

Long-term effects of DHA and/or 5-MTHF supplementation in pregnant women on their offspring's fatty-acid status, neurodevelopment and behaviour

Efectos a largo plazo de la suplementación con DHA y/o 5-MTHF en embarazadas sobre el perfil de ácidos grasos, el neurodesarrollo y el desarrollo de la conducta de los hijos

TESIS DOCTORAL

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CERTIFICAN: Que los trabajos de investigación que se exponen en la Memoria de Tesis Doctoral titulada: "*Efectos a largo plazo de la suplementación con DHA y/o 5-MTHF en embarazadas sobre el perfil de ácidos grasos, el neurodesarrollo y el desarrollo de la conducta de los hijos"* han sido realizados en el Departamento de Pediatría de la Universidad de Granada, correspondiendo fielmente a los resultados obtenidos. La presente Memoria ha sido revisada por los abajo firmantes, encontrándola conforme para ser defendida y aspirar al grado de Doctor con Mención Internacional en Medicina y Salud Pública.

Y para que conste, en cumplimiento de las disposiciones vigentes, extendemos el presente el 31 de Mayo de 2017.

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Prof. Dr. Berthold V. Koletzko

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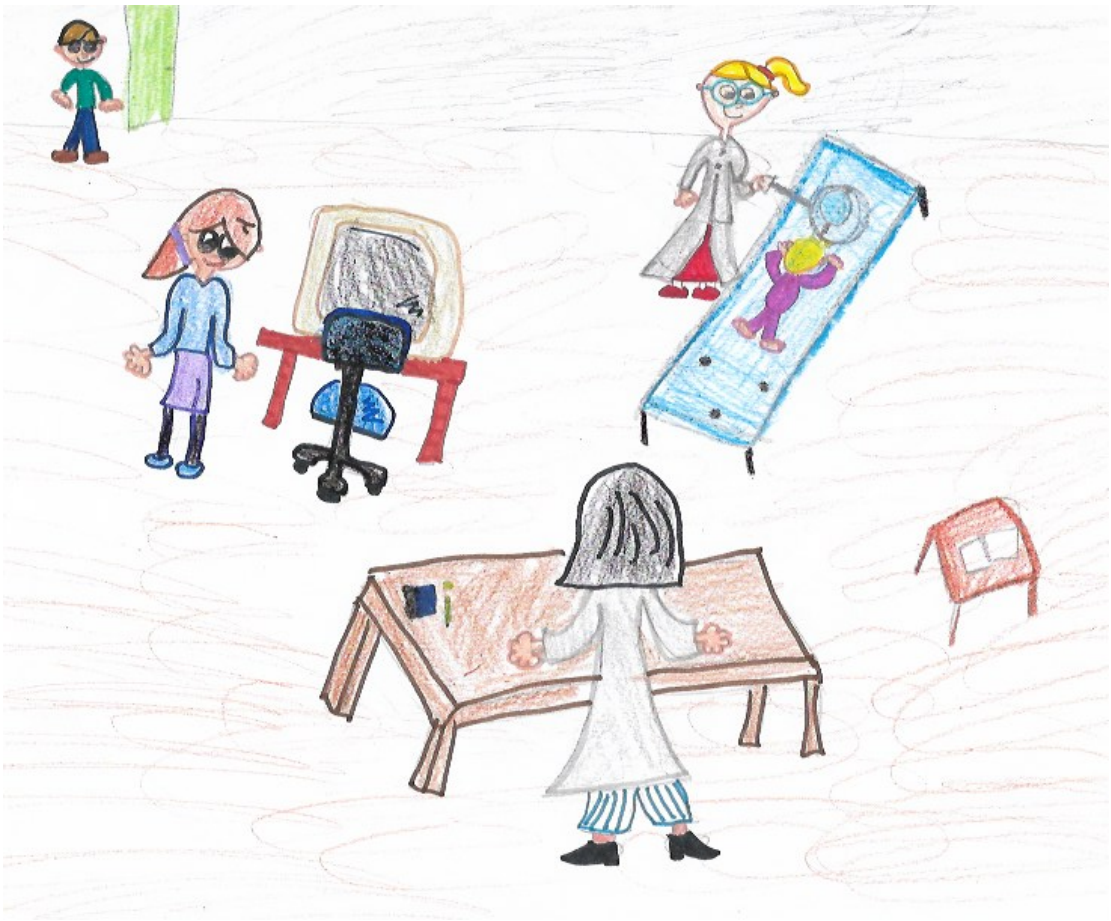

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ODA A EURISTIKOS

"Aunque nada pueda hacer
volver la hora del esplendor en la hierba,
de la gloria en las flores,
no debemos afligirnos
porque la belleza subsiste siempre en el recuerdo"
William Wordsworth, "Oda a la inmortalidad".

ODE TO EURISTIKOS

"Though nothing can bring back the hour
Of splendour in the grass,
Of glory in the flower,
We will grieve not, rather find
Strength in what remains behind"
William Wordsworth, "Ode to immortality"

Para mi será inmortal e inolvidable mi paso por EURISTIKOS. Gracias a todos mis compañeros.

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ABREVIATIONS

δ 5-D	δ 5-desaturase
δ 6-D	δ 6-desaturase
AA	Arachidonic acid
ALA	α-linolenic acid
APA	American Psychiatric Association
ASEBA	Achenbach System of Empirically Based Assessment of Cognition
BMI	Body Mass Index
CBCL	Child Behaviour Check List
CNS	Central nervous system
CSF	Cerebrospinal fluid
DGLA	Dihomo-ɤ-linolenic acid
DHA	Docosahexaenoic acid
DPA	Docosapentaenoic acid
DRI	Dietary Reference Intakes
DSM	Diagnostic and Statistical Manuals of Mental Disorders
EEG/ERP	Electroencephalography/ Event-related potential
EDI	Eating Disorder Inventory
EDTA	Ethylenediaminetetraacetic acid
EFA	Essential fatty acid
EPA	Eicosapentaenoic acid
ESPGHAN	European Society of Gastroenterology, Hepatology and Paediatric Nutrition
FA	Fatty acids
FADS	Fatty acid desaturase
FAME	Fatty acid methyl ester
FAO	Food and Agriculture Organization
FFQ	Food frequency questionnaire
FO	Fish oil
FWHM	Full width at half maximum
GLA	ɤ -linolenic acid

ABREVIATIONS

GPL	Glycerophospholipids
GMV	Gray matter volume
HC	Head circumference
H-W	Hardy-Weinberg
IMC	Índice de Masa Corporal
K-ABC	Kaufman Assessment Battery for Children
LA	Linoleic acid
LC-PUFAs	Long-chain polyunsaturated fatty acids
LD	Linkage disequilibrium
ln	Natural logarithm
MNI	Montreal Neurological Institute
MPI	Mental Processing Index
MRI	Magnetic resonance imaging
PCA	Principal component analysis
PLS-DA	Partial Least Square-Data Analysis
PUFAs	Polyunsaturated fatty acid
NMR	Nuclear Magnetic Resonance
SI	Social insecurity
SDS	Standard deviation scores
SNPs	Single-nucleotide polymorphisms
sqrt	Square root
TBSA	Total brain surface area
TIV	Total intracranial volume
VEPs	Visually evoked potentials
VIP	Variable importance in the projection
WHO	World Health Organization
WMV	White matter volume

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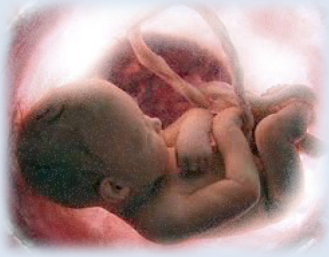
Figure 17. Relationships between regional grey matter volumes and head circumference.



1. SUMMARY

1. Summary

Introduction

The maternal intake of polyunsaturated fatty acids (PUFA) and their metabolism will determine the bioavailability of long-chain polyunsaturated fatty acids (LC-PUFAs) during the first months of life [(1)]. However, the importance of the impact of altered maternal PUFA status on the scheduling and imprinting of PUFA metabolism in the foetus is unknown, and the long-term consequences on the functionality of the metabolic pathways involved in PUFA elongation and desaturation of the PUFAs that allow the endogenous synthesis of LC-PUFAs.

The role of the genetic polymorphisms of fatty acid desaturases (*FADS*) in modulating the conversion of essential fatty acid (EFA) precursors of the n-3 and n-6 series into their consequent derivatives has been highlighted in the last 10 years [(2)]. This may explain the heterogeneity of responses found in various studies on the effect of prenatal PUFA supplementation on growth and neurodevelopment [(3-6)]. Genetic polymorphisms of *FADS1*, *FADS2* and *FADS3* (which encode the synthesis of δ5 and δ6 desaturases) have been identified as important determinants of plasma circulating LC-PUFAs [(7-10)], although due to the significant genetic variation in different populations, there is a lack of evidence of the effects of *FADS* polymorphisms on the metabolism of LC-PUFAs [(11, 12)].

There has been increasing interest in the study of the fatty acid (FA) concentrations in the cells of the cheek mucosa in recent years, since it is considered that it could be a plausible alternative for following the nutritional status of the LC -PUFAs in early stages of life, thus avoiding blood extraction with the problems that this entails in young children. Currently, there is no information about a possible association between the genetic polymorphisms of *FADS1* and *FADS2* and the concentrations of these FAs in cheek cells.

Moreover, in Europe, saturated FAs and the n-6 series have replaced the intake of n-3 FAs, in a different way in each country. Although the n-6 are also essential for health during growth and development, an appropriate balance is required with respect to n-3 PUFAs. It has been found that relative deficiencies of n-3 PUFAs are associated with a wide range of pathologies in relation to physical and mental health, which pose increasing problems in developed countries.

SUMMARY

Nutrition plays an important role in the structural and functional growth of the human brain, from conception and during childhood and adolescence to adulthood. There is scientific evidence that early nutrition may influence cognitive development and subsequent behaviour. It is known that an optimal contribution of macro and micronutrients is important for the synthesis of neurotransmitters (and their receptors and transporters), for the renewal and maintenance of the cytoskeleton of axons and myelin sheaths, for the growth of synaptic spines and, therefore, for neuronal plasticity and neuronal survival [13].

During the 9 months of gestation, from the creation of the initial stem cells the brain reaches more than 100 trillion nerve cells at birth and weighs about 400g. During the first 4 years of life, the brain continues to grow, reaching the size of 1,200 g, which is only 200g less than the brain of an adult. In the next 10-15 years, the brain continues to grow, involving different compartments differently. For example, the thickness of the cerebral cortex varies in different regions between the ages of 5 and 18 and at different times, and the regions of reasoning, planning and social communication do so later. Changes in brain volume are also accompanied by changes in the size of the cephalic perimeter. Preliminary studies have shown that the circumference of the head during the first years of life can predict brain size and volume and neurocognitive development. However, it is unknown whether this measurement has a predictive value in terms of regional and sub-cortical cerebral volumes. LC-PUFAs appear to play an essential role in these processes [14].

LC-PUFA deficiencies during the first 1,000 days of life, together with an unfavourable genetic load, may contribute to the development of alterations in brain morphology, structure and function, and alterations in cognitive development and behaviour in childhood. Therefore, it is necessary to monitor the intake of these FAs, before, during gestation and lactation, and in the first 2 years of life to prevent pathologies in the longer term.

Animal models have shown that maternal diets deficient in n-3 and n-6 LC-PUFAs alter the deposition of these FAs in the neonatal cortex, which determine changes in neurotransmitter metabolism and learning failures [15]. Docosahexaenoic acid (DHA) deficiency during gestation is associated with a reduction in dendritic arborisation, a failure in gene expression involved in the regulation of neurogenesis, neurotransmission and connectivity [1, 17, 18].

It has been demonstrated that *FADS1* and *FADS2* polymorphisms are related to important changes in FA metabolism and brain development [19]. Koletzko *et al.* have shown a consistent association of the minor alleles of *FADS1* and *FADS2* in pregnant women with lower concentrations of DHA in the phospholipids (PL) of erythrocyte membranes [20].

This doctoral thesis aims to: 1) to evaluate the influence of maternal supplementation during pregnancy and the genetic polymorphisms of *FADS1*, *FADS2* and *FADS3* on the composition of FAs in glycerophospholipid (GPL) cheek cells of children up to 9.5 years of age; 2) to analyse the relationship between the composition of FAs in the GPL of cheek cells in children up to 9.5 years, dietary intake, age, sex and country of origin; 3) to study the association between FA profile in the oral mucosa and behavioural problems in children; 4) to establish the extent to which the behaviour and the psychological characteristics of the mothers influence behaviour, internalizing and externalizing problems in their children at 8 years of age; and 5) to explore the relationship between cranial perimeter evolution from birth to 10 years, sub-cortical volumes and brain distribution of grey and white matter and cognitive development.

Material and methods

Participants and study design

147 Spanish (n=105) and German (n=42) children participating in the NUHEAL study have been included. Mothers were supplemented from week 20 of gestation until delivery with fish oil (FO) (500 g DHA + 150 mg eicosapentaenoic acid (EPA)), 5-methyltetrahydrofolate (5-MTHF), placebo or both. The children of these pregnant women were re-evaluated in different visits until 10 years of age.

Methodology

At 8, 9 and 9.5 years of age, a profile of 21 FAs was obtained from GPL of children cheek cell samples, following the technique described by Klinger *et al.* (2011). 17 Single Nucleotide Peptides (SNPs) were also analysed in these children in umbilical cord blood samples by iPLEX (Sequenom, San Diego, CA, USA) and mass spectrometry (MALDI-TOF MS, Mass Array; Sequenom). These children also did the Child Behaviour Check List Test (CBCL) at 7.5 and 9 years old and the Eating Disorder Inventory (EDI-2) questionnaire at 8 and 9.5

years old. At the age of 10, neuro-imaging (brain magnetic resonance imaging - MRI) of 74 Spanish children was obtained, studying morphometry and volumetry based on voxels, superficial extraction of the cerebral cortex and sub-cortical segmentation. The evaluation of dietary intake was obtained from food frequency questionnaires (FFQ) completed by the parents and a record of dietary intake 24 hours for 3 days, (2 on workdays and 1 on weekends). Data from the food frequency questionnaire and the 3-day 24-hour dietary recall were processed using the PCN-CESNID and "Young Adolescents' Nutrition Assessment on Computer (YANA-C)" international software [21]. The food composition tables from DIAL [22] (Spain) and the German Food Code and Nutrient Data Base (BLS) [23] (Germany) were used.

Statistical Analyses

To perform the analyses of FAs, in all cases, a descriptive and normal analysis was performed using the Shapiro-Wilk test. When the variables did not follow a normal distribution, transformations were made using logarithms (ln) and square root (sqrt). If normality was not reached, data analysis was performed using the non-parametric U Mann-Whitney test. The analysis of the continuous variables was performed using ANOVA and the Kruskal-Wallis test. In the case of categorical variables, χ^2 was used.

For the analysis of the genotypes, the Hardy-Weinberg equilibrium (H-W) was calculated using the statistical software R. The deviations of the H-W equilibrium were studied using Fisher's exact test. In addition, the Lewontin's D' test and quadratic (r^2) parity correlations between genes were used to assess imbalance. Linear regression and a mixed linear model were also performed to establish the association between *FADS* polymorphisms, type of supplementation received during pregnancy, age, sex and country of birth, and GPL-FA concentrations in the different moments in which they were analysed.

The multivariate analysis, Principal Component Analysis (PCA) and Partial Least Squares (PLS) were performed to verify the distribution of the results according to different criteria and to represent them in a more accurate way.

To obtain the possible relationships between the eating behaviour and the psychological status of the mothers on the behaviour of their children, a binary logistic regression was performed. We used an analysis of covariance (ANCOVA) approach to evaluate early supplementation (four groups: FO, 5-MTHF, FO+5-MTHF, and Placebo) effects on head circumference

measurements and cognitive abilities (MPI score), as well as on total and regional brain volumes and inner cortical surface area. Dependent variables were submitted to a 2 (FO) x 2 (5-MTHF) between subjects ANCOVA. The analyses were controlled by maternal age, maternal height and weight, maternal body mass index (BMI), family economic status, smoking (week 20 and 30 of pregnancy) and placental weight, sex, laterality and total intracranial volume (TIV), being included in the different models as confounding variables.

In general, the SPSS statistical program, version 20.0 (SPSS Inc. Chicago, USA), R statistical software (3.2.2 Version, "genetics" package), and STATA (StataCorp 2011; Stata Statistical Software: Release 12).

In all cases, the level of significance was set when $p < 0.05$.

Results

The first study showed that supplementation with FO and/or 5-MTHF during pregnancy has long-term effects on the GPL-FA profile of cheek cells in children up to 9.5 years. In addition, it could be seen that the polymorphisms of *FADS1* and *FADS2* genotypes of children influence the concentrations of the GLP-PUFAs obtained in cheek cells. Most associations were established between different SNPs *FADS1* and *FADS2* with linoleic (LA) and α-Linolenic (ALA) acids. It was also verified that the different supplements that the mothers received during pregnancy along with the genetic background of their children affect the composition of the FAs of the children.

In a subsequent analysis, it was possible to demonstrate that the FA profile in the GPL of the cheek cells analysed in school-age children, was strongly related to the country of origin, suggesting an effect of diet, lifestyle, etc. Likewise, statistical differences in the composition of GPL-FAs of cheek cells between 8 and 9.5 years old were verified in both Spanish and German children. The daily intake of AA showed a high impact on the percentage of FAs found in the cheek cells of children. The intake of lipids and fibre determines a decrease in araquidonic acid (AA). The intake of folic acid and EPA positively influences the concentrations of AA, EPA and docosapentaenoic acid (DPA) in cheek cells. Intake of DHA has a negative influence on the concentrations of DPA in the GPL of the cells of the buccal mucosa.

Associations were found between ALA concentrations in GPL and outsourcing and care problems. LA was associated with attention problems. EPA, DHA and DPA were associated with better ability of games and sports competitions.

Other analyses have shown that the type of maternal supplementation during pregnancy did not influence the behaviour of the children at 8 years old (30 children had internalization problems and 24 had externalization problems). An association was established between the internalizing problems of children and drive for thinness (DT) of their mothers. The mothers classified as pathological in the EDI-2 DT sub-scale were 1,138 times more likely to be influential in their children's internalizing problems.

Finally, it was found that prenatal supplementation was not associated with different cranial circumference sizes at any age, nor did it influence cognitive abilities or total brain volumes. At 4 years old, the most significant association of the cranial perimeter with the total volume of grey matter (GMV), the total volume of white matter (WMV) and the surface area of the brain could be demonstrated. Also, the size of the cranial perimeter at 4 years old was associated with a higher GMV content in the frontal, temporal and occipital areas, as well as the volume of the caudate nucleus, globus pallidum, putamen and thalamus [(24)].

Conclusions

1. FO and/or 5-MTHF supplementation during pregnancy have long-term effects on the GPL-FA profiles in children's cheek cells at school age; a programming effect of fatty acids metabolic pathways is suggested, which may cause differences in children's FA status.
2. Children's *FADS1* and *FADS2* gene cluster polymorphisms influence PUFA concentrations in children's cheek cells. The majority of the associations were established between *FADS1* and *FADS2* SNPs with LA and ALA.
3. The interaction between early life nutrition and children's *FADS1*, *FADS2* and *FADS3* genotype, demonstrate long-term effects on GPL concentrations of the oral mucosa in children, suggesting an early programming of the metabolic pathways involved in the status of these FAs, driven by the genetic background.

4. Differences amongst the FA profiles from check-cell samples analysed in the NUHEAL children are strongly related to the country of origin, and therefore diet, lifestyle, etc.
5. Dietary intake also appears to influence the fatty acid concentrations present in the cheek cells of the studied children, the daily intake of AA especially shows a high impact on the percentage of fatty acids found in the cheek cells of the children.
6. There is a relationship between certain characteristics of maternal eating disorders, such as the "obsession with thinness", and the subsequent development of internalizing behaviour problems in their children at 8 years of age. This relationship determines that those mothers, who have problems related to the obsession with being thin, increase the likelihood of their children having 1,138 times more risk of internalizing behaviour problems compared to those children whose mothers do not show this obsession.
7. We have verified that the measurement of the cephalic perimeter at 4 years of age serve as a marker of cognitive development at 10 years of age. In addition, this anthropometric measurement is very useful from birth to 4 years, as this period is considered the most sensitive for brain growth. Certain postnatal factors, such as diet or LC-PUFAs status, may have a more significant impact on the structural maturation of certain cortical areas and sub-cortical nuclei, independent of the effects determined by prenatal supplementation.

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Resumen

Introducción

La ingesta materna de ácidos grasos poliinsaturados (PUFA) y su metabolismo va a determinar la biodisponibilidad de ácidos grasos (FA) poliinsaturados de cadena larga (LC-PUFAs) durante los primeros meses de vida [1]. Sin embargo, por el momento se desconoce la importancia del impacto de las alteraciones del estado materno en PUFA sobre la programación e imprinting del metabolismo de los PUFA en el feto, y las consecuencias a largo plazo sobre la funcionalidad de las vías metabólicas implicadas en la elongación y desaturación de los PUFA que permiten alcanzar la síntesis endógena de LC-PUFAs.

