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Dietary supply of fish oil and folate during the second half of pregnancy and corresponding effects on the time course of plasma redox markers in three European Cohorts

Dissertation

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Abbreviations

A	Absorbance
AA	Arachidonic acid (C20:4n-6)
ABTS	2,2'-azino-bis(3-ethylbenzo-thiazoline-6-sulfonicacid) diammonium salt)
BMI	Body mass index
BMR	Basal metabolic rate
Chol	Cholesterol
cb	Cord blood
CV	Coefficient of variation
DHA	Docosahexaenoic acid (C22:6n-3)
dv	delivery
EFA	Essential fatty acids
EPA	Eicosapentaenoic acid (C20:5n-3)
ESP	Spain
FA	Fatty acids
GER	Germany
HPLC	High Pressure Liqiud Chromatography
HUN	Hungary
IQR	Interquartil range
LC-PUFA	Long-chain polyunsaturated fatty acids
LDL	low density lipoprotein
MDA	Malondialdehyde
MetMb	Metmyoglobine
MTHF	Methyltetrahydrofolate
MUFA	Monounsaturated fatty acids
NTDs	Neural tube defects
NUHEAL	Nutraceuticals for a Healthier Life
PUFA	Polyunsaturated fatty acids
ROS	Reactive oxygen species
SD	Standard deviation
SEM	Standard error of mean
SFA	Saturated fatty acids
SOD	Superoxid dismutase

TBARS	Thiobarbituric acid reactive substances
TG	Triacylglyceride
UA	Uric acid
vs	Versus
W20	Week 20 of gestation
W30	Week 30 of gestation

1 Introduction

1.1 Nutrition during pregnancy & the role of folate and docosahexaenoic acid

In Europe, women can easily meet their enhanced energy and protein needs during pregnancy to support fetal growth and expansion of maternal tissues. However, the increase in requirements of some micronutrients and of n-3 fatty acids is far higher than the enhancement in energy requirement. The recommended increase of energy intake during pregnancy is 17-22 %, whereas the reference intakes for some minerals and vitamins increase much more. For example folate requirements increase by approximately 50 % and requirements for the n-3 FA DHA increase by approximately 40 % (Table 1) (1).

Folic acid is essential for synthesis, repair, and function of DNA and the cell division (2;3). Research during the last years has established that low or inadequate folate status may contribute to congenital malformations and the development of chronic disease in later life. Neural tube defects (NTDs) are common major congenital anomalies, with folic acid as the primary known environment factor. A poor folate status during early pregnancy is associated with increased rates of neural tube defects (4-6). A similar protective effect of folic acid has been also postulated for non-neural birth defects, like congenital heart defects and oral clefts. It is widely accepted that a periconceptional folic acid supplementation decreases the occurrence of neural tube defects (5) and additional folate intake may also reduce pregnancy complications like pre-eclampsia and adverse neonatal outcomes. Mahomed showed in his metaanalysis that routine folate supplementation during pregnancy resulted in a reduction of the incidence of low serum as well as red cell folate levels (7). Pregnant women are recommended to consume synthetic folic acid from fortified foods, supplements or both, in addition to consuming folate from a varied diet. But in reality these recommendations are often not implemented. The PEGASUS study shows that less than 10 % of more than 900 women took folic acid during the critical period before conception and the first time of pregnancy. Furthermore, researches showed that mainly mothers who were not taking folic acid were less educated, from lower socio-economic groups and were not actively trying to fall pregnant at the time they became pregnant (8;9).

There for, campaigns were established during the last years to improve periconceptional folic acid use in many countries (10). As a consequence, in the Netherlands the use of folate supplements increased from 4.8 % to 21 % within 1 year (11).

A further nutrient with particular relevance for perinatal development is docosahexaenoic acid (DHA), a α n-3 long-chain polyunsaturated fatty acid (n-3 LCPUFA) (12). DHA is an essential component of all cell membranes in the brain and other tissues, with relevance for fetal neurological development (13-17). Several controlled studies found that DHA availability during gestation is associated with improved cognitive and visual development as well as reduced risk of early preterm birth (18-20). Perinatal n-3 supply is largely dependent on maternal dietary intake (21), because the fetal enzymatic system seems to be unable to supply sufficient amounts to meet the high perinatal needs (Figure 2) . Mothers may also functionally benefit from LCPUFA supplementation themselves, because a higher DHA status at delivery (22) and a better improvement of the maternal DHA status after delivery (23) are associated with less depressive symptoms in the post partum period. An average dietary DHA intake of at least 200 mg per day has been recommended for pregnant and breastfeeding women, which can be realized by consuming one to two portions of fatty fish per week (24). Because of the existing heavy metal contamination (methylmercury), pregnant women should select their dietary fish from a wide range of species (25). In the coming years, alternative strategies, such as n-3 fatty acid supplements and food enrichment will get more and more important to satisfy the demand and to solve the problems of contamination and overfishing.

The positive effects summarized above indicate that it may be beneficial to increase the intake of n-3 FA during pregnancy to optimize maternal and fetal status. However, LCPUFA are susceptible to peroxidation and even during normal pregnancy, there is an increase in lipid peroxidation products and oxidative stress (26). An excessive dietary intake of LCPUFA may enhance lipid peroxidation and reduce antioxidative capacity (27).

1.2 Oxidative Balance and Redoxsystem

Pregnancy is a physiological state associated with increased energy demands and elevated oxygen requirements. Several studies have indicated that women with normal pregnancies showed increased oxidative stress and higher lipid peroxidation products compared with non-pregnant women (26). At the beginning of pregnancy there is an increase in body fat accumulation, associated with increased lipogenesis. During advanced pregnancy a dearangement of the oxidative balance could lead to inflammatory

changes, thus triggering complications, such as preeclampsia, growth retardation or premature labour (28). A major source of oxidative stress during pregnancy is the placenta, which is rich in PUFA and thus a source of lipid peroxides for the maternal metabolism.

During the last few years, lipid peroxidation and its products (Figure 3) gained more and more attention in respect to pregnancy outcome. The multisystem disorder preeclampsia is associated with an imbalance between antioxidants and oxidants, which could lead to premature delivery and intrauterine fetal growth retardation. Kim et al found a relation between oxidative stress biomarkers (MDA and 8-Hydroxydeoxyguanosine) and reduced neonatal birth weight (29). However, the human metabolism has evolved a complex system to minimize the harmful effects of ROS. During pregnancy, not only oxidative stress increases, there is also an adapted increase of antioxidants and antioxidative enzymes (30;31). If there is no adaptation, miscarriage might be the consequence (30).

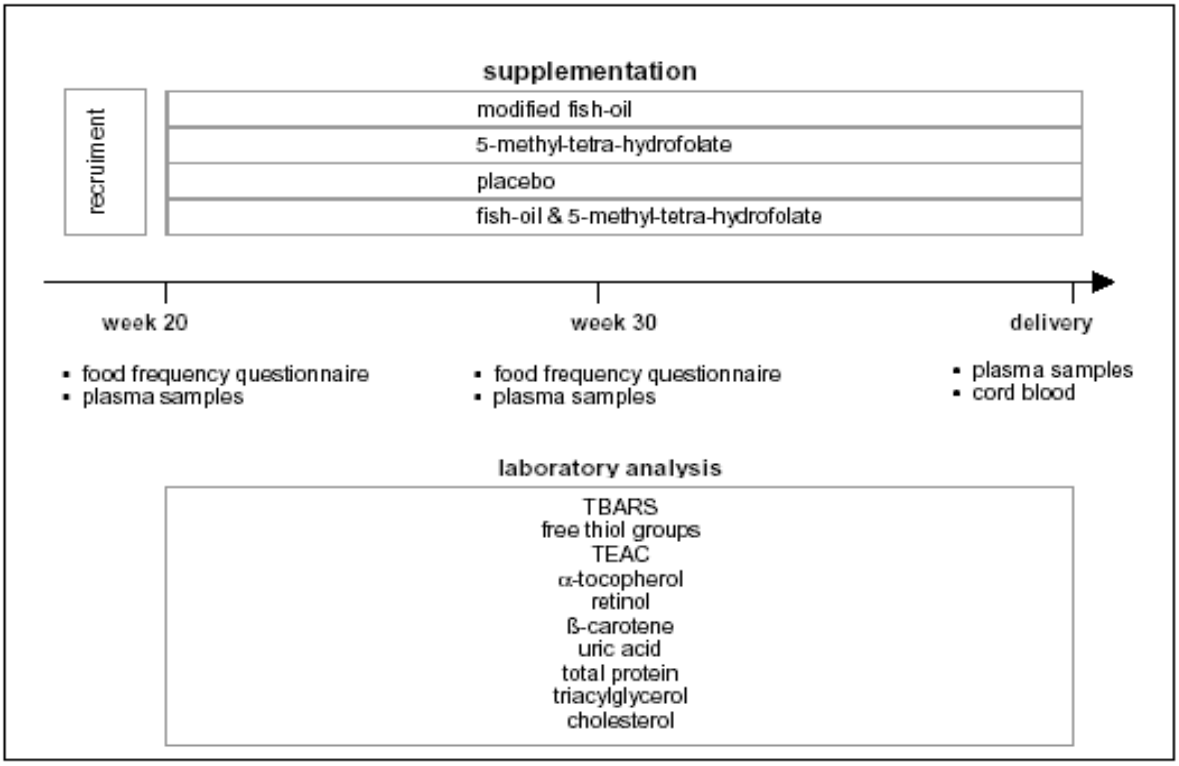

Figure 1 Course of interventions and analyses.

There are many different analyses to determine the extent of lipid peroxidation belonging to 2 main categories, the measurement of oxidative damage and / or the measurement of

antioxidative protection. We investigated longitudinal changes in the maternal redox status of plasma and the effect of fish oil and folate supplementation, using thiobarbituric acid reactive substances (TBARS) as marker of lipid peroxidation, and different non enzymatic antioxidants, because one measured lipid peroxidation marker may not provide a definite answer (Figure 1; Figure 4 & Figure 5).

Given the relevance of DHA and folate supply during pregnancy, we assessed additionally the dietary intake of these nutrients to obtain data on current intakes in the 3 European samples. Such information is the basis to identify risk groups for inadequate supply as well as to perform calculations to assess the progression by enriched food or supplements. The study population was drawn from participants of the NUHEAL Study (Nutraceuticals for a Healthier Life), a prospective randomized intervention study which compared the effects of a dietary supplementation with fish oil and/or 5-methyl-tetra-hydro-folate (MTHF) during the second half of gestation in mothers from 3 different European populations (32). The cohorts were taken from 3 European countries selected on the basis of their location and distance to the sea, which should be reflected in a different fish intake.

According to the conceptual framework in Figure 1, the research question of this thesis was subdivided into the analysis of the nutrition situation and the analysis of the plasma redox parameter, which leads to the following research questions:

(1) Characterisation of the dietary supply of folate and DHA in the 3 study populations compared to the recommendations.

(2) What are the effects of the supplementation on the maternal plasma redox status during the time course of the pregnancy?

(3) Does the redox status, and the response to supplementation, differ during the time course of the preganancy?

(4) Will the effects of the maternal plasma redox parameters be reflected in urinary markers?

The first research question about the dietary intakes of folate and DHA is addressed in an article entitled “Dietary Intake of Natural Sources of Docosahexaenoic Acid and Folate in Pregnant Women of Three European Cohorts”. A food frequency questionnaire (FFQ) was created with special focus on the sources of folate and DHA. This FFQ was completed twice during the second half of pregnancy by the NUHEAL participants and the resulting data are used for different statistical analyses.

The second research question is addressed in an article entitled “Influence of fish oil or folate supplementation on the time course of plasma redox markers during pregnancy”, which is based on survey data collected at week 20 (±1 week), week 30 (±1 week) and at the time of delivery. The research includes parameter of the plasma redox status, which were analysed regarding group differences and longitudinal changes as well.

The third research question is addressed in an article entitled “Effect of docosahexaenoic acid and eicosapentaenoic acid supplementation on oxidative stress levels during pregnancy”. Here, the urinary excretion of 8-hydroxy-2’-deoxyguanosine (8-OHdG) and malondialdehyde (MDA) was measured in a sub-group of the NUHEAL population.

In a final chapter of this cumulative dissertation, the main findings of the three articles are summarized in German and English language and a more general conclusion are drawn.

1.3 Authors contributions

Within the NUHEAL-project I was responsible for performing the following analysis, for data interpretation and for preparing of the manuscripts.

- laboratory analyses of uric acid, thiol groups, TEAC, TBARS
- analysis of the food frequency questionnaires
- statistical analysis of the above mentioned parameters

Lead Author:

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Co-Author:

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3 Publications

3.1 Publication 1 --- British Journal of Nutrition

“Influence of fish oil or folate supplementation on the time course of plasma redox markers during pregnancy.”

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Influence of fish oil or folate supplementation on the time course of plasma redox markers during pregnancy

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Maternal supplementation with long-chain PUFA, to improve infant neurological development, might cause additional increase of oxidative stress. Pregnant women aged 18–41 years were randomised into one of four supplementation groups. From week 22 on, they received supplements containing either modified fish oil (*n* 69), 5-methyl-tetrahydro-folate (*n* 65), both (*n* 64), or placebo (*n* 72). Plasma Trolox-equivalent antioxidative capacity (TEAC), concentrations of α-tocopherol, retinol, β-carotene, free thiol groups, uric acid and thiobarbituric acid-reactive substances (TBARS) were determined at weeks 20 and 30 and at delivery. The studied antioxidants showed no significant differences between the four supplementation groups. At week 30 plasma TBARS levels were found to be significantly higher in the fish oil group (0·80 (SEM 0·04) μmol/l) than in the folate (0·67 (SEM 0·03) μmol/l; $P=0·024$) and control (0·69 (SEM 0·04) μmol/l; $P=0·01$) groups. Concentrations of retinol and free thiol groups decreased during pregnancy, whereas uric acid increased and β-carotene as well as TEAC showed only minor changes. Fish oil supplementation during the second half of pregnancy appears not to decrease antioxidant status. The increased TBARS levels at week 30 may indicate a period of increased oxidative stress in plasma at this time.

Pregnancy: DHA: Oxidative stress: Thiobarbituric acid-reactive substances

Oxidative stress occurs as a result of an increase in oxidant generation, a decrease in antioxidant protection, or a failure to repair oxidative damage. Damage to cells results from reactive oxygen species-induced alteration of PUFA in membrane lipids, proteins and DNA. During pregnancy, oxidative stress increases, but there is also an increase of antioxidants and anti-oxidative enzymes[1,2]. Imbalances between oxidants and the antioxidative system may be associated with the onset of pre-eclampsia[3,4] and with an increased risk of miscarriage[1]. Furthermore, an inverse correlation between the maternal oxidative stress biomarkers malondialdehyde (for lipid peroxidation) and 8-hydroxydeoxyguanosine (for DNA peroxidation) and neonatal birth weight has been demonstrated[5].

Increased availability of *n*-3 long-chain PUFA, for example, DHA, during the perinatal period has been reported to improve cognitive and visual development of the infant[6–8]. The fetus accumulates up to 50 mg DHA per d in brain and adipose tissue during the last 3 months of gestation[9]. Fish oil supplementation in pregnancy was found to slightly prolong mean duration of gestation time and to markedly lower the risk for early preterm delivery[10,11]. However, long-chain PUFA are susceptible to peroxidation[12] and additional oxidative stress might be caused by a high dietary intake of *n*-3 fatty acids without adequate antioxidative protection[13].

In women of childbearing age adequate folate supply reduces the incidence of neural tube defects in infants and in the general population. Folate supplementation can reduce plasma concentrations of homocysteine[14]. During pregnancy this might improve placental vascularisation and hence maternal–fetal substrate transfer. In line with this hypothesis Böhles *et al.* showed a negative correlation between maternal plasma homocysteine and DHA-percentage in the erythrocyte membrane phospholipids of their newborns[15]. Thus a combined supplementation with folate and *n*-3 long-chain PUFA seems reasonable. As several reactions of homocysteine metabolism (for example, formation of homocysteine) promote the formation of reactive oxygen species, folate supplementation might beneficially influence redox markers[16].

The aim of the present study was to compare oxidative stress and antioxidant levels in pregnant women with and without an *n*-3 long-chain PUFA supplementation, considering a potentially confounding influence of folate supplementation.

Since redox status cannot be adequately assessed from a single analytical parameter, we analysed a set of biomarkers

Abbreviations: TBARS, thiobarbituric acid-reactive substances; TEAC, Trolox-equivalent antioxidant capacity; w20, week 20 ± 1 of gestation; w30, week 30 ± 1 of gestation.

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in the plasma of pregnant women participating in a randomised clinical trial on the effect of fish oil supplementation during the second half of pregnancy on pregnancy outcome[17]. Vitamin E is the major lipid-soluble, peroxidation chain-breaking antioxidant[18]. β-Carotene is an effective scavenger of peroxyl radicals[19] and it is a precursor of retinol, which is of essential importance for growth and development of cells and tissues. Uric acid is a powerful scavenger of singlet oxygen and other radicals[20]. Thiol groups, mainly from glutathione, are susceptible to oxidative changes and play an important role in antioxidative reactions[21]. Because there are more antioxidants and interactions in the aqueous phase of plasma, we measured Trolox-equivalent antioxidant capacity (TEAC) in plasma as an integrative parameter. In addition, the plasma concentration of thiobarbituric acid-reactive substances (TBARS) was determined as a marker of lipid peroxidation.

Subjects and methods

Subjects and enrolment

The present study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the local Ethical Committees of the participating centres. From November 2001 to March 2003, pregnant women were recruited in Granada (Spain), Pécs (Hungary) and Munich (Germany). Women attending antenatal care clinics for ultrasound examinations between week 12 and week 20 of gestation were approached by study personnel, informed about the aims and nature of the study and invited to participate. Subject information includes an oral explanation by the physician and a written informed consent which was given to the subject. The criteria for inclusion were: age at study entry of 18–41 years, uncomplicated singleton pregnancy and weight at study entry of 50–95 kg. Women taking folate supplements after week 16 of gestation or fish oil supplements since they became pregnant were excluded from the study. Furthermore, for this analysis all women who smoked during pregnancy were excluded because smoking may enhance oxidative stress. Details of the study protocol and execution have been previously reported[17].

Participants who agreed to participate were randomised without stratification into one of four dietary supplementation groups separately at each centre. To ensure that the different supplementation groups are nearly equally represented in each centre, randomisation was performed in blocks of twenty numbers. For this purpose twenty envelopes containing cards with one of the four numbers according to the supplementation groups were prepared and put into a closed box. By drawing envelopes, supplementation group numbers were assigned to the subject identity number. This procedure was performed identically for each study centre. After allocation to the dietary group, women were provided correspondingly with ninety sachets of 15 g, of which they had to consume one per d. At the second investigation date in week 30 ± 1 (w30) of gestation a further batch of ninety sachets was provided for the rest of pregnancy.

