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Original Article

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SUMMARY

Background&Aims: A poor maternal nutritional status in the preconception period is associated with adverse pregnancy outcomes. A valid standardized assessment period after pregnancy reflecting the preconception nutritional status is missing. Therefore, this study aimed to validate the assessment period at around 1 year after delivery in women undergoing fertility treatment.

Methods: In a prospective study including 30 women with a fertility problem, we compared nutrient intakes from a food frequency questionnaire and biomarkers related to the homocysteine pathway in blood, at two assessment periods, i.e., preconceptionally and 1 year after delivery. We used a linear mixed model and adjusted for possible confounders, such as body mass index and folic acid supplement use. *Results:* The energy-adjusted nutrient intakes were not significantly different between the two assessment periods, except for higher retinol, alcohol and vitamin B2 and lower carbohydrate intakes at around 1 year after delivery. The intraclass correlation coefficients of the nutrients ranged from 0.3 to 0.7. After adjustment, none of the biomarkers was significantly different between the two assessment periods. The intraclass correlation coefficients of the biomarkers were all ≥ 0.5 .

Conclusions: An assessment at around 1 year after delivery seems to adequately reflect the preconception nutritional status of women with a fertility problem, however larger confirmatory studies are required. © 2010 European Society for Clinical Nutrition and Metabolism. Published by Elsevier Ltd. All rights reserved.

1. Introduction

Non-standard abbreviations: FFQ, Food frequency questionnaires; tHcy, Homocysteine; RBC, Red blood cell; BMI, Body mass index; ICC, Intraclass correlation coefficients; BMR, Basal metabolic rate; EI, Energy intake; PAL, Physical activity level.

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E-mail addresses: l.vandriel@erasmusmc.nl (L.M.J.W. van Driel), l.zwolle@ erasmusmc.nl (L.J.H. Zwolle), Jeanne.deVries@wur.nl (J.H.M. de Vries), j.boxmeer@ erasmusmc.nl (J.C. Boxmeer), j.lindemans@erasmusmc.nl (J. Lindemans), e.a.p. steegers@erasmusmc.nl (E.A.P. Steegers), r.steegers@erasmusmc.nl (R.P.M. Steegers-Theunissen). Maternal nutrition plays a significant role in the pathogenesis of adverse pregnancy outcomes and may even predispose to chronic diseases in later life.¹ It is therefore worrisome that malnutrition during pregnancy is an increasing problem in both deprived and rich countries.

At the time of conception, the maternal nutritional status is a particular important determinant of embryonic and fetal growth² The growth of the placenta and fetus is most vulnerable to the maternal nutritional status during the preimplantation period and the period of rapid placentation, which takes place during the first weeks and typically before pregnancy has been confirmed.³ Most organs develop 3–7 weeks after the last menstrual period and any teratogenic effect may occur then. The nutritional status of the mother is influenced by numerous variables, including genetics,

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environment, lifestyles, illnesses, physiological factors, and drug-toxicant exposures. $\!\!\!\!^4$

The preconception period is the best time to study the role of maternal nutrition in association with adverse pregnancy outcomes. Moreover, it is also the best time period for nutritional interventions, i.e., preconception care. In most studies, however, the maternal nutritional status is assessed by food frequency questionnaires (FFO) at various time periods during and after pregnancy to determine past habitual nutritional intakes.^{5–8} At different assessment periods, varying from a few days until 24 months after delivery, mothers have been asked in retrospect to report their nutrient intake of the preconception period. The main disadvantage of this approach is its sensitivity to recall bias. A prospective pregnancy study, in which the nutritional intake is assessed and blood samples are taken for measurement of biomarkers preconceptionally and at several time periods during and after pregnancy, would be the first choice. However, it is very difficult to enrol women preconceptionally. Moreover, when studying rare reproductive outcomes, such as congenital malformations and preeclampsia, a prospective study is hardly feasible with regard to sample size and costs.

Therefore, for more than ten years, our group has used a casecontrol design with a standardized assessment period at around 15 months after the index-pregnancy to investigate associations between maternal nutritional intakes by FFQ and adverse pregnancy outcomes, in particular spina bifida, orofacial clefts and congenital heart diseases.^{9–13} We believe that the maternal nutritional status at around 1 year after delivery reflects the preconception maternal nutritional status. This assumption is based on studies by Willett and others, who stated that, in general, the individual dietary pattern is rather constant and is influenced only by episodes of temporary dieting, illnesses, nausea and increased needs due to excessive growth, such as during pregnancy and breastfeeding.^{14,15} The use of the assessment period of around 1 year after delivery as reflection of the preconception time period, however, has never been validated. Therefore, in the current prospective study, we examined whether the maternal nutritional status in the preconception period is comparable with that at around 1 year after delivery. We used FFQs and a selection of biomarkers related to the homocysteine pathway to assess the nutritional status.

