use (Table 2, model 1). To investigate possible confounding by ejaculate volume, this variable was added to the logistic regression analysis (model 2). In model 3 the analysis was repeated with a modification of the window of PPI exposure of 12-0 months preceding semen analysis. The effect of PPI use on TMSC was also analyzed using a multivariate linear mixed model analysis only in men with a TMSC ≥1, adjusted for age and clustered by GP and with a fourth root transformation to TMSC because of a left-skewed distribution (Table 3). The analysis was repeated after the exclusion of men using other medication (Table 3). Statistical analyses

were performed using SPSS (version 21.0, IBM SPSS) and SAS 9.2 (SAS Institute). P <0.05 was considered statistically significant for all analyses.

Table 2 | Proton-pump inhibitor use and the risk of low total motile sperm count.

	OR	P value	95% CI
Unadjusted model cases n= 241, controls n= 714			
PPI use in period 1	**2.90**	**0.01**	(1.24-6.76)
PPI use in period 2	0.70	0.41	(0.30-1.65)
Model 1 cases n= 241, controls n= 714			
PPI use in period 1	**2.96**	**0.01**	(1.26-6.97)
PPI use in period 2	0.72	0.47	(0.30-1.74)
Age	1.00	0.83	(0.98-1.02)
Medication use other	0.85	0.32	(0.61-1.17)
Model 2 cases n= 183, controls n= 667			
PPI use in period 1	**2.95**	**0.02**	(1.18-7.39)
PPI use in period 2	0.60	0.33	(0.22-1.67)
Age	1.00	0.77	(0.98-1.03)
Medication use other	0.77	0.14	(0.53-1.10)
Ejaculate volume	0.91	0.09	(0.82-1.01)
Model 3 cases n= 241, controls n= 714			
PPI use 1 year preceding semen analysis	1.52	0.17	(0.83-2.79)
Age	1.00	0.70	(0.98-1.03)
Medication use other	0.84	0.30	(0.61-1.17)

Note: Results from conditional logistic regression analyses in which we defined men with a total motile sperm count (TMSC) ≤1 (10^6/ejaculate) as cases and >1 as controls. Model 1: adjusted for age and other medication use. Model 2: model 1 with additional adjustment for ejaculate volume. Model 3: modification of the window of proton-pump inhibitor (PPI) exposure to 12–0 months preceding semen analysis. CI = confidence interval; OR = odds ratio.

RESULTS

General characteristics of the 241 cases and the 714 matched controls are shown in Table 1. No significant differences were observed in the general characteristics. The median abstinence period and time to semen analysis were similar, respectively 3.0 vs. 3.0 days (p= 0.78) and 40 vs. 40 minutes (p= 0.77). In cases a lower median sperm count, ejaculate volume, sperm concentration, sperm motility and TMSC was observed (all p <0.01).

Risk of low motile sperm count (TMSC ≤1)

Table 2 shows the results of the case-control analyses. Use of a PPI in period 1 (12-6 months before semen analysis) was associated with a three-fold higher risk of low TMSC (odds ratio [OR] 2.96; 95% confidence interval [CI], 1.26-6.97). Use of a PPI in period 2 was not associated with TMSC. Because of the differences in ejaculate volume between cases and controls (Table 1), we adjusted the analysis for ejaculate volume, which slightly attenuated this risk (OR

2.95; 95% CI, 1.18-7.39). When the window of PPI exposure was modified into 12-0 months preceding semen analysis, PPI use was no longer statistically significantly associated with low TMSC (OR 1.52; 95% CI, 0.83-2.79). The interaction between PPI use in the two periods was tested and showed no statistically significant interaction (OR 1.56; p= 0.64; 95% CI, 0.25-9.95).

Multivariate linear mixed model analysis

The use of PPIs in periods one and two was not statistically significantly associated with TMSC (β -0.17 and β -0.001, respectively; see Table 3) when the analysis was performed in PPI users and non-users.

After the exclusion of men using other medication (n= 786, ST1), this analysis was repeated in PPI users and men using no medication. The use of PPIs in period 1 was associated with a decrease in TMSC (β -0.33; Table 3), but not PPI use in period 2 (β 0.18). For example, a man of 30 years, using PPIs in period 1 would have a TMSC that is 17.99 (10^6/ejaculate) lower than a man of the same age who was not using any medication, when both were not using PPIs in period 2. This difference increases with age, as shown by a 22.89 (10^6/ejaculate) lower TMSC in a PPI user compared with a man aged 50 years who was not using any medication.

Table 3 | Multivariate linear mixed model analysis of total motile sperm count.

	β estimate	P value	95% CI
Effects in PPI users and non-users [a]			
Intercept	2.40	<0.001	(2.02 to 2.79)
PPI use in period 1	-0.17	0.20	(-0.44 to 0.09)
PPI use in period 2	-0.001	0.99	(-0.24 to 0.24)
Age	0.001	0.83	(-0.01 to 0.01)
Medication use other	-0.005	0.93	(-0.10 to 0.09)
Effects in PPI users and no medication users, after exclusion of men using other medication than PPIs [b]			
Intercept	2.25	<0.001	(1.75 to 2.76)
PPI use in period 1	-0.33	0.047	(-0.66 to -0.01)
PPI use in period 2	0.18	0.28	(-0.15 to 0.52)
Age	0.01	0.32	(-0.01 to 0.02)

Note: CI = confidence interval; PPI = proton-pump inhibitor; TMSC = total motile sperm count. Total motile sperm count is transformed to achieve normality $\sqrt[4]{}$ (TMSC 10^6/ejaculate).

[a] Multivariate linear mixed model analysis of TMSC in association to the use of PPIs in the two periods, adjusted for age and other medication use and using the general practice (GP) as clustering variable.

[b] Multivariate linear mixed model analysis of TMSC in association to the use of PPIs in the two periods, adjusted for age and using the general practice (GP) as clustering variable.

For 86 men the PPI dose was known. In Supplemental Figure 1 we plotted TMSC against cumulative DDD in the 12 months preceding semen analysis. Subsequently, the cumulative DDD was categorized into low dose (1-30) and high dose (>30) groups of 43 men each, and

the allocation was assessed between five clinical relevant TMSC categories; ≤1, >1 to ≤3, >3 to ≤10, >10 to ≤20 , >20. In TMSC category ≤1 we observed more men using a high dose than men using a low dose (n= 8, 66.7% vs. n= 4, 33.3%). In TMSC category >20 more men used a low dose than a high dose (n= 28, 51.9% vs. n= 26, 48.1%). However, we observed no statistically significant trend in the five TMSC categories (linear-by-linear association, p= 0.64). To evaluate selection bias, in Supplemental Table 1 we presented the general characteristics of PPI users compared with non-users, with users of other medication, with non-users who had an incomplete medication history, and with those meeting the study exclusion criteria. The general characteristics were comparable, except for a statistically significantly higher age in PPI users. Sperm parameters were statistically significantly different among the groups; the group of excluded men showed on average a lower median sperm count, concentration, motility, and TMSC. The PPI users showed a lower TMSC (median 23.8 vs. 40.0; p= 0.005 for pairwise comparison) and a lower ejaculate volume (median 2.5 vs. 3.0 mL; p= 0.02) than non-users.

DISCUSSION

To our knowledge, our study is the first to show negative associations between PPI use and semen parameters. In men of couples planning pregnancy and visiting a GP, a three-fold higher risk of a low TMSC ≤1 was observed when using PPIs between 12 and 6 months before semen analysis. This association was substantiated in men with a TMSC ≥1, showing a decrease in TMSC when PPIs were used in the same period in comparison with men using no medication. We found that PPI use in the 6 months preceding semen analysis was not associated with a low TMSC.