Thus, from week 22 of gestation onwards participants received milk-based supplements containing either modified fish oil providing 500 mg DHA and 150 mg EPA per d (fish oil group), or 400 μg 5-methyl-tetrahydro-folate (MTHF) per d (folate group), both in combination (fish oil + MTHF; fish oil + folate group) or placebo (control group). All supplements provided the estimated additional requirements for minerals and vitamins during the second half of pregnancy (Table 1).

Table 1. Nutrition, mineral and vitamin content of the supplements according to manufacturer's analysis (nutrient supply per sachet of 15 g)

Group…	Fish oil	Folate	Fish oil + folate	Control
DHA (mg)	500	–	500	–
EPA (mg)	150		150	
5-MTHF (μg)	–	400	400	–
Energy				
kJ	297	293	297	293
kcal	71	70	71	70
Protein (g)	2·5	2·9	2·5	2·9
Fat (g)	3·1	2·9	3·1	2·9
Carbohydrates (g)	8·2	8·0	8·2	8·0
Vitamin A (μg)	330	330	330	330
Vitamin D (μg)	1·5	1·5	1·5	1·5
Vitamin E (mg)	3	3	3	3
Thiamin (mg)	0·36	0·36	0·36	0·36
Riboflavin (mg)	1·5	1·5	1·5	1·5
Niacin (mg)	4·5	4·5	4·5	4·5
Vitamin B_6 (mg)	1·9	1·9	1·9	1·9
Vitamin B_{12} (μg)	3·5	3·5	3·5	3·5
Vitamin C (mg)	270	270	270	270
Ca (mg)	300	300	300	300
P (mg)	240	240	240	240
Mg (mg)	93	93	93	93
Zn (mg)	3	3	3	3
I (μg)	66	66	66	66

5-MTHF, 5-methyl-tetrahydro-folate.

Non-fasting maternal venous blood samples for the laboratory analyses were collected at week 20 ± 1 (w20) of gestation, before supplementation started, at w30 and at the time of delivery using EDTA as anticoagulant. The plasma samples were stored at −80°C until assayed. At the same time points a well-trained physician performed standardised interviews with the woman to assess data about socio-economic status, obstetrical history, intercurrent diseases and maternal smoking habits. Additionally, maternal height, weight and blood pressure were measured. At w20 and w30 participating women completed a FFQ to assess the DHA and folate intake with their habitual diet. Details of the nutritional evaluation have previously been reported[22].

Analytical procedures

TBARS, TEAC, free thiol-groups, total protein and uric acid concentrations from all samples of each woman were analysed during one and the same day.

TBARS concentrations were determined by reaction with 2-thiobarbituric acid, based on the method of Knight *et al.*[23]. Ortho-phosphoric acid (500 μl; 0·44 M), 100 μl plasma and 200 μl 2-thiobarbituric acid solution (60 mg per 10 ml water) were pipetted into reaction vials. The mixture was heated in a water-bath for 1 h to 100°C. After cooling, a 100 μl sample was added to a 100 μl methanol–NaOH mixture (0·45 ml 1 M-NaOH per 4·55 ml methanol).

After centrifugation a 50 μl portion of the supernatant fraction was used for HPLC with fluorescence detection (excitation, 550 nm; emission, 532 nm) for the measurement of TBARS[23]. External calibration with 1,1,3,3-tetraethoxypropane was used to quantify TBARS in the plasma samples. Intra- and inter-assay CV were 2·6 and 8·8 %, respectively.

Plasma α-tocopherol, retinol and β-carotene concentrations were analysed at the Department of Paediatrics, University of Frankfurt am Main (Germany) by an established HPLC method with UV detection after extraction of lipids into hexane[24]. An external standard was applied for quantification. Plasma α-tocopherol is given as concentration and in relation to plasma lipids (α-tocopherol:cholesterol + TAG ratio).

Free thiol-groups were determined using Ellman's reagent (5,5′-dithio-bis 2-nitrobenzoic acid)[25]. Micro-plate wells were filled with 165 μl water, 60 μl phosphate-saline buffer (0·1 M), 15 μl plasma or standard and 60 μl Ellman's reagent (10 mM in 0·15 M-NaCl and 0·1 M-Na_3PO_4). Blanks were measured with each plasma sample, containing distilled water instead of Ellman's reagent, and one blank containing pure water instead of plasma. The reaction was allowed to proceed during incubation at room temperature for at least 15 min on a shaker plate, before absorption was measured at 405 nm (photometer anthos ht III; Labtec Instruments, Wals, Austria). A five-point calibration curve was prepared daily using fresh cysteine solution. Intra- and inter-assay CV averaged 6·6 and 8·9 %, respectively.

The measurement of TEAC is based on the inhibition of the formation of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) ($ABTS^+$) radical cations by antioxidants[26]. PBS buffer (506 μl; 5 mM; pH 7·4), 36 μl myoglobine (70 μM), 300 μl 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (500 μM) and 8·4 μl plasma were combined. The mixture was incubated for 3 min at 30°C. The reaction was started by the addition of 150 μl H_2O_2 (450 μM), which was prepared fresh every day, and after 3 min absorbance at 734 nm was read. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid diluted in 2·5 mM-PBS) was used for calibration. The intra- and inter-assay CV averaged 1·1 and 4·7 %, respectively.

Cholesterol, TAG, total protein and uric acid were analysed with an automatic Hitachi analysis system (Fa. Boehringer, Mannheim, Germany), using enzymic assays for cholesterol, TAG, uric acid and a colour test for total protein, respectively.

Statistical analysis

Data were analysed with SPSS for Windows 12.0 (SPSS Inc., Chicago, IL, USA). Normal distribution was examined using the Kolmogorow–Smirnov test (with Lilliefors correction). One-way ANOVA with *post hoc* Bonferroni correction was used to evaluate differences between supplementation groups for normally distributed data. In the case of non-normal distribution the Mann–Whitney *U* test was applied. Statistical significance was assumed at $P<0·05$. Differences over time were evaluated using a general linear model. Correlations between parameters were estimated by computing Pearson's correlation coefficient in the case of normally distributed values and the Spearman ρ correlation coefficient in the case of other distributions, respectively.

Results

Study participants

From the 311 women enrolled into the study, forty-one women were excluded from the analyses because they did not complete the study. Reasons for dropping out were non-compliance (*n* 2), relocation (*n* 1), aversion to or bad taste of the supplement (*n* 9), and the loss of contact (*n* 2). For the remaining cases, a special reason for drop out could not be identified. From 270 study participants who completed the study[17], samples were available for sixty-five women recruited in Munich, 113 in Granada and fifty-four in Pècs, respectively. Allocation of these women to the different intervention groups was: fish oil group (*n* 69), folate group (*n* 65), control group (*n* 72) and fish oil + folate group (*n* 64). Age and BMI were not significantly different between the four supplementation groups at study entry (Table 2). The four supplementation groups differed at none of the time points in BMI and weight gain during pregnancy. The whole study population showed an average weight gain of 5·8 (SEM 3·8) kg from w20 to w30 and 4·2 (SEM 3·5) kg from w30 until the end of pregnancy. Weight development was not different between the groups.

Effects of fish oil and folate supplementation on maternal plasma

Plasma cholesterol levels were significantly different over time of pregnancy in the whole study population (w20, 5·72

Table 2. Characteristics of the participants in the four supplementation groups*
(Median values and interquartile ranges (IQR))

Group...	Fish oil		Folate		Control		Fish oil + folate		Total study population		
	Median	IQR	Median	IQR	Median	IQR	Median	IQR	Median	IQR	*P*
Age at study entry (years)	31·2	27·4–34·0	31·9	26·1–35·2	31·0	28·4–34·8	31·9	28·0–35·2	31·3	27·6–34·8	0·62
BMI (kg/m^2)											
w20	25·2	22·8–28·2	24·3	22·2–27·3	24·2	23·0–26·6	24·5	23·1–27·2	24·6	22·8–27·4	0·42
w30	27·7	25·0–31·6	26·2	24·2–28·3	26·4	25·0–29·1	26·2	24·6–29·1	26·5	24·7–29·5	0·37
Delivery	29·1	26·4–33·0	27·5	25·3–30·3	28·4	26·6–31·1	28·2	26·4–31·2	28·4	26·4–31·3	0·27

w20, Week 20 ± 1 of gestation; w30, week 30 ± 1 of gestation.
*Group-specific statistical differences were assessed by ANOVA (with Bonferroni correction) and the Kruskal–Wallis test.

Table 3. Plasma levels of total protein, TAG and cholsterol at the studied time points according to supplementation group‡ (Median values and interquartile ranges (IQR))

Group. . .§	Fish oil			Folate			Control			Fish oil + folate		
	Subjects (*n*)	Median	IQR	Subjects (*n*)	Median	IQR	Subjects (*n*)	Median	IQR	Subjects (*n*)	Median	IQR
Total protein (mg/l)												
w20	59	65·5	62·5–69·1	53	65·9	63·8–69·4	65	66·7	63·8–69·5	52	66·5	63·2–69·1
w30	59	65·5	63·0–69·2	52	66·1	61·9–68·2	63	66·4	62·0–70·0	52	65·2*	61·7–67·0
Delivery	57	61·3†	55·2–65·6	51	60·3†	57·7–65·0	59	60·7†	54·7–65·7	49	61·2†	58·1–65·3
Cholesterol (mmol/l)												
w20	59	5·52	5·02–6·11	53	5·75	5·22–6·42	65	5·46	4·93–6·40	52	5·72	5·13–6·46
w30	59	6·66*	5·98–7·49	52	6·70*	5·81–7·17	63	6·37*	5·67–7·23	52	6·85*	6·02–7·63
Delivery	57	6·01†	5·48–6·95	51	6·24	5·57–7·30	59	5·93†	4·84–6·86	49	6·68	5·88–7·36
TAG (mmol/l)												
w20	59	1·53	1·30–1·87	53	1·64	1·36–2·04	65	1·63	1·43–2·10	52	1·78	1·45–1·98
w30	59	2·09*	1·66–2·59	52	2·43*	1·98–2·83	63	2·30*	1·93–2·87	52	2·25*	1·82–2·49
Delivery	57	2·44	1·81–2·78	51	2·37	1·95–2·68	59	2·42	1·80–2·88	49	2·43†	2·03–2·91

w20, Week 20 ± 1 of gestation; w30, week 30 ± 1 of gestation.
* Median value was significantly different from that at w20 ($P<0·05$).
† Median value was significantly different from that at w30 ($P<0·05$).
‡ Statistical differences were calculated with ANOVA and the Mann–Whitney *U* test between the groups. Differences between time points were determined with Student's *t* test and the Wilcoxon test, respectively.
§ Differences between the supplementation groups were not found.

(SEM 0·06); w30, 6·64 (SEM 0·08); delivery, 6·24 (SEM 0·09) mmol/l; $P<0·001$). In all supplementation groups the highest plasma mean cholesterol value was found at w30 and the lowest at baseline, before supplementation started (Table 3). A significant difference ($P=0·003$) at w30 was found between women supplemented with fish oil (fish oil and combined groups: 6·78 (SEM 0·10) mmol/l) and non-supplemented women (folate and control groups: 6·18 (SEM 0·17) mmol/l). There were no other significant differences between the supplementation groups. Plasma TAG values increased significantly with advancing pregnancy (w20, 1·72 (SEM 0·04); w30, 2·26 (SEM 0·05); delivery, 2·30 (SEM 0·06) mmol/l; $P<0·01$) without significant group differences at the different time points ($P=0·30$; Table 3).

Supplementation did not affect plasma total protein levels (general linear model: $P=0·723$). But all supplementation groups showed time-dependent changes. With exception of the combined group ($P=0·04$), total plasma protein levels were similar at w20 and w30 and decreased towards delivery in all groups ($P<0·001$; Table 3). Between all time points we found significant correlations for plasma total protein (w20–w30, r 0·54; w30–delivery, r 0·34; $P<0·001$).

Plasma TBARS concentrations at w20 showed no significant differences between the supplementation groups. At w30 we found a significant difference between the DHA group and the folate group ($P=0·047$; Fig. 1). In all four groups, plasma TBARS increased significantly from w30 until the end of pregnancy. Subjects with *n*-3 long-chain PUFA in their supplement, with or without folate, had higher plasma TBARS concentrations at w30 ($P=0·042$) and at delivery than non-*n*-3 long-chain PUFA-supplemented groups ($P=0·030$; Fig. 2). Plasma TBARS level showed no differences from w20 to w30 in groups with the fish oil supplement, but decreased significantly without the fish oil supplement ($P=0·001$; Fig. 2). We found no significant effect of the study supplement on the plasma TBARS levels (general linear model: $P=0·305$). Plasma TBARS correlations between the different time points in the whole study group (w20–w30, r 0·35, $P<0·001$; w30–delivery, r 0·21, $P=0·03$) and in women with fish oil supplementation (fish oil and combined groups) were statistically significant (w20–w30, r 0·22, $P=0·021$; w30–delivery, r 0·28, $P=0·006$). However, there was only a significant correlation in the subjects not supplemented with fish oil (folate and control groups) between

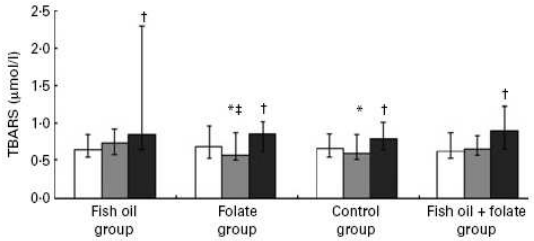

Fig. 1. Maternal thiobarbituric acid-reactive substances (TBARS) plasma levels in the different supplementation groups over time: week 20 ± 1 of gestation (w20; □); week 30 ± 1 of gestation (w30; ■); delivery (■). Values are medians, with interquartile ranges represented by vertical bars. *Median value was significantly different from that at w20 ($P<0·05$). †Median value was significantly different from that at w30 ($P<0·05$). ‡Median value was significantly different from that of the fish oil group at w30 ($P<0·05$).

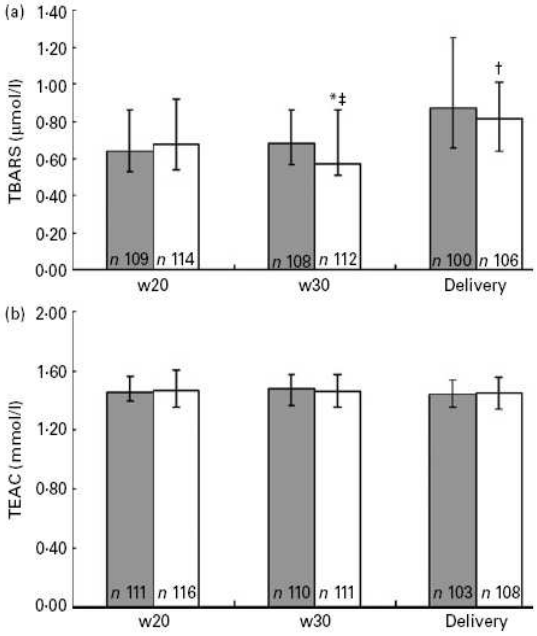

Fig. 2. Plasma thiobarbituric acid-reactive substances (TBARS) (a) and Trolox-equivalent antioxidant capacity (TEAC) (b) values in women with (■) and without (□) fish oil in their supplementation over time: week 20 ± 1 of gestation (w20); week 30 ± 1 of gestation (w30); delivery. Values are medians, with interquartile ranges represented by vertical bars. *Median value was significantly different from that of the women receiving the fish oil supplementation at the same time point (P=0·042). †Median value was significantly different from that of the women receiving the fish oil supplementation at the same time point (P=0·030). ‡Median value was significantly different from that of the women not receiving the fish oil supplementation at w20 (P=0·001).

w20 and w30 (w20–w30, r 0·37, $P<0·001$; w30–delivery, r 0·05; NS). Only at w30 were there significant negative correlations between TBARS and the plasma α-tocopherol concentration (r −0·21; P=0·002) and the α-tocopherol:lipids ratio (r −0·24; $P<0·001$).

The concentration of plasma α-tocopherol was related to total lipids (cholesterol + TAG) and rose between w20 and w30 significantly in all four supplementation groups ($P<0·001$). From w30 until the end of pregnancy the plasma α-tocopherol concentrations showed no differences in all groups (Table 4). Supplementation had no effect on the plasma α-tocopherol:lipid ratio; during the whole intervention time no differences were found between the supplementation groups. Significant intra-individual correlations of the α-tocopherol:lipid ratio in the study population were found between w20 and w30 (r 0·27; $P<0·001$) as well as w30 and delivery (r 0·27; $P<0·001$). Correlations between these time points were also significant for α-tocopherol concentrations (w20–w30, r 0·45, $P<0·001$; w30–delivery, r 0·40, $P<0·001$).

Plasma TEAC was not affected by supplementation at any time point (Table 4). There were no significant changes over time and no differences between fish oil-supplemented and non-fish oil-supplemented women (Fig. 2). Significant intra-individual correlations of TEAC at all time points were found in the total population (w20–w30, r 0·82, $P<0·001$; w30–delivery, r 0·70, $P<0·001$). Plasma TEAC levels correlated significantly with β-carotene levels at w20 (r 0·18; P=0·006) and w30 (r 0·14; P=0·034). Other correlations were obtained between TEAC and retinol (w20, r 0·32, $P<0·001$; w30, r 0·28, $P<0·001$; delivery, r 0·21, P=0·002), TEAC and α-tocopherol (w30, r 0·29, $P<0·001$; delivery, r 0·45; $P<0·001$) as well as between TEAC and total protein (w20, r 0·17, P=0·009; delivery, r 0·36; $P<0·001$). No correlations were found between TEAC and uric acid and the free thiol groups, respectively.

Maternal retinol levels decreased in the whole study population with increasing duration of pregnancy ($P<0·001$; w20, 2·74 (SEM 0·08) μmol/l; w30, 2·18 (SEM 0·07) μmol/l; delivery, 1·99 (SEM 0·07) μmol/l). Significant changes between w20 and w30 were found in all four groups (Table 4) and a significant change between w30 and delivery was found in the fish oil group (P=0·008). Splitting women into subjects with (fish oil and combined groups) and without fish oil supplementation (folate + control groups) resulted in significant differences between w20 and w30 in both groups (with fish oil: P=0·002, w20, 2·70 (SEM 0·11) μmol/l; w30, 2·2 (SEM 0·10) μmol/l; without fish oil: $P<0·001$, w20, 2·72 (SEM 0·11) μmol/l; w30, 1·99 (SEM 0·09) μmol/l) and between w30 and delivery only in fish oil-supplemented women (P=0·002; delivery, 1·86 (SEM 0·12) μmol/l). Between supplementation groups we found no significant differences at any of the time points. Intra-individual correlations of the plasma retinol levels were significant between all time points (w20–w30, r 0·40, $P<0·001$; w30–delivery, r 0·23; $P<0·001$).