2. Methods

2.1. Subjects

This study was part of the FOLFO-study, which is an ongoing prospective study focusing on the role of the homocysteine pathway in fertilization, implantation and embryo quality.¹⁶ The study protocol was approved by the Central Committee for Human Research in The Hague, the Netherlands and the Medical Ethical and Institutional Review Board of the Erasmus MC, University Medical Center in Rotterdam, the Netherlands. All participants gave their written informed consent.

2.2. Study design and protocol

At the first assessment period, women with a fertility problem were asked to fill out a general questionnaire and a validated FFQ during the in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) procedure. These questionnaires were collected at the day of the embryo transfer, then checked by two experienced researchers and, if necessary, incomplete data were supplementedafter a telephone interview. The FFQ covered the intake of the previous 4 weeks. In addition, women were invited to visit the hospital at their first week of the menstrual cycle for blood sampling. The blood samples were thus taken before the women started on the hormonal therapy of the IVF/ICSI treatment. The following biomarkers were then measured: plasma total homocysteine (tHcy), serum and red blood cell (RBC) folate, whole blood vitamin B_{6} , and serum vitamin B_{12} . We selected these biomarkers, because they are all involved in the homocysteine pathway and the design of the FOLFO-study aimed to investigate the role of the homocysteine pathway in fertilization, implantation and embryo quality.

At the second assessment period of around 1 year after delivery, the same women filled in the same questionnaires at home. They were then invited to visit the hospital at the first week of the menstrual cycle for blood sampling. The data from the questionnaires and biomarker concentrations in blood were compared with the data available from the preconception period. We made an effort to keep the conditions for the blood sampling the same for each woman in both periods, which resulted in 23 matching samples (21 non-fasting and 2 fasting) and 7 non-matching samples of which the fasting state was not clear in one of the periods.¹⁷

2.3. Data collection

The data from the general questionnaire comprised age, height, weight, ethnicity, education level, smoking, and the use of medication, recreational drugs and folic acid containing vitamin supplements in the previous four weeks. Body mass index (BMI) was calculated as reported weight divided by the square of reported height. Ethnicity was categorized as follows: Dutch natives: both parents and grandparents were born in the Netherlands, or one of the parents was born in another country, but both grandparents were born in the Netherlands; European others: one of the parents or grandparents was born in a European country, or was from European origin and living in the USA, Indonesia or Australia; non-European: all others¹⁸ Education level was categorized into low (primary/lower vocational/intermediate secondary education), intermediate (higher secondary/intermediate vocational education) or high (higher vocational/university education), according to Statistics Netherlands¹⁹ Smoking was categorized in four groups: none, 1–10 cigarettes per day, 10–25 cigarettes per day and >25cigarettes per day. Medication use was defined as prescribed daily use and recreational drug use was defined as any use in the previous four weeks. The use of folic acid containing supplements comprised both folic acid supplements and multivitamin supplements containing folic acid.

The modified version of the semiquantitative FFQ validated by Feunekes et al.²⁰ was used to estimate daily habitual intake of energy, macronutrients, and micronutrients. Based on data of the Dutch national food consumption surveys in 1992 and 1998, the FFQ has been updated twice.^{21,22} The FFQ has also been modified for the estimation of B vitamin intakes.²³ After modification, the FFQ covered the daily intake of each nutrient or food of interest for at least 90% of the population mean intake. The FFQ has been structured according to a meal pattern and consists of 166 food items. For most items, the women report the intake, preparation methods, portion sizes and additions of the previous 4 weeks. The average daily energy and nutrient intake was calculated using the 2001 electronic version of the Dutch food composition table.²⁴

Venous blood samples were drawn from each participant at both hospital visits. We measured the concentrations of plasma total homocysteine (tHcy), serum and red blood cell (RBC) folate, whole blood vitamin B_6 , and serum vitamin B_{12} .¹⁶ For the determination of serum folate and vitamin B_{12} , venous blood samples were drawn into dry vacutainer tubes and allowed to clot. After centrifugation at 2000 x g, serum was collected before being

