

## The effect of docosahexaenoic acid and folic acid supplementation on placental apoptosis and proliferation

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The hypothesis was tested that the additional dietary uptake of *n*-3 fatty acids, in particular of DHA and 5-methyltetrahydrofolate (5-MTHF), during the second half of pregnancy would influence proliferation and apoptosis in the full-term human placenta. The diets of pregnant women from Spain (*n* 55) were supplemented with modified fish oil and/or 5-MTHF or placebo, and assigned in a random, double-blind manner to one of the four groups. Immunohistochemistry and immunoblotting were used to detect placental proliferation and apoptosis with monoclonal antibodies for key proteins that reflected the extent of both processes: proliferation cell nuclear antigen (PCNA), p53, cytokeratin 18 neoepitope. The PCNA level in the fish oil/5-MTHF-treated group was higher by 66% ( $P < 0.05$ ) than that of the placebo group, whereas the levels of p53 and cytokeratin 18 neoepitope were unaffected by treatment. PCNA expression was altered only in the trophoblast compartment (placebo 11.1 (SE 0.5) % *v.* combination 21.5 (SE 1.2) %;  $P < 0.05$ ), whereas the proportion of nuclei stained in endothelial and other stromal cells was similar in the placebo and combined treatment groups. No correlation was found between fish oil or 5-MTHF supplementation and the levels of the proteins. The present data suggest that supplementation with fish oil and/or 5-MTHF had no effect on the parameters reflecting placental proliferation and apoptosis. A defined combination of DHA and 5-MTHF may, however, affect placental proliferation.

### Apoptosis: Folic acid: *n*-3 fatty acids: Placenta: Proliferation

The quality of nutrient supply to pregnant women is associated with maternal health and well-being, pregnancy outcome and the rate of complications, and fetal growth (Koletzko *et al.* 1998). In addition, the intrauterine substrate supply to the growing fetus and the extent and quality of fetal growth have important consequences for its long-term outcome and behavioural and cognitive development, as well as for disease risk in adulthood (von Kries *et al.* 1999; Waterland & Garza, 1999; Godfrey & Barker, 2000; Osmond & Barker, 2000). With the possible exception of special subgroups, pregnant women in European populations can easily meet their increased energy and protein needs. However, the pregnancy-related increase in reference intakes for a number of micronutrients is far higher than the increase in energy requirement (Elmadfa *et al.* 2000). Thus, the provision of diets with a high content of selected micronutrients may be of relevance to meet the increased needs for micronutrients.

Of special interest in this regard is the provision of long-chain *n*-3 PUFA, such as DHA, and of biologically active folic acid. DHA is an indispensable component of all cell membranes that is incorporated in relatively high

concentrations into the brain and other membrane-rich tissues (Gibson *et al.* 1996). DHA can influence neural maturation and function through the activation of a retinoid X receptor signalling pathway (de Urquiza *et al.* 2000).

Supplementation with folic acid or 5-methyltetrahydrofolate (5-MTHF), its most active and abundant form in the tissues, during the early period of pregnancy can reduce the incidence of neural tube defects (Koletzko *et al.* 1998; Scholl & Johnson, 2000). It can also contribute to activating the conversion of homocysteine to methionine, and hence to the reduction of plasma concentrations of homocysteine (Jacques *et al.* 1999). A limitation of intracellular folate may lead to uracil misincorporation into DNA and hence to DNA repair, double-stranded breaks, chromosomal damage and cancer. Although dietary supplementation during pregnancy has recently become popular, the feasibility and efficacy of such preparations need to be determined. Moreover, the safety of supplements must be documented, and any adverse effects on reproductive tissues must be ruled out.

The human placenta separates the maternal and fetal circulation and may protect the fetus from adverse changes in

**Abbreviations:** 5-MTHF, 5-methyltetrahydrofolate; PCNA, proliferation cell nuclear antigen.

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the maternal circulation. Its proper functioning is of major importance for nutritional supply to the fetus (Hay, 1994). The morphological architecture and function of the human placenta depends on an adequate balance of proliferation, differentiation and apoptosis. In placentas from early pregnancy, cell proliferation, especially of cytotrophoblasts, is very high, but this diminishes constantly with the duration of pregnancy (Ishihara *et al.* 2000). In contrast, the rate of apoptosis is low throughout early pregnancy and only increases shortly before delivery (Smith *et al.* 1997). Maintaining the homeostasis of these basic processes in the placenta is necessary for adequate growth and development of the fetus. An imbalance may result in spontaneous abortion, pre-eclampsia, preterm delivery and reduced fetal growth (Ding *et al.* 2002; Myatt, 2002; Crocker *et al.* 2003).

The influence of individual fatty acids, for example EPA and DHA, on apoptosis and proliferation is controversial. *In vitro*, EPA inhibits cell proliferation, whereas DHA increases apoptosis (Finstad *et al.* 1998). The influence of DHA and EPA on apoptosis seems to be mediated by different mechanisms (Calviello *et al.* 1998). The effects of DHA on apoptosis are attributed mainly to its incorporation into the phospholipid bilayer, whereas those of EPA are associated with triacylglycerols of the lipid bodies within the cell. DHA effects on apoptosis are also tissue-dependent. When supplemented with the diet, DHA increases apoptosis in colonic and hepatocarcinoma cells (Calviello *et al.* 1998; Narayanan *et al.* 2001), whereas apoptosis is inhibited in neuronal and lymphoma cells (Yano *et al.* 2000; Akbar & Kim, 2002).