The effect of PPI use on semen quality has scarcely been studied before. A study in mice treated with omeprazole during spermatocytes meiosis observed no effect on chromosome nondisjunction [198]. Unfortunately, sperm parameters were not assessed. Direct genotoxicity was tested by exposing purified eukaryotic DNA of salmon sperm to omeprazole and was found to be safe [199]. The only study performed in humans addressed men testing positive for Helicobacter pylori IgA antibody, and omeprazole use was combined with tinidazole and clarithromycin. Three months after triple therapy, an amelioration of sperm motility was observed [200]. This seems contradictory to our results, although a longer follow-up period up of 6 to 12 months was not achieved. The eradication of H. pylori itself could have led to a general improved wellbeing, causing a restoration of sperm motility.

To our knowledge there have been no other attempts to investigate semen parameters in PPI users. Use of H2RA to neutralize gastric acidity was previously related to a deterioration of semen parameters [172], although H2RAs in comparison with PPIs are far less frequently used [157].

Our study showed that only PPI use between 12 and 6 months preceding semen analysis was associated with a lower TMSC, not PPI use in the 6 months preceding semen analysis nor

when the two periods were combined in one window of PPI exposure 1 year preceding semen analysis. This suggests that spermatogenesis, which covers a time span of around 10 weeks, is not directly affected by PPI use. A possible mechanism explaining the long period between PPI exposure and sperm quality decline may be that PPIs induce a prolonged increase in gastric pH resulting in B vitamin and other micronutrient deficiencies as a consequence of impaired gastrointestinal absorption [12,20,46]. Apparently the resources of these vitamins are exhausted after 4 to 6 months, resulting in a subsequent dramatic decrease in semen quality. The previous studies investigating cobalamin malabsorption were not conclusive in assessing the time window of the development of these micronutrient deficiencies. Two weeks after PPI use a dose dependent decrease in cobalamin absorption was observed [160].

Lam et al. [12] investigated only the effect of a 2 or more years' supply of PPIs and also observed a dose dependent increased risk of cobalamin deficiency. Periodically assessing cobalamin levels in patients who are on long-term treatment with PPIs is therefore recommended [160]. To our knowledge deficiencies in folate absorption and the adverse effects of a shorter time span of PPI use have not been studied. Along with a lower TMSC, we also observed a lower ejaculate volume in PPI users. It is interesting to speculate about whether this is due to PPI exposure; it is more important to note that there was no confounding because ejaculate volume is used to calculate TMSC. Additional adjustment for ejaculate volume only slightly attenuated the association. In addition, confounding due to differences in abstinence or time to semen analysis can be excluded because this was similar in the cases and controls and in PPI users and non-users. To exclude selection bias we also showed that in included and excluded men the general characteristics were largely comparable. In this study we did not observe a linear association between PPI use and TMSC when the PPI users were compared with PPI non-users. However, when the PPI users were compared with relatively healthy men-after the exclusion of men using other medication, by selecting men not using any medication as nonexposed group-a statistically significant decrease in TMSC was observed. This can be explained by confounding by indication because medication use is a proxy for the presence of disease that may affect semen parameters as well [169].

Inherent to the observational character of the study is that we cannot control for the effect of gastrointestinal disease itself because all medication users will suffer from gastrointestinal symptoms. Furthermore, when interpreting these data we must keep in mind that prescription data were used to identify PPI users and non-users. The use of prescription data minimizes the risk of information bias, although misclassification could have occurred in patients with low compliance to PPI use. On the other hand, PPIs are approved by the U.S. Food and Drug Administration as over-the-counter drugs, and underreporting of PPI use might have been occurred in the non-exposed group. However, the comparable prevalence of PPI use (8%) in our study compared with a large population-based study makes underreporting or overreporting not very likely [157]. The IPCI database is a very reliable and validated data source composed of a large cohort of medical records [196].

General practitioners play a pivotal role in cases of difficulty conceiving as they are the first medical professionals to be consulted and they provide further referral to a gynecologist if needed. Our search strategy was very comprehensive; it expanded the amount of records for manual review and reduced the chance of missing cases. The manual review allowed us to classify all individuals accurately. We thus assume that these first observations serve as an acceptable representative of the general population. Ethnic background information was not available in this database, but it is related to semen quality and to PPI use [179,201]. By matching cases and controls by GP management, we attempted to reduce confounding; patients visiting the same GP are more likely to live in the same neighborhood, which is a proxy not only for ethnic background but also for socioeconomic status, health, and other characteristics [202]. Matching by GP also accounts for confounding due to interlaboratory differences in semen analyses and for differences among GPs in reporting and prescribing.

Inherent to a database study are limitations that need to be addressed. Unfortunately, the effect of body mass index, smoking, or alcohol use could not be assessed because of poor registration data (see Table 1 and Supplemental Table 1) [57,203-205]. Another study limitation was that we could not account for intraindividual variation in semen quality because only one semen analysis was used per patient. Instead of repeating the sample, it is more likely for GPs to refer the patient onward in cases of poor semen quality. Due to the study sample size, it was not possible to match cases and controls on age in addition to GP. Finally, although semen abnormalities are not observed in digestive diseases, we cannot exclude that the observations in this study are caused by the gastroesophageal reflux disease itself, because all patients who received a prescription for PPIs reported gastrointestinal symptoms [169].

The cumulative dose–effect association substantiates the findings, but we have to interpret them with caution because this analysis showed no statistically significant trend, was performed in a small sample of PPI users, and was performed without matching for GP.

CONCLUSION

This study showed in a population-based cohort, the novel finding that PPI use 12-6 months preceding semen analysis is associated with a three-fold higher risk of low TMSC in the general male reproductive population. This emphasizes the need for preconceptional research and counseling on the side effects of medication use on semen quality.

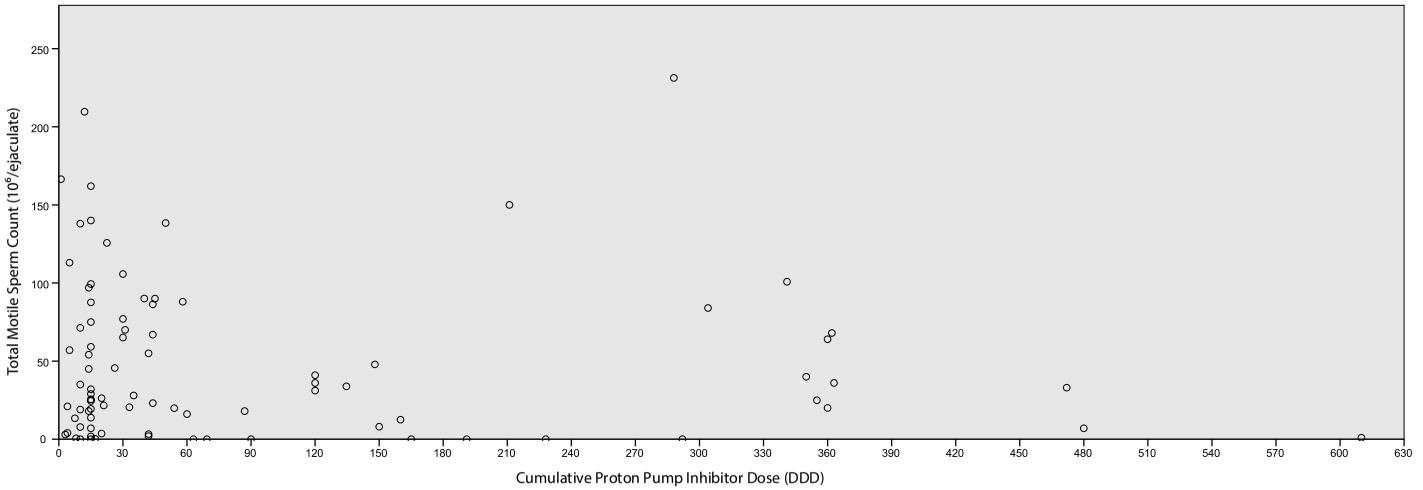
Supplemental Table 1 | General characteristics of the PPI users and non-users in the year preceding semen analysis and the groups of excluded men.