En los últimos 10 años se viene destacando el papel de los polimorfismos genéticos de las desaturasas de ácidos grasos (*FADS*) en la modulación de la conversión de los ácidos grasos esenciales (EFA) precursores de las series omega 3 (n-3) y omega 6 (n-6) en sus consiguientes derivados [2], lo que podría explicar la heterogeneidad de respuestas encontradas en diversos estudios sobre el efecto de la suplementación prenatal con PUFA sobre el crecimiento y el neurodesarrollo [3-6]. Los polimorfimos genéticos (SNPs) de las *FADS1*, *FADS2* y FADS3 (que codifican la síntesis de la δ5 y δ6 desaturasas) se han identificado como importantes determinantes de las concentraciones LC-PUFAs circulantes en plasma [7-10], aunque debido a la importante variación genética en distintas poblaciones existe una falta de evidencia de los efectos de los polimorfismos de las *FADS* sobre el metabolismo de los LC-PUFA [11, 12].

Por otra parte, el interés del estudio de las concentraciones de los ácidos grasos en las células de la mucosa de la mejilla ha aumentado en los últimos años, pues se considera que puede ser una alternativa plausible para hacer un seguimiento del status nutricional de los LC-PUFAs en etapas precoces de la vida, evitando la extracción sanguínea con los problemas que ésta conlleva en niños de corta edad. Por el momento, no existe información acerca de una posible asociación de los polimorfismos genéticos de las *FADS1* y *FADS2* y las concentraciones de dichos ácidos grasos en las células de la mejilla.

Por otra parte, en Europa, la ingesta de ácidos grasos de la serie omega 3 ha sido reemplazada, de forma diferente en cada país, por ácidos grasos saturados y de la serie omega 6. Aunque si bien estos últimos son también esenciales para la salud durante el crecimiento y desarrollo, se requiere un equilibrio apropiado respecto a los omega 3 PUFAs. Se ha comprobado que deficiencias relativas de omega

3 PUFAs se asocian a una amplia gama de patologías en relación con la salud física y mental, que plantean problemas cada vez mayores en países desarrollados.

La nutrición juega un papel importante en el crecimiento estructural y funcional del cerebro humano, desde la concepción y durante la infancia y la adolescencia, hasta la adultez. Existe evidencia científica de que la nutrición precoz puede influir sobre el desarrollo cognitivo y la conducta posterior. Se sabe que un aporte óptimo de macro- y micronutrientes es importante para la síntesis de neurotransmisores (y sus receptores y transportadores), para la renovación y mantenimiento del citoesqueleto de los axones y las vainas de mielina, para el crecimiento de las espinas sinápticas y, por tanto, para la plasticidad neuronal, y la supervivencia neuronal [13].

Durante los 9 meses de gestación, a partir de las células madres iniciales se llega a más de 100 billones de células nerviosas y el cerebro viene a pesar alrededor de 400 g al nacimiento. Durante los primeros 4 años de vida, el cerebro continúa creciendo, llegando al tamaño de 1200 g, lo que sólo se diferencia en 200 g respecto al cerebro de un adulto. En los próximos 10-15 años, el cerebro continúa creciendo, implicando a diferentes compartimentos de forma distinta. Por ejemplo, el grosor de la corteza cerebral varía en diferentes regiones entre los 5 y 18 años y en diferentes momentos, siendo las regiones del razonamiento, la planificación y la comunicación social las que lo hacen más tarde. Los cambios en el volumen cerebral también van acompañados de cambios en el tamaño del perímetro cefálico. Estudios preliminares han demostrado que la circunferencia de la cabeza durante los primeros años de vida puede predecir el tamaño y volumen cerebrales y el desarrollo neurocognitivo. Sin embargo, se desconoce si esta medición tiene un valor predictivo en cuanto a volúmenes cerebrales regionales y subcorticales. Los LC-PUFAs parecen jugar un papel esencial en estos procesos [14].

SUMMARY

Las deficiencias de LC-PUFAs durante los primeros 1000 días de vida, junto a una carga genética desfavorable, pueden contribuir al desarrollo de alteraciones en la morfología, estructura y función cerebrales, y alteraciones del desarrollo cognitivo y de la conducta en la infancia. Por tanto, es necesario vigilar la ingesta de estos ácidos grasos, antes, durante la gestación y lactancia, y en los primeros 2 años de vida para prevenir patologías a más largo plazo.

Los modelos animales han demostrado que dietas maternas deficientes en n-3 y n-6 LC-PUFAs alteran el depósito de estos ácidos grasos en el cortex neonatal, lo que determina cambios en el metabolismo de neurotransmisores y fallos de aprendizaje [15]. La deficiencia de ácido docosahexaenoico (DHA) durante la gestación se asocia a una reducción de la arborización dendrítica (16), un fallo en la expresión génica implicada en la regulación de la neurogénesis, neurotransmisión y conectividad [1, 17, 18].

Se ha demostrado que los polimorfismos de las *FADS1* y *FADS2* están relacionados con importantes cambios en el metabolismo de los ácidos grasos y el desarrollo cerebral [19]. Koletzko *et al.* [20] han mostrado en embarazadas una asociación consistente de los alelos menores de las *FADS1* y *FADS2* con concentraciones menores de DHA en los fosfolípidos de las membranas de los eritrocitos.

La presente tesis doctoral tiene como objetivos: 1) Evaluar la influencia de la suplementación materna durante el embarazo y los polimorfismos genéticos de las *FADS1*, *FADS2* y *FADS3* sobre la composición de los ácidos grasos en los glicerofosfolípidos (GPL) de las células de la mejilla de los niños hasta los 9.5 años de edad; 2) Analizar la relación entre la composición de los ácidos grasos en los GPL de las células de la mejilla en los niños hasta los 9,5 años, la ingesta dietética, la edad, el sexo y el país de origen; 3) Estudiar la asociación entre el perfil de ácidos grasos en la mucosa bucal y los problemas de comportamiento en los niños; 4) Establecer en qué medida influye el comportamiento alimentario y las características psicológicas de las madres sobre el comportamiento, problemas internalizantes y externalizantes en sus hijos a los 8 años de edad; 5) Explorar la relación entre la evolución del perímetro craneal desde el nacimiento hasta los 10 años, los volúmenes subcorticales y distribución cerebral de sustancia gris y blanca y el desarrollo cognitivo.

Material y Métodos

Participantes y diseño del estudio

Se han incluido 147 niños españoles (n=105) y alemanes (n=42) participantes en el estudio NUHEAL. Las madres fueron suplementadas a partir de la semana 20 de gestación y hasta el momento del parto, con aceite de pescado (500 g de DHA+ 150 mg de ácido eicosapentaenoico (EPA)), 5-metil-tetrahidrofolato (5-MTHF), placebo o ambos. Los hijos de estas embarazadas fueron reevaluados en diferentes visitas hasta los 10 años de edad.

Metodología

A los 8, 9 y 9.5 años de edad, se obtuvo un perfil de 21 ácidos grasos en los glicerofosfolípidos (GPL-FA) de las células de la mejilla de los niños, siguiendo la técnica descrita por Klinger et al. (2011). Igualmente, se analizaron 17 Single Nucleotide Peptides (SNPs) en los niños en muestras de sangre obtenidas en cordón umbilical mediante iPLEX (Sequenom, San Diego, CA, USA) y espectrometría de masas (MALDI-TOF MS, Mass Array; Sequenom). A estos niños también se les realizó el Test "*Lista de verificación del Comportamiento Infantil*" (Child behaviour Check List, CBCL) y el cuestionario de trastornos de la conducta alimentaria "Eating Disorder Inventory" (EDI-2) a los 8 y 9.5 años de edad. A los 10 años de edad, se obtuvieron neuroimágenes (resonancia magnética cerebral – RMN) de 74 niños españoles, estudiándose la morfometría y volumetría basada en voxels, extracción superficial de la corteza cerebral y segmentación subcortical. La evaluación de la ingesta dietética se obtuvo a partir de cuestionarios de frecuencia de consumo alimentaria (FFQ) completados por los padres y el registro de la ingesta alimentaria de 24 horas durante 3 días, 2 en días laborables y 1 en los fines de semana. Los datos del cuestionario de frecuencia alimentaria y el recuerdo dietético de 3 días de 24 horas se procesaron mediante el software internacional PCN-CESNID y "Evaluación de la nutrición de los jóvenes adolescentes en computadoras (YANA-C)" [21]. Se utilizaron las tablas de composición de alimentos de DIAL [22] (España) y el Código Alimentario Alemán y la Base de Datos de Nutrientes (BLS) [23] (Alemania).

Análisis estadísticos

En todos los casos se realizó análisis descriptivo y de normalidad mediante el test de Shapiro-Wilk. Cuando las variables no seguían una distribución normal, se realizaron transformaciones utilizando logaritmos (ln) y raíz cuadrada (sqrt). Si no se alcanzó la normalidad, el análisis de los datos se realizó mediante la prueba no paramétrica U Mann-Whitney. El análisis de las variables continuas se realizó mediante ANOVA y el test de Kruskal-Wallis; en el caso de variables categóricas se usó la χ^2.

Para el análisis de los genotipos se calculó el equilibrio de Hardy-Weinberg (H-W) mediante el software estadístico R; las desviaciones del equilibrio H-W se estudiaron mediante el test exacto de Fisher. Además, se utilizaron el Lewontin's D' test y las correlaciones cuadráticas (r^2) de paridad entre genes para evaluar el desequilibrio. También se realizó una regresión lineal y un modelo lineal mixto para establecer la asociación entre los polimorfismos de los *FADS*, el tipo de suplementación recibida durante el embarazo, la edad, el sexo y el país de nacimiento y las concentraciones de GPL-FA en los diferentes momentos en las que se analizaron.

El análisis multivariante, Análisis de Componentes Principales (PCA) y Mínimos Cuadrados Parciales (PLS) se realizaron para comprobar la distribución de los resultados según los diferentes criterios y representarlos de una forma más exacta.

Para obtener las posibles relaciones entre el comportamiento alimentario y el estado psicológico de las madres sobre el comportamiento de sus hijos, se realizó una regresión logística binaria. Se utilizó un análisis de covarianza (ANCOVA) para evaluar los efectos de la suplementación temprana (cuatro grupos: FO, 5-MTHF, FO + 5-MTHF y Placebo) sobre las mediciones de la circunferencia de la cabeza y las capacidades cognitivas (puntuación MPI), así como sobre volúmenes totales y regionales del cerebro y área de superficie cortical interna. Las variables dependientes se sometieron a un 2 (FO) x 2 (5-MTHF) entre los sujetos ANCOVA. Los análisis se controlaron por la edad materna, estatura y peso maternos, el Índice de Masa Corporal (IMC) de la madre, estado económico familiar, el tabaquismo (semana 20 y 30 de embarazo) y el peso placentario, sexo, lateralidad y volumen intracraneal total, incluyéndose en los diferentes modelos como variables confusoras.

En general se han utilizado los programas SPSS statistical program, version 20.0 (SPSS Inc. Chicago, USA), R statistical software (3.2.2 Version, "genetics" package), y STATA (StataCorp. 2011; Stata Statistical Software: Release 12).

En todos los casos, el nivel de significancia se estableció cuando $p<0.05$.

Resultados

El primer estudio realizado mostró que la suplementación con aceite de pescado (FO) y/o 5-MTHF durante el embarazo tiene efectos a largo plazo sobre el perfil de GPL-FA de las células de la mejilla de los niños hasta los 9.5 años. Además, se pudo comprobar que los polimorfismos de los genotipos *FADS1* y *FADS2* de los niños influyen en las concentraciones de los GPL-PUFAs obtenidas en las células de la mejilla; la mayoría de las asociaciones se establecieron entre diferentes polimorfismos de nucleótido simple (SNP) *FADS1* y *FADS2* con los ácidos linoléico (LA) y α-linolénico (ALA). Igualmente, se comprobó que los diferentes suplementos que recibieron las madres durante el embarazo en asociación con los antecedentes genéticos de sus hijos, afectan la composición de los FA de los hijos.

En un análisis posterior, se pudo demostrar que el perfil de ácidos grasos en los GPL de las células de la mejilla analizados en los niños escolares, está fuertemente relacionado con el país de origen, lo que sugiere un efecto de la dieta, el estilo de vida, etc.. Igualmente, se comprobaron diferencias estadísticas en la composición de los GPL-FAs de las células de la mejilla entre los 8 y 9,5 años de edad, tanto en niños españoles como alemanes. La ingesta diaria de ácido araquidónico (AA) mostró un alto impacto en el porcentaje de ácidos grasos encontrado en las células de la mejilla de los niños. La ingesta de lípidos y fibra determina una disminución del AA. La ingesta de ácido fólico y ácido EPA influye positivamente sobre las concentraciones de AA, EPA y ácido docosapentaenóico (DPA) en las células de la mejilla. La ingesta de DHA influye negativamente sobre las concentraciones de DPA en los GPL de las células de la mucosa bucal.

Se comprobaron asociaciones entre las concentraciones de ALA en los GPL y los problemas de externalización y de atención; el LA se asoció a problemas de atención, los ácidos grasos EPA, DHA y DPA se asociaron a mejor capacidad de juegos y competencias deportivas.

Otros análisis han demostrado que el tipo de suplementación materna durante la gestación no influyó sobre el comportamiento de los hijos a los 8 años; no obstante, 30 niños tuvieron problemas de internalización y 24 manifestaron problemas de externalización. Se estableció una asociación entre los problemas de internalización de los niños y la obsesión por la delgadez de sus madres; las madres clasificadas como patológicas mediante el EDI-2 en la sub-escala "*Obsesión por la delgadez*" (DT) de EDI-2 mostraron 1.138 veces más probabilidades de ser influyentes en los problemas de internalización de sus hijos.

Por último, se comprobó que la suplementación prenatal no se asoció a diferentes tamaños de la circunferencia craneal en ninguna edad, ni tampoco influyó sobre las habilidades cognitivas o los volúmenes totales cerebrales. A los 4 años se pudo demostrar la asociación más significativa del perímetro craneal con el volumen total de sustancia gris (GMV), el volumen total de sustancia blanca y el área de la superficie del cerebro; igualmente el tamaño del perímetro craneal a los 4 años se asoció a un mayor contenido de GMV en las áreas frontales, temporales y occipitales, así como al volumen del núcleo caudado, globus pallidum, putamen y tálamo [(24)].

Conclusiones

1. Los suplementos de FO y/o 5-MTHF durante el embarazo tienen efectos a largo plazo sobre el perfil de GPL-FA en las células de la mejilla de los niños en edad escolar. Se sugiere un efecto de programación de las vías metabólicas de los ácidos grasos, lo que puede causar diferencias en el estado de los ácidos grasos en los niños.
2. Los polimorfismos de los genotipos *FADS1* y *FADS2* influyen en las concentraciones de PUFAs en las células de la mejilla de los niños. La mayoría de las asociaciones se establecieron entre *FADS1* y *FADS2* SNP con LA y ALA.
3. La nutrición temprana, junto al genotipo de las *FADS1*, *FADS2* y *FADS3*, tienen efectos a largo plazo en las concentraciones de los GPL de la mucosa bucal, lo que sugiere una programación temprana de las vías metabólicas implicadas en el estado de dichos FA, impulsada por loa antecedentes genéticos
4. Las diferencias encontradas en el perfil de ácidos grasos de las muestras de células de la mejilla analizadas en los niños del estudio NUHEAL están fuertemente

relacionadas con el país de origen, lo que sugiere el efecto de la dieta, los estilos de vida, etc.

5. La ingesta dietética también parece influir sobre las concentraciones de ácidos grasos presentes en las células de la mejilla de los niños estudiados, especialmente la ingesta diaria de AA muestra un alto impacto en el porcentaje de ácidos grasos encontrado en las células de la mejilla de los niños.
6. Existe una relación entre ciertas características de los trastornos alimentarios maternos, como la "obsesión por la delgadez", y el posterior desarrollo de problemas internalizantes de conducta en sus hijos a los 8 años de edad. Esta relación determina que aquellas madres que tienen problemas relacionados con la obsesión por ser delgadas, aumentan la probabilidad de que sus hijos tengan un riesgo 1.138 veces mayor de problemas internalizantes de conducta en comparación con aquellos niños cuyas madres no muestran esta obsesión.
7. Hemos verificado que la medición del perímetro cefálico a los 4 años sirve como marcador del desarrollo cognitivo a los 10 años de edad. Además, esta medición antropométrica es muy útil desde el nacimiento hasta los 4 años, ya que este período se considera como el más sensible del crecimiento cerebral. Determinados factores posnatales, como la alimentación o el estado de LC-PUFAs, podrían tener un impacto más relevante sobre la maduración estructural de ciertas áreas corticales y núcleos subcorticales, y de forma independiente a los efectos determinados por la suplementación prenatal.

2. FINANCIAL BACKGROUND

2. Financial Background

The NUHEAL study is part of a larger multi-centre study undertaken to investigate the effects of n-3 long chain polyunsaturated fatty acid (n-3 LC-PUFA) supplementation during pregnancy on neurodevelopment and behaviour in children. The research reported herein is part of the NUHEAL follow-up within NUTRIMENTHE EU Project, supported by the Commission of the European Communities, within the RTD Programme "Quality of Life and Management of Living Resources". NUHEAL study has been supported by different EU Projects from 1999 till 2013, within:

- 5th Framework Programme (Contract No. QLK1-CT-1999-00888 NUHEAL "*Nutraceuticals for a healthier life*")
- 6th Framework programme (Priority 5.4.3.1. Food quality and safety. Contract No. 007036-EARNEST Project [*EARLY Nutrition programming-long term follow up of Efficacy and Safety Trials and integrated epidemiological, genetic, animal, consumer and economic research*].
- 7th Framework programme (Topic *KBBE-2007-2-2-01 - Effect of diet on mental performance of children*) NUTRIMENTHE: Grant agreement nº: 212652.

NUTRIMENTHE has brought together a team of leading international scientists from major research centres across Europe and beyond who are leaders in the key areas of nutrition and mental performance. The combination of expertise brought together by this project can be seen in **Figure 1**.

Figure 1. *Combination of expertise in NUTRIMENTHE Project.*

Some data from the NUHEAL cohort funded by the NUHEAL EU Project FP5 and the EARNEST EU Project FP6 have already been analysed and published. The most important ones related to my research, in chronological order, are the following:

- Decsi *et al.* [(1)] in 2005, reported that preliminary data on erythrocyte membrane fatty acid composition after fish oil (FO) and/or 5-metiltetrahydrofolate (5-MTHF) supplementation from the 20th week gestation to delivery showed a significant enhancement of erythrocyte DHA in maternal, placental and venous cord blood lipids.
- Linde J *et al* [(2)] in 2005, presented the study of the Spanish NUHEAL cohort showing higher docosahexaenoic acid (DHA) increments in the FO compared to the FO+5-MTHF group in plasma phospholipids, but when the mothers were supplemented with FO+5-MTFH a significant increment resulted in the triglyceride lipid fraction. Thus 5-MTHF may contribute to a preferential DHA incorporation into triglycerides rather than into phospholipids.
- Larqué *et al.* [(3)], in 2006, reported that correlation of the mRNA expression of the membrane placental proteins FATP-1 and especially of FATP-4 with maternal and cord DHA leads them to conclude that these lipid carriers are involved in placental transfer of long-chain polyunsaturated fatty acids.
- Krauss-Etschmann *et al.* [(3)], in 2007, published the effects of FO and/or 5-MTHF supplementation during pregnancy on plasma lipids, concluding that FO

supplementation improves foetal DHA and eicosapentaenoic acid (EPA) levels in plasma phospholipids (PLs) and attenuates depletion of maternal stores.

- Franke *et al.* [5], in 2008, analysed the dietary intake of DHA and folate in pregnant women in the three participating countries and concluded that dietary DHA and folate intake of pregnant women differs significantly across the three European samples. 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.
- Escolano-Margarit *et al.* [6], in 2011, assessed the effects of maternal DHA supplementation on the neurological development of their children. They found that higher DHA levels in cord blood might be related to a better neurological outcome at 5.5 y of age.
- Campoy *et al.* [7], in 2011, studied the long-term effects of n-3 LC-PUFA supplementation, 5-MTHF supplementation, or both in pregnant women on cognitive development of offspring at 6.5 y of age. They did not observe any significant effect of supplementation on the cognitive function of children, but maternal DHA status was related to later cognitive function in children.
- Gage H *et al.* [8], in 2011, explained that new mothers were asked about the extent to which lifelong health is influenced by diet as an infant, rather than by genetic predispositions or lifestyle and behaviour. They found that mothers considered their diet as an infant to be a less important influence on lifelong health than many lifestyle, behavioural, and environmental factors and genetics.
- Escolano-Margarit *et al.* [9], in 2013, investigated the effects of FO supplementation to pregnant women on the maternal and foetal fatty acid profile in plasma and erythrocyte phospholipids and to identify the best compartment for the assessment of fatty acid status. They concluded that FO supplementation increases maternal and foetal DHA status, and both plasma and erythrocytes appear to be suitable for evaluating the fatty acid status of mothers, but erythrocytes seem to be a more reliable marker of long term fatty acid status in neonates.
- Anjos *et al.* [10], in 2013, made a review, the objective of which was to provide a background and update on the current knowledge linking nutrition to cognition and behaviour in children, and to show how the large collaborative European Project NUTRIMENTHE was working towards this aim. These authors concluded that those

major nutritional problems that currently are having a huge impact on public health (e.g. obesity, diabetes, malnutrition) need multiple groups to work together in large national and international consortia to understand and solve the problems.