The plasma β-carotene levels showed different developments in women with and without fish oil supplementation. Both the folate and control groups showed a significant decrease between w20 and w30 (P=0·014; P=0·002), as well as all subjects without fish oil supplementation taken together (control and folate groups: w20, 0·52 (SEM 0·04) μmol/l; w30, 0·41 (SEM 0·04) μmol/l; $P<0·001$). In all groups, the lowest plasma β-carotene concentration was found at the end of pregnancy. There were no significant differences between the four supplementation groups at any of the time points. Comparing women with fish oil (fish oil and combined groups) and those without fish oil supplementation (control and folate groups), we found a significant difference at w30 (P=0·03) and delivery (P=0·04). In both cases, women with fish oil supplementation had higher β-carotene concentrations (with fish oil: w30, 0·51 (SEM 0·04) μmol/l; delivery, 0·42 (SEM 0·03) μmol/l; without fish oil: w30, 0·42 (SEM 0·04) μmol/l; delivery, 0·38 (SEM 0·04) μmol/l). Correlations between the time points were significant (w20–w30, r 0·60; w30–delivery, r 0·75; $P<0·001$) in the whole study population.

Plasma free thiol groups decreased until the end of pregnancy, but no differences were found between the four supplementation groups, as well as between fish oil-supplemented (fish oil and combined groups) and non-fish oil-supplemented (control and folate groups) subjects. A significant decrease between w30 and delivery was found in all four groups (Table 4). Correlations were significant between all time points (w20–w30, r 0·49; w30–delivery, r 0·45; each $P<0·001$). At all time points we found significant correlations between the concentrations of total protein and the free thiol groups (w20, r 0·22, P=0·001; w30, r 0·34;

Table 4. Plasma levels of redox parameters at the studied time points according to supplementation group‡
(Median values and interquartile ranges (IQR))

Group...	Fish oil			Folate			Control			Fish oil + folate		
	Subjects (*n*)	Median	IQR	Subjects (*n*)	Median	IQR	Subjects (*n*)	Median	IQR	Subjects (*n*)	Median	IQR
Thiol groups (μmol/l)												
w20	58	200	173–243	51	213	181–256	62	217	170–271	52	216	171–272
w30	58	196	157–256	51	204	140–252	61	203	172–269	52	205	145–294
Delivery	57	156†	117–209	51	161†	132–194	58	183†	126–238	49	178†	127–236
TEAC (mmol/l)												
w20	59	1·46	1·40–1·56	53	1·49	1·37–1·64	63	1·45	1·36–1·55	52	1·48	1·39–1·62
w30	58	1·49	1·41–1·57	50	1·47	1·36–1·59	61	1·46	1·37–1·57	52	1·47	1·36–1·59
Delivery	57	1·43	1·37–1·55	52	1·48	1·35–1·58	56	1·43	1·34–1·54	47	1·45	1·36–1·53
Uric acid (μmol/l)												
w20	59	178	155–208	53	190	158–217	65	186	158–214	52	173	137–201
w30	59	200*	178–232	52	194	161–225	63	203*	172–232	52	188*	155–220
Delivery	57	274†	229–312	51	259†	214–315	59	266†	226–291	49	257†	214–294
Tocopherol:TAG + cholesterol ratio												
w20	59	5·44	4·82–6·06	53	5·27	4·54–5·79	64	5·26	4·47–5·98	51	5·14	4·68–5·85
w30	57	5·99*	5·12–7·10	50	5·43*	4·50–6·98	61	6·11*	4·98–7·30	50	6·24*	5·07–7·66
Delivery	55	5·71	4·78–7·03	51	5·42	4·41–6·24	57	5·53	4·57–7·30	48	5·48	4·72–7·12
β-Carotene (μmol/l)												
w20	59	0·39	0·29–0·54	54	0·42	0·26–0·65	65	0·41	0·25–0·63	51	0·47	0·26–0·70
w30	59	0·41	0·24–0·61	51	0·30*	0·17–0·59	65	0·30*	0·19–0·47	50	0·41	0·21–0·70
Delivery	55	0·34	0·20–0·69	53	0·31	0·19–0·54	58	0·28	0·18–0·45	48	0·41	0·24–0·63
Retinol (μmol/l)												
w20	59	2·38	1·84–3·56	54	2·51	1·71–3·60	65	2·57	1·84–3·51	51	2·30	1·79–3·62
w30	58	2·04*	1·66–2·87	51	1·69*	1·20–2·51	65	1·91*	1·35–2·91	50	2·19*	1·46–2·89
Delivery	55	1·52†	1·05–2·46	53	1·74	1·13–2·58	58	1·75	1·41–2·65	48	1·81	1·39–2·79

w20, Week 20 ± 1 of gestation; w30, week 30 ± 1 of gestation; TEAC, Trolox-equivalent antioxidant capacity.
*Median value was significantly different from that at w20 ($P<0·05$).
†Median value was significantly different from that at w30 ($P<0·05$).
‡Statistical differences were calculated with ANOVA and the Mann–Whitney *U* test between the groups. Differences between time points were determined with a general linear model (Bonferroni correction) and the Wilcoxon test, respectively.

delivery, r 0·44, $P<0·001$). The ratio of thiol groups per total protein did not differ between the supplementation groups.

In the whole study population, plasma uric acid level showed a slight increase between w20 and w30 (510 (SEM 14) to 551 (SEM 8) μmol/l; $P<0·001$) and a stronger increase towards the end of pregnancy (739 (SEM 13) μmol/l; $P<0·001$). There was no significant effect of the supplementation on maternal uric acid level during intervention time (general linear model: $P=0·60$). Except the folate group ($P=0·07$), all groups showed a significant increase between w20 and w30 ($P\leq0·001$). Between w30 and delivery, all four groups showed a significant increase ($P<0·001$). Significant correlations with $P<0·001$ were found between w20 and w30 (r 0·65) as well as w30 and delivery (r 0·60) in the total study population.

Dietary intake of DHA

The analysis of the FFQ showed no significantly different DHA intake with habitual diet between the supplementation groups at w20 and w30. DHA intake in the study population was 30·1 mg per 1000 kJ (126 mg per 1000 kcal) at w20 and 29·4 mg per 1000 kJ (123 mg per 1000 kcal) at w30 on average[22]. We found no significant difference regarding the dietary intakes of folate between the four supplementation groups at w20 nor at w30. The study population showed a folate intake of about 27·7 μg/1000 kJ (116 μg/1000 kcal) at both time points with no significant difference between the groups and change with time.

Discussion

The present study shows a significant increase of plasma TBARS during the second half of pregnancy, independently from supplement allocation. An increase of lipid peroxidation was previously reported with progression of pregnancy, and thus with increasing age of the placenta[27]. While measures of peroxidation are generally higher in pregnant women than in non-pregnant controls, results on the evolution of plasma markers of peroxidation during pregnancy are quite variable[28]. The significant increase of TBARS in our subjects is in line with the reported increase of lipid hydroperoxides between first and third trimester[29], while a significant increase of plasma malondialdehyde concentration with the course of pregnancy was not observed by Patrick *et al.* in American women[30]. A reason for these divergent results might be that the TBARS test is non-specific for malondialdehyde[31]. The mid-pregnancy malondialdehyde concentrations were clearly lower in the women studied by Patrick *et al.*[30] (0·38 μmol/l in white and 0·50 μmol/l in black women) than the TBARS concentrations in the European women in the present study (0·74 μmol/l). Thus antioxidative capacity might have been exhausted earlier in our women. An association between lipid peroxidation products and lipid-soluble antioxidants is reflected in the negative correlations between TBARS and α-tocopherol in the whole study population at w30 (r −0·21; $P=0·002$). Fish oil supplementation induced both significantly higher plasma phospholipid DHA and EPA percentages[17] at w30 and at delivery, while plasma TBARS concentrations were only at w30 significantly increased in the fish oil-supplemented group. This might indicate that the antioxidant system was brought to its limits around w30, but adapted later on or at delivery other factors are the determinants of redox status.

Plasma concentrations of α-tocopherol and the α-tocopherol: lipid ratio increased after w20, which reflects the additional intake of α-tocopherol with the study supplements. While a negative correlation between percentage of total PUFA and vitamin E has been reported in pregnant Italian women[32], we did not find lower α-tocopherol concentrations in the fish oil-supplemented women. The reason seems to be the different amounts of vitamin E intake. None of the studied Italian women took nutritional supplements containing lipid-soluble vitamins[32]. The additional vitamin E intake with the Nutraceuticals for a Healthier Life (NUHEAL) study supplement might have provided α-tocopherol in an amount much higher than the minimal requirement for radical scavenging.

Plasma protection against free radical injury is offered by a wide range of antioxidants with synergistic action. Measurement of all individual antioxidants is not possible; thus we applied the integrative TEAC assay to estimate plasma antioxidative capacity. We did not find significantly lower plasma TEAC in fish oil-supplemented compared with non-supplemented women. In both groups TEAC tended to decrease towards the end of the intervention period. Comparing measured TEAC concentration with antioxidants measured in plasma, we found significant correlations to retinol, β-carotene and total protein levels, but no correlation to uric acid. TEAC was not affected by the progression of pregnancy and the significant changes in uric acid concentration over time were not reflected. Plasma albumin and uric acid are the major determinants of TEAC, but correlations to specific antioxidant concentrations are difficult to establish and depend on the applied radical-generating substrate and reaction time[33–35].

The decrease of plasma retinol concentration during pregnancy is in agreement with the results of Bruinse *et al.*[36] and Cikot *et al.*[37]. Possible reasons for a decrease could be the increasing plasma volume during pregnancy, a decrease in retinol-binding proteins or increased tissue retention. The decreased plasma retinol concentration during advanced pregnancy might also reflect enhanced fetal utilisation[32,38].

Significant differences in plasma β-carotene concentrations were found between fish oil-supplemented and non-supplemented subjects. In contrast to a short-term *n*-3 long-chain PUFA supplementation study[39], we found higher values of plasma β-carotene at w30 and at the time of delivery in fish oil-supplemented women. All groups showed the lowest concentrations at the time of delivery, which might be an indication for a higher level of oxidative stress towards the end of gestation as well as the increased maternal blood volume. Several other factors known to influence β-carotene concentration, such as age, sex, smoking status and residence location, could be excluded as they were not different between the groups ($P>0·05$)[40]. Thus, the reason for the higher β-carotene concentrations in the fish oil-supplemented women remains unclear.

Uric acid plasma concentration increased significantly throughout pregnancy, irrespective of type of supplementation. A similar increase has been reported in other studies and has been interpreted as reflecting a xanthine–xanthine oxidase pathway stimulation[41,42]. Uric acid may act as an

antioxidant, but there is also evidence that it has pro-oxidative effects[43–45]. Thus, increased levels of uric acid indicate oxidative stress, but it is unclear whether it induces oxidative stress or reflects antioxidant activity. In pregnancy elevated uric acid levels have been associated with increased rates of complications such as pre-eclampsia[42]. In non-smoking pregnant women, uric acid levels of 197 (SEM 15) μmol/l (33·1 (SEM 2·5) mg/l) in weeks 16–24 and 194 (SEM 9) μmol/l (32·6 (SEM 1·5) mg/l) in weeks 24–34 have been reported[46], which is similar to our findings. In contrast, results in smoking pregnant women (202 μmol/l (33·9 mg/l); 214 μmol/l (35·9 mg/l))[46] tended to be higher than the plasma uric acid in our *n*-3-supplemented women (w20, 179 μmol/l (30 mg/l); w30, 196 μmol/l (33 mg/l)). Thus, it appears that the increase with time found in the present study is not related to the *n*-3 supplementation, but rather with falling renal clearance towards the end of pregnancy[46].

Plasma thiol groups showed a decrease in all subjects during the intervention time. The present results confirm the results of previews studies[47,48]. The decrease in thiol groups and in total protein levels during pregnancy may reflect the increasing maternal plasma volume and physiological functions of the thiol groups in fetal metabolism, but increases of oxidative stress with pregnancy duration has also been proposed as an explanation[47,49,50]. Our data show a similar decrease in all supplementation groups, which was on average 20 % from baseline to delivery. Thus, if an *n*-3 long-chain PUFA supplementation induced oxidative stress, it was too small to influence plasma thiol concentration.

We conclude that the recommended supply of *n*-3 long-chain PUFA in pregnancy[51,52], here providing a daily supply of 500 mg DHA and 150 g EPA, did not affect water-soluble and lipid-soluble antioxidants. Higher TBARS concentrations in the fish oil group were not associated with a depletion of plasma antioxidants, and the group difference was clearly smaller than the increase with pregnancy duration. Independent of the inclusion of folate the applied dosage of *n*-3 long-chain PUFA supplementation seems to be without adverse effects on antioxidative defence in pregnant women.

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All authors have made substantive contributions to the study, and endorse the data and conclusions. The authors contributed as follows: B. K., C. C. and T. D. designed and supervised the study. C. F., K. M., H. D., M. C. and J. A. M.-F. participated in data collection and/or laboratory analyses. C. F. did the statistical analysis and wrote the first draft of the manuscript. B. K., H. D., C. C. and T. D. revised the manuscript and contributed to the final version of the manuscript.

All authors certify that there is no actual or potential conflict of interest in relation to this article.

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3.2 Publication 2 --- Annals of Nutrition & Metabolism

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Dietary Intake of Natural Sources of Docosahexaenoic Acid and Folate in Pregnant Women of Three European Cohorts

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Docosahexaenoic acid · Folate · Nutrition · Pregnancy

Abstract
Background: Folic acid plays a fundamental role in cell division and differentiation. Docosahexaenoic acid (DHA) has been associated with infantile neurological and cognitive development. Thus, optimal intrauterine development and growth requires adequate supply of these nutrients during pregnancy. ***Methods:*** Healthy pregnant women, aged 18–41 years, were recruited in Granada (Spain; n = 62), Munich (Germany; n = 97) and Pécs (Hungary; n = 152). We estimated dietary DHA and folate intake in weeks 20 (w20) and 30 of gestation (w30) using a food frequency questionnaire with specific focus on the dietary sources of folate and DHA. ***Results:*** Both w20 and w30 Spanish participants had significantly higher daily DHA intakes (155 ± 13 and 161 ± 9 mg/1,000 kcal) than the German (119 ± 9 and 124 ± 12 mg/1,000 kcal; p = 0.002) and Hungarian participants (122 ± 8 and 125 ± 10 mg/1,000 kcal; p = 0.005). Hungarian women had higher folate intakes in w20 and w30 (149 ± 5 and 147 ± 6 μg/1,000 kcal) than Spanish (112 ± 2 and 110 ± 2 μg/1,000 kcal; p < 0.001) and German participants (126 ± 4 and 120 ± 6 μg/1,000 kcal; p < 0.001), respectively. ***Conclusion:*** Dietary DHA and folate intake of pregnant women differs significantly across the three European cohorts. Only 7% of the participants reached the recommended folate intake during pregnancy, whereas nearly 90% reached the DHA recommended intake of 200 mg per day.

Introduction

Nutrient requirements increase markedly during pregnancy to support fetal growth and expansion of maternal tissues. The quality of nutrient supply during pregnancy is associated with maternal health, pregnancy outcome, rate of complications as well as fetal development and growth [1, 2]. Poor maternal nutrition is one of the key factors leading to compromised fetal growth and adverse effects on child health [3]. Pregnant women often do not meet their increased nutrient needs, particularly of folic acid [4–6]. Folate deficiency is one of the most common vitamin deficiencies worldwide [7]. This vitamin is essential for DNA synthesis, amino acid metabolism and cell division [8, 9]. Poor folate status during early pregnancy is associated with increased rates of neural tube defects [10–12]. Moreover, poor folate supply can lead to increased plasma homocysteine, an established risk factor of placental abruption, preterm delivery and increased rates of low birth weight [13, 14]. Considering these facts, an optimal supply with folate during the

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whole pregnancy – not only at the beginning – might improve pregnancy outcome [15].

Food provides two forms of folate, pteroylmonoglutamate and pteroylpolyglutamate. These two forms differ in their bioavailability, because a hydrolysis of the polyglutamate side chain is necessary before absorption. The estimated average bioavailability of folate from omnivorous diets is about 50% [15], whereas synthetic folic acid from supplements is almost completely available for the metabolism [16]. Folate is sensitive to heat and light, and is easily oxidized during food preparation. Thus, it is hard to achieve a well-balanced folate supply from food [17], and pregnant women are recommended to consume synthetic folic acid from fortified foods, supplements or both, in addition to consuming folate from a varied diet. However, there exists no single recommended value for women in childbearing age or pregnant women throughout Europe. On average, a supplementation of 400 μg/day of folic acid is recommended (e.g. Germany: 600 μg/day, Hungary and Spain: 400 μg/day) [18, 19].

A further nutrient with particular relevance for perinatal development is docosahexaenoic acid (DHA), an n-3 long-chain polyunsaturated fatty acid (FA) mainly found in fatty sea fish [20]. DHA is an indispensable component of all cell membranes in the brain and other tissues, with major relevance for fetal neurological development [21–24]. In the last 3 months of gestation, the fetus accumulates up to 50 mg DHA per day in the brain and adipose tissue [25]. After birth, breast-fed infants are provided with DHA through breast milk. Several controlled studies found that DHA availability during pregnancy is associated with improved cognitive and visual development as well as reduced risk of early preterm birth [26–28]. Therefore, an average dietary DHA intake of at least 200 mg/day has been recommended for pregnant and breast-feeding women [29].

Given the relevance of DHA and folate supply during pregnancy, we assessed the dietary intake of these nutrients in women participating in the Nutraceuticals for a Healthier Life (NUHEAL) Study in Germany, Hungary and Spain, to obtain data on current dietary intakes during pregnancy in these European cohorts.

Subjects and Methods

Subjects and Recruitment

The study population was comprised of the participants of the NUHEAL Study, a prospective cohort study, which compared the effects of dietary supplementation with DHA and/or methyltetrahydrofolate from week 21 of gestation until child birth in mothers from three different European countries [30, 31].

From November 2001 to March 2003, apparently healthy pregnant women (aged 18–41 years) attending antenatal care clinics were recruited between week 12 and 20 of gestation. Recruitment took place at three study sites: the University Hospital of Granada in Spain, the Ludwig Maximilians University in Munich, Germany, and the University of Pécs, Hungary. Further inclusion criteria were: body weight at study entry between 50 and 92 kg, uncomplicated singleton pregnancy, no participation in another clinical trial, no use of fish oil supplements from the beginning of pregnancy and no use of folate or vitamin B_{12} supplements after the 16th week of gestation [30].