assayed. Serum folate and vitamin B_{12} concentrations were routinely determined by immunoelectrochemoluminescence immunoassay (Roche Modular E170, Roche Diagnostics GmbH, Mannheim, Germany). Ethylenediamine tetra-acetate (EDTA) containing vacutainer tubes were used for the determination of plasma tHcv and RBC folate. Lithium heparin containing vacutainers were used for determining vitamin B₆. Immediately after blood sampling, 1 EDTA-tube was centrifuged at 2.000 x g for 10 min and the blood was separated. Vitamin B_6 (pyridoxal'5-phosphate) and tHcy concentration were determined during routine laboratory procedures using high performance liquid chromatography with reserved phase separation and fluorescence detection.²⁵ For the determination of RBC folate, 100 µL blood out of one EDTA tube was hemolyzed with 2 ml freshly prepared ascorbic acid directly after blood sampling. Subsequently, the hematocrit of the EDTA-blood was determined on a Sysmex XE-2100 (Groffin Meyvis, Etten-Leur, the Netherlands). The folate concentration in the hemolysate was recalculated in RBC folate using the following formula: (nmol hemolysate folate \times 21) – (nmol/L serum folate \times {1 – hematocrit})/hematocrit = nmol/L RBC folate. The between-run coefficients of variation for serum total folate were 9.5% at 8.3 nmol/L and 3.2% at 20.2 nmol/L, for tHcy 3.3% at 14.55 µmol/L and 2.3% at 34.23 µmol/L, for vitamin B₆ 1.8% at 40 nmol/L and 1.3% at 115 nmol/ L and for vitamin B₁₂ 5.1% at 125 pmol/L and 2.9% at 753 pmol/L.

2.4. Statistical analysis

The characteristics of the women are presented as medians with ranges or as a number with percentage. We compared the timedependent characteristics of the preconception period with those at 1 year after delivery. Differences between both periods were tested with the repeated measurements linear mixed model. Biochemical data and dietary energy and nutrient intakes are also presented as medians with ranges. To eliminate confounding due to variation in the amount of food consumed, we used the residual method to adjust the mean nutrient intakes for total energy intake.²⁶ This method also controls for possible under-reporting or over-reporting of food consumption. In short, the nutrient intakes were regressed on the total energy intake and the predicted mean nutrient intake was then calculated by adding the individual residuals to the predicted mean nutrient intake.

We conducted a multivariable analysis to examine the independent absolute differences of the biomarkers and dietary nutrient intakes between the two assessment period as repeated measurements using a mixed linear regression model. Within this model, the fixed-in-time and time-varying confounders are taken into account. Another advantage of the mixed linear regression model is that it handles data with missing measurements. In the first model, the biomarkers and dietary nutrient intakes were put into the model as dependent variables, with the time-factor as categorical predictor. This model computes a *p*-value for the timefactor with values <0.05 indicating a significant difference between the two measurements. In the second model, we additionally included possible confounders based on their biologically plausible association with nutritional status and not solely on their significance. The fixed-in-time confounders were education and ethnicity, which were assessed at the preconception period only. The time-varying confounders were age, BMI, smoking, and use of recreational drugs, medication and folic acid containing supplements, which were assessed at both time periods. The continuous variables BMI and age were put into the model as covariates and the categorical variables as factors.

Intraclass correlation coefficients (ICC) of the biomarkers and nutritional intakes were calculated with 95% confidence intervals to evaluate the linear associations of the biomarkers and nutritional intakes over the two periods.²⁷ This method is preferable above the Pearson correlation coefficient, because it takes the within-person variation into account. The ICC indicates whether the ranking of the data in the preconception period is comparable with those around 1 year after delivery. The ICC was computed by the following formula with measurements from the repeated measurements linear mixed model: estimated intercept variance/(estimated residual variance + estimated intercept variance).

We performed an additional method to evaluate possible underreporting by estimating the mean basal metabolic rate (BMR) using the new Oxford equation for women aged 30–60 years: BMR (MJ/ day) = 0.0407 × weight (kg) + 2.90.²⁸ The physical activity level was then calculated by dividing the mean reported energy intake (EI) by the mean BMR.²⁹ The cut-off point of EI/BMR 1.35 was used to evaluate under-reporting. Moreover, the ICCs were calculated in a separate analysis excluding the women with a PAL \leq 1.35. *P*-values <0.05 were considered statistically significant. All statistical analyses were performed using SPSS software package version 16.0 (SPSS Inc, Chicago, IL).

3. Results

From November 2004 until November 2006. 266 women started with the procedure of IVF or ICSI at the Department of Obstetrics and Gynecology, Division of Reproductive Medicine at the Erasmus MC. University Medical Center in Rotterdam, the Netherlands, Of these women, 68 achieved pregnancy, of which 2 women were diagnosed with an extra uterine gravidity and 14 with a spontaneous miscarriage. Four women were excluded because they gave birth to twins and 7 women were lost to follow-up. Therefore, at the second assessment period, which was at around 1 year after delivery of the index-pregnancy, 41 women were eligible to participate in the second part of the study. However, of these 41 women, we excluded the women who were pregnant again (n = 3), who were still breastfeeding (n = 1), or who reported and tested to have a substantially different diet than in the preconception period (n = 3). Four women were lost to follow-up, which resulted in the evaluation of the nutritional status of 30 women at both assessment periods (Fig. 1).