- Piqueras *et al.* [11], in 2014, investigated child size and dietary differences at the age of 4 between three European countries and to assess dietary adequacy. Their findings showed that Spanish children had a higher mean BMI compared with German and Hungarian children. The diet of Spanish children may be more obesogenic than those of German or Hungarian children. In this study, many children in all three countries were consuming diets that were high in protein, saturated fat and sugar.
- Catena *et al.* [12], in 2015, analysed the long-term effects of FO, 5-MTHF, or FO+5-MTHF prenatal supplementation on attention networks. They concluded that folate supplementation during pregnancy, rather than FO or FO+5-MTHF supplementation, improves children's ability to solve response conflicts. This advantage seems to be based on the higher activation of the midcingulate cortex, indicating that early nutrition influences the functionality of specific brain areas involved in executive functions.
- Gispert-Llaurado *et al.* [13], in 2016, assessed the relationship between fish consumption, estimated dietary n-3 LCPUFA intake and cognition and behaviour in childhood in a multi-centre European sample. The results showed that children who consumed 2 fish meals per week, including one of oily fish, were less likely to show emotional and behavioural problems than those who did not.
- Ortega *et al.* [14], in 2016, aimed to examine the association of the main health-related physical fitness components with the shapes of sub-cortical brain structures in a sample of 44 Spanish children aged 9.5 years of age. Their results supported the fact that cardio-respiratory fitness is the strongest predictor of brain shaping in children; yet significant and positive associations were also observed for muscular strength (more for relative than for absolute strength) and speed-agility.

3. INTRODUCTION

3. Introduction

There is enough evidence regarding the role of nutrition in early stages of life on the modulation of growth and human development that may exercise lasting effects on health and the quality of life in adulthood. Numerous experimental and epidemiological studies have shown that nutrition in the prenatal and postnatal stages will have a great impact on the development of metabolic diseases, such as obesity or type 2 diabetes, cardiovascular disease, hypertension, cancer, etc. or alterations in cognitive development and behaviour. David Barker established the theory of metabolic programming by linking low birth weight with rapid postnatal weight gain, high risk of coronary heart disease, hypertension, insulin resistance and diabetes at adult age [15-17]. Foetal exposure to maternal dietary components, such as calcium, folic acid, magnesium, high or low levels of protein and zinc, has been linked to birth weight. Other studies have shown that certain conditions such as obesity or maternal diabetes, related to the birth of children of high weight for gestational age, will affect in the early stages in the development of metabolic functions in different organs and systems of their children, increasing the risk of developing obesity, glucose intolerance, type 2 diabetes, hypertension, mental illness, cognitive deficits, etc. [18]

There is consistent evidence that LC-PUFAs are important for ensuring optimum growth and development, particularly during foetal and early postnatal life [19], especially important are n-3 LC-PUFAs [20].

Furthermore, in combination with nutrition, there are genetic and other environmental factors involved in a child's development, which will influence their cognition and behaviour. Environmental factors such as socioeconomic level, school, media or even the behaviour of other members of the family seem to have long- term effect on their behaviour.[21].

3.1. Fatty acids

Fatty acids (FA) are predominately non-polar molecules consisting of a long chain of carbons with an oxygen and a hydroxyl group at one end. Fatty acid chains differ inlength, often categorized as short to very long. Short-chain fatty acids are fatty acids

with aliphatic tails of five or fewer carbons. Medium-chain fatty acids are fatty acids with aliphatic tails of 6 to 12 carbons, which can form medium-chain triglycerides. Long-chain fatty acids are fatty acids with aliphatic tails of 13 to 21 carbons. Very long-chain fatty acids are fatty acids with aliphatic tails of 22 or more carbons.

It is said that a fatty acid is saturated if each carbon is joined to its neighbouring carbon by a single bond. If one or double bonds are present, the FA is said to be unsaturated. If more than one double bond is present, the FA is said to be polyunsaturated (PUFA).

Unsaturated fatty acids are classified based on the position of the first double bond from the end of the chain. The counting starts at the end of the fatty acid molecule without the oxygens.

3.2. Long-chain polyunsaturated fatty acids (LC-PUFA)

Metabolism

LC-PUFAs are classified into two principal families, the n-6 (or ω-6) and the n-3 (or ω-3), according to the position of the terminal double bond. The parent FAs of these families, linoleic acid (LA, 18:2n-6) and α-linolenic acid (ALA, C18:3n-3), cannot be synthesized by humans and so, they have to be provided by the diet and are therefore defined as essential fatty acids (EFA). LA and ALA serve as substrates for the synthesis of other important fatty acids and are in turn precursors of arachidonic acid (AA, 20:4n-6), docosahexaenoic acid (DHA, 22:6n-3) and eicosapentaenoic acid (EPA, 20:5n-3). [22, 23] (**Figure 2**).

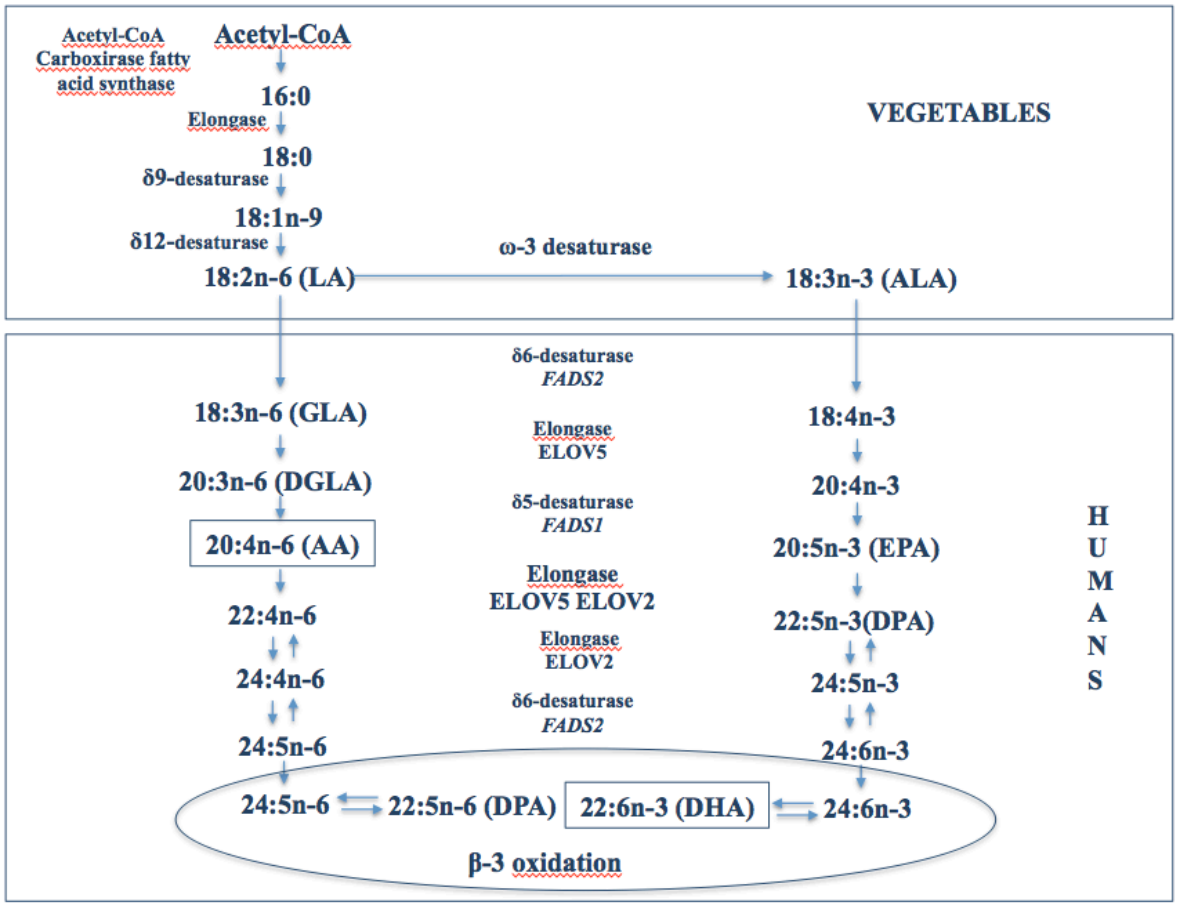


Figure 2. *Metabolic conversion of the essential fatty acids to long-chain polyunsaturated fatty acids (Modified by Koletzko et al. J Perinatal Med 2008).*

n-3 and n-6 PUFAs are desaturated and elongated via the same pathway [24-26], causing competition between the two families; so, the δ6-desaturase (δ6-D) is rate-limiting converting ALA to EPA and DHA, and LA to AA and docosapentaenoic acid (DPA, 22:5n-6), respectively [27]. The affinity of the δ6-D for ALA is greater than for LA, but the concentrations of LA are typically higher [27]. High intake of ALA usually only increases EPA [22]. The individual capability to form LC-PUFAs has been shown to depend on the genotype of fatty acid desaturase 1 (*FADS1*) and fatty acid desaturase 2 (*FADS2*), both located on chromosome 11 (11q12-q13.1), which encodes δ5-desaturase (δ5-D) and δ6-D, respectively [23, 28]. There are 18 SNPs reported in public databases in the *FADS1 FADS2* cluster as being polymorphic [29] (**Figure 3**).

On the other hand, the human desaturase-encoding genes (*FADS1* for δ5-D and *FADS2* for δ6-D) are arranged in a head-to-head orientation and build a gene cluster on chromosome 11 together with a third desaturase gene, *FADS3* [30]. FADS3 function, however, has not been completely characterized so far [31]. Park *et al.* (2009) identified several alternative splice forms of *FADS3* and hypothesized that *FADS3* might have a tissue- or PUFAs-specific role in LC-PUFAs synthesis.

Several studies have demonstrated the association between variants in the human genes *FADS1*, *FADS2* and *FADS3* and blood levels of PUFAs [30-36], provide some evidence. It seems that genetic variation in the FADS gene cluster may modify desaturase function supporting that minor alleles increase the concentrations of LA and ALA, and decrease the concentrations of AA, EPA, DPA and DHA in serum, plasma and erythrocyte. Glaser *et al.* [35] found that *FADS1* and *FADS2* gene variants modulate tracking of serum PUFA levels in glycerophospholipids (GPLs). GPLs are the main lipid constituents of cell membranes and their key functions are to give structure to the cell membranes, preservation of intracellular messengers, anchoring some proteins to cell membranes, stabilization of protein structure and they are enzyme cofactors. Some authors report that *FADS* gene variants account for up to 28.5% of the variability in the PUFAs and LC-PUFAs levels in human tissues [10].

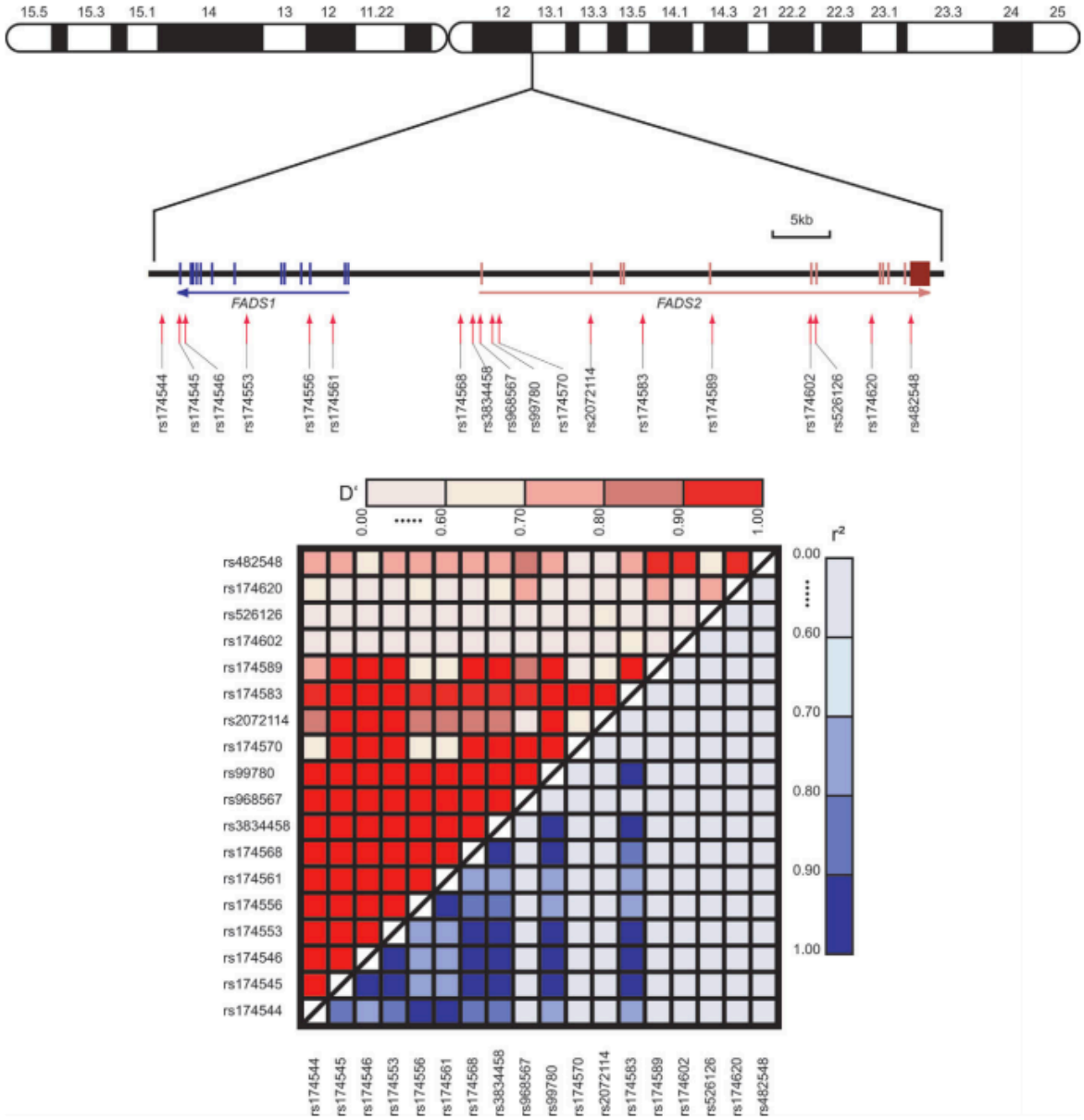


Figure 3. *Structure of FADS1 and FADS2 gene cluster, its location on chromosome 11 and pairwise LD D' and r^2 plots of 18 SNPs across the FADS1 and FADS2 gene cluster.* [29]

Biomarkers for the assessment of LC-PUFA status in humans

In recent years there has been an increasing interest in the status of fatty acids and the best way to determine them due to the importance of LC-PUFA in foetal development and the early stages of life. Fatty acids can be measured in various blood fractions and tissues [37, 38].

The fatty acid status in humans depends not only on intake, but also on non-dietary factors. Metabolic and genetic factors, as well as lifestyle determinants, may affect fatty acid concentrations in human tissues. Interventional studies show that mothers consuming n-3 PUFA supplements have higher n-3 PUFA levels in plasma and erythrocytes, both during pregnancy and at delivery [39-43].

To assess the FA status of an individual there are several options available. However, when determining which body compartment reflects a better measure of FA, the type of study is a major consideration. It has been suggested that FA composition of cell membranes is less influenced by short-term changes in FA intake, given that FA in erythrocyte phospholipids have a slower turnover than plasma lipoproteins [44-46]. Therefore, erythrocyte membrane phospholipids (PLs) may be a more reliable measure in the evaluation of long-term FA intake.

All these approaches are invasive and not well accepted in population studies, particularly in infants and young children. In this study we have evaluated, using a non-invasive technique [47], cheek-cell GPL-FA composition in children from the NUHEAL cohort.

Effects of n-3 LC-PUFA supplementation on fatty acid status

Several intervention studies with different n-3 FA have been conducted in order to increase the LC-PUFA status of individuals. Nevertheless, even high doses of ALA result in only modest increases in ALA plasma concentrations, small changes in EPA and no effect on DHA under conditions of typical western diets [22, 48]. Studies with fish-oil supplements containing both DHA and EPA have also been conducted. They all agree that FO supplementation increases EPA and DHA levels in plasma and in erythrocyte PLs, although more slowly [48, 49]. Dose-response analysis of FO supplementation suggests a near linear increase in plasma EPA concentrations and an apparent saturable increase in DHA concentrations after supplementation in normal individuals. AA concentrations also showed a dose-dependent decrement in response to FO supplementation [22].

The effects of n-3 PUFA supplementation in tissues have also been reported in animal and human studies. DHA supplementation has been demonstrated to produce a dose

dependent increase in erythrocyte membrane DHA concentrations. In addition, strong correlations have been reported between plasma PLs and erythrocyte membrane content of fatty acids, especially DHA and EPA [22, 50]. Furthermore, animal studies have shown increased content of DHA and a concomitant AA decreases in brain, retina, heart, liver, skeletal muscle, red blood cell and bone marrow of DHA supplemented animals [22]. These studies suggest that as well as plasma EPA and DHA, tissue concentrations of these fatty acids increase after supplementation.

From the above description it could be concluded that erythrocyte membrane fatty acid levels may be a better biomarker for the long-term intake of n-3 FA, whilst plasma is more sensitive to short term changes in their intake.

As I mentioned previously, the fatty acid status of humans depends not only on intake, but also on non-dietary factors, such as genetic and metabolic factors and lifestyle determinants. All of them may affect fatty acid metabolism and therefore concentrations in human tissues.

Finally, McMurchie *et al.* [51] reported that changes in dietary FA compositions were reflected in cheek cell PLs and suggested those as an alternative marker for FA status. Later studies focusing on LC-PUFA uptake confirmed these findings. Diets rich in LC-PUFAs increased infantile DHA and eicosapentaenoic acid (EPA) levels in erythrocytes, plasma and cheek cell phospholipids [52-54]. DHA contents in all compartments were significantly correlated. This was also shown in a study with infants (age\12 months) who received DHA and arachidonic acid (ARA) with an infant formula [55]. A large intervention study in school children showed a positive relationship of n-3 supplementation and cheek cell DHA levels in cheek cell total lipids [56].

3.3. Brain development

Neurobiological development

Brain development is a process that is dependent on the timely orchestration of external and internal inputs through sophisticated intra- and intercellular signalling pathways. Brain development is a complex interactive process in which early disruptive events can have long-lasting effects on later functional adaptation [57].

The primitive neural tube is formed during the first 4 weeks, followed by a period of further cell proliferation and diversification into neurons and glia (5–18 weeks). At the same time there is neuronal migration from the ventricular germinal zone to the final position. Migration has a role in establishing the identity of some neurons and may define their functional properties and future connections. Differentiation of the neurons occurs over a span that encompasses the late prenatal period as well as the postnatal period. It comprises axonal and dendritic outgrowth, as well as the expression of enzymes and receptors for specific neurotransmitters. Myelination is a late-occurring process, starting in the third trimester and extending into the third decade and possibly beyond. During the last trimester there is also what is referred to as an 'exuberant' increase in the number of synaptic contacts. This process is followed by a series of regressive events, including programmed cell death (apoptosis) and synaptic and axonal pruning. These processes, which continue through the second decade of life, are influenced strongly by environmental input, and are thought to be the mechanisms underlying the establishment of circuitry that is functional in terms of the brain's adaptive capacity, including behaviour. Where it was thought previously that neurogenesis and synapse formation occur only during brain development, there is recent evidence of neuronal generation in adult mammalian brains, including those of primates [58, 59].

Anthropometric measurements are routinely performed in paediatric settings and are used around the world as health and development indexes during infancy and childhood [60]. In particular, height, weight, and head circumference have been associated to neurodevelopmental outcomes [61]. Studies focused on head circumference have special relevance to explain neurodevelopment, where this is used as an indicator of brain size. During prenatal and early development and maturation, brain growth is the major force for the increase of intracranial volume and head size [62, 63]. This process continues throughout childhood and ends in early adulthood, when brain size begins its decline [64-66]. Thus intracranial volume is highly associated with total brain volume during early life [62] and, therefore, head circumference is used as an accurate index of brain size in normally developing children [67, 68]. For 6-year-old children and younger, the correlation between head circumference and total brain volume is very strong [67], but

decreases for subjects aged 12 and older [69]. Developmental changes associated to head growth and skull thickness probably contribute to this general decrease in correlation [67]. Moreover, typical brain maturation encompasses distinct stages with fast and slow progressions depending on age [70]. Therefore, the child's age and growth curve are crucial when estimating brain size from head circumference. Additionally, head circumference has also been associated to future cognitive performance in high-risk individuals (e.g. low birth weight or premature infants) [71], as well as for full-term and normally developing infants [68, 72]. Thus, head circumference measurements at various postnatal ages are differentially associated with either motor or cognitive outcomes [73]. Moreover, the child's behaviour repertoire, in a given time, would be related to functional activity over a specific brain region [74]. However, the ability of head circumference measurements during childhood to predict regional cortical and sub-cortical brain volumes is unknown. Actually, no study to date has demonstrated that head circumference – measured at consecutive ages – is linked to specific distributions of gray matter in healthy children.