	PPI use (n= 129)	No medication use (n= 625)	Medication use other (n= 786)	No medication and incomplete history (<12 months) (n= 505)	Excluded (n= 428)	P value
Age (years)	36 (30-39)	34 (31-38)	34 (30-39)	33 (30-37)	34 (30-38)	.02
Obesity						
Yes	13 (76.5%)	25 (78.1%)	62 (74.7%)	15 (65.2%)	29 (60.4%)	.34
No	4 (23.5%)	7 (21.9%)	21 (25.3%)	8 (34.8%)	19 (39.6%)	
(missing)	112 (86.8%)	593 (94.9%)	703 (89.4%)	482 (95.4%)	380 (88.8%)	
Smoking						
Yes	25 (65.8%)	64 (53.3%)	93 (52.2%)	59 (51.8%)	54 (53.5%)	.63
No	13 (34.2%)	56 (46.7%)	85 (47.8%)	55 (48.2%)	47 (46.5%)	
(missing)	91 (70.5%)	505 (80.8%)	608 (77.4%)	391 (77.4%)	327 (76.4%)	
Alcohol						
Yes	17 (68.0%)	41 (66.1%)	65 (72.2%)	33 (76.7%)	41 (80.4%)	.48
No	8 (32.0%)	21 (33.9%)	25 (27.8%)	10 (23.3%)	10 (19.6%)	
(missing)	104 (80.6%)	563 (90.1%)	696 (88.5%)	462 (91.5%)	377 (88.1%)	
Sperm Parameters						
pH	7.9 (7.7-8.0) (n= 92)	8.0 (7.7-8.0) (n= 331)	8.0 (7.7-8.1) (n= 519)	8.0 (7.7-8.1) (n= 315)	8.0 (7.7-8.2) (n= 255)	.76
Sperm count (10^6/ejaculate)	88.1 (30.0-176.0) (n= 58)	90.0 (31.1-187.2) (n= 258)	89.0 (31.0-190.0) (n= 349)	104.1 (40.0-222.0) (n= 210)	47.6 (19.2-112.8) (n= 173)	**<.001**
Ejaculate volume (mL)	2.5 (1.6-3.5) (n= 115)	3.0 (2.0-4.0) (n= 605)	2.9 (1.8-4.0) (n= 740)	3 (2.0-4.0) (n= 462)	2.9 (1.6-4.0) (n= 384)	.05
Sperm concentration (10^6/mL)	32.5 (15.0-61.5) (n= 110)	35.2 (15.6-65.0) (n= 561)	32.0 (13.0-75.0) (n= 698)	36.1 (15.6-66.3) (n= 440)	16.9 (5.0-42.0) (n= 340)	**<.001**
Sperm motility (A+B%)	40.5 (27.0-54.0) (n= 110)	41.0 (22.0-54.0) (n= 592)	43.0 (25.0-57.2) (n= 713)	44.9 (27.0-60.0) (n= 440)	35.0 (10.0-53.0) (n= 350)	**<.001**

Supplemental Table 1 | *(Continued)*

	PPI use (n= 129)	No medication use (n= 625)	Medication use other (n= 786)	No medication and incomplete history (<12 months) (n= 505)	Excluded (n= 428)	P value
Total motile sperm count (10^6/ejaculate)	23.8 (3.4-67.5) (n= 124)	40.0 (9.6-90.1) (n= 584)	30.1 (6.6-84.2) (n= 757)	33.1 (5.0-95.0) (n= 491)	10.8 (0.0-38.0) (n= 387)	**<.001**
Abstinence period (days)	3.0 (2.0-4.0) (n= 37)	3.0 (2.0-5.0) (n= 243)	3.0 (2.0-4.0) (n= 270)	3.0 (2.0-5.0) (n= 202)	3.0 (2.0-4.25) (n= 164)	.13
Time to semen analysis (min)	0:44 (0:30-1:00) (n= 37)	0:40 (0:30-1:00) (n= 243)	0:41 (0:30-0:56) (n= 305)	0:45 (0:35-1:01) (n= 214)	0:50 (0:30-1:00) (n= 183)	**.003**

Note: Values are expressed as median (interquartile range) or as number (%).



Supplemental Figure 1 | Scatterplot of Total Motile Sperm Count (TMSC) and cumulative proton-pump inhibitor dose.

Chapter

8

General discussion

In this thesis we investigated the role of nutrient deficiencies in human subfertility, promoted by an inadequate intake or a drug-induced malabsorption. In this chapter we will describe the main findings and discuss the methodological considerations, and the suggestions for future research.

MAIN FINDINGS

New screening tools and mHealth intervention

In chapter 2 we observed that the Preconception Dietary Risk (PDR) score is a sensitive, quick and simple screening tool to assess diet inadequacy, ideal for use in clinical practice with a sensitivity of more than 80% for inadequate intakes of bread, vegetables, fruit, meat and fish. Diet inadequacy was present in the majority of women and men and a significant correlation was observed between PDR score and serum cobalamin concentration. We are aware that dietary interventions only cover one of the modifiable risk factors and do not offer the overall solution for the treatment of reproductive disorders, but we really want to emphasize that its contribution is important. Changing your diet into a healthy and adequate diet can be achieved with personal counselling or coaching and is necessary since in the majority of couples, diet inadequacy was present.

The PDR score covered the inadequacy of the six most important food groups and was previously used for the assessment of dietary inadequacy [6,59]. However, unhealthy food groups are not included in this score and therefore only assessed inadequacy in terms of insufficient intake of the six healthy food groups. Therefore with the PDR score we cannot investigate a harmful effect of intake of unhealthy food groups and that is why in the m-health program 'Smarter Pregnancy', extra questions were added to cover the intake of unhealthy food groups as well. It is also interesting to speculate whether all six items contribute beneficially in the same quantity. Especially, since Hammiche et al. observed a beneficial effect of omega-3 polyunsaturated fatty acids on embryo morphology and maybe justifies that the item fish should account for a more significant contribution in the calculation of the summary PDR score [7]. To elaborate on this issue further, a gender specific calculation of the PDR score could be proposed since different beneficial effects are observed for women and men.

We presented a simple score for health care providers to use in clinical practice in which they can assess diet inadequacy with six short questions. For simplicity the options per PDR score item are adequate or inadequate according to the guideline, but in daily practice it might be better to nuance the degree of inadequacy. Because when a person is not eating any vegetables and a person is eating every day 195 grams of vegetable, they are both scored as inadequate for that PDR item (both <200 grams daily). Other variants of the PDR score worldwide struggle with the same issue; that adding more details to the score is actually a trade-off for being less usable for clinical use [74,206-208]. Furthermore, a cumulative effect can be achieved when other modifiable risk factors such as smoking, alcohol use, drugs use are improved as well and can be considered to add to the score as well [59].

These observations were used for the further personalization of the preconceptional counselling and treatment of couples during the periconception period. In chapter 3 we observed that M-health digital coaching is an effective tool to improve and additionally maintain healthy lifestyle behaviors in women and men in the preconception period and during pregnancy as well and is in line with the current rise in use and possibilities of mobile technology. One or two consultations in a clinical setting cannot match the effects accomplished with information send weekly by e-mail or sms messages, and can contribute to a higher compliance when dietary advice is given in the shape of tasty recipes. Although it is known that a vis a vis contact with a doctor in a white coat will propagate a paternalistic relationship and increases the confidence in the doctor's competence which can benefit the effectiveness of counselling [209].

Periconceptional diet and PCOS

In the second part of this thesis we gained new insights in the role of the diet in the development of PCOS.

In chapter 4 diet inadequacy assessed with the PDR score was associated with PCOS and in particular with the HA phenotype. Diet inadequacy was also independently associated with AMH, the marker for ovarian function, and with the free androgen index. This was substantiated when we observed the beneficial effects of strong adherence to a healthy dietary pattern on the severity of the PCOS phenotype (chapter 5). PCOS patients with a strong adherence to the healthy dietary pattern also showed a 3-fold higher chance of ongoing pregnancy, irrespective of PCOS phenotype. Again the adherence to the healthy dietary pattern was also inversely associated with AMH. The latter two chapters established the suggested association between 1-C metabolism and disease severity, suggesting that nutrition plays an important role, probably as second hit in the development of PCOS disease.