Dietary Assessment

Dietary intake was recorded using a food frequency questionnaire (FFQ) containing standard portion sizes, which was based on previous studies evaluating dietary intakes (the MONICA study [32], the nutrition protocol of Freiburg, Germany, and the GISELA study). To address reproducibility and the possibility of changes in dietary intakes, nutritional assessment was completed in week 20 ± 1 (w20) and week 30 ± 1 (w30) of gestation. Intake of nutrients was calculated from the portion size and frequency of food consumption using the German nutrient database (Bundeslebensmittelschlüssel) [33], version II.3. It was decided to use only one nutrient database because of possible systematic errors, which would increase in case of using three different databases [34]. Nutrient intake is expressed as intake per 1,000 kcal. Thirty-three food items were included in the questionnaire primary focused on dietary sources of DHA and folate. For this reason, certain food categories like milk, dairy products and beverages were disregarded. We did not assess the intake of processed food fortified with folate because the used nutrient database contained only incomplete information about those foods.

In the FFQ, details on the following supplements were also recorded: multivitamin juice/pills, beer yeast, wheat bran, flaxseeds and evening primrose oil. However, intakes from the NUHEAL study supplements were not included.

In addition to the amount and frequency of food consumption, women were asked to provide information on the mode of preparation and special dietary habits (e.g. vegetarian diet). Single food items were combined in several food groups such as meat (beef, pork, poultry, liver and processed meat including sausage); seafood (divided into lean fish, medium fat fish and fatty fish); vegetables (raw vegetables, cooked vegetables and legumes), fats (for warm dishes, cold dishes or spreads), fruits, soy products, cheese, eggs, baked goods and potatoes. The frequency of intake was categorized into never/1 or 2–3 times per day/1 or 2–3 or 4–6 times per week/1–3 times per month. Record sheets were checked by an experienced dietician for the presence of implausible amounts or inadequate description to ensure accuracy. Body height of the participants was determined in w20 of gestation and body weight in both w20 and w30. Estimated basal metabolic rate was calculated from weight and height based on the formula of Schofield [35]. Participants whose energy intake calculated from the FFQ was less than the basal metabolic rate were defined as 'under-reporters' and excluded from nutritional analyses.

Statistical Analyses

Data were analyzed with SPSS for Windows 12.0 (SPSS, Chicago, Ill., USA). Normal distribution was examined using the Kolmogorov-Smirnov analysis. One-way analysis of variance and the post hoc Bonferroni test were used to evaluate differences between study groups in normally distributed data. For non-normally distributed data, the Mann-Whitney U test was employed. A linear model was used to obtain more information regarding variables affecting dietary intake. Non-normally distributed data were logarithmized before the test. Statistical significance was considered at $p < 0.05$. Correlations between parameters were estimated by computing Pearson's correlation coefficient in the case of normally distributed values and the Spearman correlation coefficient (ρ) in the case of other distributions, respectively.

Results

Three hundred and eleven pregnant women agreed to participate in the clinical trial. A total of 271 women with an average age of 30.8 years (mean) completed the study protocol (table 1). More than 98% of the women were Caucasians and >96% were living in a partnership. Most of the women consumed omnivorous diets, <1% of the participants followed a vegetarian diet (n = 2). Forty-eight percent of the participants were living in urban areas. At the time of study entry, 42% of the participating women were not working, 32% had a full time job and 13% worked part time. Women from Munich were significantly older than those from the two other centers ($p < 0.001$). In w20 and w30, 12 and 14%, respectively, of the participants reported to smoke. An overview about socioeconomic characteristics of the three study samples is shown in table 2.

In w20, 15 women were excluded from analysis (Germany: n = 12; Spain: n = 2, and Hungary: n = 1), because of apparent under-reporting of dietary intake. Thus, 256 subjects were included in the calculations. In w30, 35 women were excluded (Germany: n = 24; Spain: n = 5, and Hungary: n = 6), because of apparent under-reporting, thus a total of 236 FFQs were included. Women who dropped out of the study did not differ in age, ethnic group, residency, family status and education from the remaining participants.

Body Weight Progress and Birth Outcomes

Body weight and weight gain (on average 5.7 kg from w20 to w30) did not significantly differ between the three cohorts, but in w20 the body mass index was lower in Munich compared to Granada ($p = 0.047$, table 1). Body mass index increased from w20 to w30 by 2.1 ± 1.3 kg/m^2 (mean ± SD). The birth outcomes, birth weight, head

Table 1. Characteristics of the study cohorts at study entry

	Germany (n = 68)	Spain (n = 147)	Hungary (n = 55)	p value
Age, years	33.5 ± 3.5[a, b]	30.1 ± 4.9[a]	29.4 ± 4.8[b]	<0.001
Weight, kg	67.8 ± 9.9	67.2 ± 8.9	69.6 ± 11.8	NS
Height, cm	166 ± 6.0	162 ± 6.0	165 ± 7.0	NS
BMI	24.4 ± 3.3[a]	25.8 ± 3.5[a]	25.5 ± 4.6	0.003
Vegetarians, n	2	0	0	NS
Smokers, n	3	27	1	NS
Cigarettes n/week	22	55	28	NS

BMI = Body mass index; NS = no significant difference. Statistical differences were tested with ANOVA and the post hoc Bonferroni test (means ± SD).

[a, b] Common superscripts indicate a significant difference.

Cigarettes n/week are related to the subgroup of smokers.

Table 2. Socioeconomic characteristics of the study participants (frequency in %)

	Spain	Germany	Hungary
Education			
None	–	1.4	–
Primary school	39.7	62.6	5.5
General qualification for university	54.4	30.6	60.0
University	5.9	4.1	30.9
Other	–	–	3.6
Graduation			
None	5.9	40.8	74.5
With graduation	44.1	25.2	–
Degree	8.8	4.8	–
University graduation	38.2	23.8	23.6
Other	2.9	2.7	1.8
Career			
Appointee	2.9	18.4	–
Employee	52.9	29.3	96.4
Manager	19.1	1.4	–
Worker	1.5	22.4	1.8
Freelancer	7.4	8.2	1.8
Current job			
None	23.5	46.3	52.7
<15 h per week	2.9	2.7	3.6
Half time	16.2	15.0	3.6
Full time	48.5	27.2	25.5
Maternity leave	8.8	–	14.5
University/education	–	0.7	–
Family status			
Single	1.5	6.1	–
Partnership	98.5	93.9	100.0
Habitat			
Urban area	57.4	31.3	80.0
Rural area	42.6	68.0	20.0

Table 3. Dietary intake in w20 and w30 of gestation (medians and interquartile ranges: P25–P75)

	Germany		Spain		Hungary		Total study population	
	1,000 kcal	day	1,000 kcal	day	1,000 kcal	day	1,000 kcal	day
w20								
Energy, kcal		2,204[a*, b*]		3,078[a*]		3,267[b*]		2,968
		1,765–3,520		2,492–3,549		2,462–3,994		2,271–3,520
SFA, g	16.0	35.4[d, e*]	15.9	48.1[d]	16.6	48.0[e*]	16	45.8
	14.2–20.3	27.9–46.7	14.4–17.9	39.5–59.3	14.4–18.7	38.1–66.7	14.3–18.3	35.2–59.3
MUFA, g	19.7[a*]	41.4[d*, e]	25.4[a*, b*]	74.2[d*, f*]	19.3[b*]	60.9[e, f*]	22.90	65.6
	17.5–23.4	32.8–57.8	22.6–28.4	61.0–93.2	17.2–21.3	44.7–72.1	19.3–26.7	47.8–85.2
PUFA, g	9.9[b*]	20.1[d*, e*]	10.2[c*]	29.5[d*, f*]	12.4[b*, c*]	39.0[e*, f*]	10.7	29.7
	8.0–12.8	16.5–31.1	9.2–11.8	24.2–40.6	10.9–16.2	29.6–51.9	9.1–12.6	21.9–42.0
DHA, mg	111[a]	235[d*, e*]	134[a, b]	413[d*, f]	107[b]	315[e*, f]	126	355
	66.3–140	154–244	103–176	297–327	86.3–151	231–444	91.5–165	240–477
n-6/n-3 ratio	9.0[a*]	9.0[d*]	9.1[b*]	9.1[e*]	12.0[a, b*]	12.0[d*, e*]	9.3	9.3
	7.9–13.1	7.9–13.1	8.0–10.3	8.0–10.3	9.1–14.5	9.1–14.5	8.1–11.5	8.1–11.5
Folate, µg	123[a, b]	271[d, e*]	110[a, c*]	324[d, f*]	152[b, c*]	429[e*, f*]	116	327
	104–147	206–354	97–124	270–403	123–166	319–610	103–139	262–424
w30								
Energy, kcal		2,203[a*, b*]		2,934[a*]		2,926[b*]		2,713
		1,774–2,612		2,301–3,402		2,354–3,662		2,205–3,365
SFA, g	17.8[a]	37.0[d, e]	16.3[a]	45.4[e]	16.1	48.0[d]	16.4	44.0
	13.9–21.2	30.4–48.2	14.5–17.6	35.4–57.9	14.5–17.8	37.8–59.5	14.5–17.9	35.4–56.7
MUFA, g	19.2[a*]	40.0[d*, e]	25.5[a*, c*]	71.9[d*, f*]	18.5[c*]	54.3[f*, e]	23.4	61.8
	17.9–22.1	32.8–61.5	23.5–28.0	55.8–89.3	17.0–21.3	42.7–73.7	19.2–26.6	48.1–85.1
PUFA, g	9.7[a]	21[d*, e*]	10.8[b*]	29.5[d*, f]	12.4[a, b*]	37.4[e*, f]	11.0	29.4
	7.7–12.8	15.8–27.2	9.5–12.3	24.0–38.7	10.4–15.8	27.9–56.1	9.5–12.9	22.6–39.2
DHA, mg	107[a]	259[d*, e]	136[a, b*]	403[d*, f]	110[b*]	317[e, f]	123	372
	68.3–180	136–363	109–185	325–494	93.3–138	239–458	98.8–175	271–465
n-6/n-3 ratio	9.5[a]	9.5	9.3[c*]	9.3[f*]	12.0[b, c*]	12.0[e, f*]	9.7	9.7
	7.0–13.0	7.0–13.0	8.1–10.8	8.1–10.8	9.4–15.2	9.4–15.2	8.2–11.9	8.2–11.9
Folate, µg	113[b*]	254[d, e*]	109[c*]	304[d*, f*]	143[b*, c*]	396[e*, f*]	116	311
	92.1–147	193–320	94.3–124	254–360	119–165	321–526	97.2–138	254–393

Statistical differences were tested using the Mann-Whitney U test. SAF = Saturated FAs; MUFA = monounsaturated FAs; PUFA = polyunsaturated FAs. [a–f] Common superscripts indicate a significant difference; * $p < 0.001$.

circumference and placental weight were not significantly different between the three cohorts (data not shown). Birth length was significantly higher in the German sample ($p < 0.001$) compared with the two other centers.

Nutrient Intake

The highest calculated energy intake was observed in Hungary (table 3). German women had the lowest energy intake from the FFQs ($p < 0.001$ vs. both other cohorts), while there was no significant difference between Spain and Hungary. Dietary fat intake contributed about 45% to energy intake. Spanish women had a higher total fat intake than both other groups at both time points (table 3). The carbohydrate intake was significantly higher in Germany than in Spain in w20 and w30, and there was also a significant difference between Spain and Hungary in w30. Analysis of the total protein intake and protein intake in grams per kilogram body weight was significantly lower in German and Spanish than in Hungarian cohorts.

Our estimate of folate intake includes natural sources of folate in food, folic acid and folic acid equivalents. The daily intake of folate differed significantly between the three centers. The highest intake was observed in Hungarian women (table 3). Significant differences were found between Hungarian and German as well as between Hungarian and Spanish women. Other variables like age, and the mother's education level and family sta-

Table 4. Important food sources of folate, and their percentage to total folate intake

w20	Germany (n = 56)	Spain (n = 146)	Hungary (n = 54)
Vegetables	31.2	23.4	30.0
Fruits	11.9	18.5	13.4
Bread	11.9	10.6	6.4
Meat products	3.5	5.8	10.0
Nuts, oil seeds	5.9	7.9	12.8
Potato products	7.4	7.6	6.7
w30	(n = 43)	(n = 143)	(n = 50)
Vegetables	25.6	23.4	28.9
Fruits	13.8	17.6	15.0
Bread	10.8	10.9	7.0
Meat products	3.8	5.2	8.1
Nuts, oil seeds	4.9	8.9	13.1
Potato products	7.0	8.3	6.6

Table 5. Important food sources of DHA, and their percentage to total DHA intake

w20	Germany (n = 56)	Spain (n = 146)	Hungary (n = 54)
Fish	42.2	48.1	22.0
Poultry	20.3	23.7	35.5
Eggs	7.1	6.9	9.4
Bread	14.9	4.3	2.5
Sweets and pastries	2.1	2.7	2.1
w30	(n = 43)	(n = 143)	(n = 50)
Fish	47.2	51.0	25.6
Poultry	18.9	25.2	37.0
Eggs	8.3	5.5	7.2
Bread	16.8	13.1	11.7
Sweets and pastries	2.4	2.7	2.5

tus had no effect on the folate intake. The correlation between the two time points was statistically significant for the whole study population ($r = 0.5$; $p < 0.001$). Only 7 and 5% of the women attained the recommended folate intake of 600 μg/day during pregnancy in w20 and w30, respectively. The folate intake of 400 μg/day, which is recommended for pregnant women in Hungary (Hungarian National Center of Epidemiology), was met by 29 and 23% of the total study population in w20 and w30, respectively.

In all study centers, the two most common sources of folate were vegetables and fruits (table 4). Bread was important for the supply in Spain and Germany, but not in Hungary, where meat products and sausages, especially liver, were the major folate sources. Additionally, nuts and oil seeds contributed to a larger extent to the folate supply in Spain and Hungary than in Germany. The correlation between the estimated daily folate intake and the vegetable and fruit intake was significant in the whole study population ($r = 0.6$; $p < 0.001$). Daily folate and liver intake showed the highest correlation coefficient in Hungary ($r = 0.7$; $p < 0.001$), while there was no correlation in Germany.

Fatty Acids

The FA composition and consumption for the three study centers is shown in table 3. Values for monounsaturated FAs were significantly higher in Spain compared to the other centers at both time points. Saturated FA intake was similar in the three centers in w20, whereas in w30 German women showed a significantly higher intake than Spanish women. The n-6 FA intake as well as the ratio of n-6 (linoleic and arachidonic acid) to n-3 (α-linolenic acid, eicosapentaenoic acid and DHA) polyunsaturated FAs was high in all centers. Women from Hungary had the highest n-6 FA intake as well as the highest n-6/n-3 ratio, whereas women from Spain showed the lowest n-6/n-3 ratio and the highest n-3 FA intake. Significant differences in the n-6 intake were found between Hungary and the two other centers at both time points.

The highest DHA intake was found in the Spanish cohort (significantly different from both Hungarian and German cohorts). DHA intake in w20 and w30 correlated with each other ($r = 0.519$; $p < 0.001$) and with daily fish intake ($r = 0.55$; $p < 0.001$). The three most common sources for DHA intake from food in all three study samples were fish, poultry and eggs (table 5). The linear model showed no effect for family status, age, education level and urban/rural residency on DHA intake.

Supplement Intake

Forty-three percent of the women took dietary supplements in w20 and 39% in w30. Hungary provided the biggest group of participants taking one or more supplements in w20 and w30 (78 and 62%, respectively). In the remainder, the number of women taking supplements was about half of those in Hungary (Germany: 36 and 28%, and Spain: 32 and 35%). Multivitamin juices and tablets were the most common supplements, followed by beer yeast, wheat bran and flaxseeds.

FFQ w20 versus FFQ w30

A comparison between the two evaluation time points showed significant differences in energy ($p = 0.01$) and retinol ($p = 0.03$) intake in the total study population. Correlations between FFQ w20 and FFQ w30 were statistically significant for all nutrients ($p < 0.001$). Spearman's rank correlation coefficient ranges from $r_{sp} = 0.49$ to $r_{sp} = 0.62$.

Discussion

This study on the nutrient intake of pregnant women from three different European cohorts indicates a poor folate supply, as well as considerable differences in nutrient supply between the three study centers. To decrease any potential effects of incomplete reporting, we expressed nutrient intakes per energy intake, i.e. compared the nutrient density between the three study centers. About one third of the folate intake was provided by vegetables. Leafy greens such as spinach, legumes, and some fruits as well as vegetables are rich food sources of folate. Staple foods such as bread or potatoes contribute only moderately, but consumed in large amounts they can provide a significant portion of the total folate intake [36]. The relative contribution of other folate sources varied from sample to sample. Nuts and meat products played a particularly important role in the Hungarian cohort, where women showed the highest liver consumption (median; w20: 88 g/week; w30: 59 g/week). German participants obtained their folate supply primarily from vegetables, pastries, fruits and cheese, whereas meat products played only a minor role and liver consumption was negligible. In all centers, about 7% of total dietary folate was supplied by potatoes and potato products, which confirms that staple food can significantly contribute to the total folate intake [36]. While our results indicate that vegetables and fruits were the primary sources of dietary folate intake, Siega-Riz et al. [37] reported that folate-fortified grains and ready-to-eat cereals followed by different kinds of juices were the most important sources for folate intake in pregnant women in the US, where cereals and grains are generally fortified with folic acid.

The average dietary folate intake of the study population was 327 μg/day in w20 and 311 μg/day in w30. Thus, only 6% of the participants reached the intake of 600 μg/day recommended in Germany, Austria and Switzerland, and only 26% of the participants reached 400 μg/day. However, we may have underestimated total folate intake, because we could not account for some fortified foods. The consumption of a folate-rich diet and folic acid supplements is recommended for women of childbearing age [38–40]. Folic acid fortification of foods is an alternative option to cover the needs of women who get pregnant [41–43]. Nationwide fortification programs of staple foods such as flour are well established in many countries around the world [7, 41, 44, 45], primarily because folic acid supplementation during the first weeks of pregnancy decreases the incidence of neural tube defects. For example, cereal fortification with folic acid in Canada has reduced the prevalence of neural tube defects >50% [46]. The rather low folate intake in pregnant women found in this study was also reported in other studies [5, 37]. The folate intake of the Hungarian subjects reported in the present study exceeded the results of a previous Hungarian nutritional survey carried out between 1990 and 1994 [47]. This trial revealed a mean daily folate intake of 166 μg in w20 and 149 μg in w30 of gestation, and hence folate intake may have increased in pregnant Hungarian women during the last decade. However, the 3rd Hungarian dietary survey showed that folic acid intake of adult women did not meet the criteria of the Hungarian recommendations [48]. German non-pregnant women aged 25–51 years were reported to have a mean daily folate intake of about 225 μg/day [49] and, thus, to have a lower intake than the NUHEAL participants, potentially due to an increased health consciousness of a population participating in a dietary intervention trial during pregnancy and different data collection methods.