The first assessment period was up to 2 weeks before pregnancy and the second assessment period was at a median of 12.9 months (range 9.5–18.9) after delivery of the index-pregnancy; thus, approximately 21 months in between. The population comprised 70% Dutch natives, 10% European others and 20% non-Europeans. The majority of women had an intermediate 57%, 11% a low and 32% a high education level. Table 1 shows the characteristics of the women in the preconception period and at approximately 1 year after delivery. BMI was slightly, though significantly lower preconceptionally than at 1 year after delivery. Smoking, use of recreational drugs and medication were comparable between both periods. Use of folic acid containing supplements was significantly higher in the preconception period than after delivery.

In Table 2, the daily energy-adjusted dietary nutrient intakes are shown. The intake of vitamin B₂, alcohol and retinol was significantly higher after delivery than in the preconception period. The energy-adjusted intake of carbohydrates was significantly lower. All differences became non significant after adjustment, except for alcohol. Interestingly, after adjustment, intake of vitamin B₃ and zinc was significantly different. The daily energy-adjusted dietary intake of all other nutrients was comparable between the two assessment periods.

The ICCs for the energy-adjusted nutrient intakes are shown in Table 3, ranging from 0.3 to 0.7.



Fig. 1. Patient flowchart, Abbreviations: EUG, extra uterine gravidity.

The mean BMR was calculated for the preconception period and at 1 year after delivery with the Oxford equation. BMR (preconception period) = 0.0407×67 (weight) + 2.90 = 5627 MJ/day. BMR (after delivery) = 0.0407×68 (weight) + 2.90 = 5668 MJ/day. The estimation of physical activity level in both periods was calculated as mean reported El/mean BMR, which were 8697/5627 = 1.55 in the preconception period and 8694/5668 = 1.53 after delivery.

Serum and RBC folate concentrations were significantly higher preconceptionally than after delivery. However, serum folate was

Table 1

Comparison of the characteristics of the women in the preconception period ar	nd 1
vear after delivery.	

Characteristics	Preconception $n = 30$	1 year after delivery $n = 30$	p-value ^b
Age (years)	33.4 (28.5–43.7)	35.4 (30.3-45.4)	< 0.001
BMI (kg/m ²)	23.1 (18.2-34.3)	23.2 (18.2-35.1)	0.028
Smoking ^a			0.517
None	23 (79)	24 (80)	
1 - 10 cigarettes per day	6 (21)	4 (13)	
10 - 25 cigarettes per day	0(0)	2 (7)	
> 25 cigarettes per day	0(0)	0(0)	
Recreational drug use	3 (10)	-	0.088
Medication use	3 (10)	6 (20)	0.216
Use of folic acid	25 (83)	3 (10)	< 0.001
containing supplements			

Results are presented as number (percentage) or median (range).

^a Smoking, n = 29.

^b *P*-value calculated with a repeated measurements linear mixed model – variance components.

not significantly different after adjustment for education, ethnicity, age, BMI, use of folic acid containing supplements, medication, tobacco and drugs. The other biomarkers were comparable between the two assessment periods (Table 4).

As shown in Table 5, the ICCs of all biomarkers were significant, ranging from 0.5 to 0.99.

In a separate analysis, we calculated the ICCs excluding the women with a PAL \leq 1.35 (n = 9). This did not substantially change our results (data not shown).

4. Discussion

The aim of this study was the validation of the maternal nutritional status at the assessment periods of around 1 year after delivery and the preconception period. The nutritional status was assessed by FFQ to estimate the dietary energy and nutrient intakes and by measurement of a selection of biomarkers in blood related to the homocysteine pathway. To our knowledge, this is the first study on this subject with a unique prospective design.

The first part of the results showed that daily dietary intake of energy and the energy-adjusted nutrients were comparable between the two assessment periods. The only exceptions were vitamin B_2 , alcohol and retinol intake, which were significantly lower, while the intake of carbohydrates was significantly higher preconceptionally compared with the assessment period after delivery. After adjustment for possible confounders, none of the differences were significant, except for alcohol. The ICCs for the intake of energy and the energy-adjusted nutrients ranged from 0.3 to 0.7.