It is very interesting to speculate whether the effect of sufficient intake of healthy foods alone is different to sufficient intake of healthy foods next to additional intake of unhealthy food. In contrast to the first study, in chapter 5 we investigated the effect of an unhealthy intake. However, these analyses showed no associations with PCOS disease severity or chance of ongoing pregnancy. This might be best explained by the principal component analysis (PCA) itself to extract dietary patterns. PCA organizes and pools food groups in order to obtain patterns dominated by certain food groups. The pattern labeled as unhealthy was dominated by the unhealthy intake of fats, snacks, sugars and refined grains but might be more or less sufficient in the intake of the healthy food items as well. On the long term, this type of an unhealthy diet will promote obesity due to high total energy intake and stimulation of excessive oxidative stress responses and metabolic disturbances, with or without a deficiency of nutrients [210]. Obesity alone has a strong impact on fertility since it is associated with a reduced probability of successful fertility treatment, with more complications during pregnancy and with higher costs [211,212].

In part 2 all analyses were adjusted for BMI and total energy intake and because the studies in this thesis were observational and assessed data of one visit we did not investigate the associations of weight loss alone, irrespective to a specific dietary intervention. In the majority of couples a switch to a healthy diet will go hand in hand with weight loss, which in turn makes it difficult to tease out the cause of this weight reduction. In other words is it due to either the intervention itself or is it due to an adequate intake of healthy nutrients or a combination of both?

The general recommendation for PCOS patients is promoting weight loss regardless dietary composition [33,213]. Weight loss intervention with a 20 weeks hypocaloric dietary program showed improvements in reproductive function without a change in AMH levels [213] whereas others found a decrease in AMH and androgen levels [107].The latter study also investigated the effect of exercise and the combined effect of exercise and a dietary intervention. They observed the most pronounced effects in the group with diet alone, with regards to a decrease in BMI, levels of free T and AMH [107]. Unfortunately, this implies that there is no decisive answer on our question what part of the intervention did lead to the improved outcome, the weight loss or the dietary intervention?

The results of the dietary pattern analyses are essential steps in unravelling dietary habits in PCOS patients and contribute to simple advices for PCOS patients in daily life. For clinical practice a motivational advice should be given to PCOS patients to achieve weight loss with a healthy diet which can be assessed with the 6 items of the PDR score. Subsequently the use for specific coaching programs should be encouraged since it is developed to coach and support patients and gives extensive background on all modifiable risk factors (Chapter 3).

Periconceptional medication use and semen parameters

In part three we observed adverse effects of medication use on male fertility, suggesting a drug-induced malabsorption of nutrients important for 1-C metabolism. The use of medication for gastric acid-related symptoms was significantly associated with a 2-fold higher risk of a low total motile sperm count and a decreased sperm concentration. This was observed in men visiting our tertiary hospital (Chapter 6) and we validated this finding in men visiting the general practitioner (Chapter 7). Although we did not observe significant differences in biomarkers of 1-C metabolism between medication users and non-users in chapter 6, we presume this was due to the small sample size since the overall biomarker profile tended to be worse in medication users.

In chapter 7 the use of proton-pump inhibitors, the most commonly used and most effective medication for gastric acid-related symptoms use, in the period of 12-6 months preceding semen analysis was associated with a three-fold higher risk of a low motile sperm count. Biomarkers of 1-C metabolism were not available in this study, however the observation that a time window of >6 months is necessary for adverse effects on semen parameters is very confirmative for our hypothesis since it would take a certain time to develop B vitamin

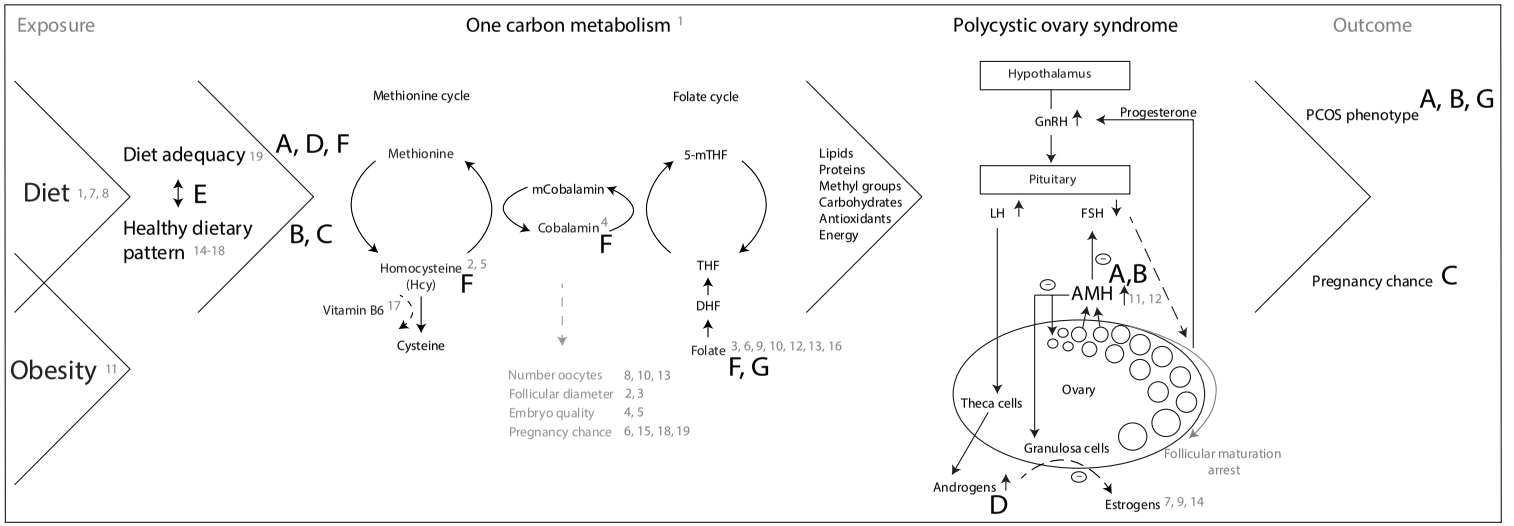


Figure 1 | Effects of the role of the diet in the pathophysiology of PCOS

Abbreviations; PDR score = Preconception Dietary Risk score, FFQ = Food Frequency Questionnaire, GnRH = Gonadotropin Releasing Hormone, LH = Luteinizing Hormone, FSH = Follicle Stimulating Hormone, AMH = Anti-Müllerian hormone, tHcy = total Homocysteine.
References A-G depict the effects observed in this thesis.

deficiencies that derange the 1-C pathway. In chapter 6, current use of medication during the preconception visit resulted in adverse outcome as well. This is conflicting with this latter hypothesis, although we think this might be due to the fact that the majority of the medication users in our study could have been chronic users, but unfortunately this issue will remain indecisive since only current medication was assessed in this cohort.

The high prevalence of medication use in men and women stresses the importance of this part of the thesis further [214]. Especially since the effect of commonly used medication is not often studied yet. The FDA did wrote in 2011 a recommendation for guidance of applicants of new drug applications towards developmental and reproductive risks [215]. However this guide actually means that it is suggested or recommended, but not required which now leads to the issue that conclusive evidence is often limited [216]. Furthermore, the adverse effects of medication use are mostly correlated to central hormonal effects, direct gonadotoxicity, to sperm function or to sexual function [216] but not yet to malabsorption of nutrients. In our studies we could not exclude that the adverse effects observed were due to a central hormonal mechanism or due to direct gonadotoxicity. Although the long period between exposure and outcome is more directive towards our hypothesis of nutrient malabsorption. Finally, the studies reporting adverse effects of medication use on male fertility will hopefully raise awareness of this deteriorating effect and will emphasize why the contribution of men in preconception counselling is important as well.