Throughout the lifespan, but also during foetal development, biological (e.g., sex) and environmental (e.g., family status) factors affect head and brain growth [75, 76] through genetic mechanisms or distinct nutrition and/or stimulation [77]. However, most of the studies relating head circumference and brain size have not taken into account these factors. In particular, prenatal micro-nutrients, such as LC-PUFAs and folate, are important for the development of the intrauterine central nervous system (CNS) [78], and their effects on head and brain growth might go beyond the foetal life [79]. A great deal of evidence shows that supplementation with LC-PUFAs [80] and folate [81] not only impact during the prenatal stage, but also effect head growth during the postnatal stage. Moreover, these micronutrients might enhance cognitive development, even beyond early infancy [7, 82]. In addition to these confounding factors, previous studies focused on the age-dependent estimates of brain size from head circumference have been cross-sectional in nature. Head growth, similarly to what happens with brain maturation, occurs in a variable sequence of relatively slow and fast periods [83]. Therefore, to properly examine the predictive value of head circumference to know later brain size,

multiple measurements from the same individual should be acquired and several confounding factors should be taken into account.

Behavioural development

The brain is not a homogeneous structure, and the timing and course of maturation varies considerably among brain regions [(84)]. The anatomical and functional specialisation of neurons proceeds from the last trimester until early adulthood, and it has been suggested that this protracted period of brain development may be the origin of human behavioural flexibility [(85)]. The function of the brain depends ultimately on the operations of neural systems that are established by the patterns of synaptic connections among neurons. Behavioural neuroscience addresses the factors that, at an organismic level, influence the establishment of these neural systems. This endeavour cannot be accomplished independently of a developmental perspective, because events (both internal and external to the organism) that occur early in the development of the nervous system influence the final pattern of connection between nerve cells.

Emotional and behavioural problems in childhood

Emotional and behavioural problems are common issues in childhood and adolescence. Epidemiological studies estimate a prevalence of between 10 and 20% of childhood psychopathology [(86)]. The family and the child's environment, especially school and peers have to be assessed throughout the diagnostic and therapeutic process. Some of the psychosocial issues which may affect children behavioural development are [(87)]:

- *Socio-cultural environment*: creates the false impression that everything is possible with current scientific and technical resources (drugs, machines, etc.). The value of effort and time needed to manage adaptation and overcome conflicts is underestimated. This helps children to grow up with a low tolerance to frustration and accept effort with difficulty.
- *Family structure:* this is changing rapidly, with more and more separations and divorces in many European countries, there are more stepfamilies, more varieties and types of family than in the past.
- *Body cult and female models* (slim and slender women) increase internal displeasure pathologies, such as anorexia nervosa and bulimia.

- *Current difficulties to think independently*: this symbolizes and releases the internal stress in the form of emotions and feelings. The body is then used as the preferred means of expression of conflict.

Childhood psychopathological disorders can be seen as a continuum from mild cases to very severe processes. Psychological disturbances in children have traditionally been classified into two basic patterns of psychological problems: behavioural and feelings [87]. Alterations of emotions or internalizing syndromes are related to mood instability, obsessions, somatic problems, nervousness, insecurity, fears, phobias, sadness, apathy, dysphoria, anxiety, tension, worry and guilt amongst other symptoms. The American Psychiatric Association (APA), based on the various proposals made by successive Diagnostic and Statistical Manuals of Mental Disorders (DSM) [88], used to diagnose internalizing disorders with the presence of three pure syndromes resembling the disorders in adults (emotional problems, anxiety problems and somatization). However, empirical taxonomies show that the internalizing symptoms do not appear as clearly defined in childhood and adolescence, and propose mixed groups of symptoms. Achenbach *et al*. [89] have isolated three empirical internalizing syndromes that define these types of behavioural problems during these ages: anxiety/depression, withdrawal/depression and somatic complaints [86].

The internalizing symptoms can and usually manifest in childhood with externalizing behaviour disorders, such as these types: irritability, impulsivity, aggression, inattention, disruptive behaviour, etc., being less visible to adults. Externalizing problems, however, are more visible in the family and school context, expressed by disruptive behaviour that interferes with the functioning of children in these environments, testing tolerance and ability to regulate this behaviour [89].

Other important behavioural problems nowadays are eating disorders (ED), which are complex disorders comprising two types of behavioural disorders: some directly related to food and weight, and other derivatives of the relationship with oneself and others. These disorders have become emerging problems in developed and developing countries [90].

The development of this behaviour influences biological, psychological, familial and socio-cultural factors, although the relative contribution of each factor can vary greatly between heterogeneous population groups affected. However the “beauty model” of our society has influenced the habits of eating behaviour (advertising, media, "miracle diets", fashion models); they are multidimensional disorders and have multiple causes. These disorders are due to the sum of clinical aspects considered characteristic of such pathologies: obsession with thinness, bulimia, body dissatisfaction, perfectionism, inefficiency, interpersonal distrust, interoceptive awareness, asceticism fear of maturity, impulsiveness and social insecurity [91].

Before offering a formal diagnosis of a disorder of eating behaviour, it is interesting to consider the observation and accurate assessment of certain psychological traits or sets of symptoms that are supposed to be relevant to understand and to treat these eating disorders.

Different studies have correlated the behaviour of mothers with their children [92, 93]. In this paper, which is part of the NUHEAL-follow-up, paternal and maternal factors are also taken into account as well as supplementation in pregnancy, which may influence the results on neurodevelopment, behaviour and other secondary studied variables. Eating behaviour and the psychological status of mothers is evaluated, considering some characteristic factors of eating disorders, although the potential impact on their children’s behaviour in the long term has not yet been studied. Other studies demonstrate an influence on the development of typical behaviour linked to eating disorders in children whose mothers are have previously been diagnosed with any of these disorders [94]. Amongst the typical behaviour patterns are: perfectionism, intraceptive awareness, impulsivity, asceticism, fear of maturity, social insecurity, interpersonal distrust, inefficiency and other weight-related food and body appreciation.

The progressive increase in cases of eating disorders and the clear influence of mothers on their children brings up an incipient question as to whether the existence of some of the typical characteristics of these food and psychopathological problems in mothers may also influence their children. These and other factors, such as diet before and during pregnancy, breastfeeding or formula and family environment, amongst others,

have been proven as influential in the development of children's behaviour in the long term.

3.4. Nutritional neuroscience

There is a substantial literature that indicates that the chemistry and function of both the developing and mature brain can be influenced by diet [95]. Direct effects on development are likely to be related to alterations in levels or timing of the various necessary growth factors, including the neurotransmitters. In the adult nervous system neurotransmitters mediate intercellular communication by activating specific receptors and second-messenger systems. In the developing system, however, they also function as trophic factors to regulate neurogenesis, neural migration and synaptogenesis [96, 97]. Many of these neurotransmitters are derived from nutrient precursors; tyrosine is the amino-acid precursor of dopamine and noradrenaline, tryptophan of serotonin and choline of acetylcholine. In some cases they may also require the presence of micronutrient cofactors such as minerals and vitamins. Dietary components frequently interact. This process can be seen in the studies, which show that the levels of tyrosine and tryptophan in the brain are related to the relative levels of protein and carbohydrate in the diet [95].

On the other hand, it is interesting for our study to mention that there are two possible strategies when studying nutritional effects on behaviour. The first is based on the assumption that dietary-induced changes at a molecular or cellular level will necessarily have functional consequences. The challenge then becomes that of identifying the appropriate functional outcome to be measured. In the interests of hypothesis-driven enquiry, the best approach is to measure an outcome known to be associated with a particular biochemical pathway. For example, the literature on the role of the prefrontal dopamine system suggests that tests of short-term or 'working memory', i.e. memory relevant to solving a unique problem embedded in a particular task (as opposed to reference memory, which refers to learning the rules associated with the task generally) might prove particularly informative [98]. In the second strategy the approach used is similar to that used in neurotoxicity testing, where comprehensive batteries of tests are used to assess performance across a wide range of behavioural outcomes. In addition to

tests of sensory capacity and emotional reactivity, these batteries include various measures of learning and memory, as well as species-typical behavioural adaptations. Such test batteries are usually not based on a specific hypothesis, because they were designed as a screening tool. This approach, together with the number of tests they encompass, leads to a concern with respect to an increased probability of false positive findings. However, they may serve to direct attention to the possible neural systems involved.

Moreover, there has been a renewed interest in the role of specific nutrients, e.g. choline or Fe, on brain and behavioural development [(99)]. The following section addresses the role of dietary lipids in this regard, specifically that of the LC-PUFA.

Importance of LC-PUFA in brain growth and development

All nutrients are important in neuronal growth and brain development although some appear to be particularly important, the LC-PUFA. They play important functions during pregnancy, lactation and infancy as they are the constituents of the phospholipids of cell membranes and the synthesis of eicosanoid precursors [(100)].

Lipids comprise 50–60 % of the dry weight of the adult brain, of which approximately 35 % are in the form of LC-PUFA, mainly AA and DHA, as I mentioned before. These LC-PUFAs are derived through biosynthesis from their respective dietary essential fatty acid (EFA) precursors, LA and ALA, or they can be obtained directly from dietary sources such as eggs, fish and meat or, more recently, from single-cell oils. AA is found at relatively high levels in many tissues, whereas DHA is found at high levels in only a few tissues outside the central nervous system, such as the testes, but it represents a high proportion of the lipids of the retina and grey matter of the brain. AA and DHA accrue rapidly in the human brain during the third trimester and the early postnatal period, when there is the maximum rate of brain growth. There is controversy over whether infant formulas that contain only LA and ALA are sufficient for optimum brain development, or whether additional preformed AA and DHA, such as found in human milk, are necessary for ensuring the long-term integrity of functional outcomes [(101, 102)].

Some animal models address the relationship between dietary FA composition, brain growth and FA composition and behaviour. Severe combined deficiency of both n-3 and

n-6 FA, i.e. EFA deficiency, results in aberrant physical and behavioural development. It has been demonstrated in mice that not only EFA deficiency, but also an extreme dietary fatty acid imbalance, in terms of high levels of DHA with low levels of n-6 FA, also resulted in growth retardation, accompanied by impaired swimming ability in the Morris water maze, unless a portion of the n-6 fatty acids were provided in the form of AA. A subsequent study showed that the growth retardation was accompanied by behavioural retardation of a magnitude equivalent to that resulting from moderate malnutrition [(103)]. These effects may be related to the role played by AA in the signalling actions of neuro-endocrine cells, where there is evidence for its involvement in the release of both growth hormone and prolactin [(104)]. The message to be taken from this information is that not only absolute, but also relative, dietary FA content is important to developmental status. This factor is particularly important to bear in mind as the technology of food production changes, as seen in genetically-engineered oils with very much higher concentrations of specific biologically active FAs than have so far been available.

Although dietary deficiency of n-3 FA during development does not appear to affect growth, it does lead to characteristic changes in brain fatty acid composition, which include a decrease in DHA and a reciprocal increase in DPA. There is also a reciprocal relationship between levels of dietary AA and DHA and those in the brain. There is also a non-linear relationship between DHA in brain and erythrocyte phospholipids such that there was a levelling off of brain levels at the higher levels of DHA in the blood. This finding would lead one to expect that functional outcomes might show similar dose-response effects, where the largest differences would be between animals fed deficient and adequate diets, with little or no variation above the threshold that could be said to define an adequate diet [(104)].

3.5. Folic acid and brain development

Another important nutrient in the early stages of development is folic acid or vitamin B9, necessary for the formation of structural proteins, maintenance of new cells and an important cofactor of homocysteine methylation for producing methionine.

The correct intake of folic acid during pregnancy is critical during periods of cell division, affecting amongst other things, the proper development of the nervous system. Maintaining adequate levels of folic acid in women in the pre-conception period and during the first trimester of pregnancy protects the baby against birth defects, with neural tube defects being the most well known [105]. Poor dietary folate supply leads to increased plasmatic concentration of homocysteine, also related to placental fissure, miscarriages, high levels of low birth weight, premature disorders [106] and neuro-conductual development [107].

Although folic acid is found in various foods (spinach, endive, peas, dried beans, fortified cereals, nuts, sunflower seeds and so on), it is difficult to get the necessary contribution required to meet the high demands during pregnancy, so there is a broad consensus on the need for both pre-conceptional and first trimester of gestation nutritional supplementation of 400 µg/day [108].

A recent study about attention network performed behavioural assessment, neurophysiological and specialised neuroradiological investigations in 136 children aged 8.5 years who were born to mothers supplemented with 5-MTHF and/or fish oil (FO) during pregnancy, has shown that folate supplementation during pregnancy, rather than FO or FO+5-MTHF supplementation, improves children's ability to solve response conflicts. This advantage indicates that early nutrition influences the functionality of specific brain areas involved in executive functions [12].

Some studies have indicate that the recommendations for folate intake in toddlers, in particularly that of 150 µg folate/day (US RDA & WHO RNI)[109, 110] , may be unnecessary high. Recently, it has been reported that 35% of healthy Norwegian toddlers at 24 months of age did not meet the age appropriate Norwegian recommendations for folate intake (80 µg folate/day) [111]. Despite the overall low dietary intake of folate, the proportion of children with low serum folate concentrations (<10 nmol/L) was only 5.7%, and they had a modest rise in tHcy concentrations, a sensitive marker of folate status in children ≥24 months of age.

In children, folate deficiency is related to impaired cognitive development [112] and increased diarrhoeal and respiratory disease [113, 114]. However, folic acid supplementation does not help to decrease these pathologies [115].

4. AIMS

4. Aims

The aims of this thesis are divided into 3 issues:

Children's composition of fatty acids in cheek-cell glycerophospholipids was determined at school age with the following aims:

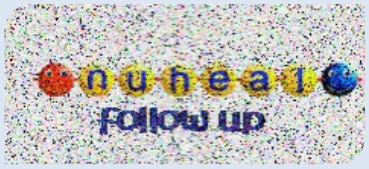
1) To analyse the long-term effects of maternal supplementation during pregnancy with DHA and/or 5-MTHF on LC-PUFA concentrations in their offspring cheek-cell glycerophospholipids at 8, 9 and 9.5 years.
2) To evaluate whether *FADS1*, *FADS2* and *FADS3* gene clusters may modify PUFA concentrations in children's cheek cells.
3) To explore the interaction between the described intervention in the mothers during pregnancy and their offspring's FADS polymorphisms on children's LC-PUFA concentrations in cheek-cell glycerophospholipids.
4) To examine the associations between diet and cheek-cell GPL-FA composition, and how these associations vary in relation to age, sex, country of origin and *FADS*.

Evaluation of the relationship between children FAs status, mothers eating behaviour and children behavioural problems at 8 years of age. The EDI-2 was performed on the mothers and CBCL on their offspring, in order to achieve the following objectives:

5) To demonstrate potential associations between fatty acid status and behavioural problems in children.
6) To establish to what extent the mother's feeding behaviour and psychological characteristics may influence the behaviour, both internal and external problems, of their children at eight years of age.

Finally, at 9.5 years of age, the Spanish children participating in the NUHEAL study were examined by MRI to obtain brain neuro-imaging, with the final objective:

7) To explore the relationship between several head circumference measurements from birth till 10 years old and brain distributions of grey matter volume and sub-cortical volumes in later childhood.



5. MATERIAL AND METHODS

5. Material and Methods

5.1. Study design

The study participants were the cohort of women taking part in the NUHEAL study and their respective children. The subjects taking part in study consisted of a prospective cohort of pregnant women from three different European countries: the University of Granada (Spain), the Ludwig Maximilians University in Munich (Germany) and the University of Peçs (Hungary). Eligible for the study were pregnant women who fulfilled the inclusion criteria and were not participating or were going to participate in any other clinical trial:

- Apparently healthy women with uncomplicated singleton pregnancies in < 20 week of gestation before enrolment, the expected delivery date and gestation week were determined by using Naegeles's rule.
- Intention to give birth in one of the centres participating in the study.
- Aged between 18 and 40 years at study entry.
- Body weight between 50 and 92 kg at study entry.
- No use of any n-3 fatty acid supplements since the beginning of pregnancy.
- No regular use of folate or vitamin B12 supplements after the 16th week of gestation.

The women were recruited between November 2001 and March 2003, between the 12th and the 20th week of gestation. Briefly, the NUHEAL study is multi-centre (Spain, Germany, Hungary), randomized, double-blind placebo controlled study [(4)]. From week 22 and up to the time of delivery, 315 pregnant women received 4 different nutritional supplements, together with vitamins and minerals in amounts meeting the recommended intakes during the second half of pregnancy for European mothers (109), that were assigned randomly: 500 mg/day of DHA + 150 mg/day of EPA, or 400 µg/day of 5-MTHF, or both or the placebo (**Figure 4**).

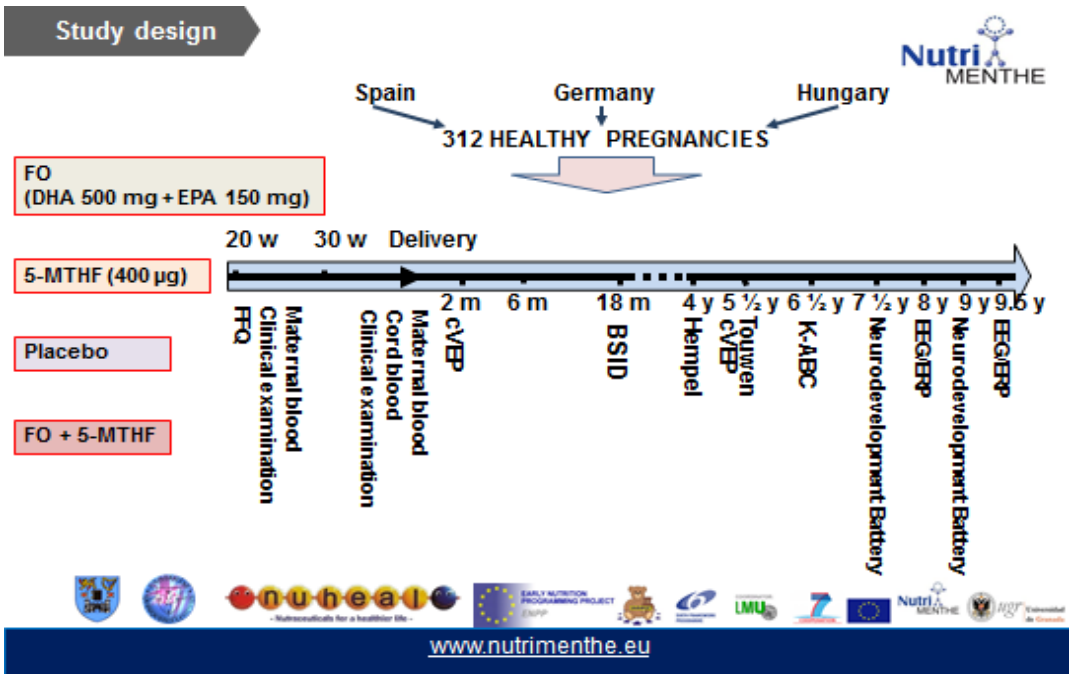


Figure 4. *NUTRIMENTHE Study design.*

From week 20th of pregnancy until 6.5 years of age several information were collected and examinations were performed (**Table 1** and **Figure 4**).

At the age of 7.5, age in which start the study in the present thesis, participating mothers and their infants were again approached and asked to participate (NUHEAL into NUTRIMENTHE EU Project). 156 NUHEAL children were back for a new examination at 7.5, 8, 9, 9.5 and 10 years of age. A summary of the tests and examination are showed in **Table 1**.

From 7.5 to 9.5 years old the drop-out rates were 5.41% at 8 and 54.21% at 9 and 87.23 at 9.5 years of age. The physician lost contact with 5 children, 22 renounced to continue, one mother died and one family moved to another city.

Medical Ethics Committees of the 3 centres participating in the study approved the study protocol. After careful explanation of the study details, written informed consent was obtained from all participants at study entry, at 4 and 7.5 and at 10 years old (only Spanish) years of age.