The effect of folate on the human placenta has been observed only indirectly in an *in vitro* study. Folate deficiency induces apoptosis in human trophoblasts (Stegers-Theunissen *et al.* 2000), which would suggest a protective role of folate for the human placenta. However, no direct data are available.

The present study investigated the potential *in vivo* effect of supplementing the diet of pregnant women with modified fish oil and 5-MTHF on proliferation and apoptosis in the full-term placenta by measuring key proteins of both processes. These include proliferating cell nuclear antigen (PCNA) as marker for proliferation, the product of the tumour suppressor gene *p53* as a cellular master switch for both processes, and cytokeratin 18 neopeptide, which is detectable only in apoptotic cells. An enhanced placental proliferation rate in the supplemented groups might be hypothesised as indicative of a prolongation of pregnancy owing to a high intake of fish oil.

## Methods

### *Subjects and study design*

Healthy women between 18 and 40 years of age with an uncomplicated singleton pregnancy were recruited before week 20 of pregnancy in the University Hospital 'San Cecilio' of Granada, Spain. They were then assigned in a random, double-blind manner to one of four groups. Inclusion criteria were no use of fish oil supplements since the beginning of pregnancy and no regular use of folate and vitamin B<sub>12</sub> supplements after week 16 of gestation. All women habitually consumed an omnivorous diet.

From week 20 of pregnancy onwards, the mothers received daily either: modified fish oil containing 500 mg DHA and

150 mg EPA (Pronova Biocare, Lysaker, Norway; *n* 40); 400 µg 5-MTHF (delivered as 800 µg 6,RS,5-methyltetrahydrofolate; BASF, Ludwigshafen, Germany; *n* 36), a combination (500 mg DHA, 150 mg EPA, 400 µg 5-MTHF; *n* 37); placebo (*n* 41) without DHA, EPA or 5-MTHF (Blemil plus; Laboratorios Ordesa, Barcelona, Spain). The components were supplied as 15 g milk-based portions containing other vitamins and minerals in amounts meeting the estimated additional requirements during the second half of pregnancy (Elmadfa *et al.* 2000).

The study protocol was approved by the local Ethical Committee. After a detailed explanation of the study, a written consent was obtained from all participating women.

### *Placental tissue and venous blood collection*

Placental tissue was obtained within 15 min after delivery, thus minimising metabolic changes in the tissue. Because of the heterogeneity of this organ, samples were collected from different locations, including central as well as peripheral locations, following a strict sampling protocol. The amnion and the chorionic plate were excluded. After several washings in NaCl (0.9%, 4°C) to eliminate blood residues, the tissue pieces were frozen in liquid N and stored at -80°C. For analysis, placental samples from the different locations were pooled as pilot studies showed no influence of sampling site. For immunohistochemistry, placental tissues were fixed in neutral buffered 4% paraformaldehyde for 12 h, dehydrated and paraffin-embedded. Blood samples were collected from the umbilical cord and maternal peripheral vein and centrifuged at 1500 g for 5 min. EDTA plasma for folate analysis was stored at -20°C until analysis.

### *Placental tissue fatty acids and plasma folate*

Fatty acid compositions (% w/w) of placental tissue and plasma folate concentrations (ng/ml) were determined as previously described (Molloy & Scott, 1997; Klingler *et al.* 2003).

### *Tissue culture, western blotting and immunohistochemistry*

**Induction of apoptosis.** Culture media, fungizone and penicillin/streptomycin were obtained from Invitrogen (Carlsbad, CA, USA), and fetal calf serum was purchased from HyClone (South Logan, UT, USA). To generate a positive control, apoptosis was induced in placental explants by incubation with 1.5 mM-MgCl<sub>2</sub> (Black *et al.* 2001). Briefly, small pieces of villous tissue from the centre of the placental parenchyma were incubated in medium M199 supplemented with 1% (v/v) fungizone and 1% (v/v) penicillin/streptomycin for 1 h. After two washings in PBS, villi were placed in 1.5 mM-MgCl<sub>2</sub> DMEM medium, supplemented with 10% (v/v) fetal calf serum, 1% (v/v) fungizone and 1% (v/v) penicillin/streptomycin at 37°C under 5% CO<sub>2</sub> for 48 h (Polliotti *et al.* 1995).

**Protein analysis and quantification using western blotting.** All chemicals were obtained from Merck (Darmstadt, Germany) unless stated otherwise. Positive controls were included in all blots. Chemicals for western blotting were obtained from Invitrogen. Chemiluminescence western

blottings were performed according to the manufacturer's protocol (Invitrogen).

Briefly, approximately 200 mg placental tissue was washed three times in ice-cold PBS and subsequently heated to 95°C for 5 min in lyses buffer containing 0.01 M-Tris, pH 7.4, 1% SDS (w/v), 1 mM-Na-Orthovanadate and complete protease inhibitor cocktail (Roche, Mannheim, Germany). The mixture was homogenised carefully with a metal-blade homogeniser (IKA, Staufen, Germany), chilled on ice and centrifuged at 1000 g at 4°C for 15 min. The aliquots of supernatant were then transferred into Eppendorf cups. Protein concentration was measured according to the method of Lowry *et al.* (1951).