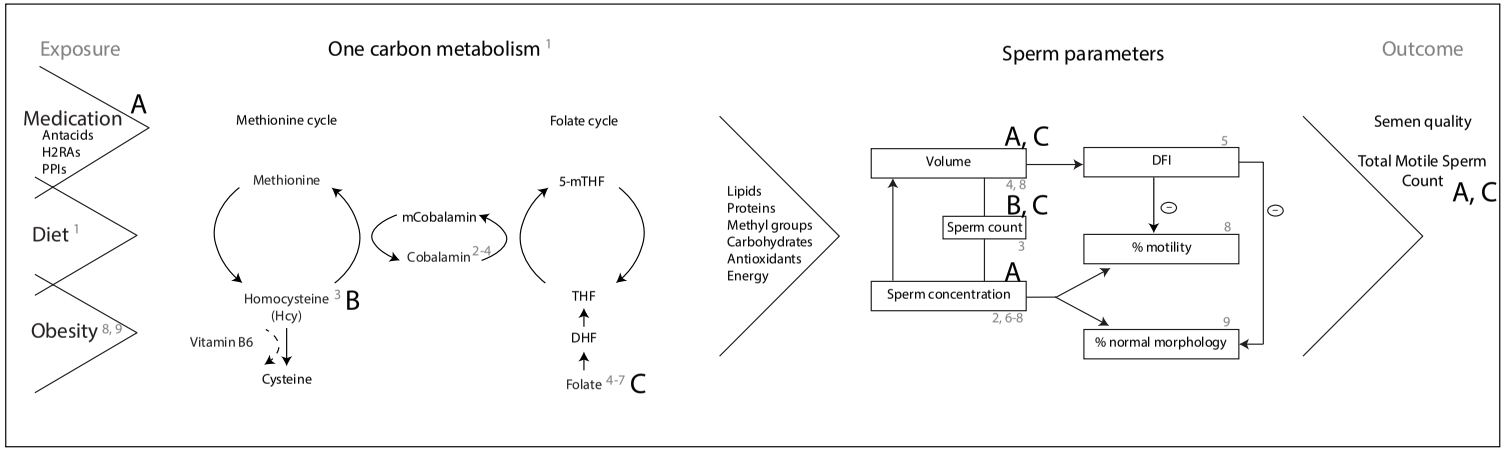


Figure 2 | Effects of the diet and medication use on semen parameters.

Abbreviations; tHCY = total Homocystein, DFI = DNA fragmentation index.
References A-C depict the effects observed in this thesis.

METHODOLOGICAL CONSIDERATIONS

Inherent to the observational nature of the studies in this thesis there are several methodological considerations to address.

Study design

Observational studies without randomization and interventions show associations but are not designed to demonstrate causality. Nevertheless, the prospective cohort design combined with case control analyses and the analogy of the observations with previous studies results, approximate causality in the observed associations. Observational studies are necessary as first attempt to generate evidence for a new hypothesis and eventually initiate a shift in the current thinking, resulting in the development of new interventions studies proving the causation of the hypothesis. Furthermore the criteria of Hill [217] are commonly used to provide evidence of causality in observational studies. In this thesis we complied to these criteria for as much as possible. In all studies for example, we critically evaluated the effect size of the association and we attempted to observe a biological gradient in the observed associations with regard to the outcome variable. We tested the consistency of the findings in different study populations and in similar factors and we tried to find coherence between epidemiological and laboratory findings. Lastly, causality is more plausible when a distinct mechanism is present between cause and effect and in this thesis derangements in 1-C metabolism were the fundament of all study hypotheses.

Study population

The majority of the data used in this thesis were derived from the preconception outpatient clinic 'Achieving a Healthy Pregnancy', the clinic for cycle disturbances 'COLA' and the Predict study [59,63] and investigated the effect of nutrition and lifestyle factors on fertility outcome, embryonic and fetal growth and development. Eventually, these new insights are used to coach couples on individual risk factors, to improve the chance of a healthy pregnancy outcome for mother, child and future offspring [59]. When interpreting these results we have to keep in mind that all of the studies in this thesis were performed in subfertile couples visiting the tertiary hospital of the Erasmus Medical Center or in couples seeking first consultation at the general practitioner because of the wish to conceive. Moreover, in the studies investigating dietary inadequacy and the severity of the PCOS phenotype the control group consisted of subfertile women instead of healthy women. Therefore caution is necessary when translating these results to other, maybe 'healthier' populations or to patients with a different ethnic background since dietary intake will be cultural defined.

Accuracy of data

All biomarker measurements were performed in one laboratory of the Erasmus MC, University Medical Center which diminishes the chance of measurement errors. As previously stated,

inter-assay coefficients of variation for serum folate were 4.5% at 13 nmol/L and 5.7% at 23 nmol/L, for serum cobalamine 3.6% at 258 pmol/L and 2.2% at 832 pmol/L and for plasma tHcy were 4.8% at 14.6 µmol/L and 3.3% at 34.2 µmol/L. Intra- and interassay coefficients of variation were, <5% and <15% for LH, <3% and <18% for FSH, <3% and <5% for T, <8% and <11% for AD, <4% and <5% for SHBG, <9% and <11% for DHEAS, <5% and <8% for AMH, and <6% and <8% for insulin.

For the semen analyses performed in the Erasmus MC, University Medical Center, the between technician variation for sperm concentration was 1.6% (Intra class correlation coefficient (ICC) 0.992, 95 % CI 0.978 to 0.997). The between technician variation for sperm motility was 2.4% (ICC 0.988, 95% CI 0.972 to 0.996).

The between technician variation (ICC) for semen analyses recorded in the IPCI database were unknown, although all hospital-affiliated laboratories in the Netherlands are obliged to participate in the external quality control scheme of the Dutch foundation for quality assessment in clinical laboratories (www.SKML.nl). Which ensures that the staff is trained and tested annually according to the certified European Society of Human Reproduction and Embryology (ESHRE) course for performing semen analyses in order to receive the ISO15189 certificate (the worldwide standard for medical laboratories).

Questionnaires versus biomarkers in blood

We very much assume that our recorded dietary intake and medication use are a valid resemblance of the actual intake or use, as well as for the recordings of all other general characteristics [218].

Previous research from our group showed that FFQ estimated levels of micro- and macronutrients were highly correlated to actual blood measurements of serum folate, RBC folate and serum vitamin B12. Thereby concluding that it is a sufficient tool for the investigation of nutrient-disease associations [64].

Nevertheless, when data is gathered with self-administered questionnaires recall and information bias could have occurred. Underreporting in for example medication use probably could have led to a stronger association between medication use and low semen quality, if the control group also comprised medication users. On the other hand a low medication compliance could have occurred as well, and in that case the observed association between medication use and low semen quality was also underestimated, since the deteriorating effect was found in less frequently PPI users. The high participation and response rates in our studies plead against the occurrence of selection bias and because we observed mainly similarities in general characteristics between included and excluded groups in all sensitivity analyses.

The studies from this thesis are explorative and reveal further insights of the importance of 1-C metabolism and disease outcome, therefore confounding due to other factors have to be studied in future such as effects of psychological stress, work-related effects, pesticides etc. [219-221].

CLINICAL IMPLICATIONS AND FUTURE RESEARCH

The aim for this thesis originated from previous studies such as the 'The Food Lifestyle and Fertility Outcome study' (FOLFO) which was conducted between 2004 and 2007 and examined the influence of preconception lifestyle exposures in subfertile couples on fertility parameters and pregnancy outcome [7-9]. The observed associations in this study and others, together with the food-based dietary guidelines of the Dutch Nutrition Center in the Netherlands were the reason to develop the 'Achieving a healthy Pregnancy' outpatient clinic in 2007 [59]. The implementation of this counselling was one of the first initiatives to help health care professionals in the guidance of couples planning pregnancy into a healthier lifestyle and raise awareness for the adverse effects of risk factors on pregnancy outcome. Due to the subsequent observation that an improvement in the PDR score resulted in a 65% increased chance of ongoing pregnancy in couples attending this preconception counselling [6] and due to the work of Boxmeer et al. [41-43,45,46], we were intrigued by the possible effect of deranged 1-C metabolism and fertility outcome.