These results confirm previous assumptions that pregnancy planning does not significantly affect the maternal nutritional intakes³⁰ and that women return to their nutritional habits of the preconception period.¹⁴ There are, however, a few aspects to consider.

The first point is the significantly different intake of vitamin B₂, alcohol, retinol and carbohydrates. After adjustment for the included confounding factors, only alcohol intake remained significantly different. Moreover, after adjustment for multiple comparisons, again, only alcohol intake remains significantly different (data not shown). This may be not surprising, since women who want to become pregnant are advised not to consume alcoholic beverages before and during pregnancy. The mean difference of energy-adjusted alcohol intake between both periods was 4839 mg/day, which might be explained by the fact that these women with

Table 2

Energy-adjusted ^a	dietary nutri	ent intakes ir	n the preco	nception pe	eriod and 1	year after delivery.
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Nutrient	Preconception	1 year after delivery	Median difference (SD) ^b	<i>p</i> -value ^c	Adjusted P-value ^d
Energy (MJ)	8.0 (4.1–29.8)	8.8 (3.7-16.3)	-0.57 (3.2)	0.944	0.759
Total FA (g)	80.8 (53.3-104.8)	83.1 (66.2-107.8)	1.1 (8.6)	0.295	0.535
Saturated FA (g)	30.1 (23.4-39.6)	30.4 (21.6-42.7)	-0.4 (5.5)	0.743	0.219
Total Unsaturated FA (g)	41.6 (32.2-59.8)	43.2 (24.8-67.3)	1.8 (8.0)	0.372	0.111
- MUFA (g)	26.2 (19.4-36.4)	25.8 (19.3-42.7)	2.1 (5.0)	0.972	0.238
- PUFA (g)	16.0 (11.4-24.9)	17.0 (19.3-41.8)	2.09 (4.0)	0.158	0.089
Linoleic acid (g)	13.3 (8.6-20.1)	13.3 (2.8-25.2)	1.2 (4.2)	0.270	0.113
ALA (mg)	1214 (500-2660)	1101 (470-2180)	-84 (423)	0.230	0.101
EPA (mg)	39 (0-180)	44 (0-810)	21 (131)	0.553	0.950
DHA (mg)	64 (0-270)	69 (0-1050)	-11 (165)	0.673	0.967
Cholesterol (mg)	164.9 (106.7-280.8)	166.8 (87.9-273.1)	13 (60.3)	0.587	0.064
Proteins (g)	75.3 (55.4–91.7)	74.0 (43.8-104.2)	0.2 (11.2)	0.854	0.101
Carbohydrates (g)	257.4 (214.6-306.2)	248.1 (185.5-299.0)	-9.7 (21.3)	0.044	0.142
Dietary fibers (g)	22.3 (13.9-30.9)	22.0 (14.2-35.2)	0.3 (4.1)	0.687	0.369
Vitamin B1 (mg)	1.3 (0.8–2.1)	1.2 (0.7-2.0)	-0.09 (0.3)	0.406	0.150
Vitamin B2 (mg)	1.5 (0.8–1.9)	1.5 (0.7–2.6)	0.1 (0.3)	0.029	0.361
Vitamin B3 (mg)	15.3 (9.2-20.1)	15.2 (7.9–23.3)	0.1 (3.0)	0.908	0.010
Vitamin B6 (mg)	1.6 (1.1–2.3)	1.7 (0.8–2.3)	0.06 (0.3)	0.637	0.692
Folate (µg)	190.8 (118.9-302.3)	190.7 (118.6-338.7)	1.1 (43.9)	0.735	0.573
Vitamin B12 (µg)	3.5 (1.3-7.9)	3.7 (2.1-11.6)	0.8 (1.8)	0.078	0.904
Alcohol (mg)	2211 (0-16430)	5429 (204-31440)	3540 (6133)	< 0.001	0.047
Zinc (mg)	8.5 (5.8-10.9)	8.7 (5.1-12.7)	0.2 (1.2)	0.137	0.022
Retinol (µg)	474.5 (192.3-1368.6)	557.1 (268.5-2335.8)	132.0 (402.6)	0.027	0.988
Vitamin C (mg)	109.0 (32.1-231.4)	100.7 (32.0-173.5)	-9.7 (41.2)	0.083	0.403
Vitamin E (mg)	13.2 (7.3–19.0)	12.7 (6.0–22.2)	0.6 (3.3)	0.429	0.945

MUFA, mono unsaturated fatty acids; PUFA, poly unsaturated fatty acids; ALA, alpha linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid. ^a Nutrient intake adjusted for energy intake according to the residual method of Willett et al., except for total energy.

^b Difference = 1 year after delivery – preconception.