Equal amounts of protein from each sample (40 µg per lane) were separated on 4–12% Bis-Tris polyacrylamide gradient gels (NuPAGE; Invitrogen) under reducing conditions and transferred to nitrocellulose membranes. Membranes were treated with primary antibodies, as listed in Table 1, and incubated with an alkaline phosphatase-labelled goat-anti-mouse chemiluminescent kit (Western Breeze; Invitrogen) according to the manufacturer's protocol. High-performance chemiluminescence films (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK) were used for exposure. Bands corresponding to specific proteins were quantified densitometrically using AlphaEaseFC software, version 3.2.3 (Alpha Innotech Corp., Cannock, UK).

For negative control experiments, the primary antibodies were replaced by isotype-specific mouse IgG negative control (DAKO, Glostrup, Denmark). The intensity (arbitrary units) of the analysed samples was normalised to a positive control sample that was run on each blot to make interblot comparison possible.

*Localisation and semi-quantification by immunohistochemistry.* Paraffin sections (5 µm) were deparaffinised in xylene and rehydrated in a graded series of alcohol solutions, incubated in 10 mM-citrate buffer (pH 6.0) and boiled under pressure in an autoclave (Decloaking chamber; Biocarta, Hamburg, Germany) at 120°C for 3 min. Unspecific protein binding was blocked with activated human serum (20%), diluted in V-blocking reagent (Lab Vision, Fremont, CA, USA). Primary antibodies (Table 1) were diluted in background reducing agent (DAKO), and their binding was detected by biotinylated goat-anti-mouse antibody and streptavidin-horseradish peroxidase (Lab Vision). After treatment with AEC the sections were slightly counterstained with Mayer's haemalum and mounted with Kaiser's glycerol gelatine.

The number of stained cells, for example trophoblasts, endothelial and other stromal cells such as blood cells or macrophages, was determined under a light microscope by counting three representative visual fields for each placenta (*n* 3) per group (original magnification (% 400). The proportions of labelled nuclei were expressed relative to the total number of cells in the field.

### Statistical analysis

Results are given as means with their standard errors. Group comparisons were carried out using ANOVA and a subsequent *post hoc* test with Bonferroni's correction. Correlation coefficients were calculated according to Pearson. All statistical tests were performed using SPSS for Windows, release 11.5 (SPSS Inc., Chicago, IL, USA); *P*<0.05 was considered statistically significant.

## Results

### Clinical results

Out of the total of 154 women studied, eight did not finish the clinical trial. Because of logistical restrictions during sample collection, only 100 samples were available for fatty acid analysis. Apoptosis and proliferation were analysed for only sixty-six pieces of placental tissue that could be collected in the strict random sampling mode. Eleven of the samples contained too little protein to allow an analysis of all parameters. The full set of parameters was, therefore, investigated for fifty-five women. No differences in characteristics of the study population were found between the groups (Table 2).

### Fatty acid analyses

Fatty acid compositions were determined in the placental phospholipid, NEFA and triacylglycerol fractions. The fatty acid patterns of the fifty-five subjects evaluated were representative of the total samples analysed (data not shown). With the exception of the supplemented fatty acids, DHA and EPA, the proportion of all other fatty acids did not differ between the four treatment groups (Fig. 1).

The proportion of DHA in the placental phospholipids was similar in both the fish oil treatment groups (fish oil 5.9 (SE 0.3) %, combined treatment 6.2 (SE 0.3) %), but higher (*P*<0.05) than in the 'non-fish oil' supplemented groups (placebo 4.8 (SE 0.2) %, 5-MTHF 4.9 (SE 0.2) %). Differences (*P*<0.05) were also measured for EPA (fish oil 0.20 (SE 0.03) % and combined 0.26 (SE 0.03) % *v.* placebo 0.11 (SE 0.01) % and 5-MTHF 0.10 (SE 0.01) %). In the cytosolic NEFA pool, EPA content differed (*P*<0.05) between the placebo (0.4 (SE 0.1) %) and combined treatment (0.6 (SE 0.1) %) groups. Differences in the triacylglycerol fraction were observed for DHA content in the triacylglycerol fraction between the combination (7.9 (SE 0.7) %) and folate-treated (5.2 (SE 0.5) %) groups, and for EPA content (*P*<0.05) between the placebo (0.21 (SE 0.05) %) or the 5-MTHF (0.28 (SE 0.07) %) treated group and the fish oil (0.56 (SE 0.12) %) or combined (0.43 (SE 0.03) %) supplemented group. There was no effect on the