There are several studies, testing the effect of dietary compositions on clinical, and metabolic features of PCOS, only most of them are controversial [32,33,35-40]. The study populations differ in composition, struggle with small sample sizes, with different control groups and with different ways of adjustments. Hence, it is almost impossible to compare and interpret those results and additionally constitute simple advice for PCOS patients in daily life. Therefore, it is necessary to first understand and evaluate in large observational cohorts whether there are differences in eating behavior underlying PCOS before we initiate randomized controlled trials aimed to investigate the effects of specific nutrients.

Together with this thesis, the amount of evidence is numerous, however more important is the challenge of making health care providers and couples planning pregnancy aware of these results. In the future, the key initiative therefore should be to investigate the most effective method to reach as much couples as possible, and preferably starting <14 weeks before conception [20]. M-health emerged as the new medium for counselling and personal coaching on nutrition and lifestyle and seems very promising, since this is an impressive new field offering many opportunities in the digital era we are in [90]. Our survey with the mHealth web based coaching 'Smarter Pregnancy' showed impressive abilities in improving inadequate behavior effectively, which already resulted in the start of two large randomized controlled trials testing this coaching tool in different populations with follow up into pregnancy. The compliance is high when participants enjoy personally tailored healthy recipes and involve the partner in this program as well. In future we presume that this coaching will improve the chance of ongoing pregnancy, prevent adverse pregnancy outcome and will be cost effective in reducing the costs for subfertility treatment and pregnancy complications [222].

The emerging interests in the developmental origins of health and disease (DOHaD) is driven by the evidence that nutrigenomics not only affect parents to be, but also disease occurrence in adulthood of the offspring due to heritable changes in the gene expression [21,223-225].

A periconceptional improvement of inadequate behavior can therefore contribute to the prevention of disease, in later life and may alter fertility outcome and disease prevalence in offspring as well. For future research it is important to follow offspring into adolescence and into adulthood and support them to maintain a healthy lifestyle. Therefore a new program was already launched to guide new parents and provide recipes and advice for a healthy start after birth.

The studies in this thesis will hopefully stimulate that in new phase 3 studies the testing of drug safety will require the investigation of adverse effects on fertility parameters as well. The FDA could contribute to this issue by changing the current recommendation into a mandatory guideline for safety studies. All medication that is used commonly in men and women in reproductive age should therefore additionally be tested and evaluated for safety. When couples attend preconception counselling in future, extra advice to stop or switch certain medicines can be given. Or in case of impaired 1-C metabolism, the use of additional vitamin supplements can be motivated, although new studies have to investigate the effect of this intervention.

To conclude, it will be very interesting to investigate the role of diet and 1-C metabolism in other reproductive diseases as well, for example in couples with unexplained cause of infertility and in comparison to healthy, fertile control women. Moreover, in this thesis we started observing adverse effects on semen parameters of only one group of medication and more work has to be done to elucidate the adverse effects on human fertility of other groups of medication that is commonly used in the reproductive population.

GENERAL CONCLUSION

The studies performed in this thesis demonstrate the importance of diet and medication use and their impact on human subfertility.

Firstly, we showed that diet inadequacy is very common in subfertile couples and that it promotes derangements in 1-C metabolism. For clinical practice we designed an accurate and easy tool to measure the diet inadequacy, which was further developed to the M-health tool 'Smarter pregnancy' and we demonstrated that it is effective in improving and maintaining a healthy lifestyle in women and men. This exciting new development can be seen as an additional tool for future personal preconception counselling and coaching on nutrition, lifestyle, and medication use.

Secondly, in women, dietary inadequacy was related to the clinical spectrum of polycystic ovary syndrome and the chance of ongoing pregnancy.

Lastly, in men the use of medication, interfering with the absorption of nutrients important for 1-C metabolism, was associated to male subfertility.

Chapter

9

Summary
Samenvatting

Summary

Subfertility is a reproductive disorder defined by the inability to achieve a clinical pregnancy after 12 months which affects 10-15% of couples worldwide. Causes can be attributed to a male factor, female factor, a combination of both and in 30% of these couples no medical cause can be identified. Deranged 1-C metabolism can contribute to reproductive failures since this pathway is essential for developing oocytes and spermatocytes and adequate nutrition provides for the necessary cofactors (cobalamin, folate). The aim of this thesis was to study the influences of 1-C metabolism on female and male subfertility, more specifically in women the effect of diet on PCOS disease and in men the effect of drug-induced malabsorption of nutrients on semen parameters. In chapter 1, we provide background information on 1-C metabolism, PCOS and male subfertility and we clarify the aim, objectives and methodology of this thesis.

PART 1 | New screening tools and mHealth intervention

In the first part of this thesis , in chapter 2, we evaluate the use of the Preconception Dietary Risk score as a tool to determine the inadequacy of the habitual diet. We investigated couples planning pregnancy at the outpatient clinic and a summary Preconception Dietary Risk score was calculated from seven questions to assess the inadequacy of the diet using the six Dutch guidelines for the consumption of bread, oils/fats, vegetables, fruit, meat and fish. An inadequate habitual diet was present in 55.4% of women and 54.2% in men. The tool revealed a sensitivity of more than 80% and a linear positive trend for saturated fat intake, and negative trends for the intake of protein, EPA, DHA, fiber, folate and vitamin B6, B12 and C intake. The Preconception Dietary Risk score was also significantly correlated to serum vitamin B12. From these results we concluded that the PDR score is a sensitive, quick and simple tool which can be used in clinical practice to assess the inadequacy of the habitual diet.

The implementation of the digital, m-health, coaching tool 'Smarter Pregnancy' is discussed in Chapter 3. A compliance of 64.9% and a positive or very positive usability of 54.7% were assessed. After 6 months of coaching the lifestyles improved by 26.3% for vegetables, 38.4% for fruits, 56.3% for folic acid use, 35.1% for smoking and 41.9% for alcohol use. Participating couples showed the strongest effectiveness, emphasizing the importance of including women and men together in preconception counselling. This novel m-Health platform is promising for future preventive medicine by showing a high compliance, usability and effectiveness in improving lifestyle behaviors.

PART 2 | Periconceptional diet and PCOS

Subsequently the PDR score was used in chapter 4, to assess diet inadequacy in women with PCOS. Our hypothesis was that dieting and diet inadequacy in the reproductive life course leads to an impaired programming of ovarian follicles and contributes to the severity of the PCOS phenotype. We observed that PCOS patients, in particular the HA phenotype reported more often the use of a self-initiated diet than controls. The PDR score was also higher in PCOS than in controls and every point increase was associated with a higher risk of the HA phenotype. The PDR score was also independently associated with AMH concentration, a marker for ovarian function, and with free androgen index. This suggests that the use of a self-initiated diet and diet inadequacy is associated with PCOS, in particular with the severe HA phenotype.

In chapter 5, our aim was to investigate whether a specific dietary pattern is related to the HA PCOS phenotype and if this dietary pattern is associated to the chance of ongoing pregnancy. Principal component factor analysis was used to summarize dietary patterns in PCOS patients and a strong adherence to the healthy dietary pattern (comprising fruits, vegetables, fish and whole grains) was inversely associated to the severe HA PCOS phenotype. A higher chance of ongoing pregnancy was observed in the PCOS patients with strong adherence to the healthy dietary pattern. The healthy dietary pattern was also inversely associated with AMH concentration. This affirms the influence of nutritional composition on disease severity.

PART 3 | Periconceptional medication use and semen parameters

In the third part of this thesis we investigate if the use of medication interfering with the absorption of nutrients, contributes to impaired semen quality. Medication for gastric acid-related symptoms is often used in the general population and affect the gastrointestinal pH.