^c *P*-value is computed with a repeated measurements linear mixed model.

^d P-value is computed with a repeated measurements linear mixed model, adjusted for education, ethnicity, age, BMI, use of supplements, medication, tobacco and drugs.

Table 3 Intraclass correlation coefficients for dietary nutrient intakes comparing the preconception study moment with around 1 year after delivery.

Nutrient	Unadjusted ρ ^a	Adjusted ρ ^b
	(95% CI)	(95% CI)
Energy	0.606 (0.315-0.793)	0.418 (0.068-0.676)
Total FA	0.667 (0.403-0.828)	0.710 (0.470-0.852)
Saturated FA	0.317 (-0.049-0.608)	0.341 (-0.022-0.624)
Total Unsaturated FA	0.557 (0.246-0.764)	0.673 (0.413-0.832)
-MUFA	0.672 (0.412-0.831)	0.693 (0.443-0.842)
-PUFA	0.351 (-0.011-0.631)	0.550 (0.236-0.760)
Linoleic acid	0.322 (-0.043-0.611)	0.549 (0.236-0.759)
ALA	0.400 (0.046-0.664)	0.447 (0.103-0.695)
EPA	0.314 (-0.053-0.605)	0.918 (0.834-0.961)
DHA	0.356 (-0.005-0.635)	0.873 (0.748-0.938)
Cholesterol	-	-
Proteins	0.412(-0.060-0.672)	0.525 (0.203-0.745)
Carbohydrates	0.667 (0.403-0.828)	0.705 (0.462-0.850)
Fibers	0.636 (0.358-0.811)	0.708 (0.467-0.851)
Vitamin B1	0.445 (0.101-0.694)	0.577 (0.274-0.776)
Vitamin B2	0.741 (0.519-0.869)	0.747 (0.528-0.872)
Vitamin B3	0.420 (0.070-0.678)	0.644 (0.369-0.815)
Vitamin B6	0.695 (0.447-0.844)	0.672 (0.412-0.831)
Folate	0.552 (0.239-0.761)	0.559 (0.249-0.765)
Vitamin B12	0.348 (-0.014-0.629)	0.781 (0.585-0.890)
Alcohol	0.270 (-0.100-0.574)	0.136 (-0.236-0.473)
Zinc (mg)	0.639 (0.362-0.812)	0.686 (0.433-0.839)
Retinol	0.318 (-0.048-0.609)	0.387 (0.031-0.656)
Vitamin C	0.483 (0.149-0.718)	0.551 (0.238-0.760)
Vitamin E	0.465 (0.126-0.707)	0.385 (0.029-0.655)

MUFA, mono unsaturated fatty acids; PUFA, poly unsaturated fatty acids; ALA, alpha linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

^a The ρ (rho) was computed by the following formula with measurements from the repeated measurements linear mixed model: estimated intercept variance/ (estimated residual variance + estimated intercept variance).

^b Adjusted for education, ethnicity, age, BMI, use of supplements, medication, tobacco and drugs.

a fertility problem were highly motivated to get pregnant and/or that they gave a socially desirable answer to this specific question.

Another aspect that needs to be considered related to epidemiological studies is the ICC, which ranged from 0.3 to 0.7 for the nutrient intakes. The lowest ICC was for alcohol ($\rho = 0.270$), which was not surprising considering the aforementioned explanation. Interestingly, the ICCs for vitamin B₂ ($\rho = 0.741$) and carbohydrates ($\rho = 0.667$) were relatively high, in contrast to the significant absolute differences. In other words, it might be that in the preconception period, overall, women equally eat more food items with vitamin B₂, for example dairy products, and less carbohydrates, such as soft drinks.

The results of this study are difficult to compare with those from other reproducibility studies, because of differences in study populations, number of days of dietary recording, intervals between the dietary assessments, and different correlation coefficients that were used. Some studies have reported higher ranges of correlation coefficients than in our study (from 0.5 to 0.7), but these studies used intervals of 1 month to a maximum of 1 year. 31-33 Overall, the mean unadjusted ICC was 0.483 and the mean adjusted ICC was 0.593, which are comparable to the mean correlation coefficients of the aforementioned studies. We calculated the ICCs also after exclusion of the 8 women (27%) who responded positively to the following question, "Has your diet changed since the preconception period?". Excluding this group of women did not change the results (data not shown). This was previously reported by Ajani et al.³¹ and it indicates that this question is not very important. Nevertheless, we did exclude the women who reported to have changed their diet substantially, for example a specific low-fat or low-caloric diet to lose weight (Fig. 1).