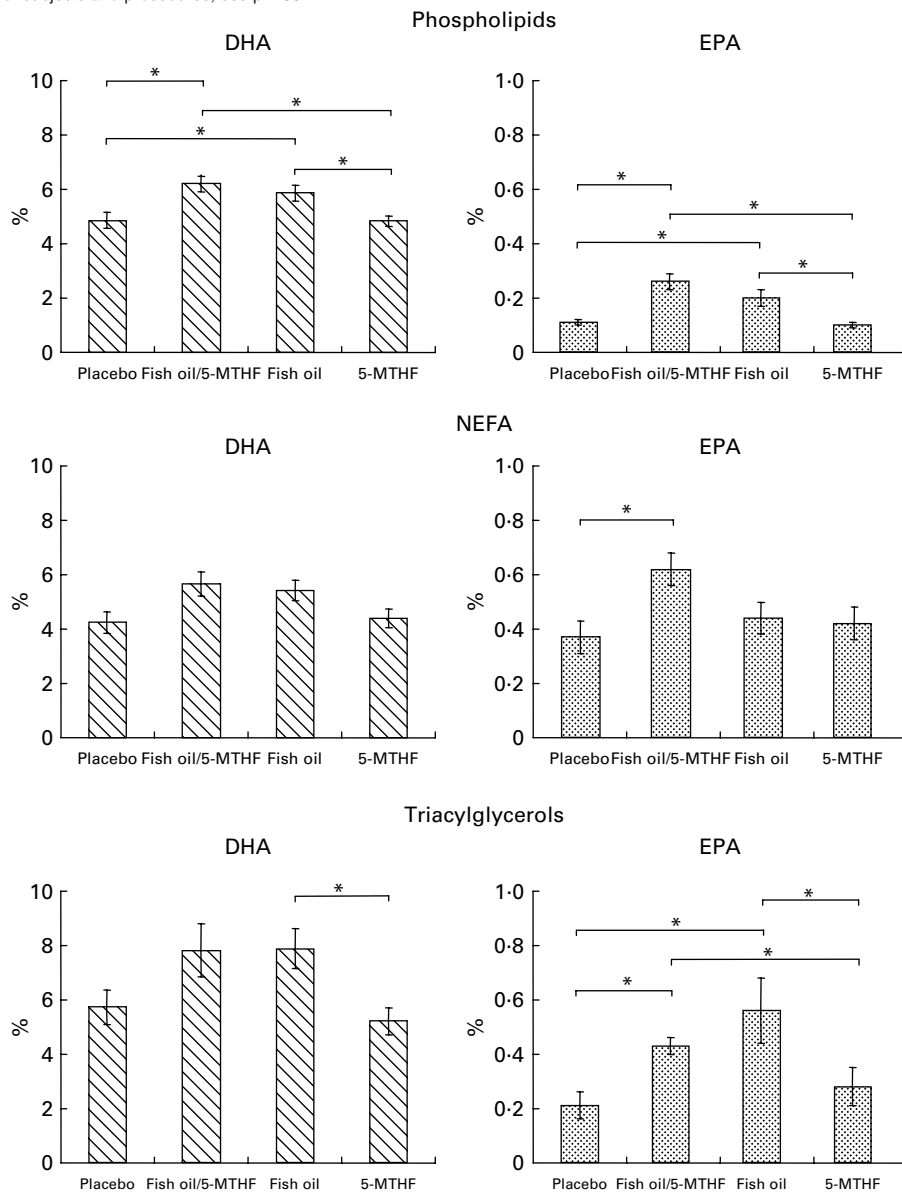
**Table 1.** Primary antibodies used in western blotting and immunohistochemistry

Primary antibody	Clone	Isotype	Dilution western blot	Dilution immunohistochemistry	Source
Proliferation cell nuclear antigen	PC10	Mouse IgG <sub>2A</sub>	1:10 000	1:1000	Santa Cruz Biotech, Santa Cruz, CA, USA
p53	DO-1	Mouse IgG <sub>2A</sub>	1:500	1:100	Santa Cruz Biotech, Santa Cruz, CA, USA
Cytokeratin 18 neopeptide	M30	Mouse IgG <sub>2B</sub>	1:500	1:50	Roche, Mannheim, Germany

**Table 2.** Characteristics of the study population  
(Values are means with their standard errors)

SE	Treatment group							
	Placebo (n 12)		Fish oil/5-MTHF (n 11)		Fish oil (n 16)		5-MTHF (n 16)	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Length of gestation (d)	281.5	2.0	274.0	3.9	279.4	2.3	276.1	9.8
Maternal age (years)	31.6	0.8	30.8	1.3	28.1	1.3	29.0	1.3
BMI at study entry (kg/m <sup>2</sup> )	25.2	1.1	25.8	0.9	25.2	1.1	25.1	1.0
BMI at delivery (kg/m <sup>2</sup> )	29.7	1.5	29.1	0.8	29.1	0.5	27.6	2.5
Placental weight (g)	586	24	551	52	553	34	527	41
Infant's birth weight (g)	3316	71	3110	191	3288	127	3215	129
Infant's birth length (cm)	51.0	0.6	49.9	0.7	50.6	0.5	50.9	0.6
Smoking during pregnancy (n)	0		3		2		3	

5-MTHF, 5-methyltetrahydrofolate.  
For details of subjects and procedures, see p. 183.



**Fig. 1.** Proportion of DHA and EPA (% w/w) in placental tissue phospholipids, NEFA and triacylglycerols. The groups were supplemented with either placebo (n 12), modified fish oil and 5-methyltetrahydrofolate (5-MTHF; n 11), modified fish oil (n 16) or 5-MTHF (n 16). Mean values were significantly different: \*P < 0.05. For details of subjects and procedures, see p. 183.

arachidonic content of any of the lipid fractions analysed (Table 3).