Also Ortega et al. [50] showed in their survey that in 319 Spanish women aged 18–35 years none of them reached the recommended 400 μg/day of folate.

DHA is primarily contained in fatty sea fish such as salmon, mackerel and herring, as well as in certain micro algae [51]. In the used FFQ, women were asked to separate their intake into lean fish, medium fat fish and fatty fish as well as into the kind of preparation (cooked, fried, conserved, crumbed or soup). Eighty-five percent of the Spanish women ate fatty fish at least once a month. In comparison, only 4% of the Hungarian women consumed fatty fish with high DHA contents, they rather consumed red and sea bass, swordfish and trout, with moderate fat and lower DHA contents. German participants tended to eat more lean fish (e.g. halibut, cod or sole pike) with low DHA contents, which possibly explains their lower DHA intake.

Our results agree with previous data reporting a higher availability of fish in Spain (75 g/day) than Hungary (4 g/day) and Germany (12 g/day) [52]. Authors reported also that cooking and frying are the main types of pre-

paring fish, followed by the use of canned fish, while fish soups play only a minor role. Other possible dietary DHA sources are meats and eggs. A European Commission-funded evidence-based consensus recommendation recently advised that pregnant and lactating women should reach an average intake of at least 200 mg DHA per day [29]. Nearly 90% of the participating women achieved this intake. The WHO has recommended an n-6/n-3 FA ratio between 3:1 and 4:1 [53]. The subjects in this study reached an n-6/n-3 ratio of 10:1, i.e. much higher than the WHO goal. The ratio in Spanish women (9.4:1) is very similar to the 9.8 ± 7.3 previously reported in 162 Spanish women [54]. A somewhat higher dietary DHA intake would contribute to lowering the n-6/n-3 ratio.

While mean macronutrient intake of our study population seems quite adequate, our findings clearly show shortcomings particularly in folate intake, and to a lesser extent also in the amount of DHA intake, in pregnant women in the study population. While there were differences between the three cohorts studied, intake in w20 and w30 within study populations was very similar. Other variables like age, family status or education level did not have significant effects either on folate or on DHA intake from food. An increased dietary micronutrient density and micronutrient supplementation could enhance micronutrient intake [37].

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3.3 Publication 3 --- Free Radical Research

Effect of docosahexaenoic acid and eicosapentaenoic acid supplementation on oxidative stress levels during pregnancy

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Effect of docosahexaenoic acid and eicosapentaenoic acid supplementation on oxidative stress levels during pregnancy

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Abstract

Docosahexaenoic acid (DHA) is an indispensable component of cell membranes with high requirements during pregnancy. DHA supplementation is thought to enhance oxidative stress because of increased likelihood of lipid peroxidation. We estimated the oxidative stress levels in two groups of pregnant women who received daily supply of required vitamins with ($n = 23$) or without ($n = 23$) 500 mg of DHA and 150 mg of eicosapentaenoic acid (EPA) from 20 weeks of gestation to the time of delivery. Urinary excretions of 8-hydroxy-2′-deoxyguanosine (8-OHdG), a marker of oxidative DNA damage and of malondialdehyde (MDA), a marker of lipid peroxidation, were measured at 20, 30 weeks and at the time of delivery. Urinary MDA excretion remained unchanged throughout the study period in both groups. Urinary 8-OHdG excretion at delivery was significantly higher than at 20 and 30 weeks ($p < 0.05$), but there were no group differences at the three time points. There were no differences between the two groups in plasma α-tocopherol levels. We conclude that under the conditions studied, a daily supplementation of 500 mg DHA and 150 mg EPA with vitamins to pregnant women did not enhance lipid peroxidation or oxidative DNA damage.

Keywords: *Long-chain polyunsaturated fatty acids, docosahexaenoic acid, malondialdehyde, 8-hydroxy-2′-deoxyguanosine, oxidative stress, pregnant woman*

Introduction

Evidence accumulates that the quality of nutrient supply to pregnant women affects maternal health and well-being, pregnancy outcome, the rate of complications and fetal growth [1]. Long-chain polyunsaturated fatty acids (LCPUFA) are required in relatively large amounts during pregnancy. Docosahexaenoic acid (DHA; C22:6$n-3$), a major $n-3$ LCPUFA in fish oil, is an indispensable component of all cell membranes that is incorporated in relatively high concentrations into the brain and other membrane rich tissues of the fetus [2]. Depletion of dietary DHA is associated with adverse neurological outcomes in animals [3], suggesting that variations in maternal LCPUFA stores have the potential to affect fetal development [4]. A series of controlled studies has demonstrated that DHA availability during early development is associated with long-term cognitive and visual development [5–7]. Furthermore, $n-3$ LCPUFA have a wide range of biological effects, including beneficial effect on lipoprotein metabolism, platelet function, endothelial function, vascular reactivity, cytokine production and coagulation

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[8,9]. However, there are concerns that an added supply of $n-3$ LCPUFA might enhance oxidative stress with potential untoward effects during pregnancy. Oxidative stress can be defined as the imbalance between free radical damage and antioxidant protection [10]. Levels of peroxidation markers, such as lipid peroxides and malondialdehyde (MDA), are higher in pregnant than in non-pregnant women [11], and the placenta has been identified as an important source of lipid peroxides [12].

LCPUFA including DHA are susceptible to peroxidation because of their high degree of unsaturation [13,14], and they might enhance peroxidative damage also in proteins and DNA. 8-Hydroxy-2′-deoxyguanosine (8-OHdG) is produced by oxidation of the nucleoside deoxyguanosine and is subsequently excreted directly into urine. Urinary 8-OHdG excretion has been identified as a sensitive marker for oxidative DNA damage [15]. Excessive oxidative stress was suggested to be causally involved in the development of pre-eclampsia and fetal growth retardation [16–18], but there is only limited information on the potential effects of DHA supplementation on oxidative stress in pregnant women. Therefore, we measured urinary 8-OHdG and MDA excretion as markers of oxidative damage in pregnant women with or without DHA supplementation. Additionally, plasma α-tocopherol, the major lipid soluble antioxidant vitamin and the fatty acid composition of plasma phospholipids were quantified.

Materials and methods

Study population and intervention

The study was performed as a double-blind randomized controlled trial. Pregnant women were enrolled prior to the 20th week of gestation at the University Hospital of Granada, Spain, between 15 November 2001 and 15 July 2002. Healthy women with an uncomplicated singleton pregnancy between 18 and 40 years of age at study entry, who did not use fish oil supplements since beginning of pregnancy, were eligible for this study. Women who smoked were excluded.

Pregnant women were randomized double blind at 20 ± 1 weeks of gestation to receive one of the dietary supplements. The supplements *Blemil plus* (Laboratories Ordesa, Barcelona, Spain) are milk based and contain vitamins in amounts meeting the estimated additional requirements during the second half of pregnancy [19] and were provided as sachets of 15 g for mixing with water. The DHA group received the supplement with 500 mg DHA and 150 mg eicosapentaenoic acid (EPA; C20:5$n-3$) from modified fish oil (Pronova Biocare, Lysaker, Norway), whereas, the other group was given a placebo with negligible contents of DHA or EPA (control group) (Table I).

Table I. The nutrient content, fatty acid and vitamin composition of the two daily supplements.

Nutrient content (per sachet)	Control	DHA
Energy (kcal)	70	71
Protein (g)	2.9	2.9
Carbohydrate (g)	8.0	8.2
Fat (g)	2.9	2.9
DHA (C22: 6$n-3$) (mg)	0.0	500
EPA (C20: 5$n-3$) (mg)	0.0	150
α-tocopherol (mg)	3.0	3.0
Vitamin A (μg)	240	240
Vitamin D (μg)	0.50	0.50
Vitamin C (mg)	30	30

The supplements were taken daily from week 20 until delivery. Their habitual diet of the subjects was not restricted, but they were asked to follow the recommendations for pregnant women.

After enrollment in the study, obstetrical history, urine and blood samples were obtained basally, before supplementation start (time point 20 week), at week 30 ± 1 of gestation (time point 30 week) and at the time of delivery (time point delivery). Blood samples were drawn into vacutainers containing EDTA and centrifuged immediately. Plasma and urine samples were stored at -80°C until assay.

Analytical methods

Urinary MDA was determined by high performance liquid chromatography (HPLC) of the adduct obtained with thiobarbituric acid (TBA) [14]. Urinary MDA concentrations were expressed as nmol MDA-TBA adduct/mg creatinine. The concentration of 8-OHdG was determined using an enzyme linked immunosorbent assay kit (8-OHdG check, Japan Institute for the Control of Aging, Shizuoka, Japan). The specificity of the assay has been established [20], and the determination range is 0.64–2000 ng/ml. All urine samples were analyzed in duplicate. The coefficient of variation of this assay was 4.5%. Urinary 8-OHdG concentrations are expressed as nanogram 8-OHdG/mg creatinine. Plasma α-tocopherol was determined by HPLC and plasma phospholipid fatty acids were quantified by gas liquid chromatography. Fatty acids were calculated as weight percent of all detected plasma phospholipids fatty acids with 14–24 C atoms.

Statistical analyses

Results are given as mean ± standard error of mean (SEM). Differences between groups were analyzed with Student's-*t* test. Difference from baseline was tested by two-way repeated measures analysis of variance and *post hoc* Tukey–Kramer test if indicated.

Table II. General characteristics of the pregnant women and offspring (mean ± SEM).

	Control group (n=23)	DHA group (n=23)
Pregnant women		
Age at entry (years)	30.42 ± 0.94	29.97 ± 1.10
Previous number of pregnancies	1.65 ± 0.20	1.78 ± 0.23
Gestational age (week)		
Entry	19.73 ± 0.16	19.80 ± 0.17
Delivery	39.89 ± 0.30	39.42 ± 0.36
Body weight (kg)		
20 week	66.31 ± 1.66	67.05 ± 1.74
30 week	72.34 ± 1.86	72.81 ± 1.88
Delivery	76.55 ± 1.98	77.54 ± 2.05
Body mass index at 20 week (kg/m^2)	25.32 ± 0.65	25.62 ± 0.80
Weight of placenta (g)	533.91 ± 20.33	527.73 ± 24.36
Offspring		
Sex (M/F)	7/16	9/14
Birth weight (kg)	3.39 ± 0.74	3.26 ± 0.96
Body length (cm)	50.84 ± 0.48	50.47 ± 0.45
Head circumference (cm)	34.94 ± 0.43	34.28 ± 0.41

Correlations were evaluated according to Pearson correlation. Statistical significance was considered at $p < 0.05$. All statistical analyses were performed with StatView 5.0, (Abacus Concepts, Inc., Berkeley, CA).

Results

Samples of 46 pregnant women were analyzed. There were no significant differences in clinical characteristics of pregnant women and offspring between the control group and the DHA group (Table II).

At study entry there were no differences between the two groups in plasma α-tocopherol level and plasma phospholipid DHA and EPA levels. After supplementation, the levels of DHA were significantly higher in the DHA supplemented group than in the control group. EPA levels at 30 week were significantly higher in the supplemented group than in the control group but not at delivery (Table III).

Urinary MDA excretions in both groups at 30 week (control: 1.31 ± 0.21, DHA: 1.27 ± 0.10 nmol/mg Cr) tended to be higher than those at 20 week (control: 1.03 ± 0.08, DHA: 1.12 ± 0.12 nmol/mg Cr) and at delivery (control: 1.01 ± 0.16, DHA: 1.03 ± 0.12 nmol/mg Cr) but were not significantly different, nor were there group differences (Figure 1). Urinary 8-OHdG excretion in both groups at delivery (control: 12.38 ± 0.72, DHA: 13.19 ± 1.03 ng/mg Cr) were significantly higher than at 20 week (control: 9.29 ± 0.69, DHA: 9.81 ± 0.79 ng/mg Cr) and at 30 week (control: 8.97 ± 0.69, DHA: 7.73 ± 0.63 ng/mg Cr) but there were no significant differences between the groups at any of three time points (Figure 1).

At the time of study entry there was a significant correlation between urinary 8-OHdG and MDA excretion ($r = 0.34$, $p = 0.02$) (Figure 2) but not at 30 week and at delivery. The measured urinary biomarker levels did not correlate with DHA and EPA levels as well as plasma α-tocopherol level.

Table III. Plasma α-tocopherol level, plasma phospholipid DHA and EPA composition (% by wt) at 20, 30 weeks of gestation and at delivery (mean ± SEM).

	Control group (n=23)	DHA group (n=23)
α-tocopherol (μmol/l)		
20 week	17.58 ± 0.64	18.44 ± 0.78
30 week	25.36 ± 1.26	25.12 ± 1.15
Delivery	23.24 ± 1.53	25.11 ± 1.22
DHA (C22: 6n − 3) (% by wt)		
20 week	6.14 ± 0.31	6.26 ± 0.31
30 week	5.72 ± 0.19	7.76 ± 0.42*
Delivery	6.02 ± 0.39	7.65 ± 0.35*
EPA (C20: 5n − 3) (% by wt)		
20 week	0.44 ± 0.09	0.39 ± 0.09
30 week	0.37 ± 0.04	0.58 ± 0.05*
Delivery	0.33 ± 0.05	0.39 ± 0.03

* $p < 0.01$ compared with control group. Student's-t test.

Discussion

Pregnancy is a physiological state accompanied by a high metabolic demand and elevated requirements for tissue oxygen [21] and causes an increase of ROS production [22]. Moreover, the placenta is a major source of oxidative stress because of its enrichment with PUFA [23]. Falkay et al. suggested that the increase in the lipid peroxide levels was due to the increased prostaglandin synthesis in the placenta [24]. Lipid peroxidation is enhanced in the second trimester and tapers off later in gestation and decrease after delivery [25]. Monitoring of the oxidative stress in pregnant women is important to enable an understanding of the relationship between oxidative stress and pregnancy

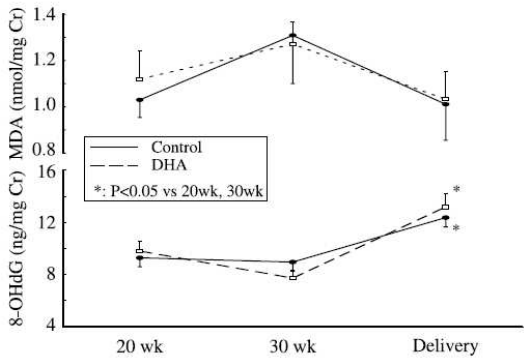

Figure 1. Effect of the DHA and EPA supplementation on urinary MDA and 8-OHdG excretion (mean ± SEM). * $p < 0.05$, two-way repeated measures analysis of variance with the *post hoc* analysis by the Tukey–Kramer test.

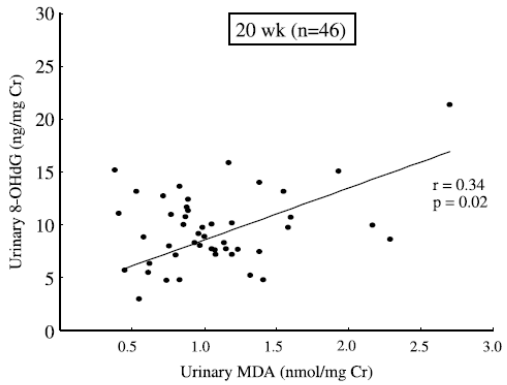

Figure 2. Relationship between urinary 8-OHdG and urinary MDA excretion at 20 weeks of gestation ($r = 0.34$, $p = 0.02$)

outcome [26]. Placental oxidative stress was suggested to play a role in the pathogenesis of pre-eclampsia [12,16] and fetal growth retardation [16–18]. On the other hand, the placenta is a source of antioxidative enzymes to control placental lipid peroxidation during healthy pregnancy. Placental production of lipid peroxides decreases as normal gestation advances, most likely because of an increase in the activity of superoxide dismutase and catalase [27]. Placental antioxidant defense is considered sufficient to control lipid peroxidation in healthy pregnancy [23].

Our data showed that there was a discrepancy between the levels of oxidative DNA damage and lipid peroxidation at delivery. Urinary 8-OHdG excretions at delivery were significantly higher than those at 20 and 30 weeks of gestation whereas urinary MDA excretions did not differ significantly between the time points. A previous study suggested that oxidative stress may influence the placenta or trophoblastic cells differently in respect to DNA damage and to lipid peroxidation [16]. Oxidative DNA damage appears to occur mainly in rapidly growing cells, such as those of the trophoblast of the cell columns, while lipid peroxidation occurs mainly in the superficial cell layers, such as those of the syncytiotrophoblast [16]. Further investigations will be needed to understand the mechanism of the discrepancy, but one possible explanation is that the significant increase of 8-OHdG excretion at the time of delivery may be associated with apoptosis in placenta. Kim et al. [28] reported that bcl-2, an anti-apoptotic protein, expression significantly decreases in placenta after 32 weeks of gestation. Bcl-2 gene expression can protect DNA from oxidative stress [29,30].

In our study, daily supplementation of 500 mg DHA and 150 mg EPA to pregnant women did not significantly influence oxidative DNA damage and lipid peroxidation during the second half of pregnancy. Several human nutritional studies have indicated that supplementation with $n-3$ LCPUFA enhanced the marker of lipid peroxidation at dietary intakes of $n-3$ LCPUFA higher than those supplied in our study [31–33]. However, the influence of $n-3$ LCPUFA supplementation on the oxidative stress is still controversial. Recent studies showed that $n-3$ LCPUFA supplementation reduces oxidative stress level *in vivo* [34,35]. $n-3$ LCPUFA may have both pro- and antioxidant properties depending on experimental conditions, dosage and the antioxidant content of the supplement or background diet. We supplied $n-3$ LCPUFA to pregnant women with vitamins in amounts meeting the estimated additional requirements [19] including Vitamin E (α-tocopherol, 3 mg/day). Vitamin E is known as most important lipid-soluble antioxidant and plays an essential role in protecting cell membrane LCPUFA against oxidation [36]. Vitamin E also has a decreasing effect on 8-OHdG [37] and lipid peroxide production [38] in placenta. We speculate that added supply of Vitamin E may prevent the increase of oxidative stress levels after $n-3$ LCPUFA supplementation in pregnant women.