Table 1. *List of tests performed in children along the examinations programed in the NUHEAL Follow up.*

AGES	MEASURMENTS
1, 2, 3, 4, 6, 9, 12, 18 months	Anthropometry
2, 18 months	Visual Evoked Potentials
18 months	Bayley Mental Development Test
4, 5.5, 6.5 years	Hempel, Touwen, Kaufman Tests Allergy Questionnaires Anthropometry Health Questionnaires
4, 5.5, 6.5 7.5, 9 years	Food Frequency Questionnaire
7.5, 9 years	Neurodevelopment Assessment Child Behavior Check List (CBCL) Medical History Food Frequency Questionnaire Body Figure Perception
8, 9.5 years	Cognitive Potentials (EEG/ERP) Anthropometry Bioimpedance Armband Senseweare: Physical Activity Registry Physical Activity Questionnaire Dietary Intake (3 days) Questionnaire of food habits and parental attitudes to child feeding Eating Disorder Inventory (EDI-2)
10 years (only Spanish)	Magnetic Resonance Imaging (MRI)

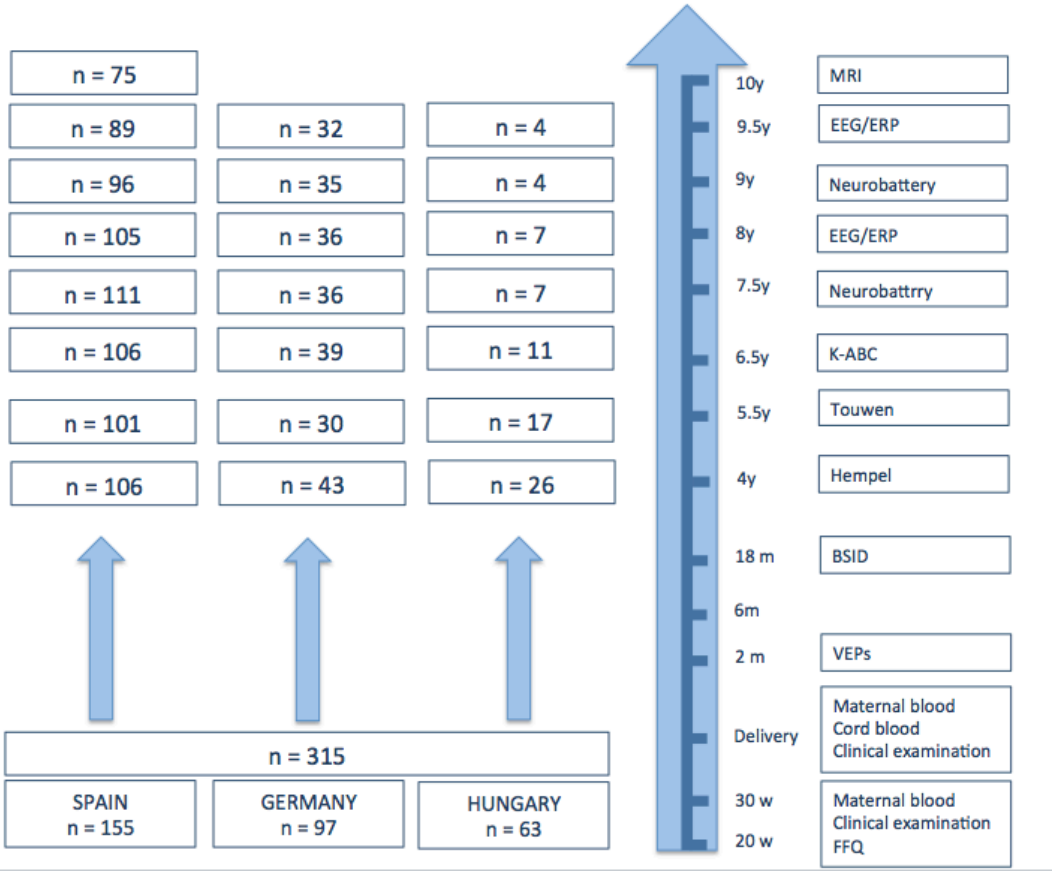


Figure 5. *Summary of drop-out rate until 10 years of age.*

5.2. Data and biological sample collection

Data and biological samples from participating women were collected at study entry, prior to the beginning of supplementation in the 20th week of pregnancy, as well as in the 30th week of gestation and at delivery. Socio-demographic data were obtained at study entry with standardized questionnaires, which included information about maternal age, parental ethnicity, residence area, education level, type of work and maternal smoking habits as well as information about parental diseases. Dietary information was collected by FFQ at the 20th and the 30th week of gestation. A trained physician who performed standardized examinations and interviews during the 20th and 30th week of gestation collected clinical information on the course of pregnancy. Besides, biological material was obtained from mothers at the 20th and 30th week of pregnancy and at delivery.

Data on the infants of the participating women were collected at birth, at the age of 8 weeks, at the age of 6 months and at the age of 18 months (Spain). At birth a trained physician examined the babies and obtained data on the infants in standardized reports. At the age of 2 and 6 months a physical examination and anthropometric measurement of the infants were performed and data on post-natal diseases and visits to paediatrician were also obtained.

The children were again evaluated at 4, 5.5 and 6.5 years of age. The children's neurological development was assessed at the age of 4 with the Hempel, at 5.5 with the Touwen examination and at 6.5 with the K-ABC. In addition, a trained physician explored and measured the children and obtained information on the children's health status in standardized health screening questionnaires during each visit.

The children were also evaluated at 7.5, 8, 9 and 9.5 years of age, when a trained physician explored and measured the children and obtained information on the children's anthropometric measurements and bio-impedance. At these four ages the physician collected check cell samples following the method developed by Klinger *et al.* [116]. The NUTRIMENTHE Neurodevelopment Battery [117] was assessed at the ages of 7.5 and 9. In addition, the Medical History, Food Frequency Questionnaire (FFQ) and Body Figure Perception were assessed. At the ages of 8 and 9.5, the following measurements were carried out: cognitive potential (EEG/ERP) Armband Sensewear (physical activity registry), physical activity questionnaire, dietary intake (3 days, questionnaire of food habits and parental attitudes to child feeding, questionnaire of food habits and parental attitudes to child feeding, eating disorder inventory EDI-2 and nuclear magnetic resonance measurements (NMR) (only for Spanish children) (**Table 1**).

5.3. Cheek-cell sampling procedure

The sampling procedure for children at 8, 9 and 9.5 years of age consisted of mouth cleaning with 200 ml tap water three times prior to sampling, followed by rubbing each inner cheek side with a Bio Brush (Ref. 73506013). Subsequently, the mouth was rinsed with 10 ml pure water, and the rinsing solution containing cells washed off from the inner cheeks was collected in a tube. The brush used for scraping was inserted in this

tube for a few seconds and after removing the brush, this tube was closed and centrifuged at 1,400 x g (2710 rpm) for 10 min at 4ºC. The supernatant was discarded. The cell pellet was either processed immediately or stored at -80 ºC until analysis [116] (**Figure 6**).

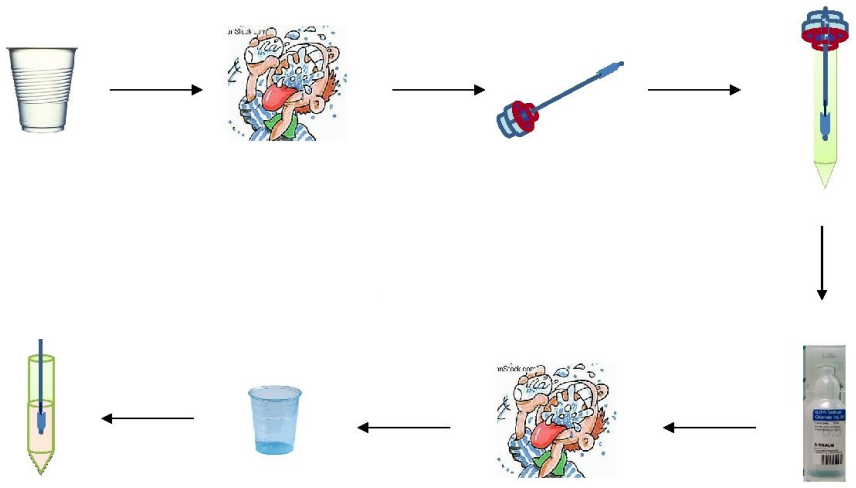


Figure 6. *Collection of buccal mucosa sample.*

5.4. Fatty acid measurement from cheek-cell glycerophospholipids

Cheek-cell glycerophospholipid fatty acids for children at 8, 9 and 9.5 years of age were analysed as described by Klingler *et al*. [116]. Briefly, the GPL were selected, then dissolved in a water methanol mixture and the GPL bond fatty acids were transferred into their methyl esters. The methyl esters were extracted into hexane and qualified by gas chromatography. Quality control samples indicated analytical precision from 2.29 (LA) to 12.53% (ALA) (coefficient of variance) depending on the percentage contribution of each individual fatty acid. After quality control and plausibility checking, cheek-cell GPL-FAs percentages were compiled in an MS Excel Table.

The FAs used for the analyses as control were Myristic acid (C14:0), Palmitic acid (C16:0), Palmitoleic acid (C16:1n-7), Margaric acid (C17:0), Stearic acid (C18:0), Oleic acid (C18:1n-9), Vaccenic acid (C18:1n-7), LA, ɤ-linolenic acid (GLA, 18:3n-6),

ALA, Arachidic acid (C20:0), Eicosaenoic acid (C20:1n-9), Dihomo-ɤ-linolenic acid (DGLA, C20:3n-6), AA, Behenic acid (C22:0), EPA, DPA, Lignoceric acid (C24:0), Nervonic acid (C24:1), DPA and DHA. These analyses were performed on samples from school age children.

5.5. SNP selection and genotyping

Seventeen single-nucleotide polymorphisms (SNPs) from the *FADS1* (rs174548, rs174556 and rs174561), *FADS2* (rs174570, rs174574, rs174575, rs174576, rs174578, rs174579, rs174602, rs498793, rs968567, rs2727271and rs3834458) and *FADS3* (rs174448, rs174449 and rs174455) gene cluster from the children's umbilical cord were genotyped. The genotyping efficiency was 10.7 fold, although the two further SNPs rs174561 and rs3834458 could not be included as these two were not included in the HapMap database. Genotyping of SNPs was carried out with the iPLEX (Sequenom, San Diego, CA, USA) method by means of matrix-assisted laser desorption ionization–time of the flight mass-spectrometry method (MALDI-TOF MS, Mass Array; Sequenom) according to the manufacturer's instructions. Standard genotyping quality control included 10% duplicate and negative samples. The genotyping discordance rate was below 0.3%.

5.6. Assessment of the children's dietary intake

The evaluation of the dietary intake was carried out on Spanish and German NUHEAL children, using a food frequency questionnaire when the child was 7.5 years old and 24-hour dietary recall for3 days (2 week days and 1 weekend day) at 8 years old. Data from food frequency questionnaire and the 3-day 24-hour dietary recall were processed using the PCN-CESNID and "Young Adolescents' Nutrition Assessment on Computer (YANA-C)" international software [(118)]. The food composition tables from DIAL [(119)] (Spain) and the German Food Code and Nutrient Data Base (BLS) [(120)] (Germany) were used.

The analysis of FFQ included coding the data from questionnaires, depuration dataset, calculation of nutritional composition of each question of the FFQ and the estimation of the nutritional composition of the diet intake for each child. The estimation of the nutritional composition was analysed in two phases: first we analysed the frequency

data (daily, weekly, monthly) which were recoded into factors, i.e. the frequency of intake per day; and second, we multiplied the new recoded frequency per day, the quantity of food and the value of the nutrient contained in this question. Finally, all the values obtained for each specific nutrient were added up and we obtained the total nutrient intake per day for each child.

Moreover, we created food groups and their nutritional composition to compare the different tendencies of children's dietary intake between countries. Those foods with similar nutritional composition were aggregated into the same question and only the frequency was asked about but not the quantity. Although in general the values for the energy and nutrients calculated were lower than the first ones, those which were still too high were re-analysed; actions taken to solve these problems consisted of making the nutritional analysis of the 24-hour dietary recall for 3 days and establishing new portion sizes for the items contained in the FFQ based on the real information which was provided by the diet records. A total of 35,000 records from the 140 children aged 7.5 years old, whose parents had completed the questionnaire, were obtained.

Dietary intake of water, proteins, lipids, carbohydrates, fiber, folate, AA, EPA, DHA, PUFAs-n3 and PUFAs-n6 derived from the protocols were analyzed and recoded. Quality control data were then compiled in an MS Excel Table.

5.7. "Eating Disorder Inventory" (EDI-2)

Figure 7. *Eating Disorder Inventory*

Hundreds of studies with the EDI-2 (**Figure 7**) have been conducted [121], both within the clinical setting and out of it, detecting in a quick and economic way those subjects with sub-clinical eating disorders or who are at risk of suffering these disorders in the future. It is important to distinguish between the formal diagnosis of an eating behaviour disorder and the evaluation of their symptoms. The EDI-2 is not intended to lead to a specific diagnosis of anorexia nervosa or bulimia nervosa, but rather to observe and accurately assess certain sets of symptoms that are supposed

to have relevance to the understanding and treatment of these behavioural eating disorders.

Many characteristic features of these disorders are reflected in the scales of the EDI-2 findings, the obsession with thinness, bulimia, body dissatisfaction, perfectionism, inefficiency, interpersonal distrust, interoceptive awareness, asceticism, fear of maturity, impulsiveness and social insecurity [122]. There is a validated Spanish version of this questionnaire, which also allows the use of some of its scales independently in order to conduct studies concerning satisfaction or body dissatisfaction, weight preoccupation and perfectionism [123].

The current Spanish version of EDI-2 consists of 11 scales, 8 major ones: drive for thinness (DT), bulimia (B), body dissatisfaction (BD), ineffectiveness (I), perfectionism (P), interpersonal distrust (ID), interoceptive awareness (IA) and fear of maturity (MF), and 3 additional ones: asceticism (A), impulsivity (IR) and social insecurity (SI), clinically relevant in the case of eating disorders. It consists of 91 items that are answered on a Likert Scale of six points and in which subjects must indicate whether each situation happens "never," "rarely", "sometimes", "often", "almost always" or "always". Some scales of the EDI-2 can be used separately for studies concerning satisfaction or body dissatisfaction, weight preoccupation and perfectionism:

- Drive for Thinness (DT) [124]: This construct was prepared by Bruch (1973,1982), who described the obsession for thinness or the "relentless pursuit of thinness" as the essential characteristic of an eating disorder. The elements of this scale refer to the concern about weight, diets and fear of gaining weight.
- Body Dissatisfaction (BD): This scale measures the dissatisfaction of the subject with the general shape of their body or those parts of it that most concern those with an eating disorder (stomach, hips, thighs, buttocks, etc.). Body dissatisfaction is considered one of the aspects of "disturbance of body image" which is a feature of patients with eating disorders.
- Perfectionism (P) [125, 126]: This scale measures the degree to which the subject believes that their personal results should be better. The elements of this scale assess to what extent the subject believes that only excellent levels

of personal performance are acceptable and they are convinced that others (parents and teachers, for example) expect exceptional results from them.

Since the ultimate purpose of the use of EDI-2 in this study is not to detect possible eating disorders, but only to evaluate certain characteristics, just 22 items included, belonging to the scales recently analysed and presented in **Table 2**.

Both the father and the mother of the child filled out the questionnaire individually in a time of about 5-10 minutes. Once EDI-2 was filled out, it was corrected by giving each of the items values between 0 and 3 as indicated in **Table 3** and adding the values to have a "Raw-Scoring" of each one of the scales.

As for the internal consistency of the instrument reliability evaluated through Cronbach's alpha in the study of Garner [127] ranges between 0.83 and 0.93 in the various categories, throwing similar values in the clinical sample in the validation of Corral *et al.,* with slightly lower values in the normal sample [128].

Table 2. *Eating Disorder Inventory Scales.*

SCALE	ELEMENTS
DT	1. I like sweets and carbohydrates without worrying
	3. I think about going on a diet
	5. I feel very guilty when I eat too much
	8. I dread the idea of getting fat
	10. I exaggerate or give too much importance to my weight
	13. I am concerned I'd want to be a thinner person.
	17. If I gain one kilogram I can keep going
BD	2. I think my stomach is too big
	4. I think my thighs are too big
	6. I think my stomach is the right size
	9. I am satisfied with my figure
	12. I like the shape of my bum
	16. I think my hips are too wide
	19. I think the size of my thighs is too wide
	20. I think my bum is too big
	21. I think my hips are the right size
P	7. In my home only outstanding results are considered good enough
	11. When I was a child, I diligently tried not to disappoint my parents and teachers
	14. It annoys me not being the best in everything
	15. My parents expected outstanding results from me
	18. I think I have to do things perfectly or not do them at all
	22. I set seriously ambitious goals

Table 3. *Eating Disorder Inventory Scoring.*

	Never	Rarely	Sometimes	Often	Almost always	Always
DT	3	2	1	0	0	0
BD	0	0	0	1	2	3
P	0	0	0	1	2	3

5.8. Child Behaviour Checklist (CBCL)

The Child Behaviour Checklist (CBCL / 6-18) [89] is a standardized Achenbach System of Empirically Based Assessment of (ASEBA) instrument (**Figure 8**).

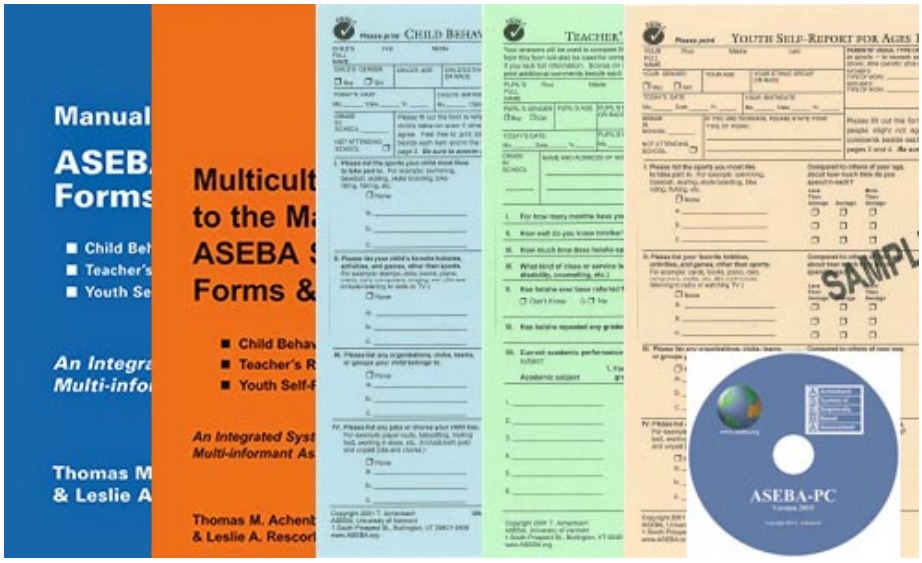


Figure 8. *Child Behaviour Checklist 6-18 years.*

Using the Child CBCL [129] it is possible to explore three internalizing syndromes equivalent to three diagnoses described in official classifications [130]: affective disorders, anxiety disorders and somatic problems (**Table 4**). However, empirical taxonomies show that the internalizing symptoms do not appear as clearly defined in childhood and adolescence, and proposes mixed groups of symptoms. Achenbach *et al.* have done 27 different factorial analysis of the items of the CBCL, and have isolated three empirical internalizing syndromes: anxiety/depression, withdrawal/depression and somatic complaints [86].

Through this inventory parents evaluate child psychopathology in the last 6 months. The questionnaire consists of two parts: one on social skills and the other on behaviour problems. Behaviour problems, the part that we are interested in for this study, consist of 113 items with three response options (possible values: 0 = not true; 1 = sometimes true or in some way; 2 = very true or often true), referring to different behavioural problems that can be found in children and adolescents aged 6-18 years [131].

The list of items on behavioural problems provides qualifications of 8 empirical scales or syndromes (anxiety/depression, withdrawal/depression, somatic complaints, social problems, thought problems, attention problems, rule-breaking behaviour and aggressive behaviour), and two wider scales (internalizing and externalizing disorders) in addition to the total score. The large scales are: "internalizing" (made up of "withdrawal/depression", " anxiety/depression" and "somatic complaints") presented in Table 4, and "externalizing" (consisting of "anti-social behaviour" and "aggressiveness"). The total score is obtained from the sum of the partials [132].

In a previous study of behaviour problems in adolescents [133] feasibility is attributed with a Cronbach's alpha of 0.88 for internalizing problems, 0.93 for the scale of externalizing problems and 0.89 for the total scale. The instrument displays a good validity criterion by differentiating between clinical samples of children and others within normal limits.

ASEBA (Assessment Data Manager) Software, specially designed for the processing of these data, was used to obtain results on possible behavioural problems of children differentiating between "normal", "borderline clinical range" or "clinical range". Once obtained, the results were transferred to a database using SPSS statistical program, version 20.0 (SPSS Inc. Chicago, USA). When we classified internalizing and externalizing the behaviour as pathological or not, a raw score of 60 obtained in the questionnaire as a cut off based on validated and published studies was used [134].