#### Folate concentrations

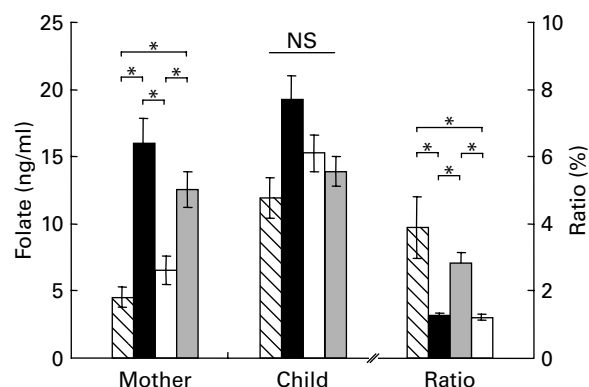
A total of 142 samples were analysed for folate concentration. The evaluated subgroup ( $n = 55$ ) was representative of the total population (data not shown). 5-MTHF intake was reflected in maternal blood plasma concentrations; that is, the 5-MTHF-supplemented groups (5-MTHF 12.6 (SE 1.3) ng/ml, combined 16.0 (SE 1.8) ng/ml) had higher levels than the untreated groups (placebo 4.5 (SE 0.8) ng/ml and fish oil 6.6 (SE 1.1) ng/ml;  $P < 0.05$ ). In contrast, no differences were found in fetal plasma (Fig. 2). The fetal:maternal ratio of plasma folate concentration decreased after 5-MTHF supplementation (5-MTHF 1.2 (SE 0.1) % and combined 1.3 (SE 0.1) % *v.* placebo 4.2 (SE 0.9) % and fish oil 3.0 (SE 0.3) %;  $P < 0.05$ ).

#### Western blotting and immunohistochemistry

The proliferation marker PCNA, the tumour suppressor protein p53 and the apoptosis marker cytokeratin 18 neopeptide (42 kDa) were quantified to determine proliferation and apoptosis in the term placenta (Figs 3 and 4). The level of PCNA in the fish oil/5-MTHF-treated group was 66 % higher ( $P < 0.05$ ) than that in the placebo group (Fig. 3(A)), whereas levels of p53 and cytokeratin 18 neopeptide were unaffected by treatment (Fig. 3(B), 3(C)). The western blotting results were confirmed by immunohistochemical analysis (Fig. 4).

In order to determine whether the changes in PCNA expression were due to changes in the maternal (trophoblast) or fetal (endothelial cells, other cells) side of the placenta, the proportion of cells immunolabelled for PCNA was further determined for the combined treatment group (Fig. 4(A)) and the placebo group (Fig. 4(B)). The level was higher in the combined treatment group (12.8 (SE 0.6) % *v.* 7.3 (SE 0.3) %;  $P < 0.05$ ). Clearly, PCNA was altered only in the trophoblast compartment (11.1 (SE 0.5) % placebo *v.* 21.5 (SE 1.2) % combined treatment group;  $P < 0.05$ ), whereas similar proportions of nuclei were stained in endothelial and other stromal cells (Fig. 5).

The density of p53-labelled nuclei was similar in all four study groups (Fig. 4(C)), which is in agreement with similar



**Fig. 2.** Effect of 5-methyltetrahydrofolate (5-MTHF) supplementation on fetal and maternal plasma folate concentrations. In maternal plasma, higher concentrations were measured in the folate-supplemented groups. In fetal plasma, no differences were found between the four groups. The ratio of maternal to fetal plasma folate concentration was reduced by additional 5-MTHF uptake. Mean values were significantly different: \* $P < 0.05$ . □, Placebo; ■, fish oil/5-MTHF; ▨, fish oil; □, 5-MTHF. For details of subjects and procedures, see p. 183.

apoptosis rates found by cytokeratin 18 neopeptide staining. In addition, the immunoreactive cells for cytokeratin 18 neopeptide also contained apoptotic bodies, as identified by their characteristic morphology including a round, regular and condensed appearance and dark blue staining. Apoptotic cells were found almost exclusively among extravillous trophoblasts (Fig. 4(D)). These results did not alter when women who smoked were excluded from the analysis.

#### Discussion

One of the important questions concerned the potential effect of supplementation with modified fish oil (DHA and EPA) and/or 5-MTHF on the proliferation and apoptosis of different cells in the human placenta. To address this, key proteins of both processes were selected. PCNA is a well-known cell-cycle marker that has, in different studies, been proved as a useful tool for determining the proportion of proliferating cells (Kurki *et al.* 1988). It was chosen because it can be used in both immunohistochemistry and immunoblotting. PCNA can be immunolocalised mainly in the nuclei of cytotrophoblasts (Ishihara *et al.* 2000). Although its long half-life of about 20 h may lead to absolute overestimations (Ishihara *et al.* 2000), this is unlikely to affect group comparisons.

Protein p53 is a master-switch regulator in the cell, plays an important role as a cell-cycle checkpoint protein and may determine the apoptotic fate of a cell. The p53 protein is localised mainly in nuclei of extravillous trophoblasts, being found in low proportions also in villous cytotrophoblasts and in the syncytiotrophoblast (Haidacher *et al.* 1995). The PCNA:p53 ratio determines PCNA function. A low ratio leads to DNA repair, whereas DNA replication occurs when the ratio is high (Paunesku *et al.* 2001).