The measurement of MDA is widely applied as peroxidation marker, but MDA might be absorbed from the diet [39] and it is an unspecific product of peroxidation [40]. Analysis of urinary isoprostans would have provided more specific information on LCPUFA peroxidation [40]. Nevertheless, MDA excretion seems to be a reliable indicator for LCPUFA peroxidation, as there were no other differences in dietary intake according to the dietary protocols (data not shown). Measurement of urinary 8-OHdG has become a well accepted marker of oxidative DNA damage in the human body [15] and it has been measured by several methods such as gas chromatography with mass spectrometric detection [41] and HPLC with electrochemical detection (ECD) [20]. Recently, an ELISA based on monoclonal IgG (N45.1 clone) was developed for estimation of 8-OHdG in urine [15], which made urinary 8-OHdG measurement much easier. Yoshida et al. [42] found a good correlation ($r = 0.88$) between urinary 8-OHdG levels obtained with using ELISA (same kit as in our study) and HPLC-ECD. Urinary 8-OHdG excretion is not affected by the dietary intake of 8-OHdG [43].

In conclusion, under the conditions studied, the daily supplementation of 500 mg DHA and 150 mg EPA with required vitamins to pregnant women from 20 weeks of gestation to the time of delivery did not enhance lipid peroxidation or oxidative DNA damage.

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4 Summary (English)

This cumulative dissertation was prepared within the NUHEAL-trial (NUHEAL = **Nu**traceutical for a **Hea**lthier **L**ife“, EU FP5, CLK1-CT-1999-00888), a prospective randomised interventional trial in pregnant women, which evaluates the beneficial roles and interactions of long-chained n-3 polyunsaturated fatty acids (n- 3 LC PUFA) and 5-methyl-tetra-hydro-folate (5-MTHF) in cardiovascular health and infant development. Samples were available from women participating in Germany, Spain and Hungary. The participants were randomised into 4 different supplementation groups receiving milk based supplements (Blemil plus, Laboratorios Ordesa, Barcelona, Spain) containing modified fish oil (500 mg docosahexaenoic acid, 150 mg eicosapentaenoic acid daily) and/or 400 µg 5-methyl-tetrahydrofolate or placebo. All supplements provided 270 mg vitamin C and 3 mg α-tocopherol daily. The importance of an optimised supply of long chain polyunsaturated fatty acids (LCPUFA) during pregnancy for fetal growth and development is widely accepted. A fish oil supplementation can enhance DHA blood levels during pregnancy. However, LCPUFA are susceptible to peroxidation and require adequate antioxidative protection.

This dissertation investigates in three articles the effects of a supplementation with fish oil and / or folate on redox-related biomarkers during pregnancy as well as the supply with dietary folate and DHA in pregnant women to identify possible inadequateness. The article “Influence of fish oil or folate supplementation on the time course of plasma redox markers during pregnancy” compares the effects of a dietary supplementation with fish-oil and / or 5-methyl-tetrahydrofolate (5-MTHF) from week 20 of gestation until delivery on plasma redox-status. As redox status can not be adequately assessed from a single analytical parameter, a set of marker substances was analyzed; α-tocopherol, free thiol groups, uric acid and total antioxidant capacity (TEAC) as well as thiobarbituric acid reactive substances (TBARS) in blood samples collected at gestational weeks 20, 30 and at the time of delivery. Studied antioxidants showed no significant differences between the 4 supplementation groups. At week 30 plasma TBARS levels were found to be significantly higher in the fish oil group than in the folate and control group. Until the end of pregnancy TBARS increased intensely in all groups without any significant group differences at

delivery. Concentrations of retinol and free thiol groups decreased during pregnancy, whereas uric acid increased and ß-carotene as well as the antioxidative capacity showed only minor changes.

The article entitled “Effect of docosahexaenoic acid and eicosapentaenoic acid supplementation on oxidative stress levels during pregnancy” analyzes the oxidative stress levels in a subgroup of the NUHEAL subjects, who received daily supplementation with or without fish-oil. The urinary excretion of 8-hydroxy-2′-deoxyguanosine (8-OHdG), a marker of oxidative DNA damage and of malondialdehyde (MDA), a marker of lipid peroxidation was estimated at 3 time points. In both groups the urinary MDA excretion remained unchanged throughout the study period. In contrast, the urinary 8-OHdG excretion was significantly higher at delivery than at week 20 and 30, but no group differences were found at the three time points.

On the background of these results it can be concluded that redox markers change over time during pregnancy. Under the conditions studied, a daily supplementation of 500 mg DHA and 150 mg EPA with appropriate vitamins to pregnant women did not enhance lipid peroxidation or oxidative DNA damage. Antioxidant protection seems adequate from the data at the time of delivery, but the different TBARS concentrations at week 30 might indicate a period of increased oxidative stress, which is overcome by endogenous antioxidant response or small compared to other influencing factors. This conclusion is also reflected in a wide range of supplementation products available for pregnant women, which increasingly contain DHA to close the gap between recommendations and real dietary intake.

The article entitled “Dietary Intake of Natural Sources of Docosahexaenoic Acid and Folate in Pregnant Women of Three European Cohorts” analyzes the supply with dietary folate and DHA in the participants. An optimal intrauterine development and growth as well as maternal health require adequate supply of these two nutrients during pregnancy. A comparison with the recommendations should identify possible inadequate supplies. The analysis was based on data from dietary intake assessed at week 20 and week 30 of gestation using a food frequency questionnaire (ffq) focused on the dietary sources of folate and DHA. Intake of the nutrients was calculated using the German nutrient data base `Bundeslebensmittelschlüssel´ (BLS). Participants whose energy intake was less than the estimated basal metabolic rate (BMR) were defined as “under-reporter” and excluded from nutritional analysis. While mean macronutrient intakes of our study population was found to be quite adequate, our findings clearly show areas of

improvement particularly in folate, and to some extent also in DHA supplies. Only 6 % of the participating women reached the recommended folate intake during pregnancy (600 µg/day), whereas their DHA intakes are in the order of 50-75 % of intakes considered desirable. Furthermore, the results agree with other studies, reporting a higher availability of fish in Spain than in Hungary and Germany. The main sources for dietary folate were vegetables. Other possible dietary DHA sources in addition to the different kinds of fish were meat and eggs.

On the basis of these results and other researches it seems that the core message applies not only to the studied cohorts but also to the European women in general. Possible ways to improve the supply and to enhance the micronutrient intake is an increase in dietary micronutrient density and / or nutrient supplementation.

5 Summary (German)

Diese kumulative Dissertation wurde als eigenständiges Projekt im Rahmen der NUHEAL-Studie („**Nu**traceutical for a **Hea**lthier **L**ife“, EU FP5, CLK1-CT-1999-00888) durchgeführt. Bei dieser Studie handelt es sich um eine prospective, randomisierte, interventionelle Studie, die den positiven Einfluss einer Supplementierung mit Fischöl und Folsäure während der Schwangerschaft auf verschiedene Parameter des mütterlichen und fetalen Organismus untersucht. Für diese Analysen standen Proben von Frauen aus Deutschland, Ungarn und Spanien zur Verfügung. Die Probandinnen wurden doppelblind in vier verschiedene Gruppen randomisiert und erhielten täglich ein Nahrungsergänzungsmittel (Blemil plus, Laboratorios Ordesa, Barcelona, Spanien), welches je nach Gruppen-Zugehörigkeit modifiziertes Fischöl (500 mg Docosahexaensäure DHA, 150 mg Eicosapentaensäure EPA), und/oder 400 µg 5-Methyl-Tetrahydrofolsäure oder ein Placebo enthielt. Alle Supplemente enthielten zusätzlich 270 mg Vitamin C sowie 3 mg α-Tocopherol. Die optimale Versorgung Schwangerer mit langkettigen mehrfach ungesättigten Fettsäuren ist von großer Bedeutung für die fetale Entwicklung und allgemein anerkannt. So erhöht eine Fischöl-Supplementierung während der Schwangerschaft die DHA-Werte im Blut. Auf der anderen Seite sind die LCPUFAs jedoch anfällig für Peroxidationsreaktionen und benötigen ausreichenden antioxidativen Schutz. Die vorliegende Arbeit analysiert in 3 Artikeln den Effekt einer Supplementierung mit Fischöl und/oder Folat auf die Biomarker des Redoxsystems im Verlauf der Schwangerschaft sowie die Versorgung der Probandinnen mit Folat und DHA aus der Nahrung, um mögliche Defizite in der Versorgung dieser speziellen Bevölkerungsgruppe zu identifizieren.

Der Artikel “Influence of fish oil or folate supplementation on the time course of plasma redox markers during pregnancy” untersucht die Effekte der Supplementierung mit Fischöl und/oder 5-MTHF in der 2. Schwangerschaftshälfte auf den mütterlichen Plasma-Redoxstatus. Da der Redoxstatus nicht durch einen einzelnen analytischen Parameter bewertet werden kann, wurde ein weites Spektrum an Redoxmarkern an 3 verschiedenen Zeitpunkten (w20, w30, Geburt) untersucht: α-Tocopherol, freie Thiol-Gruppen, Harnsäure, die antioxidative Kapazität (TEAC) sowie die Thiobarbitursäure-reaktive Substanzen (TBARS). Die untersuchten Antioxidantien zeigten keinen signifikanten Unterschied zwischen den 4 Supplementierungsgruppen. Im Gegensatz dazu gab es in

Woche 30 signifikant höhere TBARS Werte in der Fischölgruppe im Vergleich zur Folat- und Kontrollgruppe. Bis zum Ende der Schwangerschaft stiegen die TBARS Werte in allen Gruppen an, wobei es zum Zeitpunkt der Geburt keinen signifikanten Gruppenunterschied mehr gab. Die Plasmakonzentrationen an Retinol und freien Thiol-Gruppen fielen mit fortschreitender Schwangerschaft ab, während die Harnsäurewerte anstiegen. Die ß-Carotin Werte und die antioxidative Kapazität zeigten nur minimale Veränderungen im Verlauf der Schwangerschaft und keine Gruppenunterschiede.

Der Artikel mit dem Titel “Effect of docosahexaenoic acid and eicosapentaenoic acid supplementation on oxidative stress levels during pregnancy” analysiert die oxidativen Stresslevel in einer Subgruppe der NUHEAL Probanden, welche eine Supplementierung mit und ohne Fischöl erhalten haben. Zur Beurteilung der Stresslevel wurde die Ausscheidung von 8-Hydroxy-2′-deoxyguanosin (8-OhdG), einem Marker für oxidative DNA Schädigung und von Malondialdehyd (MDA), einem Marker für Lipidperoxidation, im Urin an 3 verschiedenen Zeitpunkten bestimmt. Während des Untersuchungszeitraums war die MDA Ausscheidung in beiden Gruppen unverändert. Die Ausscheidung von 8-OhdG war jedoch zum Zeitpunkt der Geburt signifikant höher im Vergleich zu den beiden anderen Zeitpunkten (w20, w30). Signifikante Unterschiede zwischen den beiden Gruppen konnten an keinem der 3 Untersuchungszeitpunkte gefunden werden.
Zusammenfassend kann man feststellen, dass sich die untersuchten Redoxmarker im Verlauf der Schwangerschaft verändern und dass der antioxidative Schutz zum Zeitpunkt der Geburt als ausreichend eingestuft werden kann. Unter den angegeben Studienbedingungen, Supplementierung schwangerer Frauen mit 500 mg DHA, 150 mg EPA und entsprechender Vitamingabe, wurde kein Anstieg der Lipidperoxidation oder oxidativer DNA Schäden beobachtet. Die unterschiedlichen TBARS Konzentrationen in der Woche 30 deuten möglicherweise auf einen Zeitraum mit erhöhtem oxidativen Stress hin, der jedoch durch endogene Antioxidantien ausgeglichen werden kann oder im Vergleich zu anderen Einflussfaktoren eine unerhebliche Rolle spielt.
Diese Schlussfolgerung spiegelt sich auch in der Produktpalette der Nahrungs-ergänzungsmittel für Schwangere wieder, welche zunehmend DHA enthalten, um die Lücke zwischen der tatsächlichen Aufnahme und den Empfehlungen für Schwangere zu schließen.

Der Artikel mit dem Titel “Dietary Intake of Natural Sources of Docosahexaenoic Acid and Folate in Pregnant Women of Three European Cohorts” analysiert die Versorgung der NUHEAL-Probandinnen mit Folat und DHA aus der Nahrung. Ein Vergleich mit den Empfehlungen soll eine mögliche unzureichende Versorgung aufzeigen. Die Analyse

basiert auf Daten die mit Hilfe eines auf Folat und DHA fokussierten Verzehrshäufigkeits-Fragebogens in Woche 20 und 30 erfasst wurden. Die Nährstoffaufnahme wurde mit Hilfe der deutschen Nährstoffdatenbank `Bundeslebensmittelschlüssel´ (BLS) berechnet. Probandinnen, deren Gesamtenergieaufnahme pro Tag unter ihrem berechneten Grundumsatz (BMR) lag, wurden von der Auswertung ausgeschlossen.
Während die Zufuhr an Makronährstoffen adäquat war, zeigen die Auswertungen eindeutige Mängel in der Versorgung mit Folsäure und zu einem gewissen Teil auch in der DHA-Aufnahme. Nur 6 % der Probanden erreichten die während der Schwangerschaft (600 µg / d) empfohlene Folsäurezufuhr, wohingegen die DHA-Aufnahme im Bereich von 50-75 % der wünschenswerten Zufuhr lag. Des Weiteren stimmen die Ergebnisse mit denen anderer Studien hinsichtlich eines höheren Fischverzehrs in Spanien im Vergleich zu Deutschland und Ungarn überein. Als potentielle andere DHA Quellen neben Fisch wurden Fleisch und Eier identifiziert. Die Hauptquelle für Nahrungsfolat ist in allen Kohorten Gemüse gewesen.

Die Ergebnisse dieser Ernährungserhebung und anderer Studien lassen die Schluss-folgerung zu, dass die Kernaussage bzgl. der defizitären Versorgung, insbesondere mit Folsäure, nicht nur die untersuchten Kohorten betrifft, sondern sich auch auf die Europäischen Frauen im Allgemeinen ausweiten lässt. Möglichkeiten, die Zufuhr der Mikronährstoffe zu verbessern und damit diesem Trend entgegen zu wirken, wäre ein Anstieg der Mikronährstoffdichte in der Nahrung und/oder eine Nährstoffsupplementierung der Frauen sowie auch weiterhin eine offensive Aufklärung durch Ärzte und anderes medizinisches Personal.

6 Attachment

6.1 Tables and Figures of the Introduction

6.2 Chemicals and Equipment

6.3 Study questionnaires

6.4 Publications and Presentations

6.5 Acknowledgements

6.6 Curriculum vitae

6.1 Tables and Figures of the Introduction

Table 1 Comparison of recommended daily energy and nutrient intakes of adult and pregnant women.

Energy / Nutrients	Adult women	Pregnancy
Energy	19-50 y	+ 340 kcal/d 2nd trimester + 450 kcal/d 3nd trimester
Protein (g)[1]	46	71
DHA (mg)[2]	220	300
Vitamin C (mg)[1]	75	85
Thiamin (mg)[1]	1.1	1.4
Riboflavin (mg)[1]	1.1	1.4
Niacin (ng)[1]	14	18
Vitamin B_6 (mg)[1]	1.3	1.9
Folate (µg)[1]	40	600
Vitamin B_{12} (µg)[1]	2.4	2.6
Pantothenate (mg)[2]	5	6
Biotin (µg)[2]	30	30
Vitamin A (µg)[1]	30	30
Vitamin D (µg)[2]	5	5
Vitamin E (mg)[1]	15	15
Vitamin K (µg)[2]	90	90
Calcium (mg)[2]	1000	1000
Phosphorus (mg)[2]	700	700
Magnesium (mg)[1]	310	350
Iron (mg)[1]	18	27
Zinc (mg)[1]	8	11
Iodine (µg)[1]	150	220
Selenium (µg)[1]	55	60
Fluoride (mg)[2]	3	3

[1]Recommend Dietary Allowance (RDA), average daily dietary intake level that is sufficient to meet the nutrient requirements of almost all (97-98%) individuals in a life stage and gender group based on Estimated Average Requirements (EAR).

[2]Adequate Intake (AI), the value used instead of RDA, if adequate scientific evidence is not available to calculate EAR.(1)

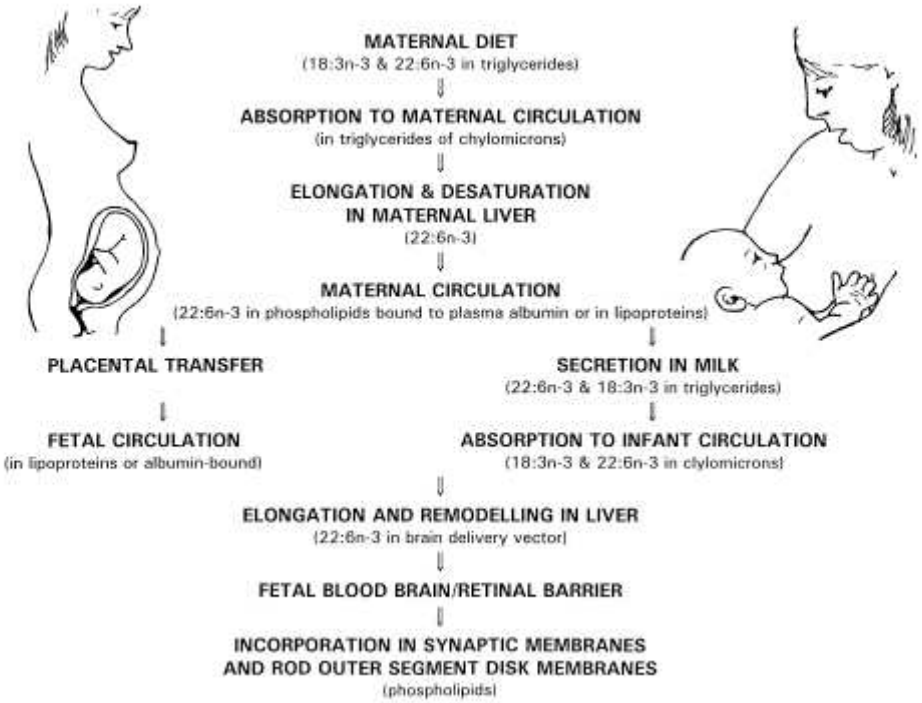

Figure 2 Process of DHA accretion in the fetal / infant brain and retina.
(according to Lauritzen et al. (33))

Oxidative stress	Oxidative stress
↓	↓
lipid peroxidation	cell damage
↓	↓
cell damage	secondary increased lipid peroxidation of damaged cells

Figure 3 Different sequences of reaction initiated by oxidative stress.
(according to Halliwell and Chirico (34)).

6.2 Chemicals and Equipment

Table 2 List of chemicals used for the several laboratory analyses.