Table 4. *Children Behavioural Check List (CBCL-DSM Empirical internalizing syndromes).*

SYNDROME	SYMTOM
Anxiety/depression	14. He/she cries a lot
	29. He/she fears certain situations
	30. He/she is afraid to go to school
	31. He/she is afraid they might think or do something wrong
	32. He/she believes they have to be perfect
	33. He/she believes or complains that no one wants them
	35. He/she feels inferior or feels worthless
	45. He/she is overeager
	50. He/she is too afraid
	52. He/she feels guilty about anything
	71. He/she is embarrassed easily
	91. He/she speaks of wanting to die
	112. He/she worries too much about things
Withdrawal/depression	5. There are very few things that he/she enjoys
	42. He/she prefers to be alone
	65. He/she refuses to speak
	69. He/she is very reserved, shutting out everything
	75. He/she is very shy
	102. He/she is lazy, slow, or lacks energy
	103. He/she is unhappy, sad or depressed
	111. He/she is isolated, not related to other people
Somatic complaints	47. Nightmares
	49. Constipation
	51. Feeling dizzy
	54. Too tired
	56a. Pain or discomfort without medical cause
	56b. Headaches without medical cause
	56c. Nausea, feeling bad without medical cause
	56d. Eye problems without medical cause
	56e. Rashes or skin problems without medical cause
	56f. Stomach pains or cramps without medical cause
	56g. Vomiting without medical cause

From López-Soler C. 2010 [86]

5.9. Head circumference assessment

Head circumference was obtained within 24 h of birth, at 4 years old, and at 10 years old following The World Health Organization (WHO) criteria [83] of passing a tape measure around head, placed above the ears and midway between the eyebrows and the hairline to the occipital prominence at the back of the head and touching two head landmarks: the most anterior protuberance (around 0.5 cm above the eyebrows) and the most posterior one. This variable was measured three times by two trained researchers. The largest of the three measurements was considered the actual head circumference. Discrepancies between the two experts were always less than 4 mm.

Figure 9. *Measure of head circumference at birth, 4 years and 10 years old.*

5.10. Cognitive ability assessment

We used the Spanish adaptation of the Kaufman Assessment Battery for Children – Second Edition (KABC-II) [135] to assess cognitive abilities when the children were 6.60±1.06 years old.

The K-ABC is designed for children aged 2.5 to 12.5 years old and measures intelligence and achievement. This is the method that we used in our study to evaluate cognitive function at 6.5 years of age. The K-ABC is composed of 16 sub-tests but children should only perform those appropriate for their age (**Figure 10**). The time needed for the evaluation varies with the age of the children; it takes about 60 minutes to perform the test at 6.5 years of age.

K-ABC	Age (years) 2-6	3	4	5	6	7	8	9	10	11	12
Book 1 Mental Processing											
1. Magic window	■	■	■								
2. Face Recognition	■	■	■								
3. Hand Movements	■	■	■	■	■	■	■	■	■	■	■
4. Gestalt closure	■	■	■	■	■	■	■	■	■	■	■
5. Number Recall	■	■	■	■	■	■	■	■	■	■	■
6. Triangles			■	■	■	■	■	■	■	■	■
Book 2 Mental Processing											
7. Word Order			■	■	■	■	■	■	■	■	■
8. Analogous Matrix				■	■	■	■	■	■	■	■
9. Spatial Memory				■	■	■	■	■	■	■	■
10. Photo Series					■	■	■	■	■	■	■
Book 3 Achievement											
11. Expressive Vocabulary	■	■	■								
12. Faces and Places	■	■	■	■	■	■	■	■	■	■	■
13. Arithmetic		■	■	■	■	■	■	■	■	■	■
14. Riddles		■	■	■	■	■	■	■	■	■	■
15. Reading/Decoding				■	■	■	■	■	■	■	■
16. Reading/Comprehension						■	■	■	■	■	■

Figure 10. *Summary of sub-tests in the Kaufman-ABC depending on the children's age in order of performance (Spanish Kaufman Neurobattery for children, 1997).*

The results of the K-ABC comprise 4 scales: the Sequential Processing Scale, the Simultaneous Processing Scale, the Achievement Scale and the Non-verbal Scale. The Sequential Processing Scales measures children's ability to solve problems that require the arrangement of stimuli in sequential or serial order. The Simultaneous Processing Scale was designed to measure children's ability to solve spatial, analogical or organizational problems that require processing of many stimuli simultaneously. These two scales are hypothesized to reflect the children's style of problem solving and information processing. The Sequential and Simultaneous Processing Scales are combined to form the Mental Processing Composite, which serves as a measure of intelligence. The Non-verbal Scale is composed of the sub-tests of the Sequential and the Simultaneous Processing Scales that do not require words. The Achievement Scales evaluate the knowledge of the facts, language and skills learned at home or at school. Raw scores are transformed into standard scores with means of 100 and SD of 15. These scores can also be transformed to percentile scores [(136)].

Raw scores are transformed into standard scores with a mean of 100 and a standard deviation of 15.

5.11. Magnetic resonance imaging (MRI): image acquisition

Resonance images were obtained at 10 years old (**Figure 11**), when the brain has typically attained 95% of its adult volume [137]. Children were scanned on a Philips Achieva 3T MRI scanner operating with an 8-channel phased-array head coil for reception. T1-weighted 3D volumes were acquired using a T1-weighted 3D-turbo-gradient-echo sequence (3D-TFE), in sagittal orientation with 0.94 × 0.94 × 1.0 mm resolution (160 slides, FOV = 240 × 240, matrix 256 × 256 × 160) with repetition time of 8 ms, echo time 4 ms, inversion delay = 1022.6264 ms, flip angle of 8°, band with 191 Hz/pixel.

Figure 11. *Girl and boy in a Magnetic Resonance Imaging (MRI) machine.*

The sequence was optimal for reducing motion sensitivity, susceptibility artefacts, and field in homogeneities. Total volumes for grey and white matter (GMV and WMV, respectively) and total intracranial volume (TIV, obtained from GM, WM, and cerebrospinal fluid (CSF) voxels in native space) were computed for each child adding up the corresponding voxel values.

5.12. Image Processing

Voxel-Based Morphometry. The structural images were first checked for artefacts and manually re-aligned to the AC-PC line. Automatic processing was done with the default parameters of the DARTEL toolbox [138] implemented in SPM8 (http://www.fil.ion.ucl.ac.uk/spm). Briefly, images were corrected for intensity in homogeneities, tissue was then clustered into GM, WM, CSF, and registered using

linear and non-linear transformations. Sample-specific GM and WM templates were created by an iterative process starting from an initial template estimates using the DARTEL high dimensional warping procedure. Next, the GM and WM individual maps were non-linearly normalized to the sample final template, and Jacobian-modulated to preserve tissue amount [138]. The final sample GM and WM templates were registered to the Montreal Neurological Institute (MNI) space, and the individual maps were co-registered to the MNI space using the affine transformation estimate for the GM and WM templates. Statistical analyses were done on GM and WM maps after smoothing them with an 8-mm full-width at half-maximum.

Cortical Surface Extraction. Total brain surface area (TBSA) for each child was estimated using the BrainSuite 13a (available at http://www.loni.ucla.edu/Software/BrainSuite13) [139, 140] that provides an automatic sequence to estimate cortical surface area: skull stripping, bias-field inhomogeneity correction, tissue classification, topological abnormalities correction, extraction of inner and pial cortical surfaces, and brain registering and labelling. Default parameters were used for each stage.

Sub-cortical segmentation and volumetric analysis. Seven sub-cortical structures for each hemisphere (hippocampus, dorsal striatum [i.e. caudate nucleus and putamen], ventral striatum [i.e. accumbens nucleus], amygdala, globus pallidus, thalamus, and brainstem+4th ventricle) were segmented for each child's scan using a semi-automated, model-based sub-cortical tool (FMRIB's Integrated Registration and Segmentation Tool; FIRST v1.2) [141] in FMRIB's Software Library (FSL) version 4.1.4 (available at http://www.fmrib.ox.ac.uk/fsl) [142]. A two-stage affine registration to a standard space template (MNI space) with 1 mm resolution using 12 degrees of freedom and a sub-cortical mask to exclude voxels outside the sub-cortical regions was performed on each participant's MPRAGE scan. The sub-cortical structures were then segmented. Erickson and colleagues have reported a high test–retest reliability of this segmentation algorithm [143]. Segmentations were visually checked for errors. The volume of each participant's sub-cortical structures was measured in mm^3, and the average of bilateral sub-cortical brain volume values were used in subsequent analyses.

5.13. Ethical considerations

The dietary intervention could be considered without risk, even for pregnant women. Women were supplemented with natural constituents of common European diets and the applied dosages did not have any known side effects. The dosage of DHA is 500 mg per day does not involve any risk, either for pregnant women or for infants. Studies on healthy adult volunteers have reported no side effects with DHA doses up to 6 g per day (169), furthermore studies in pregnant women with supplementation doses of more than 1 g DHA daily did not report any negative side effects in women or infants [41, 144, 145]. With respect to folic acid, the recommendations for daily folic acid intake are below 1,000 μg, because a higher intake might mask a vitamin B 12-deficiency [146]). For this reason we use MTHF which does not mask this deficiency. Additionally, the dosage of 400 μg is less than the maximum recommended intake of folic acid.

The only invasive test in the study was maternal blood sampling, which involved a minimum risk of minor complications. In addition, no invasive tests were performed on the children, the only samples collected from the children consisted of cord blood at delivery and urine and cheek cells. Thus, there was no risk for the mothers or the babies. During the enrolment pregnant women and their partners were informed about the study and a written informed consent was obtained prior to entry into the study and before the beginning of the neurological follow-up of the children and NMR for the Spanish ones. It was made clear to all the subjects that they could withdraw their consent at any time without any consequences for their medical treatment.

The information collected was treated strictly confidentially and was used only for the project.

The local ethical committees of each of the participating clinical study centres approved the study protocol.

Although no risks were anticipated, insurance coverage for any injury to the subjects was provided, Laboratorios Ordesa with Catalana Occidente SA SEG signed the insurance contract. REAS, 08190 Sant Cugat, Barcelona (Contract number 83115540).

This study was also conducted according to the guidelines laid down in the Declaration of Helsinki [147].

6. CHAPTERS

This section is organized in three chapters, that are in concordance with the three issues involving the 7 aims of this doctoral thesis:

- **Chapter 1**: Children's composition of fatty acids in cheek-cell glycerophospholipids. It covers aims from 1 to 4.
- **Chapter 2**: Evaluation of the relationship between children FAs status, mothers eating behaviour and children behavioural problems at 8 years of age. It covers aims 5 and 6.
- **Chapter 3**: Relationship between several head circumference and brain structure. It covers aim 7.

CHAPTER 1

Children's composition of fatty acids in cheek-cell glycerophospholipids.

For the first time, we explored if maternal supplementation during pregnancy and children's genetic variation in the *FADS1*, *FADS2* and *FADS3* gene cluster influence the FA composition in their cheek cells glycerophospholipids up to 9.5 years old.

Moreover, this section aims to examine the associations between diet and cheek-cell GPL-FA composition, and how these associations vary in relation to the supplementation group, age, sex and country of birth of the school-age children.

1.1. Statistical analyses

Histograms of FAs levels were studied and assessed for normality using Shapiro-Wilk tests with the help of SPSS statistical program, version 20.0 (SPSS Inc. Chicago, USA). Some LC-PUFAs were extremely right skewed and were transformed using the natural logarithm (ln) and Square root (sqrt). If no normal distribution was achieved, non-parametric tests were used. Descriptive results were expressed as means and standard deviation. Categorical outcomes were assessed with $\chi 2$ tests and the continuous variable with Kruskal-Wallis / one-way ANOVA tests to explore the potential association between FADS polymorphisms and type of supplementation.

Hardy-Weinberg (H-W) equilibrium for the genotypes was calculated using the R statistical software (3.2.2 Version, "genetics" package). Deviations from the H-W equilibrium where studied by using the Fisher's exact test. Further, to test the linkage disequilibrium, Lewontin's D' and pairwise squared correlations r^2 were performed.

After selecting the most representative SNP's of our sample, the data was transformed into longitudinal form, by using StataCorp. 2011; Stata Statistical Software: Release 12. College Station, TX: StataCorp LP, to perform a linear mixed model with the aim to study the relationships between *FADS* and FA concentrations in cheek cells. The outcomes were the FA concentrations, the fixed part was each one of the selected genotypes (homozygous major allele carriers were coded as 0, heterozygous as 1, and homozygous minor allele as 2), and the random effect was the time variable (8, 9 and 9.5 years).

Furthermore, we have built several linear mixed models to asses each fatty acid considering as co-founders the eight selected SNP's, group of supplementation, maternal age at delivery, mother's BMI at weeks 20 and 30 of pregnancy, mother smoking at week 20 and 30 of pregnancy, placental weight and sex.

On the other hand, descriptive results were expressed as means and standard deviation. Categorical outcomes were assessed with $\chi 2$ tests and the continuous ones with Kruskal-Wallis / one-way anova tests to explore the potential significations between groups. Linear regression and mixed model were performed to establish the associations between diet received during pregnancy and GPL-FAs concentrations at the different time-points. Age, sex and country of birth, maternal age, mother's BMI, smoking, placental weight, were considered as confounding variables

Moreover, to obtain the figures, the first approximation was Principal Component Analysis (PCA) and, depending on the significant results Partial Least Squares (PLS) and pursued model were performed.

We achieved a statistical power of 85 % to detect 0.9% (difference between any of the groups of supplementation) if 132 pairs of mother and children were studied. For all estimations an error level of 0.05 is assumed.

1.2. Results

To analyse the long-term effects of maternal supplementation during pregnancy with DHA and/or 5-MTHF on LC-PUFA concentrations in their offspring cheek-cell glycerophospholipids at 8, 9 and 9.5 years 147 children were included. Their baseline characteristics depending on the group of supplementation are shown in **Table 5**. There were no significant differences between the groups with respect to the confounding variables.

Fatty acids from cheek cells (by group of supplementation)

Depending on mother's supplementation during pregnancy, percentages of GPL-FAs (LA, ALA, AA, EPA, DPA and DHA) in cheek cells measured in the NUHEAL children are presented in **Table 6.** There were no significant differences between the four study groups of children at any individual time point. The evolutionary analysis showed a statistical difference in the AA GLP concentrations in cheek cells from 8 y to 9.5 y ($p = 0.002$). As we can see in **Figure 12** AA in GPL significantly decreases from 8 y to 9.5 y in those children whose mothers were supplemented with FO or FO+5-MTHF.

At 8 years old, children born to mothers who received the FO+5-MTHF supplementation during pregnancy had higher concentrations of behenic ($p=0.037$) and docosapentaenoic acid (C22:5n-6) ($p=0.007$) in cheek-cell GPL than those whose mothers were supplemented with FO or the placebo, after Bonferroni post-hoc analysis. At 9 years old, oleic acid concentrations in GPL measured in cheek cells were higher in those children born to mothers who received FO during pregnancy compared to those born to mothers from the 5-MTHF or FO+5-MTHF groups ($p=0.035$), after Bonferroni post-hoc analysis (**Table 6**).

Table 5. *General characteristics of the children depending on the group of supplementation included in this study.*

Variables	5-MTHF (n=41) Mean SD	5-MTHF (n=30) Mean SD	Placebo (n=40) Mean SD	FO + 5-MTHF (n=36) Mean SD	*P*
Country					0.096
Spain	28 (32)	22 (27)	30 (25)	25 (30)	
Germany	13 (68)	8 (63)	10 (75)	11 (70)	
Maternal					
Age	31.13 ± 4.85	32.08 ± 5.42	31.67 ± 4.09	30.99 ± 4.33	0.762
BMI week 20	25.37 ± 3.58	25.41 ± 3.06	24.48 ± 2.74	25.08 ± 2.65	0.529
BMI week 30	27.00 ± 5.08	27.70 ± 3.75	26.05 ± 5.05	26.35 ± 5.25	0.533
Hematocrit at week 20	35.04 ± 6.16	35.83 ± 2.41	34.54 ±2.21	35.69 ± 2.78	0.479
Hematocrit at week 30	34.74 ± 3.58	33.26 ± 4.96	33.80 ± 2.50	34.27 ± 2.99	0.349
Parity					0.410
<2	39 (95)	26 (86)	34 (85)	31 (86)	
=2	2 (5)	3 (10)	5 (12)	3 (8)	
>2	0 (0)	1 (4)	1 (3)	2 (6)	
Smoking M20					
no	35 (85)	26 (86)	37 (92)	31 (86)	0.764
yes	6 (15)	4 (14)	3 (8)	5 (14)	
Smoking M30					
no	34 (82)	24 (80)	36 (90)	30 (83)	0.691
yes	7 (18)	6 (20)	4 (10)	6 (17)	
Gravidity					0.803
0	9 (25)	8 (28)	14 (36)	9 (27)	
>1	27 (75)	21 (72)	24 (63)	24 (73)	
Age at delivery	31.51 ± 4.85	32.45 ± 5.43	32.06 ± 4.10	31.37 ± 4.33	0.758
Newborns					
Weeks gestation	39.22 ± 2.19	39.96± 4.04	39.83 ± 1.68	38.83 2.24	0.219
Sex					0.556
Male	21 (51)	13 (43)	22 (55)	14 (40)	
Feale	20 (49)	17 (57)	18 (45)	21 (60)	
Placental Weight	533.29 ± 123.15	534.32 ± 113	553.21 ± 103.2	527.27 ± 125.95	0,798
Apgar at 1 min	8.49 ± 1.30	9.03 ± 0.63	8.75 ± 0.98	8.29 ± 1.94	0.122
Apgar at 5 min	9.68 ± 0.65	9.76 ± 0.64	9.77 ± 0.43	9.49 ± 1.07	0.335
Birth weight (g)	3250.48 ± 602.54	3363.1 ± 390.25	3406.21 ± 409.21	3221.42 ± 510.2	0.894
Birth length (cm)	50.83 ± 3.59	51.38 ± 2.03	51.32 ± 2.13	51.10 ± 3.09	0.602
Birth head	34.38 ± 2.05	34.51 ± 1.47	34.82 ± 1.26	34.61 ± 1.63	0.549

Variables	5-MTHF (n=41) Mean SD	5-MTHF (n=30) Mean SD	Placebo (n=40) Mean SD	FO + 5-MTHF (n=36) Mean SD	*P*
circumference (cm)					
Infant feeding					0.682
Breast	15 (47)	10 (36)	16 (50)	14 (52)	
Formula	7 (22)	10 (36)	10 (31)	7 (26)	
Mixed	10 (31)	8 (28)	6 (16)	6 (22)	
BMI at 4 y	16.32 ± 2.06	15.82 ±1.11	10.03 ±1.36	15.73 ±1.36	0.266
BMI at 5.5 y	16.17 ± 1.76	16.12 ± 1.77	16.56 ± 1.71	16.25 ± 1.83	0.803
BMI at 6.5 y	17.07 ± 2.86	16.50 ± 1.98	16.83 ± 2.30	16.80 ± 2.40	0.801
BMI at 8 y	17.77 ± 3.16	16.75 ± 2.42	17.94 ± 2.67	17.60 ± 2.84	0.339
Maternal education					0.321
None	0 (0)	0 (0)	0 (0)	1 (2)	
Primary	24 (58)	18 (60)	25 (63)	17 (48)	
Secondary	17 (42)	11 (36)	15 (37)	15 (42)	
University	0 (0)	1 (4)	0 (0)	3 (10)	
Paternal education					0.183
None	0 (0)	0 (0)	0 (0)	1 (2)	
Primary	20 (48)	19 (63)	27 (67)	24 (68)	
Secondary	20 (48)	8 (26)	13 (32)	9 (25)	
University	1 (2)	3 (10)	0 (0)	1 (2)	

*: *Values expressed as n (%); #: Values expressed mean and standard deviation*
P: level of significance
Bold: *P values*<0.05

Table 6. *Mean concentrations of polyunsaturated fatty acids in glycerophospholipids, measured in children's cheek cells at 8y, 9y and 9.5 years old, depending on their mother's supplementation group established during pregnancy.*