In chapter 6, we observed that the use of medication for gastric acid-related symptoms was significantly associated with a higher risk of a low total motile sperm count and a decreased sperm concentration. RBC folate was positively associated with TMSC, sperm count and ejaculate volume and tHcy was negatively associated with sperm count. Although serum and RBC folate tended to be higher and cobalamin and tHcy tended to be lower in medication users, this did not reach the statistical level of significance. This emphasizes the importance of preconception counselling of subfertile men and that medication should be evaluated for safety of use in couples planning pregnancy.

To validate these results we investigated in chapter 7, in men visiting the general practitioner if the use of proton pump inhibitors was associated to semen quality as well, since PPIs are the most commonly used medication for gastric acid-related symptoms and are also the most effective in decreasing gastric acidity. Men planning pregnancy with a recorded semen analysis were identified in the Integrated Primary Care Information database, comprising medical records of 1.5 million patients.

The use of PPIs 12-6 months preceding semen analysis was associated with a three-fold higher risk of a low TMSC, whereas PPI use in the 6 months before semen analysis was not associated with low TMSC. Apparently spermatogenesis is not directly affected by PPI use, since only after 6 months exhausted resources of vitamins might result in the dramatic decrease of semen quality.

In chapter 8 we discuss the clinical implications and the methodological considerations of this thesis, and we elaborate on recommendations for future research. The results of these studies affirm that a healthy lifestyle with adequate intake of nutrients and without medication interfering the absorption of these nutrients, promote a good functioning of the 1C pathway, which contributes to an improvement in fertility outcome.

Samenvatting

Subfertiliteit is een stoornis in de vruchtbaarheid en is gedefinieerd als het onvermogen om een zwangerschap te bewerkstelligen na meer dan een jaar onbeschermde gemeenschap. Het komt wereldwijd voor in ongeveer 10-15% van de paren. Oorzaken kunnen worden toegeschreven aan de vrouw, aan de man of aan de combinatie van beide en in ongeveer 30% van de gevallen blijft de oorzaak geheel onbekend. Een verstoring in het cellulaire 1-C metabolisme kan bijdragen aan het ontstaan van subfertiliteit omdat dit proces essentieel is voor zich ontwikkelende eicellen en zaadcellen. Gezonde voeding bevat de nodige cofactoren voor dit proces zoals vitamine B12, B6, B1 en foliumzuur.
Het doel van dit proefschrift is het bestuderen van de invloed van het 1-C metabolisme op vrouwelijke en mannelijke onvruchtbaarheid. Meer specifiek onderzoeken we in vrouwen het effect van voeding op de ontwikkeling van het polycysteus ovarieel syndroom (PCOS) en in mannen het effect van medicatie afhankelijke malabsorptie van nutriënten. In hoofdstuk 1 beschrijven we de achtergrond informatie ten behoeve van het 1-C metabolisme, PCOS en mannelijke subfertiliteit en we verhelderen de doelstellingen en methodologie van dit proefschrift.

DEEL 1 | Nieuw screeningsinstrument en mHealth interventie

In het eerste deel van dit proefschrift, in hoofdstuk 2, evalueren we het gebruik van de preconceptionele voedingsrisico score (de PDR score) als instrument voor het determineren van inadequate inname van gezonde voeding. In de subfertiele paren die het 'Gezond Zwanger Worden' spreekuur in het Erasmus MC bezochten werd de PDR score berekend op basis van de 6 Nederlandse richtlijnen Goede Voeding voor een inadequate inname van brood, vloeibare vetten, groente, fruit, vlees of vis. Een inadequate inname was aanwezig in 55,4% van de vrouwen en in 54,2% van de mannen.
De sensitiviteit van de PDR score was meer dan 80% en was positief geassocieerd met de inname van verzadigd vet en negatief geassocieerd met de inname van eiwitten, met de omega 3-visvetzuren EPA en DHA, vezels, foliumzuur, vitamine B6, B12 en C. De PDR was ook significant gecorreleerd aan het serum vitamine B12 gehalte. Concluderend is de PDR score een sensitief, snel en simpel instrument voor gebruik in de dagelijkse praktijk waarmee de mate van voedingsinadequaatheid kan worden ingeschat.
Hoofdstuk 3 van dit proefschrift omvat de eerste resultaten van het digitale, mHealth coachingsprogramma 'Slimmer Zwanger'. Ongeveer 65% van de gebruikers voltooiden het complete 26 weken durende programma en 54.7% was positief tot zeer positief over de gebruiksvriendelijkheid.
Het programma verbeterde de inname van groente (26.3%), fruit (38.4%) en foliumzuur (56.3%) en verminderde het roken (35.1%) en alcoholgebruik (41.9%). In gebruikers die

deelnamen als koppel bleek het programma het meest effectief, daarmee benadrukkend dat preconceptionele counseling gericht moet zijn op zowel de vrouw als de man. Dit nieuwe mHealth coachingsprogramma is veelbelovend voor de preventieve geneeskunde van de toekomst met niet alleen een goede compliantie als ook een goede gebruiksvriendelijkheid en effectiviteit in het verbeteren van leefstijlrisicofactoren.

DEEL 2 | Periconceptionele voeding en PCOS

Vervolgens hebben we in hoofdstuk 4, de PDR score bepaald in vrouwen met PCOS. Onze hypothese was dat bepaalde diëten en dieet inadequaatheid kunnen leiden tot een minder goede programmering van ovariële follikels en daarmee kunnen bijdragen aan de ernst van het PCOS fenotype. PCOS patiënten en met name de patiënten met hyperandrogenisme volgden vaker een dieet dan de controle groep. De PDR score was ook hoger in PCOS patiënten en een stijging van deze risicoscore met 1 punt was geassocieerd met een verhoogd risico op het hyperandrogene fenotype. De PDR score was ook onafhankelijk geassocieerd met de AMH concentratie in het bloed, welke ook wel wordt gebruikt als indicatie voor de ovariële functie. Dit bevestigt het vermoeden dat dieet en voedingsinadequaatheid geassocieerd zijn met PCOS, en in het bijzonder met het hyperandrogene PCOS fenotype. In hoofdstuk 5, hebben we onderzocht of er specifieke dieetpatronen gerelateerd zijn aan het hyperandrogene PCOS fenotype en of deze patronen ook geassocieerd zijn met de kans op een doorgaande zwangerschap. Door middel van principale component factor analyse konden de verschillende dieetpatronen worden geëvalueerd. Patiënten waarbij het eetpatroon sterke overeenkomsten vertoonde met het gezonde dieetpatroon (inname van voornamelijk fruit, groente, vis en volkoren producten) hadden minder vaak het hyperandrogene PCOS fenotype en vaker een doorgaande zwangerschap. Het gezonde patroon was eveneens omgekeerd evenredig geassocieerd met de AMH concentratie in het bloed. Dit bevestigt de associatie van voedingssamenstelling en PCOS fenotype.

DEEL 3 | Periconceptioneel medicatiegebruik en semenkwaliteit

In het derde deel van dit proefschrift hebben we onderzocht of medicatiegebruik de opname van nutriënten kan beïnvloeden, en zo kan bijdragen aan een verminderde semenkwaliteit. Medicatie voor zuurbanden wordt veel gebruikt in de algemene populatie en beïnvloedt de pH balans in de maag. In hoofdstuk 6 beschrijven we dat het gebruik van zuurremmers geassocieerd is met een hoger risico op een laag aantal goed bewegende zaadcellen (VCM) en met een verlaagde concentratie zaadcellen. Het foliumzuurgehalte in de rode bloedcellen was positief geassocieerd met het aantal goed bewegende zaadcellen, het totale aantal zaadcellen en het ejaculaat volume. De homocysteineconcentratie in het bloed was negatief geassocieerd met het aantal zaadcellen. Er was geen verschil in het rode bloed cel foliumzuur- of homocysteineconcentratie tussen de gebruikers en niet-gebruikers.