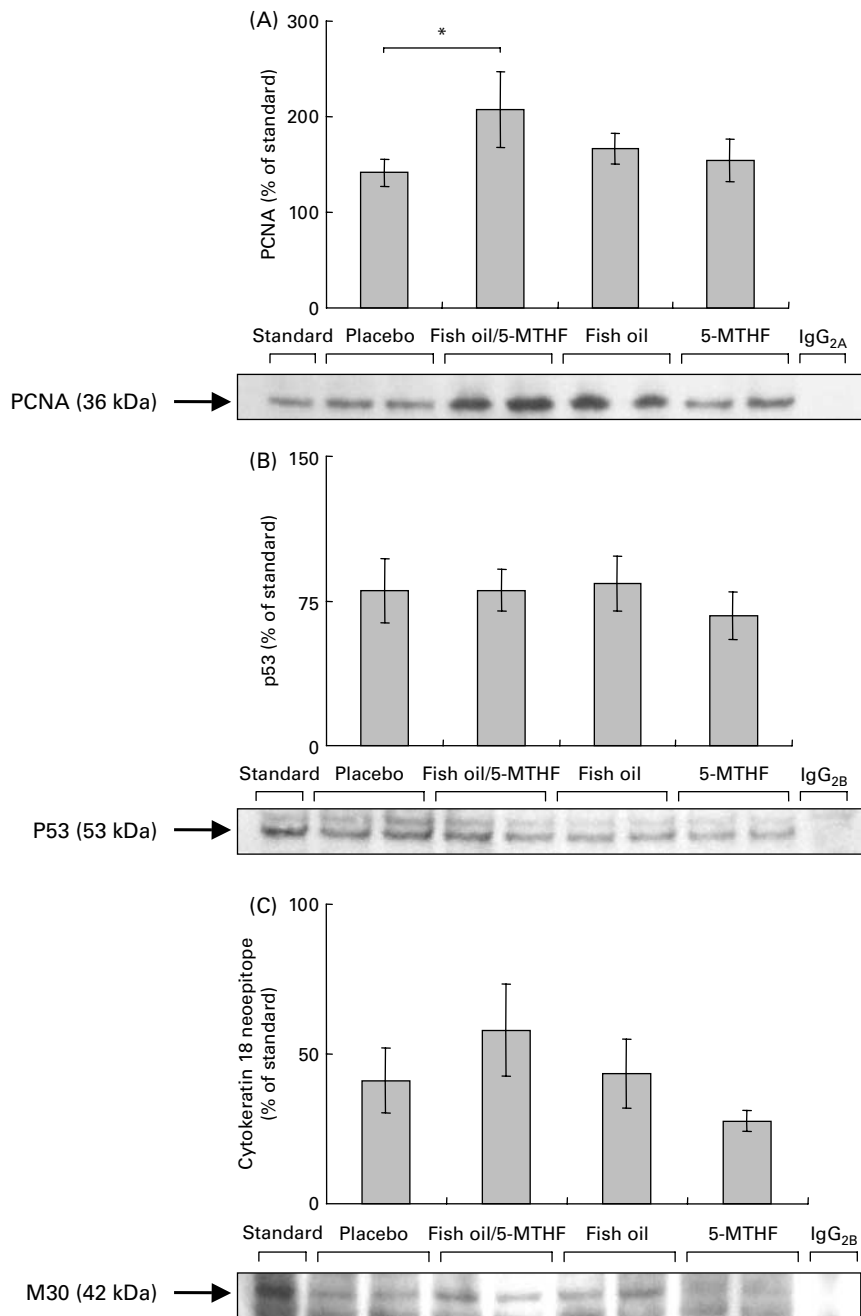
Apoptotic cells were detected using an antibody (clone M30) for the cytokeratin 18 neopeptide (Austgulen *et al.* 2002; Huppertz *et al.* 2003). The detection of cytokeratin 18 neopeptide is more reliable than the conventional terminal deoxynucleotidyl transferase-mediated deoxyuridine

**Table 3.** Arachidonic acid content in different placental lipid fractions (wt/wt in %)

(Values are means with their standard errors)

Lipid fraction	Supplement							
	Placebo ( $n = 12$ )		Fish oil/5-MTHF ( $n = 11$ )		Fish oil ( $n = 16$ )		5-MTHF ( $n = 16$ )	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Phospholipids	21.3	0.4	21.8	0.4	21.3	0.4	22.2	0.3
NEFA	17.8	0.8	17.9	0.5	18.5	0.5	18.4	0.6
Triacylglycerols	12.7	0.6	12.3	0.6	12.9	0.6	12.1	0.8

5-MTHF, 5-methyltetrahydrofolate.