The second part of the results shows that all biomarkers were comparable between the two periods. The ICCs of all biomarkers were highly significant, ranging from 0.5 to 0.99. It is difficult to compare these ICCs to those reported in other studies, since most studies comprise a maximum study period of 12 months, and they

Biomarkers	Preconception $n = 30^{a}$	1 year after delivery $n = 26^{b}$	Median difference (SD)	<i>p</i> -value ^c	Adjusted <i>p</i> -value ^d
Homocysteine (µmol/L)	9.4 (6.6–21.8)	9.3 (5.9-19.2)	-0.60 (2.2)	0.530	0.200
Folate, serum (nmol/L)	30.8 (7.5–908.0)	16.0 (8.2–79.1)	-11.4 (22.6)	0.003	0.682
Folate, RBC (nmol/L)	1111 (459–2768)	690 (320-1848)	-469 (404)	< 0.001	0.017
Vitamin B6 (µmol/L)	79.0 (43.0-310.0)	72.5 (45.0-310.0)	0.0 (58.5)	0.811	0.661
Vitamin B12 (pmol/L)	312 (155–775)	354 (22-717)	36 (156)	0.887	0.455

Table 4			
Concentrations of biomarkers in blood collected in the	preconception	period and around 1	year after delivery.

Results are presented as median (range) or as indicated otherwise. RBC, red blood cell.

^a RBC folate, n = 24; serum folate, n = 29; homocysteine, n = 29.

^b RBC folate, n = 25.

^c *P*-value calculated with a repeated measurements linear mixed model.

^d P-value calculated with a repeated measurements linear mixed model, adjusted for education, ethnicity, age, BMI, use of supplements, medication, tobacco and drugs.

mostly use different statistical measurements, different populations and laboratory methods. There are only a few studies on the withinperson variability of these biomarker concentrations^{34–36} which are mainly on tHcy and folate and comparable to the ICCs we found. To our knowledge, there are no studies on the long-term inter-person variability of serum vitamin B₁₂ or vitamin B₆.

Even with a single measurement and with a period of two years in between, these results indicate an acceptable reproducibility of tHcy, vitamin B₆ and vitamin B₁₂ concentrations. Previous studies have shown that these concentrations are rather constant over time³⁴ with no seasonal variation.³⁷ From this study and from the study of McKinley et al., we may conclude that a single measurement reflects the individual average biomarkers concentrations in blood. However, there are two things that have to be considered. First, in our study, it is not just the two measurements over a certain period: the first measurement was taken before pregnancy and the second measurement was taken around 1 year after the delivery. The question is whether the pregnancy state, which influences metabolic and hormonal processes, may have irreversibly affected the biomarkers measured at 1 year after delivery. At least two studies have shown that biomarkers change during pregnancy.^{38,39} However, as soon as 6 weeks after delivery, the concentrations returned to the preconceptional values³⁸ except for those women who were breastfeeding.³⁹ Therefore, in the current study, we took these confounding effects into account by excluding women who were pregnant again, breastfeeding, or had an ectopic pregnancy in the previous 4 months. Thus, a single measurement of these biomarkers around 1 year after delivery seems useful for studying the relationship between the biomarkers and birth defects or adverse pregnancy outcome.

The results show that folate in RBC and serum, on the other hand, varied significantly over a two-year period. However, after adjustment for the preconception use of folic acid containing supplements, serum folate was not significantly different anymore, and the *p*-value for RBC folate changed from <0.001 to 0.017. In an

Table 5

Intraclass correlation coefficients for biomarkers comparing the preconception study moment with around 1 year after delivery.

Biomarkers	Unadjusted ρ ^a (95% Cl)	Adjusted ρ ^b (95% Cl)
Homocysteine (µmol/L) Folate, serum (nmol/L) Folate, RBC (nmol/L) Vitamin B6 (µmol/L)	0.741 (0.519–0.869) 0.990 (0.979–0.995) 0.601 (0.307–0.790) 0.606 (0.314–0.793)	0.679 (0.423–0.835) 0.989 (0.977–0.995) 0.647 (0.374–0.817) 0.747 (0.530–0.873)
Vitamin B12 (pmol/L)	0.529 (0.209–0.747)	0.318 (-0.047-0.609)

RBC, red blood cell.

^a The ρ (rho) was computed by the following formula with measurements from the repeated measurements linear mixed model: estimated intercept variance/ (estimated residual variance + estimated intercept variance).