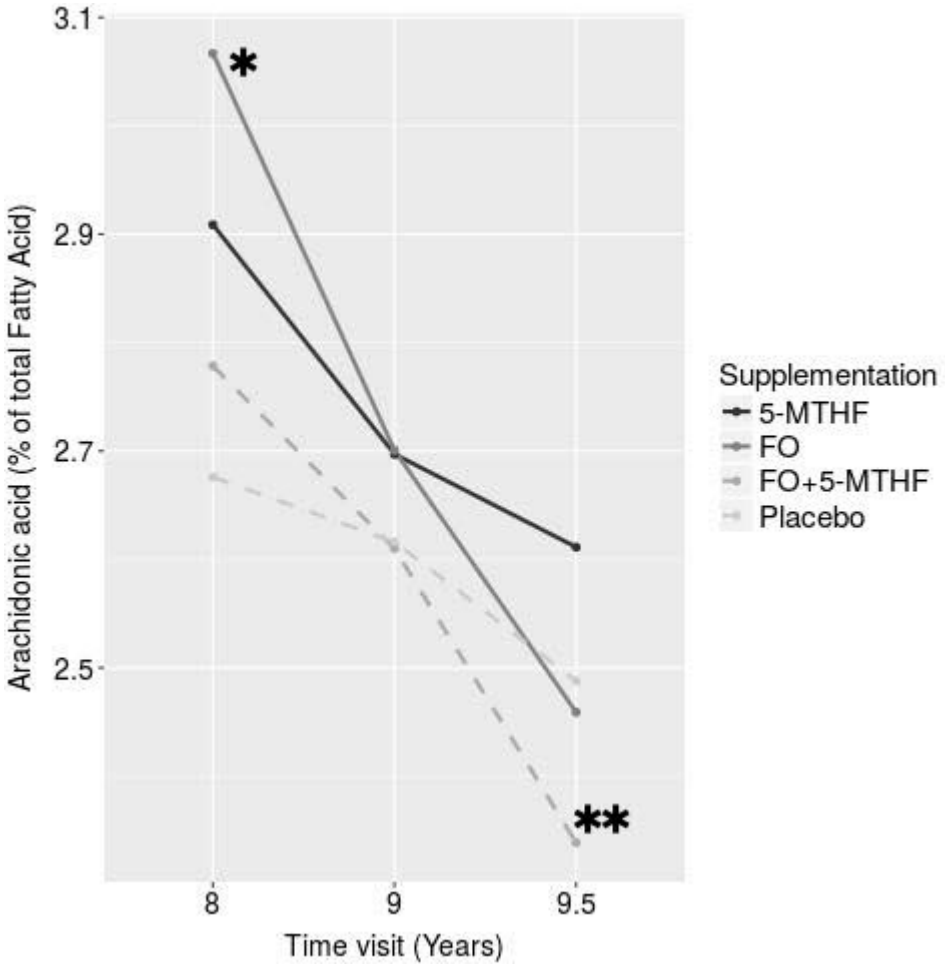
Fatty acids (%)	Supplementation group	8 years				9 years				9.5 years				P	
		n	Mean	SD	*P*	n	Mean	SD	*P*	n	Mean	SD	*P*		
Myristic acid (C14:0)	FO	38	0.89	0.40		33	1.14	0.63		33	1.24	0.79			
	5-MTHF	27	1.05	0.43		26	1.13	0.75		25	1.10	0.38			
	Placebo	38	1.16	0.97	0.405	35	0.95	0.49	0.481	35	1.15	0.68	0.658	0.554	
	FO+5-MTHF	32	1.07	0.83		26	1.01	0.40		29	1.04	0.67			
	Total	135	1.04	0.71		120	1.05	0.58		122	1.14	0.66			
Palmitic acid (C16:0)	FO	38	19.22	2.93		33	19.06	3.32		33	19.17	3.68			
	5-MTHF	27	19.45	3.14		26	19.50	3.80		25	19.57	2.91			
	Placebo	38	20.47	3.48	0.360	35	19.67	3.39	0.798	35	19.97	3.59	0.565	0.324	
	FO+5-MTHF	32	19.72	3.15		26	19.90	3.01		29	20.49	4.64			
	Total	135	19.74	3.19		120	19.52	3.36		122	19.80	3.75			
Palmitoleic acid (C16:1n-7)	FO	38	5.93	1.47		33	5.42	1.44		33	5.59	1.40			
	5-MTHF	27	5.74	1.56		26	5.35	1.49		25	5.67	1.57			
	Placebo	38	5.54	1.72	0.641	35	5.49	1.38	0.961	35	5.80	2.08	0.792	0.221	
	FO+5-MTHF	32	5.48	1.73		26	5.55	1.47		29	5.36	1.94			
	Total	135	5.68	1.61		120	5.45	1.42		122	5.61	1.77			
Margaric acid (C17:0)	FO	38	0.78	0.14		33	0.82	0.19		33	0.83	0.18			
	5-MTHF	27	0.80	0.22		26	0.79	0.29		25	0.80	0.19			
	Placebo	38	0.76	0.20	0.794	35	0.81	0.17	0.958	35	0.79	0.17	0.611	0.412	
	FO+5-MTHF	32	0.76	0.23		26	0.79	0.17		29	0.77	0.16			
	Total	135	0.77	0.20		120	0.81	0.20		122	0.80	0.18			
Stearic acid (C18:0)	FO	38	21.06	7.59		33	17.62	5.24		33	20.27	7.08			
	5-MTHF	27	20.26	8.16		26	19.00	6.14		25	20.59	7.88			
	Placebo	38	21.40	8.08	0.949	35	19.73	5.59	0.331	35	21.07	9.29	0.930	0.221	
	FO+5-MTHF	32	20.90	7.04		26	20.09	5.98		29	21.60	8.63			
	Total	135	20.96	7.65		120	19.07	5.72		122	20.88	8.20			
Oleic acid (C18:1n-9)	FO	38	24.80	4.22		33	27.21	5.52		33	24.69	5.87			
	5-MTHF	27	24.82	4.62		26	23.81	4.78		25	24.68	6.56			
	Placebo	38	23.85	6.61	0.829	35	25.11	4.35	**0.035**	35	23.76	5.65	0.571	0.325	
	FO+5-MTHF	32	24.76	5.08		26	24.22	4.60		29	22.86	5.02			
	Total	135	24.53	5.22		120	25.21	4.96		122	23.99	5.75			
Vaccenic acid (C18:1n-7)	FO	38	4.34	0.83		33	3.49	0.78		33	3.48	0.73			
	5-MTHF	27	4.31	0.92		26	3.66	1.23		25	3.52	0.81			
	Placebo	38	5.02	5.09	0.610	35	3.58	0.73	0.907	35	3.51	1.01	0.725	0.741	
	FO+5-MTHF	32	4.24	0.96		26	3.54	0.83		29	3.28	0.99			
	Total	135	4.50	2.80		120	3.56	0.88		122	3.45	0.89			

Fatty acids (%)	Supplementation group	8 years				9 years				9.5 years				P	
		n	Mean	SD	*P*	n	Mean	SD	*P*	n	Mean	SD	*P*		
Linoleic acid (LA) (C18:2n-6)	FO	38	16.45	3.28		33	16.77	2.43		33	16.26	3.61			
	5-MTHF	27	16.99	4.28		26	15.73	3.26		25	15.60	3.12			
	Placebo	38	15.95	3.48	0.692	35	16.14	2.97	0.558	35	15.79	3.54	0.834	0.391	
	FO+5-MTHF	32	16.53	2.98		26	16.05	2.92		29	16.29	3.40			
	Total	135	16.44	3.47		120	16.21	2.88		122	16.00	3.42			
Gamma-linolenic acid (C18:3n-6)	FO	38	0.10	0.05		33	0.14	0.04		33	0.15	0.05			
	5-MTHF	27	0.10	0.05		26	0.12	0.05		25	0.13	0.03			
	Placebo	38	0.11	0.05	0.958	35	0.13	0.04	0.671	35	0.12	0.05	0.163	0.221	
	FO+5-MTHF	32	0.10	0.05		26	0.13	0.05		29	0.14	0.04			
	Total	135	0.10	0.05		120	0.13	0.04		122	0.13	0.05			
Alpha-linolenic acid (C18:3n-3)	FO	38	0.32	0.21		33	0.35	0.20		33	0.32	0.17			
	5-MTHF	27	0.37	0.39		26	0.37	0.46		25	0.29	0.17			
	Placebo	38	0.30	0.14	0.638	35	0.31	0.24	0.804	35	0.28	0.13	0.729	0.662	
	FO+5-MTHF	32	0.31	0.15		26	0.30	0.18		29	0.30	0.13			
	Total	135	0.32	0.23		120	0.33	0.28		122	0.29	0.15			
Arachidic acid (C20:0)	FO	38	0.45	0.08	0.224	33	0.41	0.10	0.614	33	0.46	0.12			
	5-MTHF	27	0.45	0.09		26	0.38	0.09		25	0.44	0.11			
	Placebo	38	0.45	0.09		35	0.40	0.11		35	0.43	0.09	0.401	0.332	
	FO+5-MTHF	32	0.50	0.18		26	0.40	0.13		29	0.47	0.11			
	Total	135	0.46	0.12		120	0.40	0.11		122	0.45	0.11			
Eicosaenoic acid (C20:1n-9)	FO	38	0.21	0.06		33	0.24	0.09		33	0.21	0.06			
	5-MTHF	27	0.20	0.07		26	0.23	0.06		25	0.21	0.05			
	Placebo	38	0.23	0.09	0.328	35	0.26	0.07	0.550	35	0.21	0.07	0.865	0.521	
	FO+5-MTHF	32	0.24	0.11		26	0.24	0.06		29	0.23	0.13			
	Total	135	0.22	0.08		120	0.24	0.07		122	0.21	0.08			
Dihomo-gamma-linolenic acid (C20:3n-6)	FO	38	1.12	0.32		33	1.05	0.36		33	1.05	0.35			
	5-MTHF	27	1.10	0.37		26	1.06	0.38		25	1.07	0.33			
	Placebo	38	0.98	0.36	0.342	35	1.03	0.27	0.987	35	1.00	0.31	0.741	0.667	
	FO+5-MTHF	32	1.03	0.39		26	1.04	0.38		29	0.98	0.39			
	Total	135	1.05	0.36		120	1.05	0.34		122	1.03	0.34			
Arachidonic acid (AA) (C20:4n-6)	FO	38	3.05	0.81		33	2.66	0.73		33	2.45	0.80			
	5-MTHF	27	2.97	0.93		26	2.73	1.08		25	2.58	0.87			
	Placebo	38	2.66	1.04	0.255	35	2.61	0.71	0.951	35	2.50	0.87	0.962	**0.002**	
	FO+5-MTHF	32	2.76	0.94		26	2.69	0.81		29	2.49	0.98			
	Total	135	2.86	0.93		120	2.67	0.82		122	2.50	0.87			
Behenic acid (C22:0)	FO	38	0.22	0.10		33	0.34	0.16		33	0.34	0.13			
	5-MTHF	27	0.24	0.12		26	0.30	0.14		25	0.31	0.11			
	Placebo	38	0.20	0.11	**0.037**	35	0.33	0.17	0.751	35	0.30	0.09	0.625	0.325	
	FO+5-MTHF	32	0.29	0.20		26	0.34	0.17		29	0.33	0.15			
	Total	135	0.23	0.14		120	0.33	0.16		122	0.32	0.12			

Fatty acids (%)	Supplementation group	8 years				9 years				9.5 years				P	
		n	Mean	SD	*P*	n	Mean	SD	*P*	n	Mean	SD	*P*		
Eicosapentaenoic acid (EPA) (C20:5n-3)	FO	38	0.12	0.07		33	0.14	0.06		33	0.17	0.09			
	5-MTHF	27	0.12	0.08		26	0.80	3.20		25	0.17	0.07			
	Placebo	38	0.11	0.07	0.928	35	0.18	0.10	0.318	34	0.16	0.07	0.919	0.211	
	FO+5-MTHF	32	0.13	0.17		26	0.22	0.18		29	0.16	0.09			
	Total	135	0.12	0.10		120	0.31	1.49		121	0.16	0.08			
Lignoceric acid (C24:0)	FO					33	0.37	0.28		33	0.45	0.29			
	5-MTHF					26	0.41	0.25		25	0.45	0.24			
	Placebo					35	0.40	0.25	0.839	35	0.41	0.23	0.899	0.789	
	FO+5-MTHF					26	0.42	0.27		29	0.42	0.27			
	Total					120	0.40	0.26		122	0.43	0.25			
Docosapentaenoic acid (C22:5n-6)	FO	38	0.10	0.08		33	0.24	0.28		33	0.20	0.21			
	5-MTHF	27	0.08	0.04		26	0.16	0.19		25	0.19	0.25			
	Placebo	38	0.07	0.03	**0.007**	35	0.20	0.28	0.643	35	0.18	0.20	0.939	0.652	
	FO+5-MTHF	32	0.16	0.20		26	0.17	0.21		29	0.21	0.20			
	Total	135	0.10	0.11		120	0.20	0.25		122	0.19	0.21			
Nervonic acid (C24:1)	FO					33	0.08	0.03		33	0.11	0.04			
	5-MTHF					26	0.09	0.04		25	0.11	0.02			
	Placebo					35	0.10	0.03	0.165	35	0.10	0.03	0.878	0.845	
	FO+5-MTHF					26	0.10	0.03		29	0.10	0.04			
	Total					120	0.09	0.03		122	0.11	0.03			
Docosapentaenoic acid (DPA) (C22:5n-3)	FO	38	0.20	0.08		33	0.19	0.07		33	0.23	0.10			
	5-MTHF	27	0.20	0.09		26	0.80	3.05		25	0.22	0.08			
	Placebo	38	0.18	0.09	0.698	35	0.21	0.13	0.319	35	0.21	0.10	0.871	0.368	
	FO+5-MTHF	32	0.21	0.11		26	0.23	0.09		29	0.23	0.13			
	Total	135	0.20	0.09		120	0.34	1.42		122	0.22	0.11			
Docosahexaenoic acid (DHA) (C22:6n-3)	FO	38	0.63	0.26		33	0.56	0.18		33	0.58	0.24			
	5-MTHF	27	0.64	0.21		26	1.29	3.47		25	0.67	0.25			
	Placebo	38	0.57	0.23	0.590	35	0.64	0.30	0.336	35	0.61	0.24	0.541	0.324	
	FO+5-MTHF	32	0.62	0.28		26	0.69	0.38		29	0.62	0.25			
	Total	135	0.61	0.25		120	0.77	1.63		122	0.62	0.25			

Values expressed mean standard deviation of the percentage of each fatty acid measured in cheek cells; P: level of significance; Bold: *P values*<0.05

Figure 12. *Evolution of araquidonic acid concentrations in children's cheek-cell glycerophospholipids from 8 to 9.5 years of age.*



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**: P-value<0.05*

***: P-value <0.001*

FADS polymorphisms on fatty acid composition in cheek cells

Genotype and allele frequencies of 17 polymorphisms in *FADS1*, *FADS2* and *FADS3* genes from NUHEAL children are presented in **Table 7**. The analysed genotypes were in the Hardy-Weinberg equilibrium, except for rs174574, rs174576 and rs174578, which were excluded from the following analyses.

Based on the linkage disequilibrium data from Lewontin's D' and pairwise-squared correlations r2 (**Figure 13** and **Table 8**) 8 SNPs out of the initial 17 SNPs determined (rs174556, rs174570, rs174575, rs174579, rs174602, rs498793, rs2727271, rs174448) were selected for further analyses. Lewontin's D' ranged between 0.95 and 1.0. We analysed the effects of *FADS* gene cluster polymorphisms on GPL-FA concentrations in children. **Table 9** represents the linear mixed model between the main six GPL-FAs in the children's cheek cells and the eight *FADS* gene cluster polymorphisms selected. The analysed *FADS1* and *FADS2* polymorphisms showed different associations with LA, ALA, AA, EPA, DPA and DHA concentrations. The majority of the associations were established between *FADS1* and *FADS2* SNPs with LA and ALA. *FADS3* rs174448 did not have any effects on the analysed FA concentrations.

The frequencies of major and minor alleles according to genotypes are presented in **Table 10**. Among all groups of supplementation, the major allele carriers present the highest frequencies for FADS2 and FADS3. No statistical differences were found in the frequencies of major or minor homozygous and heterozygous for FADS1, FADS2 or FDAS3 between, depending on their mothers' group of supplementation.

When analysing the effects of SNPs on FA concentrations depending on the maternal supplementation groups, some patterns have been identified (**Table 11**).

Table 7. *Characteristics of the 17 analysed variants in FADS 1/2/3 gene of studied population.*

SNP	Possible Functional Region	Alleles (Major/Minor) and Frequency 0/1	Number (%) of subjects with Genotype 0	1	2	P-value (Fisher exact test)
rs174548	*FADS1*	G/C (0.73/0.27)	47 (0.51)	41 (0.45)	4 (0.04)	0.284
rs174556	*FADS1*	G/A (0.74/0.26)	49 (0.53)	39 (0.42)	5 (0.05)	0.595
rs174561	*FADS1*	A/G (0.75/0.25)	49 (0.54)	37 (0.41)	4 (0.04)	0.572
rs174570	*FADS2*	C/T (0.90/0.10)	75 (0.81)	18 (0.19)	-	0.595
rs174574	*FADS2*	C/A (0.68/0.32)	38 (0.41)	50 (0.54)	2 (0.05)	**0.034**
rs174575	*FADS2*	C/G (0.73/0.27)	47 (0.51)	42 (0.45)	4 (0.04)	0.195
rs174576	*FADS2*	C/A (0.68/0.32)	38 (0.41)	50 (0.54)	5 (0.05)	**0.034**
rs174578	*FADS2*	T/A (0.67/0.33)	36 (0.39)	51 (0.55)	5 (0.05)	**0.020**
rs174579	*FADS2*	C/T (0.81/0.19)	59 (0.63)	33 (0.35)	1 (0.01)	0.179
rs174602	*FADS2*	T/C (0.77/0.23)	53 (0.57)	38 (0.41)	2 (0.02)	0.143
rs498793	*FADS2*	C/T (0.55/0.45)	30 (0.32)	42 (0.45)	21 (0.23)	0.406
rs968567	*FADS2*	C/T (0.83/0.17)	61 (0.66)	32 (0.34)	-	0.064
rs2727271	*FADS2*	A/T (0.89/0.11)	73 (0.78)	20 (0.22)	-	0.591
rs3834458	*FADS2*	T/Z (0.70/0.30)	42 (0.45)	46 (0.49)	5 (0.05)	0.138
rs174448	*FADS3*	T/C (0.69/0.31)	42 (0.45)	45 (0.48)	6 (0.06)	0.229
rs174449	*FADS3*	T/C (0.69/0.31)	41 (0.44)	46 (0.49)	6 (0.06)	0.225
rs174455	*FADS3*	T/C (0.68/0.32)	41 (0.45)	43 (0.47)	8 (0.09)	0.633

0=Homozygous major , 1= Heterozygous, 2 = Homozygous minor
P: level of significance
Bold: P values<0.05

Figure 13. ***a)*** *Pairwise LD measured in D' for the complete 17 SNP's children participated in the NUHEAL Study;* ***b)*** *Pairwise LD measured in r^2 for complete the complete 17 SNP's children participated in the NUHEAL Study.*

a) ***b)***

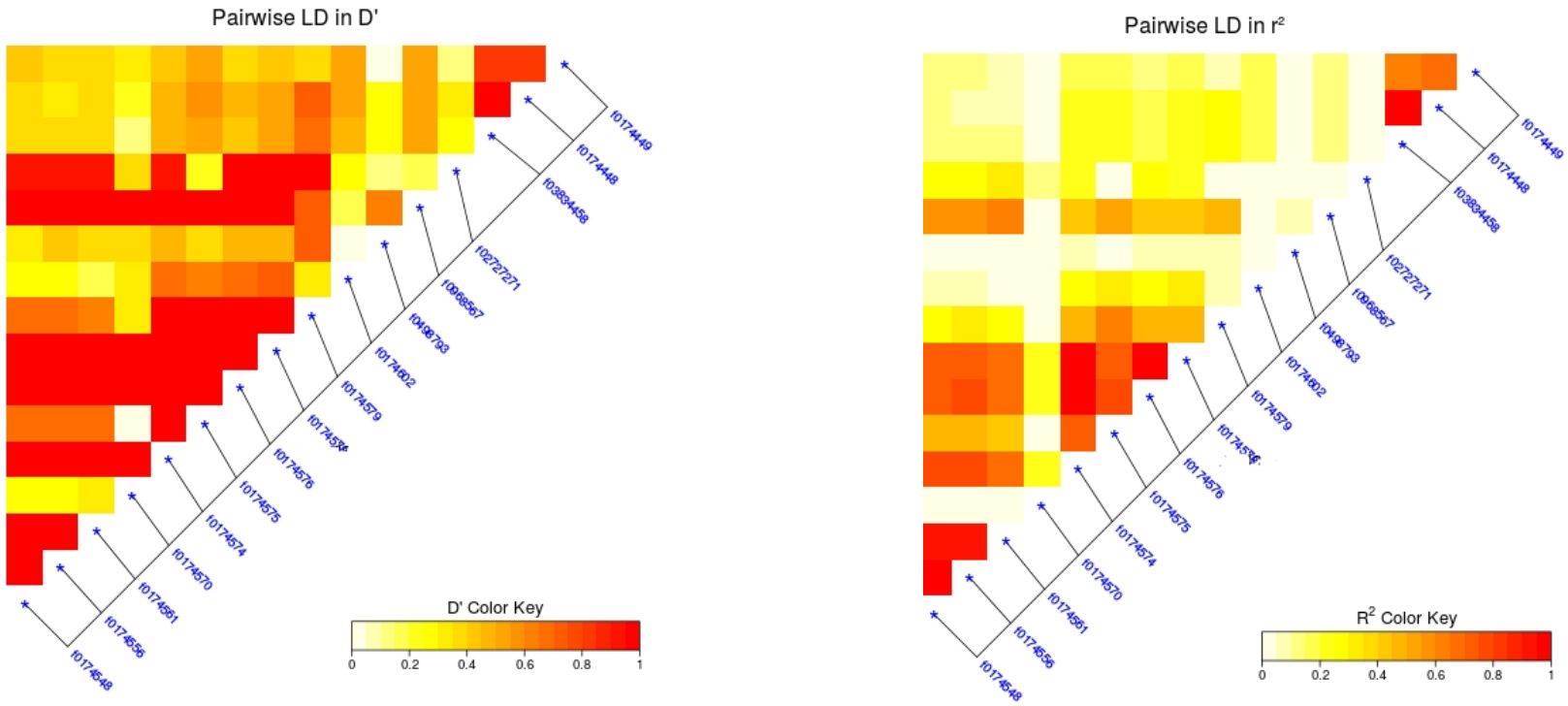


Table 8. *Pairwise linkage disequilibrium measured by Lewontin's D' for all selected SNPs.*