Preconceptionele counseling is daarom belangrijk voor subfertiele mannen en deze uitkomsten benadrukken dat bij het testen van gebruiksveiligheid van medicatie ook gekeken moet worden naar het effect op de vruchtbaarheid. Voor het valideren van deze bevindingen in een andere onderzoekspopulatie, hebben we in hoofdstuk 7 onderzocht of het gebruik van protonpompremmers is geassocieerd met semenkwaliteit in mannen die vanwege een (onvervulde)kinderwens de huisarts bezochten. We hebben gekeken naar het gebruik van protonpompremmers omdat dit de meest gebruikte en meest effectieve zuurremmer is. Het gebruik van protonpompremmers in de periode van 12 maanden tot 6 maanden voorafgaand aan de semenanalyse was geassocieerd met een 3 keer verhoogd risico op een laag aantal goed bewegende zaadcellen. Het gebruik in de periode van 6 maanden tot aan de semenanalyse was niet geassocieerd met een laag aantal goed bewegende zaadcellen. Deze bevindingen suggereren dat de spermatogenese niet direct beïnvloed wordt door het gebruik van protonpompremmers, maar dat pas na 6 maanden een vermindering van semenkwaliteit optreed, mogelijk als late termijneffect op een daling in de B vitaminereserves.

In hoofdstuk 8 bediscussiëren we de klinische consequenties en de methodologische overwegingen van dit proefschrift. Daarnaast beschrijven we onze aanbevelingen voor toekomstig onderzoek.

A d d e n d u m

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Authors and affiliations
List of abbreviations
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PhD portfolio
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Acknowledgements

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List of abbreviations

1-C	One-Carbon
AD	Androstenedione
ALA	Alpha linolenic acid
AMH	Anti-Müllerian Hormone
ATC	Anatomical Therapeutic Chemical
BMI	Body Mass Index
DDD	Defined Daily Dosage
DHA	Docosahexaenoic acid
DHEAS	Dehydroepiandrosterone sulfate
EDTA	Ethylenediamine tetraacetate
EPA	Eicosapentaenoic acid
FAI	Free Androgen Index
FFQ	Food Frequency Questionnaire
FSH	Follicle Stimulating Hormone
H2RA	H2-receptor antagonist
HA	Hyperandrogenism
HOMA-IR	Homeostatic model assessment - insulin resistance.
HPG	Hypothalamic-pituitary-gonadal
ICSI	Intra cytoplasmatic sperm injection
IPCI	Integrated Primary Care Information
IQR	Interquartile range
IVF/ICSI	*In-vitro* fertilization
LH	Luteinizing Hormone
MESA	Micro chirurgic epididymal sperm aspiration
MUFA	Monounsaturated fatty acids
OAT	Oligoasthenoteratozoospermia
OD	Ovulatory Dysfunction
PCA	Principal component factor analysis
PCOM	Polycystic Ovarian Morphology
PCOS	Polycystic Ovary Syndrome
PDR	Preconception Dietary Risk score
PESA	Percutan epididymal sperm aspiration
PPI	Proton-pump inhibitor
PQ	Preconception Questionnaire
PUFA	Polyunsaturated fatty acids
RBC folate	Red blood cell
RIA	Radioimmunoassay
SHBG	Sex Hormone-Binding Globulin
SKML	Dutch Foundation for Quality Assessment in Clinical Laboratories
T	Testosterone
tHcy	Total Homocysteine
TMSC	Total Motile Sperm Count

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PhD portfolio

Name PhD student:	Nicole Antoinette Huijgen
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PhD period:	August 2011- August 2015
Promotors:	Prof.Dr. R.P.M.Steegers-Theunissen and Prof.Dr. J.S.E.Laven

General Courses	Year
Basic introduction course (Institute of Molecular medicine; Mol Med)	2011
Biostatistical Methods I: Basic Principles (NIHES)	2012
Basic principles of Nutritional Epidemiology	2012
Methodologie van Patiëntgebonden onderzoek en Voorbereiding van Subsidieaanvragen	2013
Repeated Measurements (NIHES)	2013
Endnote course	2013
Systematic literature retrieval in PubMed	2013
Biomedical English Writing and Communication (David Alexander)	2013

Oral presentations at (inter)national conferences	
2nd European Congress on Preconception Care and Health (ECPCH), Rotterdam, The Netherlands	2012
Wladimiroff Award Meeting (RCOG), Rotterdam, The Netherlands	2013
8th World Congress on Developmental Origins of Health and Disease, Singapore	2013
Wladimiroff Award Meeting (RCOG), Rotterdam, The Netherlands	2014
Annual meeting of the Society for Gynecologic Investigation (SGI), Florence, Italy. ***(President's Presenter Award)***	2014
Sophia Research day, Rotterdam, The Netherlands	2015
21e Nederlands-Vlaams Doelencongres Infertiliteit, gynaecologie en obstetrie	2017

Poster presentations	
Annual meeting of the Society for Gynecologic Investigation (SGI), Florence, Italy	2014
David Barker Commemorative meeting, Southampton, United Kingdom	2014
Sophia Research day, Rotterdam, The Netherlands	2014
9th World Congress on Developmental Origins of Health and Disease, Cape Town, South Africa	2015

Seminars and workshops

Wladimiroff Award Meeting (RCOG), Rotterdam, The Netherlands.	2012-2015
Erasmus PhD day (portfolio & PhD training, submitting papers do's and don't's)	2012
Symposium Jonge Zwangerschap, NVOG werkgroep	2013
Erasmus PhD day (writing articles, storytelling for researchers)	2013
Wetenschapsmiddag van de Arts Assistenten Vereniging (AAV)	2013
SCEM, Symposium on E-health, de Doelen, Rotterdam	2013
Two-weekly research meeting of the department of Reproductive Medicine	2011-2015
Weekly research meeting of the department of Obstetrics and Gynaecology	2011-2015

Teaching activities

Supervising Master's theses medical students	
Hedwig Gooijen, elective research programs (21 weeks) Erasmus University Medical Center	2012
Rivka Koedooder, elective research programs (21 weeks) Erasmus University Medical Center	2012
Sylvia Snippe, elective research programs (21 weeks) Erasmus University Medical Center *(substitute supervisor)*	2012
Olivera Djuric medical doctor, exchange Master Student, NIHES Health Sciences	2013
Chantal Labee, elective research programs (21 weeks) Erasmus University Medical Center ***(Wladimiroff Research Award 2015)***	2013
Megan Jordaan, elective research programs (21 weeks) Erasmus University Medical Center *(substitute supervisor)*	2014
Supervising Master's theses Human Nutrition	
Mirjam van de Kamp, Student, Division Human Nutrition Wageningen University	2011
Daniela Briceno Noriega, Student, Division Human Nutrition Wageningen University *(substitute supervisor)*	2012
Supervising Master's theses Dietics	
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Other skills

Preconception counselling of subfertile couples	2011-2012
Coordinator of 'Achieving a Healthy Pregnancy' preconception outpatient clinic	2011-2013
Substitute coordinator of the Rotterdam Periconception Cohort Study (Predict Study)	2014-2015

About the author

Nicole Huijgen was born on August 23rd, 1986 in Dordrecht. Growing up, she developed a passion for music and playing the clarinet. In 2004 she followed her other passion and started medical school at the Erasmus University in Rotterdam. After her graduation in the summer of 2010 she started to work as a resident at the department of Obstetrics and Gynaecology in the Albert Schweitzer hospital, Dordrecht. In this year she developed from being a student to being a doctor and she enjoyed working on the obstetric care unit and outpatient clinic. In 2011 she was appointed as PhD student, performing the studies described in this thesis, at the department of Obstetrics and Gynaecology of the Erasmus MC University Hospital, under the supervision of prof.dr. Régine Steegers-Theunissen and prof. dr. Joop Laven. In 2015 she began her clinical training in Obstetrics and Gynaecology at the Ikazia Hospital in Rotterdam, which was switched to the Sint Franciscus Gasthuis Hospital in Rotterdam under the supervision of Dr. R. van der Weiden. Nicole is married to Mark Otte and they live together in Dordrecht.