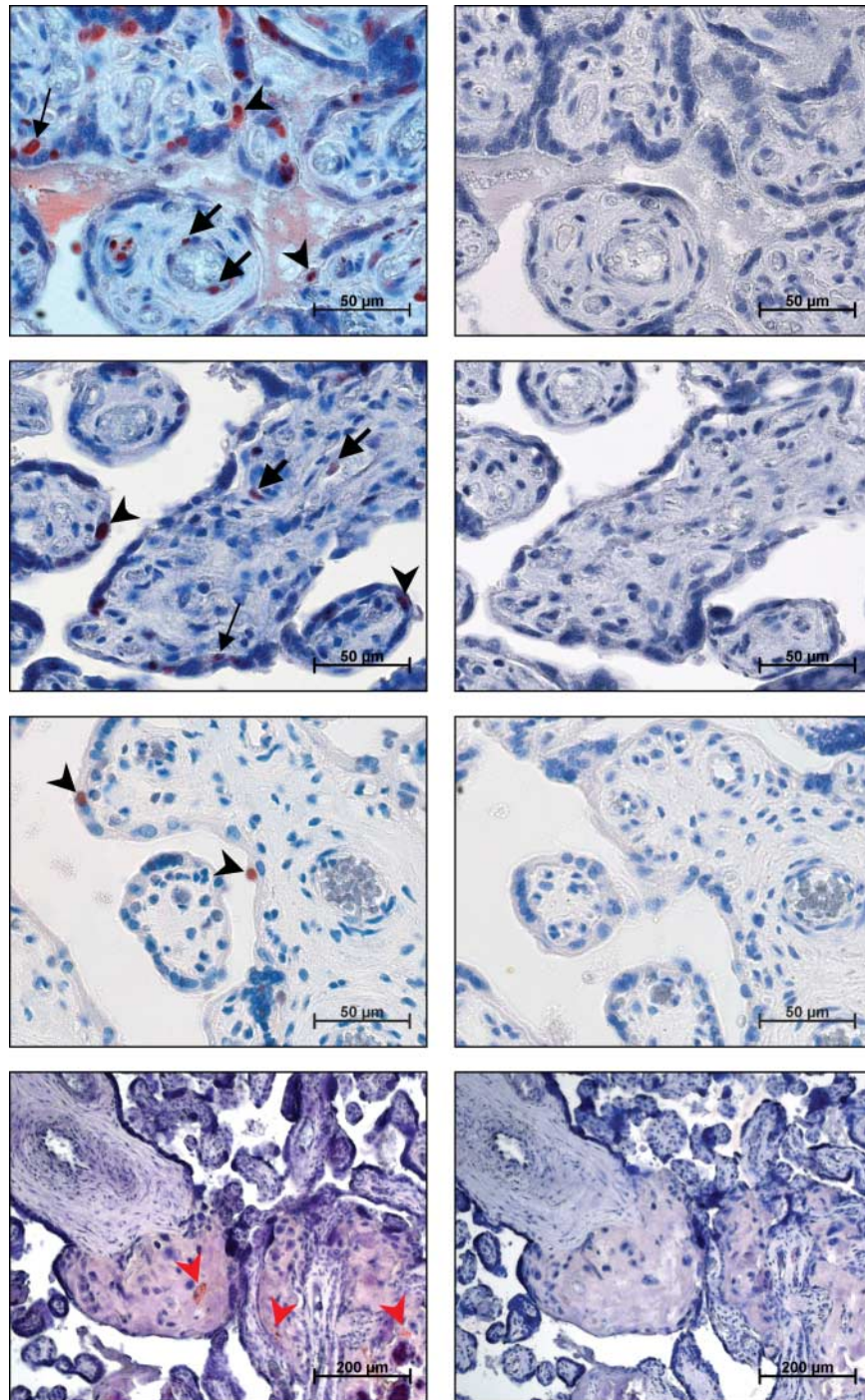
**Fig. 3.** Immunoblot and densitometric analysis of the proteins of proliferation cell nuclear antigen (PCNA; A), p53 (B) and cytokeratin 18 neopeptide (C) in placental tissue. There were no differences between the study groups except for PCNA in the placebo and the combined modified fish oil/folate-supplemented groups. Data are expressed relative to an internal standard (100%) run on each gel. 5-MTHF, 5-methyltetrahydrofolate. Mean values were significantly different: \* $P < 0.05$ . For details of subjects and procedures, see p. 183.

triphosphate nick-end labelling method for monitoring of apoptotic cells in placental tissue (Leers *et al.* 1999; Austgulen *et al.* 2002; Huppertz *et al.* 2003). In addition, the antibody for the neopeptide can be used in both immunohistochemistry and western blotting (Leers *et al.* 1999), which was another reason for choosing this method.

In some studies, a high intake of fish oil, especially DHA, during pregnancy could lead to prolonged pregnancy and increased birth weight (Olsen *et al.* 1992; Smuts *et al.* 2003). We therefore expected an enhanced proliferation rate

in the placenta at the end of pregnancy. In the present study, the additional uptake of a modified fish oil (or 5-MTHF) showed no effect on the course or outcome of pregnancy. In keeping with data from others (Montgomery *et al.* 2003), supplementation with low amounts of DHA during pregnancy does not affect the length of gestation.

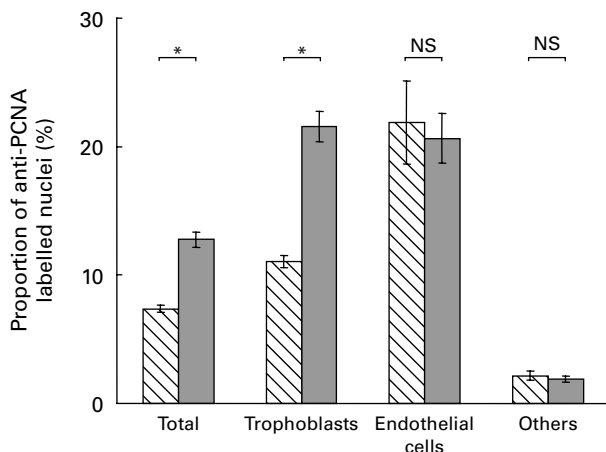
The proliferation rate of trophoblastic cells was increased in women who were supplemented with modified fish oil and 5-MTHF in combination, but not in women who were supplemented singly with either fish oil or 5-MTHF.