Chemicals	Source	Quality
TBARS		
ortho-phosphoric acid	Merck, Darmstadt	85%, extra pure
2-thiobarbituric acid	Fluka, CH-Buchs	purum >98%
1,1,3,3-Tetraethoxy-propane	Sigma,	approx. 97%
Sulphoric acid	Merck, Darmstadt	98%
trizma base	SIGMA,	reagent grade
Methanol	Merck, Darmstadt	LiChrosolv gradient grade
NaOH	Merck, Darmstadt	1N
Potassium dihydrogen phosphate	Merck, Darmstadt	GR for analysis
Potassium hydroxide pellets	Merck, Darmstadt	GR for analysis
Water	Braun, Melsungen	ad injectabilia
Thiol-groups		
DTNB 5,5'-Dithiobis(2-nitrobenzoic acid)	SIGMA	99%, per analysis
Di-sodium hydrogen phosphate dihydrate	MERCK, Darmstadt	GR for analysis
Potassium dihydrogen phosphate	Merck, Darmstadt	GR for analysis
Sodium chlorid	Fluka, CH-Buchs	
L-Cysteine	Aldrich, Steinheim	97%
Water	Braun, Melsungen	ad injectabilia
TEAC		
Potassium dihydrogen phosphate	Merck, Darmstadt	GR for analysis
Di-sodium hydrogen phosphate dihydrate	Merck, Darmstadt	GR for analysis
Sodium chlorid	Fluka, CH-Buchs	
6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid	SIGMA , Steinheim	97%
2,2'-Azinobis(3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt	SIGMA , Steinheim	~ 98%
Myoglobine equine	SERVA, Heidelberg	lyophile, research grade
Hydrogen peroxide	Merck, Darmstadt	30%, stabalised for
Water	Braun, Melsungen	ad injectabilia

Table 3 List of the equipment used for the laboratory analyses.

Equipment	Source
Analytical balance, R-200 D	Sartorius, Göttingen
Centrifuge, Universal 30 F	Hettrich, Tuttlingen
Magnet-mover M33001K	Heidolph
Peristaltic Pump, Variopex	LKB
Photometer, anthos hat III	Anthos Labtech Instr., Salzburg
Pipette 10µl, 100µl	Abimed, Langenfeld
Pipette 10-100µl, 50-250µl, 200-1000µl, 500-2500 µl	Eppendorf, Wesseling-Berzdorf
Pipette 10-100 µl, 100-1000µl (Transferpette)	Brand, Wertheim
Pipette 5ml	Brand, Wertheim
Ultrasonic bath, Sonorex Super	Badelin, Berlin
UV-visible Spectrophotometer, CARY 1E	VARIAN
Vortexter, VF 2	IKA, Heitersheim
Waterbath	GFL, Burgwedel
HPLC	
Autosampler, AS-4000	Merck-Hitachi, Darmstadt
Intelligent pump, L-6200	Merck-Hitachi, Darmstadt
Fluorescence spectrophotometer, F-1050	Merck-Hitachi, Darmstadt
Column, R10 LiChrocart 250-4	Merck-Hitachi, Darmstadt
Column thermostat	Gynkotek, Germering
Software	
Microsoft Office Excel 2003	Microsoft GmbH, Unterschleißheim
Microsoft Office Power Point 2003	Microsoft GmbH, Unterschleißheim
Microsoft Office Word 2003	Microsoft GmbH, Unterschleißheim
SPSS, Version 12.0	SPSS GmbH, Software
Reference Manager 10	ISI Research soft, St. Jones, USA
EZChrom Elite Client, Version 2.61	Scientific Software, CA, USA

Table 4 List for consumables

Consumables	Source
Brown glass bottle G1, G4	CS-Chromatographie Service, Langerwehe
Micro inlay G30/5	CS-Chromatographie Service, Langerwehe
Pipette tip CP100, CP250, CP1000	Gilson, Villiers-le-Bel, France
Pipette tip 50-1000 µl	Eppendorf, Hamburg
Pipette tip 10-100 µl, 100-1000 µl	Greiner bio-one, Frickenhausen
Screw cap G8-L, G13	CS-Chromatographie Service, Langerwehe
Sealing disc G13	CS-Chromatographie Service, Langerwehe
Silicone-PTFE septum, slitted	Merck, Darmstadt
Micro plate, 96-wells	BD Falcon, Heidelberg
UV cuvette semi micro 12.5 x 12.5 45 mm	Brand, Wertheim
Nylon 66 membranes, 0.2 µm x 47 mm	SUPELCO, Supelco Park - Bellefonte

Table 5 Reproducibility of the kit analyses according to manufacturers' declarations

	Intra-assay			**Inter-assay**		
	n	Mean	CV (%)	n	Mean	CV (%)
Triglycerides (mg/dl)						
Level 1	20	84.9	1.6	20	84.9	1.9
Level 2	20	143.0	1.6	20	143.0	1.9
Total cholesterol (mg/dl)						
Level 1	20	205	1.3	20	205	2.2
Level 2	20	259	1.1	20	259	2.5
Uric acid (mg/dl)				n.s.		
Human serum	21	5.57	0.5		7.21	1.7
Precinorm U	21	4.67	0.5		4.86	1.3
Precipath U	21	10.18	0.4		9.39	1.6
Total protein (g/dl)				n.s.		
Human serum	21	4.4	0.60		6.4	0.95
Precinorm U	21	5.0	0.47		5.1	1.21
Precipath U	21	4.8	0.70		4.9	1.22

Figure 4 HPLC system for the measurement of TBARS plasma concentrations.

1 - autosampler
2 - column heater
3 - pump
4 - fluoreszenz detector
5 - computer with EZ-chrom Elite
6 - mobile phase

Figure 5 Equipment used for the TEAC measurement.

1 - water bath
2 - peristaltic pump
3 - UV-VIS Spectrophotometer
4 - computer

6.3 Study Questionnaires

- Health and lifestyle questionnaire - week 20 (2 pages)
- Health and lifestyle questionnaire - week 30 (1 page)
- Health and lifestyle questionnaire - delivery (2 pages)
- Food frequency questionaire in German language (11 pages)

Health and lifestyle questionnaire – week 20 (baseline)

NUHEAL-Code: __ __ __

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• Nutraceuticals for a healthier life •

Nutraceutical Code: 1 2 3 4 Estimated date of delivery: __ __/__ __/__ __ (D D M M Y Y)

Week 20

A01	Gravida and Para	__ __ Gravida	__ __ Para
A02	Ethnic group	O Caucasian O Asian O Roma O African O Arabian O Others – Specify:_____	
A03	Gravity risk(s)	O Family history (diabetes, genetic disorders, hypertension, others) O Bleeding disorders or tendency of thrombosis O Complications in earlier pregnancy O Bleeding in pregnancy O Hydramnios O Oligohydramnios O Cervical incompetence O Anaemia O Rhesus incompatibility O Severe past illness – Specify: _____ O Others – Specify: _____	
A04	Maternal weight and height	__ __ , __ weight in kg	__ __ __ , __ height in cm
A05	Maternal blood pressure	___/___ mmHg	
A06	Known allergies of the parents	Mother O None O Allergic asthma O Atopic dermatitis O Hay fever O Others –Specify: _____	Father O None O Allergic asthma O Atopic dermatitis O Hay fever O Others –Specify:

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A07	Does the mother smoke?	O yes O no	If the mother smokes, average number of cigarettes during the last week: _____
A08	Routine laboratory	_____ x 1012/l Hemoglobine _____ x 109/l Leucocytes _____ X 109/l Thrombocytes _____ % Haematocrit (PCV) _____ U/l GOT, ASAT (Glutamat-Oxalacetate-Transaminase) _____ U/l GPT, ALAT (Glutamat-Pyruvate-Transaminase) _____ U/l γ-GT (γ-Glutamyl-Transferase)	
A09	Laboratory samples collected	O 10 ml EDTA blood for NUHEAL analyses O 10 ml spontaneous urine for NUHEAL analyses	
A10	Food frequency collected	O yes O no	
A11	Date of completion of the form	__/__ /__ DD/MM/YY	

Physician's name:

Physician's signature:

Health and lifestyle questionnaire – week 30

NUHEAL-Code: _ _ _ _ _

Nutraceutical Code: 1 2 3 4 Estimated date of delivery: _ _ / _ _ / _ _ (D D M M Y Y)

Week 30

B01	Woman is feeling well?	O	yes	O	no – Specify:_____	
B02	Maternal weight and blood pressure	_ _ _ , _ _ weight in kg	___/___ mmHg			
B03	Gravity risk(s)	O O O O O O O O O	Bleeding disorders or tendency of thrombosis Bleeding in pregnancy after week 28 of gestation Hydramnios Oligohydramnios Cervical incompetence Anaemia Rhesus incompatibility Severe past illness –Specify:_____ Others – Specify:_____			
B04	Does the mother smoke?	O O	yes no	If the mother smokes, average number of cigarettes during the last week	____	
B05	Maternal nutritional compliance	O O O	Full compliance, every day Total missed days <5 Total missed days 6 or more			
B06	Routine laboratory	____ x 10^{12}/l ____ x 10^{9}/l ____ x 10^{9}/l ____ % ____ U/l ____ U/l ____ U/l	Hemoglobine Leucocytes Thrombocytes Haematocrit (PCV) GOT, ASAT (Glutamat-Oxalacetate-Transaminase) GPT, ALAT (Glutamat-Pyruvate-Transaminase) γ-GT (γ-Glutamyl-Transferase)			
B07	Laboratory samples collected?	O O	10 ml EDTA blood for NUHEAL analyses 10 ml spontaneous urine for NUHEAL analyses			
B08	Food frequency collected?	O O	yes no			
B09	Date of completion of the form	__/__/__ DD/MM/YY				
Physician's name:						
Physician's signature:						

Maternal and newborns health questionnaire - delivery

NUHEAL-Code: _ _ _ _ _

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- Nutraceuticals for a healthier life -

Nutraceutical Code: 1 2 3 4 Estimated date of delivery: _ _ / _ _ / _ _ _ _ (D D M M Y Y)

Delivery

D01	Woman is feeling well?	O yes	O no – Specify:_____		
D02	Maternal weight and blood pressure	_ _ _ , _ _ weight in kg	_ _ _ / _ _ _ _ mm Hg		
D03	Parity risks	O CTG-pathology O Amnionitis O Premature rupture of the membranes O Preeclampsia-/eclampsia-syndrome O Cephalo-pelvic disproportion O Small for gestational age (SGA) O Others – Specify: _____			
D04	Maternal nutritional compliance	O Full compliance, every day O Total missed days < 5 O Total missed days 6 or more			
D05	Mode of anaesthesia	O Local O Peridural/ spinal O General O None			
D06	Fetal position and mode of delivery	O Cephalic O Breech O Transverse		O Spontaneous O Forceps O Vacuum extraction O Caesarean section	
D07	Maternal postpartal period	O No complications O Fever > 38°C O Infection O Others – Specify:_____			
D08	Placental weight and maternal blood loss	_ _ _ _ _ _ Placental weight in gramm	_ _ _ _ _ _ blood loss in ml		

NUHEAL-Code: _ _ _ _ _

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- Nutraceuticals for a healthier life -

Nutraceutical Code: 1 2 3 4 Estimated date of delivery: _ _ / _ _ / _ _ _ _ (D D M M Y Y)

D09	Baby's last name		
D10	Baby's first name		
D11	Fetal date and time of birth	_ _ / _ _ / _ _ DD / MM / YY	_ _ / _ _ HH/MM
D12	Fetal sex	O Female O Male	
D13	Fetal APGAR after 1 and 5 minutes	_ _ 1 min	_ _ 5 min
D14	Umbilical artery pH and base excess	_ . _ _ pH	_ _ _ , _ mmol/
D15	Birth weight, length and head circumference	_ _ _ _ _ weight in gramm	_ _ , _ length in cm _ _ , _ head circumference in cm
D16	Fetal perinatal morbidity	O Premature birth O Perinatal infection O Asphyxia O Others – Specify:_____	
D17	Laboratory samples collected from mother	O 12 ml EDTA blood for NUHEAL-analyses O 2 ml EDTA blood for blood count O 3 ml colostrum from mother	
D18	Cord blood samples collected	O 10 ml EDTA blood for NUHEAL analyses O 2 ml EDTA blood for blood count O 5 ml full blood (heparine tube) for NUHEAL analyses	
D19	Placenta tissue collected and frozen in liquid nitrogene	O yes O no	
D20	Date of completion of the form	_ _ / _ _ / _ _ D D / M M / Y Y	
Physician's name:			
Physician's signature:			

Food frequency questionnaire in German language

NUHEAL-Code: _ _ _ _ _

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• Nutraceuticals for a healthier life •

Nutraceutical Code: 1 2 3 4 Datum: _ _ / _ _ / _ _ _ _ (D D M M Y Y)

Liebe NUHEAL-Teilnehmerin!

Der folgende Fragebogen soll uns einen Überblick über Ihre übliche Ernährungsweise geben. Dabei liegt der Schwerpunkt auf der Zufuhr von Vitamin Folsäure und langkettigen ungesättigten Fettsäuren. Die Fragen beziehen sich auf die letzten Monate ihrer Schwangerschaft. Lesen Sie den Fragebogen bitte sorgfältig durch und füllen Sie ihn anschließend genau aus.

Wie füllen Sie den Fragebogen richtig aus?

- Lesen Sie die Fragen sowie die aufgeführten Lebensmittel sorgfältig durch.
- Überlegen Sie bei jedem Lebensmittel, ob Sie es verzehrt haben und wie häufig.
- Geben Sie danach die durchschnittliche Menge/Anzahl an Portionen bei den entsprechenden Lebensmitteln an, die Sie üblicherweise bei einer Mahlzeit zu sich nehmen (z.B. 2 EL, ½ Scheibe, 2 Suppenteller, ¼ Teetasse).
- Falls Sie ein Lebensmittel verzehren, das nicht aufgeführt ist, geben Sie es bitte bei der jeweiligen Frage unter „wie oft andere: ..... falls andere – bitte angeben?" an.
- Wenn Sie ein angegebenes Lebensmittel nicht essen, kennzeichnen Sie es in der Spalte „Esse ich nicht" oder „Verwende ich nicht" .
- „TL" bedeutet Teelöffel, „EL" bedeutet Esslöffel.

Bitte beachten Sie: Machen Sie auf jeden Fall bei jeder Frage bzw. bei jedem Lebensmittel eine Angabe. Berücksichtigen Sie nicht nur die Lebensmittel, die Sie zu Hause verzehren, sondern denken Sie auch an den Außer-Haus-Verzehr, z.B. in der Kantine, im Restaurant oder bei Freunden. Selbstverständlich werden Ihre Daten vertraulich behandelt und unterliegen strengem Datenschutz. Sollten Probleme oder Fragen beim Ausfüllen des Fragebogens auftreten, können Sie uns gerne unter der NUHEAL-Hotline 089/5160-7767 anrufen.

Herzlichen Dank für Ihre aktive Teilnahme und die tatkräftige Unterstützung!

Ihr NUHEAL-Team

NUHEAL-Code: _ _ _ _ _

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• Nutraceuticals for a healthier life •

Nutraceutical Code: 1 2 3 4 Datum: _ _ / _ _ / _ _ _ _ (D D M M Y Y)

NUHEAL-Verzehrshäufigkeiten-Fragebogen für Schwangere in der 30. Woche

1. Wie häufig verwenden Sie üblicherweise folgende Speisefette/-öle für die Zubereitung von warmen Gerichten (wie z.B. Fisch, Fleisch, Gemüse, Eier)?

	Verwende ich nicht	mal/Tag		mal/Woche			mal/ Monat	Portion	Menge/ Anzahl
		1	2-3	1	2-3	4-6	1-3		
Butter, Schmalz	O	O	O	O	O	O	O	TL	_____
Palm-, Kokosfett/-öl	O	O	O	O	O	O	O	EL	_____
Sonnenblumen-, Distel-, Maiskeimöl, Kürbis-, Traubenkernöl	O	O	O	O	O	O	O	EL	_____
Oliven-, Erdnussöl	O	O	O	O	O	O	O	EL	_____
Soja-, Raps-, Weizenkeim-, Walnussöl	O	O	O	O	O	O	O	EL	_____
Wie oft andere:	O	O	O	O	O	O	O	EL	_____
Falls andere - bitte angeben?	_____								

2. Wie häufig verwenden Sie üblicherweise folgende Speisefette/-öle für die Zubereitung von kalten Gerichten (wie z.B. Salat)?

	Verwende ich nicht	mal/Tag		mal/Woche			mal/ Monat	Portion	Menge/ Anzahl
		1	2-3	1	2-3	4-6	1-3		
Mayonnaise	O	O	O	O	O	O	O	EL	_____
Sonnenblumen-, Maiskeim-, Distelöl, Kürbis-, Traubenkernöl	O	O	O	O	O	O	O	EL	_____
Oliven-, Erdnussöl	O	O	O	O	O	O	O	EL	_____
Soja-, Raps-, Weizenkeim-, Walnussöl	O	O	O	O	O	O	O	EL	_____
Leinöl	O	O	O	O	O	O	O	EL	_____
Wie oft andere:	O	O	O	O	O	O	O	EL	_____
Falls andere - bitte angeben?	_____								

Food frequency questionnaire in German language (cont. 1)

NUHEAL-Code: _ _ _ _

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3. Wie häufig nehmen Sie gewöhnlich die angegeben Fette als Brotbelag ?

	nehme ich nicht	mal/Tag		mal/Woche			mal/ Monat	Portion	Menge/ Anzahl
		1	2-3	1	2-3	4-6	1-3		
Butter	O	O	O	O	O	O	O	TL	_____
Margarine	O	O	O	O	O	O	O	TL	_____
Wie oft andere:	O	O	O	O	O	O	O	TL	_____
Falls andere - bitte angeben?	_____								

4. Wie häufig essen Sie gewöhnlich die folgenden Fischarten ?

a) Magerer Fisch, wie Kabeljau, Scholle, Schellfisch, Hecht, Steinbutt, Seezunge, Dorata (Goldbrasse) Seelachs, Heilbutt, Katfisch, Tintenfisch, Muscheln, Krabben, Zander

	Esse ich nicht	mal/Tag		mal/Woche			mal/ Monat	Menge/Anzahl
		1	2-3	1	2-3	4-6	1-3	Filet ~ 150 g
	O	O	O	O	O	O	O	_____

b) Wie zubereitet, essen Sie den Fisch am liebsten?

O gebraten, gegrillt, gekocht
O paniert
O aus der Konserve
O Suppe

c) Mittelfetter Fisch, wie Seehecht, Rot-, Seebarsch (Lubina), Sardelle, Schwertfisch, Sardine, Forelle

	Esse ich nicht	mal/Tag		mal/Woche			mal/ Monat	Menge/Anzahl
		1	2-3	1	2-3	4-6	1-3	Filet ~ 150 g
	O	O	O	O	O	O	O	_____

d) Wie zubereitet, essen Sie den Fisch am liebsten?