^b Adjusted for education, ethnicity, age, BMI, use of supplements, medication, tobacco and drugs.

additional analysis, excluding all supplement users, both serum folate (p-value 0.735) and RBC folate (p-value 0.227) were comparable between the two assessment periods. This indicates that the variation in serum and RBC folate was mainly due to the significantly higher use of folic acid containing supplements in the preconception period than after delivery. The high percentage of women using folic acid containing supplements in the preconception period does not reflect the 37% use in the Dutch population.⁴⁰ This difference is probably due to the fact that women participating in an IVF/ICSI program are more motivated to follow the recommendation to use folic acid containing supplements. When taking these supplements into account, at least the serum and probably also the RBC folate concentrations at 1 year after delivery can be used as preconception values. Moreover, in epidemiological studies, the classification of women is of greater importance than the absolute values. In this case, the ICC is more meaningful than the absolute difference. We have shown that the ICCs of serum and RBC folate were indeed very high (0.990 and 0.601, respectively), indicating that these biomarkers are also useful for studying the relationship with birth defects or adverse pregnancy outcome.

Since folate concentrations are correlated with tHcy concentrations.³⁷ we would have expected an accompanying significant change in tHcy concentration. However, tHcy was not significantly different between both periods, despite the significantly higher use of folic acid supplements in the preconception period. Therefore, we performed an additional analysis to test the correlation between the biomarkers in both periods and in the supplement users and non-supplement users separately. In all analyses neither serum nor RBC folate was correlated with tHcy (data not shown). It is known that the response of tHcy on folic acid supplementation depends on 1) the baseline concentration of tHcy; there is a threshold of tHcy in terms of ability to respond to folic acid^{41,42} and 2) the time effect and dosage effect of the supplementation; only dosages >0.8 mg/day will further reduce tHcy concentrations and chronic use of supplements has more lowering effects on tHcy concentrations than acute use⁴¹ whereas acute use has a greater impact on serum folate. Apparently, the women in our study already had an optimal tHcy status. Moreover, of the women who reported to have used folic acid containing supplements in the previous 4 weeks, we do not know whether they used the supplements for more than 4 weeks, which would have had a greater impact on the tHcy concentration.

Although we may conclude that both the nutrient intakes and the biomarkers are useful in studies on the relationship with adverse pregnancy outcomes, some other important aspects have to be considered. First of all, this study was done in women undergoing fertility treatment. Therefore, these results may not directly be applicable to fertile women who conceive spontaneously. However, these women were not counseled on nutrition and lifestyle habits which were comparable with the prevalence in the general Dutch population⁴³ It is known that people tend to change their dietary habits after being diagnosed with a disease^{31,44} This might also apply to women who gave birth to a child with a serious birth defect. Moreover, it is very important to keep in mind the upcoming preconception counselling and recommendations for a healthy maternal nutritional status. This could imply changes towards a substantial healthier diet, while it is questionable whether these women would adhere to the healthier diet after delivery.

We also have to consider the strengths and weaknesses of the study. The main strength of this study is the unique prospective design and the limitation is the rather small sample size. Power calculations showed that with a sample size of 30, an alpha of 0.05 and standard deviations differing for each variable, the power varied from 5 to 55%. Therefore, studies with larger sample sizes are recommended to confirm our results. Dietary assessment methods are known to have a bias towards underestimating habitual energy intake. Therefore, we investigated the overall under-reporting bias by determination of the physical activity level (PAL), which was 1.55 in the preconception period and 1.53 at 1 year after delivery. These PALs are very similar and when comparing these levels with the cut-off value of 1.35, under-reporting was not likely to play a role in our study.²⁹ Moreover, when we excluded the individual PALs that were <1.35, the results remained the same (data not shown). Moreover, the FFQ covered a four week period and therefore, the day-to-day variability of food intake is minimised. The findings of this study do not address validity, since this FFQ has shown to be a valid method to estimate the included dietary energy and nutrient intakes in Dutch women.^{20,23}

In summary, we showed that the determination of the maternal nutritional status at around 1 year after delivery largely seems to reflect the preconceptional nutritional status in women undergoing fertility treatment. In addition, when using this standardized assessment period in case-control studies to investigate associations between the maternal nutritional status and adverse pregnancy outcomes, lifestyle factors such as the use of alcohol and folic acid containing supplements should be taken into account.

Statement of authorship

(LvD) carried out the studies and data analyses and drafted the manuscript; (LZ) helped to draft the manuscript and carried out some of the data analyses; (JdV) participated in the design of the study and advised on the statistical analyses; (JB) carried out the studies and data analyses; (JL) advised on and carried out the samples analyses; (ES) supervised the study and participated in its design; (RS) conceived and supervised the study, developed its design, contributed to all phases of the manuscript. All authors read and approved the final manuscript.

Conflict of interest statement

There are no conflicts of interest.

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