**Fig. 4.** Immunolocalisation of proliferation cell nuclear antigen (PCNA), cytokeratin 18 neopeptide and p53 in full-term placenta. PCNA immunostaining was stronger in placental tissue from the fish oil/folate-supplemented group (A) than in the placebo group (B). PCNA in endothelium and other cells showed no differences between the two groups. p53 was only rarely detected in all study groups and was found mainly within trophoblasts (C). Cytokeratin 18 neopeptide (D) was rarely labelled in trophoblast cells. (E–H) Ig G isotype matched negative controls as parallel section.  $\blacktriangleright$ , syncytiotrophoblast;  $\longrightarrow$ , cytotrophoblast;  $\longrightarrow$ , endothelial and other cells;  $\blacktriangleright$ , apoptotic extravillous trophoblast cells. For details of subjects and procedures, see p. 183.

The enhanced proliferation was found only in villous cytotrophoblasts, whereas endothelial and other stromal cells were not affected. These findings are notable because, in fish-oil supplementation studies, enhanced DHA contents have been measured in the fetal circulation (van Houwelingen *et al.* 1995). Thus, if the effect depended on the additional intake of DHA, proliferation rates would also be higher in

endothelial and other stromal cells. One potential confounder is smoking during pregnancy, which can affect the level of PCNA in the placental trophoblasts (Gruslin *et al.* 2001). In the present study, however, there was no difference in the proportion of smokers in the four groups ( $X^2 = 0.680$ ), and the exclusion of women who smoked did not change the results. Therefore, the exclusive upregulation of PCNA production



**Fig. 5.** A higher proportion of nuclei were immunolabelled in the fish oil/PCNA group (▨;  $n$  3) than the placebo group (■;  $n$  3), because of an exclusive increase in the trophoblast compartment. The labelling of endothelial cells and other cells, such as fibroblasts, blood cells and macrophages, remained unchanged. PCNA, proliferation cell nuclear antigen. Mean values were significantly different: \* $P$  < 0.05. For details of subjects and procedures, see p. 183.

in the trophoblast probably reflects a differential susceptibility of the cells to changes in ambient DHA level.

The specific mechanism(s) accounting for the effects of DHA and 5-MTHF in combination compared with DHA alone are unknown. 5-MTHF may have sensitised the trophoblast for a DHA effect or vice versa, or both nutrients may have to cooperate to achieve the effect; thus, the effect would be absent with an elevated concentration of only one of the nutrients because of limited availability of the other. The nutrients may alter nutritionally sensitive hormones in the mother that will then lead to the changes in the placenta, or they may have a direct effect on trophoblast cell-cycle regulation. In addition, local factors derived from the nutrients may directly or indirectly stimulate trophoblast proliferation. Further *in vivo* and *in vitro* studies are needed and are ongoing in our laboratory.

The consequence of increased trophoblast proliferation as a result of the dietary intervention is unknown. It is usually found in placentas when mothers are anaemic (Kosanke *et al.* 1998), diabetic (Desoye & Kaufmann, 2005) or in pre-eclampsia (Arnholdt *et al.* 1991). However, unlike the results in the present study, pre-eclampsia is also associated with increased apoptosis of the trophoblast, i.e. trophoblast turnover. Large nutritional intervention studies will be needed to decide whether the diet-induced placental changes have a protective or promoting effect for the development of severe pathologies of pregnancy such as pre-eclampsia and intrauterine growth retardation.

The higher DHA contents measured in membrane phospholipids as well as the slightly enhanced DHA proportions in cytosolic triacylglycerols and NEFA had no effect on apoptosis in full-term placentas. The uptake of high EPA concentrations decreases the arachidonic acid content of plasma phospholipids and triacylglycerols (van Houwelingen *et al.* 1995; Helland *et al.* 2001; Rodriguez *et al.* 2003). The increase in arachidonic acid NEFA or altered arachidonic acid levels in membrane phospholipids may induce apoptosis in different cell types (Rudolph *et al.* 2001). In the present study, we did not observe any effect of EPA on the arachidonic acid content of any of the placental lipid fractions

analysed. The absence of such a change might also be an explanation for the lack of effect on placental apoptosis.

In summary, maternal supplementation with fish oil enhanced the DHA and EPA contents of placental membrane phospholipids. When pregnant women were supplemented singly either with modified fish oil or 5-MTHF, the proliferation did not change compared with placebo. In the group receiving both supplements, however, an enhanced proliferation rate was shown in the trophoblast compartment but not in endothelial or other stromal cells. In contrast, the degree of apoptosis was unaltered in all treatment groups. The present data suggest that the combination of folic acid and DHA could alter placental proliferation in the full-term placenta, but the detailed mechanisms have to be elucidated.

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