O gebraten, gegrillt, gekocht
O paniert
O aus der Konserve
O Suppe

e) Fetter Fisch, wie Makrele, Lachs, Thunfisch, Hering, Schwarzer Heilbutt, Schillerlocke

	Esse ich nicht	mal/Tag		mal/Woche			mal/ Monat	Menge/Anzahl
		1	2-3	1	2-3	4-6	1-3	Filet ~ 150 g
	O	O	O	O	O	O	O	_____

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f) Wie zubereitet, essen Sie den Fisch am liebsten?

O gebraten, gegrillt, gekocht
O paniert
O aus der Konserve
O Suppe

5. Wie häufig verzehren Sie in der Regel Müsli?

	esse ich nicht	mal/Tag		mal/Woche			mal/ Monat	Portion	Menge/ Anzahl
		1	2-3	1	2-3	4-6	1-3		
	O	O	O	O	O	O	O	Teetasse	_____

6. Wie häufig essen Sie normalerweise die aufgeführten Brotsorten/Brötchen?

	Esse ich nicht	mal/Tag		mal/Woche			mal/ Monat	Portion	Menge/ Anzahl
		1	2-3	1	2-3	4-6	1-3		
Weißbrot, Toast, weiße Semmeln, Baguette	O	O	O	O	O	O	O	Scheibe	_____
Weizen-, Roggenbrot, Roggensemmeln	O	O	O	O	O	O	O	Scheibe	_____
Vollkornbrot, Vollkornsemmeln	O	O	O	O	O	O	O	Scheibe	_____
Croissant	O	O	O	O	O	O	O	Stück	_____
Wie oft andere:	O	O	O	O	O	O	O	Scheibe/ Stück	_____
Falls andere - bitte angeben?	_____								

7. Wie häufig verzehren Sie die folgenden Lebensmittel?

	Esse ich nicht	mal/Tag		mal/Woche			mal/ Monat	Portion	Menge/ Anzahl
		1	2-3	1	2-3	4-6	1-3		
Teigwaren, gekocht als Hauptgericht	O	O	O	O	O	O	O	Suppen-teller	_____
Reis, gekocht als Hauptgericht	O	O	O	O	O	O	O	Suppen-teller	_____
Pizza	O	O	O	O	O	O	O	ein Viertel	_____

Food frequency questionnaire in German language (cont.2)

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8. Wie häufig verzehren Sie die folgenden Kartoffelprodukte?

	Esse ich nicht	mal/Tag 1	mal/Tag 2-3	mal/Woche 1	mal/Woche 2-3	mal/Woche 4-6	mal/Monat 1-3	Portion	Menge/ Anzahl
Kartoffel gekocht, Kartoffelbrei	O	O	O	O	O	O	O	Dessert-schale	_____
Kartoffelknödel	O	O	O	O	O	O	O	Stück(e)	_____
Bratkartoffel	O	O	O	O	O	O	O	3 mittlere Kartoffel	_____
Kartoffelpuffer	O	O	O	O	O	O	O	3 mittlere Kartoffel	_____
Pommes frites	O	O	O	O	O	O	O	Dessert-schale	_____
Kartoffelchips	O	O	O	O	O	O	O	¼ Tüte	_____

9. Wie häufig essen Sie gewöhnlich folgendes Gemüse gekocht?

	Esse ich nicht	mal/Tag 1	mal/Tag 2-3	mal/Woche 1	mal/Woche 2-3	mal/Woche 4-6	mal/Monat 1-3	Portion	Menge/ Anzahl
Aubergine, Zucchini, Paprika, Mais	O	O	O	O	O	O	O	Schöpflöffel (= 150 g)	_____
Tomaten	O	O	O	O	O	O	O	Schöpflöffel	_____
Blumenkohl, Grünkohl, Kohlrabi	O	O	O	O	O	O	O	Schöpflöffel	_____
Wirsing, Weißkohl, Broccoli Rosenkohl, Rote Beete	O	O	O	O	O	O	O	Schöpflöffel	_____
Chinakohl	O	O	O	O	O	O	O	Schöpflöffel	_____
Spinat	O	O	O	O	O	O	O	Schöpflöffel	_____
Fenchel	O	O	O	O	O	O	O	Schöpflöffel	_____
Schwarzwurzel, Lauch, Artischocke	O	O	O	O	O	O	O	Schöpflöffel	_____
Ratatouille, Mischgemüse	O	O	O	O	O	O	O	Schöpflöffel	_____
Wie oft andere:	O	O	O	O	O	O	O	Schöpflöffel	_____
Falls andere - bitte angeben?	_____								

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10. Wie essen Sie gekochtes Gemüse üblicherweise?

als Beilage	O
als Hauptgericht (z.B. Eintopf)	O
als Rahmsuppe – Vorsuppe	O
als klare Suppe – Vorsuppe	O

11. Wie häufig essen Sie Gemüsesuppe als Hauptgericht?

	Esse ich nicht	mal/Tag 1	mal/Tag 2-3	mal/Woche 1	mal/Woche 2-3	mal/Woche 4-6	mal/Monat 1-3	Portion	Menge/ Anzahl
	O	O	O	O	O	O	O	Suppen-teller	_____

12. Wie häufig verzehren Sie gewöhnlich folgendes Gemüse roh (z.B. Salat)?

	Esse ich nicht	mal/Tag 1	mal/Tag 2-3	mal/Woche 1	mal/Woche 2-3	mal/Woche 4-6	mal/Monat 1-3	Portion	Menge/ Anzahl
Paprika, Gurke, Radieschen	O	O	O	O	O	O	O	Dessert-schale	_____
Tomate	O	O	O	O	O	O	O	Stück-mittel	_____
Blattsalat	O	O	O	O	O	O	O	Schale-mittel	_____
Chinakohl	O	O	O	O	O	O	O	1/5 Kopf	_____
Avocado	O	O	O	O	O	O	O	½ Stück	_____
Oliven	O	O	O	O	O	O	O	Stücke	_____
Soja-, Bohnensprossen	O	O	O	O	O	O	O	EL	_____
Wie oft andere:	O	O	O	O	O	O	O	Stück/ Schale	_____
Falls andere -bitte angeben?	_____								

13. Wie häufig verzehren Sie Hülsenfrüchte?

	Esse ich nicht	mal/Tag 1	mal/Tag 2-3	mal/Woche 1	mal/Woche 2-3	mal/Woche 4-6	mal/Monat 1-3	Portion	Menge/ Anzahl
Bohnen, Erbsen	O	O	O	O	O	O	O	Schöpflöffel	_____
Kichererbsen, Weiße Bohnen	O	O	O	O	O	O	O	Schöpflöffel	_____
Sojabohnen, Linsen	O	O	O	O	O	O	O	Schöpflöffel	_____
Wie oft andere:	O	O	O	O	O	O	O	Schöpflöffel	_____
Falls andere - bitte angeben?	_____								

Food frequency questionnaire in German language (cont. 3)

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Datum: _ _ / _ _ / _ _ _ _ (D D M M Y Y)

14. Wie essen Sie die Hülsenfrüchte üblicherweise?

als Hauptgericht (z.B. Eintopf)	O
als Suppe – Hauptgericht	O
als Beilage, Salat	O

15. Wie häufig verzehren Sie Sojaprodukte (z.B. Sojateigwaren, Tofu)?

	Esse ich nicht	mal/Tag 1	mal/Tag 2-3	mal/Woche 1	mal/Woche 2-3	mal/Woche 4-6	mal/ Monat 1-3	Portion	Menge/ Anzahl
Sojateigwaren, gekocht	O	O	O	O	O	O	O	Suppen-teller	_____
Tofu (Sojakäse)	O	O	O	O	O	O	O	Teetasse	_____
Sojafleisch/-bratlinge	O	O	O	O	O	O	O	Stück(e)	_____
Sojasauce	O	O	O	O	O	O	O	EL	_____
Sojamilch	O	O	O	O	O	O	O	Glas (=200 ml)	_____
Wie oft andere:	O	O	O	O	O	O	O	Teetasse/ Stück	_____
Falls andere - bitte angeben?	_____								

16. Wie essen Sie die Sojaprodukte üblicherweise?

als Hauptgericht (z.B. Eintopf)	O
als Suppe – Hauptgericht	O
als Beilage	O

17. Wie häufig essen Sie Nüsse oder Ölsamen (z.B. Sonnenblumen-, Kürbiskerne) als Snack (z.B. beim Fernsehen)?

	Esse ich nicht	mal/Tag 1	mal/Tag 2-3	mal/Woche 1	mal/Woche 2-3	mal/Woche 4-6	mal/ Monat 1-3	Portion	Menge/ Anzahl
Pistazien, Hasel-, Walnüsse, Paranüsse	O	O	O	O	O	O	O	Teetasse	_____
Mandeln, Cashew-, Macadamianüsse	O	O	O	O	O	O	O	Teetasse	_____
Erdnüsse	O	O	O	O	O	O	O	Teetasse	_____
Ölsamen (z.B. Sonnenblumen-, Kürbiskerne)	O	O	O	O	O	O	O	Teetasse	_____
Wie oft andere:	O	O	O	O	O	O	O	Teetasse	_____
Falls andere - bitte angeben?	_____								

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Datum: _ _ / _ _ / _ _ _ _ (D D M M Y Y)

18. Verzehren Sie die Nüssen und Ölsamen gewöhnlich geröstet und gesalzen?

ja	O
nein	O
weiß nicht	O

19. Wie häufig essen Sie in der Regel die folgenden Lebensmittel?

	Esse ich nicht	mal/Tag 1	mal/Tag 2-3	mal/Woche 1	mal/Woche 2-3	mal/Woche 4-6	mal/ Monat 1-3	Portion	Menge/ Anzahl
Hefeteilchen mit Nüssen	O	O	O	O	O	O	O	Stück	_____
Kuchen/Kaffeeteilchen	O	O	O	O	O	O	O	Stück	_____
Rahmeis	O	O	O	O	O	O	O	Kugel klein	_____
Schokolade	O	O	O	O	O	O	O	Riegel (=20g)	_____
Marzipan	O	O	O	O	O	O	O	Stück/Praline (=10g)	_____
Kekse	O	O	O	O	O	O	O	Stück	_____
Vollkornkekse	O	O	O	O	O	O	O	Stück	_____

20. Wie häufig verzehren Sie gewöhnlich folgende Obstsorten?

	Esse ich nicht	mal/Tag 1	mal/Tag 2-3	mal/Woche 1	mal/Woche 2-3	mal/Woche 4-6	mal/ Monat 1-3	Portion	Menge/ Anzahl
Erdbeere, Himbeere	O	O	O	O	O	O	O	Teetasse	_____
Orange, Kiwi, Banane, Maracuja	O	O	O	O	O	O	O	Stück	_____
Zuckermelone, Mango	O	O	O	O	O	O	O	Hälfte	_____
Brombeere	O	O	O	O	O	O	O	Teetasse	_____
Trockenfrüchte	O	O	O	O	O	O	O	Stücke	_____
Obstsalat, frisch	O	O	O	O	O	O	O	Dessert-schale	_____
Wie oft andere:	O	O	O	O	O	O	O	Stück/ Teetasse	_____
Falls andere - bitte angeben?	_____								

Food frequency questionnaire in German language (cont. 4)

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Datum: _ _ / _ _ / _ _ _ _ (D D M M Y Y)

21. Wie häufig essen Sie üblicherweise Fleisch?

	Esse ich nicht	mal/Tag 1	mal/Tag 2-3	mal/Woche 1	mal/Woche 2-3	mal/Woche 4-6	mal/Monat 1-3	Portion	Menge/Anzahl
Kalb	O	O	O	O	O	O	O	Scheibe	_____
Rind	O	O	O	O	O	O	O	Scheibe	_____
Schwein	O	O	O	O	O	O	O	Scheibe	_____
Lamm/Hammel	O	O	O	O	O	O	O	Scheibe/ Stück	_____
Geflügel	O	O	O	O	O	O	O	Scheibe/ Stück	_____
Leber	O	O	O	O	O	O	O	Scheibe	_____
Wurst, Wurstaufschnitt	O	O	O	O	O	O	O	Scheibe	_____
Leberwurst	O	O	O	O	O	O	O	EL	_____
Wie oft andere:	O	O	O	O	O	O	O	Scheibe/EL	_____
Falls andere - bitte angeben?	_____								

22. Wie zubereitet, essen Sie das Fleisch am liebsten?

gebraten, gegrillt	O
paniert	O

23. Wenn Sie Leber essen, von welchem Tier in der Regel? _____

24. Wie häufig verzehren Sie die folgenden Käsesorten?

	Esse ich nicht	mal/Tag 1	mal/Tag 2-3	mal/Woche 1	mal/Woche 2-3	mal/Woche 4-6	mal/Monat 1-3	Portion	Menge/Anzahl
Weichkäse (z.B. Brie, Roquefort, Gorgonzola)	O	O	O	O	O	O	O	Scheibe	_____
Schnittkäse (z.B. Gouda, Edamer, Parmesan)	O	O	O	O	O	O	O	Scheibe	_____
Quark, Frischkäse	O	O	O	O	O	O	O	EL	_____
Wie oft andere:	O	O	O	O	O	O	O	EL/Scheibe	_____
Falls andere - bitte angeben?	_____								

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Datum: _ _ / _ _ / _ _ _ _ (D D M M Y Y)

25. Welchen Käse bevorzugen Sie in der Regel?

fett	O
fettreduziert	O
weiß nicht	O

26. Wie häufig essen Sie gewöhnlich Eier?

	Esse ich nicht	mal/Tag 1	mal/Tag 2-3	mal/Woche 1	mal/Woche 2-3	mal/Woche 4-6	mal/Monat 1-3	Menge/Anzahl
	O	O	O	O	O	O	O	_____

27. Was ist Ihre bevorzugte Zubereitungsart?

gekocht	O
gebraten	O
Rührei, Omelette	O

28. Wie häufig trinken Sie durchschnittlich alkoholische Getränke?

	Trinke ich nicht	mal/Tag 1	mal/Tag 2-3	mal/Woche 1	mal/Woche 2-3	mal/Woche 4-6	mal/Monat 1-3	Portion	Menge/Anzahl
Bier normal/stark	O	O	O	O	O	O	O	Glas (250 ml)	____
Bier leicht	O	O	O	O	O	O	O	Glas (250 ml)	____
Wein/Sekt/Prosecco	O	O	O	O	O	O	O	Glas (150 ml)	____
Spirituosen – (mehr als 35% Alkohol, z.B. Brandy, Cognac)	O	O	O	O	O	O	O	Glas (2 cl)	____
Spirituosen – (weniger als 35% Alkohol, z.B. Eierlikör, Sherry)	O	O	O	O	O	O	O	Glas (2 cl)	____
Wie oft andere:	O	O	O	O	O	O	O	Glas (100 ml)	____
Falls andere - bitte angeben?	_____								

29. Ernähren Sie sich vegetarisch?

weiß nicht	O
nein	O
ja	O

Food frequency questionnaire in German language (cont. 5)

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30. Wenn ja, wie ernähren Sie sich vegetarisch?

Pisco-vegetarisch (einschl. Fisch, Eier, Milch und Milchprod.)	O
Ovo-lacto-vegetarisch (einschl. Eier, Milch und Milchprod.)	O
Lacto-vegetarisch (einschl. Milch und Milchprod.)	O
Streng vegetarisch (nur Lebensmittel pflanzlicher Herkunft)	O

31. Nehmen Sie d e r z e i t eines der folgenden Präparate regelmäßig (mehrmals pro Woche oder täglich) ein?

Bierhefe, Hefeflocken	O
Kleie, Weizenkeime	O
Leinsamen	O
Multivitamintabletten, -säfte	O
Nachtkerzenöl	O
Fischölpräparate, -kapseln	O
ich nehme keines	O

32. Welche Präparate (wie z.B. Folsäure, Vitamin B12, Fischölkapseln, Multivitamintabletten, Jod, Eisen) haben Sie regelmäßig (mehrmals pro Woche bzw. täglich) b i s zur 16. Schwangerschafts-woche eingenommen?
Bitte schreiben Sie Namen und Hersteller der Präparate sowie die Mengen, die Sie eingenommen haben auf.

ich habe keine genommen O

	Name und Hersteller des/r Präparate/s	Menge/Tabletten	
		pro Tag	pro Woche

6.4 Publications and Presentations

Publications

2010 Franke C, Demmelmair H, Decsi T, Campoy C, Cruz M, Molina-Font JA, Mueller K, Koletzko B.: *Influence of fish oil or folate supplementation on the time course of plasma redox markers during pregnancy.* Br J Nutr. 2010 Jun;103(11):1648-56. Epub 2010 Mar 9.

2009 Shoji H, Franke C, Demmelmair H, Koletzko B.*Effect of docosahexaenoic acid on oxidative stress in placental trophoblast cells.* Early Hum Dev. 2009 Jul;85(7):433-7. Epub 2009 Mar 26.

2008 Franke C, Verwied-Jorky S, Campoy C, Trak-Fellermeier M, Decsi T, Dolz V, Koletzko B. Dietary intake of natural sources of docosahexaenoic acid and folate in pregnant women of three European cohorts. Ann Nutr Metab. 2008;53(3-4):167-74. Epub 2008 Nov 11.

2006 Shoji H, Franke C, Campoy C, Rivero M, Demmelmair H, Koletzko B. *Effect of docosahexaenoic acid and eicosapentaenoic acid supplementation on oxidative stress levels during pregnancy*. Free Radic Res. 2006 Apr;40(4):379-84.

Presentations at conferences and summer schools

2006 Franke C., Demmelmair H., Campoy C., Decsi T., Müller K·. Molina-Font J. A.. Shoji H.: *Impact of fish oil & folic acid supplementation during pregnancy on oxidative stress markers in maternal and cord blood.* European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN), Dresden, Germany (oral presentation)

2005 Franke C., Decsi T., Campoy C., Demmelmair H., Koletzko B.: *Indicators of the redoxstatus in pregnancy.* Nutrition Summer School of the European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN), Athens, Greece (oral presentation)

Franke C., Demmelmair H., Decsi T., Campoy C., Müller K., Koletzko B.: *Influence of long-chain polyunsaturated fatty acid on vitamin E status of pregnant women.* Congress of the German Society of Nutritional Medicine (DGEM). Ernährung 2005, Geneva, Switzerland (poster)

2004 Franke C., Verwied-J. S., Trak-F. M., Desci T.: *Aufnahme von DHA und Folsäure über die Nahrung bei Schwangeren der 20. SSW.* Congress of the German Society of Nutritional Medicine (DGEM), Ernährung 2004, Munich, Germany (poster)

6.5 Acknowledgements

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