	rs174548	rs174556	rs174561	rs174570	rs174574	rs174575	rs174576	rs174578	rs174579	rs174602	rs498793	rs968567	rs2727271	rs174448	rs174449	rs174455
rs174548																
N	-	92	89	92	92	92	92	91	92	92	92	92	92	92	92	92
D	-	0.1906	0.1833	0.0182	0.1803	0.1339	0.1752	0.1779	0.0916	0.0433919	-0.0370818	0.1261493	0.0717130	0.0733636	0.0706267	0.0726834
D'	-	0.9864	0.9996	0.2577	0.9995	0.6877	0.9711	0.9995	0.6639	0.2619126	0.3083299	0.9993824	0.9090027	0.3972151	0.3853841	0.4017580
Correlation	-	0.9791	0.9579	0.1400	0.8726	0.6833	0.8478	0.8551	0.5305	0.2347841	-0.1685724	0.7561626	0.5237150	0.3600098	0.3449181	0.3523098
X²	-	176.4192	163.3446	3.6071	140.1329	85.9270	132.2809	133.0898	51.7998	10.1427337	5.2286629	105.2078618	50.4670368	23.8476997	21.8902002	22.8384852
p-value	-	< 2.2204e-16	< 2.2204e-16	0.05753	< 2.2204e-16	< 2.2204e-16	< 2.2204e-16	< 2.2204e-16	6.146195e-13	0.001448712	0.02221761	< 2.2204e-16	1.211808e-12	1.042664e-06	2.887045e-06	1.762018e-06
rs174556																
N	-	-	90	93	93	93	93	92	93	93	93	93	93	93	93	92
D	-	-	0.1840	0.0188	0.1783	0.1347	0.1783	0.1760	0.0948	0.0416520	-0.0488026	0.1266419	0.0726922	0.0652886	0.0626563	0.0654380

	rs174548	rs174556	rs174561	rs174570	rs174574	rs174575	rs174576	rs174578	rs174579	rs174602	rs498793	rs968567	rs2727271	rs174448	rs174449	rs174455
D'	-	-	0.9996	0.2651	0.9995	0.6993	0.9995	0.9995	0.6843	0.2504335	0.4101977	0.9993848	0.9178317	0.3573365	0.3456088	0.3656409
Correlation	-	-	0.9650	0.1451	0.8662	0.6898	0.8662	0.8488	0.5509	0.2261507	-0.2226233	0.7617450	0.5327043	0.3214938	0.3070532	0.3182889
X²	-	-	167.6275	3.9172	139.5881	88.5046	139.5881	132.5881	56.4565	9.5128140	9.2183669	107.9275207	52.7819462	19.2246379	17.5363847	18.6406443
p-value	-	-	< 2.2204e-16	0.04779	< 2.2204e-16	< 2.2204e-16	< 2.2204e-16	< 2.2204e-16	5.750955e-14	0.0020404 2	0.0023959 92	< 2.2204e-16	3.727019e-13	1.162039e-05	2.818619e-05	1.578198e-05
rs174561																
N	-	-	-	90	90	90	90	90	90	90	90	90	90	90	90	89
D	-	-	-	0.0220	0.1692	0.1253	0.1692	0.1670	0.0914	0.0313687	-0.0403312	0.1289543	0.0743351	0.0631560	0.0602596	0.0594847
D'	-	-	-	0.3042	0.9995	0.6857	0.9995	0.9995	0.6481	0.1852246	0.3572195	0.9993959	0.9217557	0.3642483	0.3502587	0.3502457
Correlation	-	-	-	0.1724	0.8363	0.6529	0.8363	0.8194	0.5405	0.173261	-0.1871605	0.7890650	0.5541628	0.3163693	0.3004134	0.2943345
X²	-	-	-	5.3559	125.8958	76.7486	125.8958	120.8822	52.5859	5.4035351	6.3052279	112.0722551	55.2773622	18.0161181	16.2446805	15.4206408
p-value	-	-	-	0.020	<	<	<	<	4.117817	0.020096	0.012038	< 2.2204e-	1.04694	2.19042	5.566567	8.604331e

	rs174548	rs174556	rs174561	rs174570	rs174574	rs174575	rs174576	rs174578	rs174579	rs174602	rs498793	rs968567	rs2727271	rs174448	rs174449	rs174455
				65	2.2204e-16	2.2204e-16	2.2204e-16	2.2204e-16	e-13	01	2	16	e-13	5e-05	e-05	-05
rs174570																
N	-	-	-	-	93	93	93	92	93	93	93	93	93	93	93	92
D	-	-	-	-	0.0654858	0.0035192	0.0654858	0.0646205	0.0254180	0.0249309	-0.0156850	-0.0165784	0.0340739	0.0080033	0.0155103	-0.0106912
D'	-	-	-	-	0.9981	0.9989177	0.9989033	0.3235320	0.9989177	0.3327582	0.3588877	0.9957386	0.3945184	0.1192425	0.2328971	0.3445345
Correlation	-	-	-	-	0.4738	0.4738283	0.4642936	0.2199649	0.4738283	0.2016819	-0.1066055	-0.1485736	0.3720386	0.0587179	0.113249 6	-0.0774792
X²	-	-	-	-	41.7594	41.759464 7	39.664610 7	8.999524 4	41.759464 7	7.565661 7	2.113841 0	4.1057844	25.7447 620	0.6412885	2.385537 1	1.1045577
p-value	-	-	-	-	1.032213e-10	3.015403e-10	0.002700499	3.015403e-10	0.002700499	0.005949081	0.145972 5	0.0427367 6	3.896832e-07	0.4232446	0.122462 8	0.2932681
rs174574																
N	-	-	-	-	-	93	93	92	93	93	93	93	93	93	93	92
D	-	-	-	-	-	0.1762953	0.2130958	0.2155885	0.1274268	0.0994867	-0.0693864	0.1164673	0.0655696	0.0996896	0.104078 2	0.0909473
D'	-	-	-	-	-	0.968113	0.975167	0.999769	0.999650	0.650386	0.476288	0.9993311	0.90017	0.48020	0.492705	0.4186952

	rs174548	rs174556	rs174561	rs174570	rs174574	rs174575	rs174576	rs174578	rs174579	rs174602	rs498793	rs968567	rs2727271	rs174448	rs174449	rs174455
							1	8	4	0	1		64	92	4	
Correlation	-	-	-	-	-	0.8506508	0.9751671	0.9796660	0.6974344	0.5090075	-0.2982632	0.6601364	0.4527914	0.4625756	0.4806246	0.4168489
X²	-	-	-	-	-	134.5908546	176.8768585	176.5931522	90.4731357	48.1904932	16.5467344	81.0550972	38.1337353	39.7995655	42.9659949	31.9723858
p-value	-	-	-	-	-	< 2.2204e-16	< 2.2204e-16	< 2.2204e-16	< 2.2204e-16	3.867573e-12	4.746566e-05	< 2.2204e-16	6.605808e-10	2.814077e-10	5.569967e-11	1.563798e-08
rs174575																
N	-	-	-	-	-	-	93	92	93	93	93	93	93	93	93	92
D	-	-	-	-	-	-	0.1820267	0.1796232	0.1375436	0.1036194	-0.0472965	0.1196585	0.0169172	0.0975929	0.1024332	0.0919072
D'	-	-	-	-	-	-	0.9995866	0.9995811	0.9996761	0.6275941	0.3895880	0.9512187	0.2151721	0.5234610	0.5537152	0.5032693
Correlation	-	-	-	-	-	-	0.8783056	0.8606394	0.7937602	0.5589935	-0.2143684	0.7151209	0.1231775	0.4774823	0.4987616	0.4441657
X²	-	-	-	-	-	-	143.4842397	136.2888280	117.1902798	58.120106	8.5474068	95.1200109	2.8221217	42.4060241	46.2699448	36.3000989
p-value	-	-	-	-	-	-	<2.2204e-16	<2.2204e-16	<.2204e-16	2.464695e-14	0.003460148	< 2.2204e-16	0.0929734 3	7.416123e-11	1.03032e-11	1.691553e-09

	rs174548	rs174556	rs174561	rs174570	rs174574	rs174575	rs174576	rs174578	rs174579	rs174602	rs498793	rs968567	rs2727271	rs174448	rs174449	rs174455
rs174576																
N	-	-	-	-	-	-	-	92	93	93	93	93	93	93	93	92
D	-	-	-	-	-	-	-	0.2155885	0.1274268	0.0994867	-0.0693864	0.1164673	0.0727620	0.0921714	0.0965918	0.0828106
D'	-	-	-	-	-	-	-	0.9997698	0.9996504	0.6503860	0.4762881	0.9993311	0.9989177	0.4439933	0.4572648	0.3812362
Correlation	-	-	-	-	-	-	-	0.9796660	0.6974344	0.5090075	-0.2982632	0.6601364	0.5024587	0.4276896	0.4460529	0.3795551
X²	-	-	-	-	-	-	-	176.5931522	90.4731357	48.1904932	16.5467344	81.0550972	46.9584341	34.0228197	37.0071576	26.5074160
p-value	-	-	-	-	-	-	-	< 2.2204e-16	< 2.2204e-16	3.867573e-12	4.746566e-05	< 2.2204e-16	7.250867e-12	5.446949e-09	1.176964e-09	2.625283e-07
rs174578																
N	-	-	-	-	-	-	-	-	92	92	92	92	92	92	92	91
D	-	-	-	-	-	-	-	-	0.1257	0.1094708	-0.0731503	0.1149291	0.0718006	0.1030200	0.1073564	0.0916065
D'	-	-	-	-	-	-	-	-	0.9996	0.7252283	0.4885826	0.9993222	0.9989033	0.5028892	0.5150219	0.4273707

	rs174548	rs174556	rs174561	rs174570	rs174574	rs174575	rs174576	rs174578	rs174579	rs174602	rs498793	rs968567	rs2727271	rs174448	rs174449	rs174455
Correlation	-	-	-	-	-	-	-	-	0.6834	0.556167	-0.3122410	0.6468563	0.4923478	0.4746817	0.4922915	0.4169302
X²	-	-	-	-	-	-	-	-	85.9362	56.9153501	17.9389797	76.9898397	44.6027751	41.4593775	44.5925706	31.6372112
p-value	-	-	-	-	-	-	-	-	< 2.2204e-16	4.551914e-14	2.281013e-05	< 2.2204e-16	2.413492e-11	1.203458e-10	2.426104e-11	1.858344e-08
rs174579																
N	-	-	-	-	-	-	-	-	-	93	93	93	93	93	93	92
D	-	-	-	-	-	-	-	-	-	0.0493435	-0.0595126	0.0991867	-0.0201547	0.0902638	0.0959555	0.0484157
D'	-	-	-	-	-	-	-	-	-	0.3387080	0.7003053	0.7101539	0.9961039	0.6916423	0.7409995	0.3787380
Correlation	-	-	-	-	-	-	-	-	-	0.3019448	-0.3059655	0.6723905	-0.1664605	0.5009386	0.5299739	0.2654076
X²	-	-	-	-	-	-	-	-	-	16.9577441	17.4123730	84.0922623	5.1538910	46.6747431	52.2422526	12.9611834
p-value	-	-	-	-	-	-	-	-	-	3.822109e-05	3.008611e-05	< 2.2204e-16	0.02319434	8.380185e-12	4.906076e-13	0.0003180161
rs174602																

	rs174548	rs174556	rs174561	rs174570	rs174574	rs174575	rs174576	rs174578	rs174579	rs174602	rs498793	rs968567	rs2727271	rs174448	rs174449	rs174455
N	-	-	-	-	-	-	-	-	-	-	93	93	93	93	93	92
D	-	-	-	-	-	-	-	-	-	-	0.0004600	0.0254258	0.0209381	0.0765411	0.0819000	0.0808547
D'	-	-	-	-	-	-	-	-	-	-	0.0037148	0.1908920	0.2515185	0.4887440	0.5270485	0.5270800
Correlation	-	-	-	-	-	-	-	-	-	-	0.0022107	0.1611235	0.1616544	0.3970839	0.4228487	0.4143325
X²	-	-	-	-	-	-	-	-	-	-	0.0009091	4.8287064	4.8605794	29.3276670	33.2569872	31.5875377
p-value	-	-	-	-	-	-	-	-	-	-	0.975947	0.0279896 2	0.02747711	6.111683e-08	8.074909e-09	1.906492e-08
rs498793																
N	-	-	-	-	-	-	-	-	-	-	-	93	93	93	93	92
D	-	-	-	-	-	-	-	-	-	-	-	-0.0481745	-0.0058743	-0.0370414	-0.0380407	0.0064760
D'	-	-	-	-	-	-	-	-	-	-	-	0.6200320	0.1209685	0.2676450	0.2701261	0.0368289
Correlation	-	-	-	-	-	-	-	-	-	-	-	-0.2564887	-0.0381042	-0.1614512	-0.1650119	0.0278817

	rs174548	rs174556	rs174561	rs174570	rs174574	rs174575	rs174576	rs174578	rs174579	rs174602	rs498793	rs968567	rs2727271	rs174448	rs174449	rs174455
X²	-	-	-	-	-	-	-	-	-	-	-	12.2362845	0.2700589	4.848365	5.064576	0.1430399
p-value	-	-	-	-	-	-	-	-	-	-	-	0.0004686907	0.6032923	0.0276723	0.0244164	0.7052774
rs968567																
N	-	-	-	-	-	-	-	-	-	-	-	-	93	93	93	92
D	-	-	-	-	-	-	-	-	-	-	-	-	0.0168215	0.0624041	0.0607241	0.0637491
D'	-	-	-	-	-	-	-	-	-	-	-	-	0.1889465	0.5229975	0.5128937	0.5454374
Correlation	-	-	-	-	-	-	-	-	-	-	-	-	0.1438750	0.3586507	0.3473226	0.3619000
X²	-	-	-	-	-	-	-	-	-	-	-	-	3.8502010	23.9252347	22.4377313	24.0987719
p-value	-	-	-	-	-	-	-	-	-	-	-	-	0.0497403	1.001503e-06	2.170676e-06	9.151864e-07
rs2727271																
N	-	-	-	-	-	-	-	-	-	-	-	-	-	93	93	92
D	-	-	-	-	-	-	-	-	-	-	-	-	-	-0.0092506	-0.0107992	0.0102435

	rs174548	rs174556	rs174561	rs174570	rs174574	rs174575	rs174576	rs174578	rs174579	rs174602	rs498793	rs968567	rs2727271	rs174448	rs174449	rs174455
D'	-	-	-	-	-	-	-	-	-	-	-	-	-	0.2807323	0.3220756	0.1402295
Correlation	-	-	-	-	-	-	-	-	-	-	-	-	-	-0.0647733	-0.0752537	0.0708483
X²	-	-	-	-	-	-	-	-	-	-	-	-	-	0.7803778	1.0533401	0.9235836
p-value	-	-	-	-	-	-	-	-	-	-	-	-	-	0.3770256	0.3047391	0.3365358
rs174448																
N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	93	92
D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.2108463	0.1726864
D'	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.9997861	0.8294763
Correlation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.9872807	0.8025564
X²	-	-	-	-	-	-	-	-	-	-	-	-	-	-	181.2985067	118.5137927
p-value	-	-	-	-	-	-	-	-	-	-	-	-	-	-	< 2.2204e-16	< 2.2204e-16

	rs174548	rs174556	rs174561	rs174570	rs174574	rs174575	rs174576	rs174578	rs174579	rs174602	rs498793	rs968567	rs2727271	rs174448	rs174449	rs174455
rs174449																
N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	92
D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1765301
D'	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.8333193
Correlation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.8164874
X^2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	122.6639018
p-value	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	< 2.2204e-16

Table 9. *Associations between PUFA concentrations in GPL from all cheek cells samples obtained and the selected SNPs of FADS gene cluster polymorphism.*

		Linoleic acid (LA) (C18:2n-6)				Arachidonic acid (AA) (C20:4n-6)				Alpha-Linolenic (ALA) (C18:3n-3)				Eicosapentaenoic acid (EPA) (C20:5n-3)				Docosapentaenoic acid (DPA) (C22:5n-3)				Docosahexaenoic acid (DHA) (C22:6n-3)			
Gene	**SNP**	**Intercept**	**Beta**	**CI (95 %)**	**p-value**	**Intercept**	**Beta**	**CI (95 %)**	**p-value**	**Intercept**	**Beta**	**CI (95 %)**	**p-value**	**Intercept**	**Beta**	**CI (95 %)**	**p-value**	**Intercept**	**Beta**	**CI (95 %)**	**p-value**	**Intercept**	**Beta**	**CI (95 %)**	**p-value**
FADS1	**rs174556**	15.79	1.19	(0.57, 1.81)	**0.0002**	2.71	-0.06	(-0.25, 0.12)	0.4894	0.29	0.07	(0.01, 0.12)	**0.0087**	0.17	0.10	(0.12, 0.32)	0.3736	0.23	0.08	(-0.12, 0.30)	0.4104	0.65	0.09	(-0.18, 0.31)	0.5952
	rs174570	16.30	0.28	(-0.64, 1.24)	0.5581	2.74	-0.31	(-0.60, -0.03)	**0.0275**	0.32	0.02	(-0.05, 0.10)	0.5268	0.33	0.16	(-0.04, 0.67)	0.053	0.21	0.30	(-0.01, 0.62)	0.0635	0.62	0.35	(-0.01, 0.73)	0.0579
	rs174575	15.80	1.19	(0.55, 1.84)	**0.0003**	2.77	-0.18	(-0.38, 0.005)	0.0570	0.28	0.08	(0.02, 0.13)	**0.0045**	0.27	-0.08	(-0.32, 0.44)	0.4549	0.32	-0.09	(-0.31, 0.12)	0.4120	0.76	-0.13	(-0.39, 0.11)	0.2933
FADS2	**rs174579**	16.02	1.14	(0.38, 1.90)	**0.003**	2.27	-0.31	(-0.53, -0.08)	**0.0072**	0.30	0.07	(0.01, 0.14)	**0.020**	0.26	-0.10	(0.37, 0.17)	0.4753	0.31	-0.10	(-0.36, 0.15)	0.4285	0.73	-0.11	(-0.41, 0.18)	0.4368
	rs174602	16.07	0.73	(0.03, 1.43)	**0.0396**	2.74	-0.13	(-0.34, 0.07)	0.1929	0.31	0.04	(-0.01, 0.10)	0.1771	0.27	-0.10	(-0.35, 0.14)	0.4156	0.33	-0.11	(-0.35, 0.12)	0.3362	0.77	-0.17	(-0.45, 0.09)	0.1996
	rs498793	16.91	-0.51	(-1.04, -0.01)	**0.0567**	2.82	-0.15	(-0.30, 0.006)	0.068	0.33	-0.01	(-0.05, 0.03)	0.6587	0.34	-0.12	(-0.31, 0.06)	0.2010	0.40	-0.12	(-0.30, 0.05)	0.1612	0.78	-0.09	(-0.30, 0.11)	0.3606
	rs2727271	16.22	0.85	(-0.05, 1.75)	0.065	2.63	0.20	(-0.06, 0.47)	0.1432	0.32	0.02	(-0.05, 0.10)	0.4941	0.15	0.33	(0.09, 0.65)	**0.0437**	0.21	0.30	(0.001, 0.60)	**0.0485**	0.61	0.34	(-0.01, 0.70)	0.0579
FADS3	**rs174448**	16.35	0.09	(-0.54, 0.74)	0.7654	2.74	-0.10	(-0.30, 0.08)	0.2720	0.34	-0.02	(-0.08, 0.02)	0.3137	0.31	-0.14	(-0.37, 0.08)	0.2244	-0.13	0.36	(-0.35, 0.08)	0.2221	0.79	-0.15	(-0.40, 0.09)	0.2241

Intercept: mean fatty acid concentration in homozygous carriers of the major allele
Beta: Regression coefficient β from linear regression of the fatty acid (outcome) on the respective single SNP
N: number of subjects used in analysis.
P: level of significance
Bold: P values<0.0

Table 10. *Characteristics of the selected SNPs from FADS1, FADS2 and FADS3 considering the different group of supplementation.*

SNP	Possible Functional Region	Supplementary Group	Alleles (Major/Minor) and Frequency	Number (%) of subjects with Genotype			P-value (Fisher exact test)	X^2 test
			0/1	0	1	2		
rs174556	*FADS 1*	Total	G/A (0.74/0.26)	49 (0.53)	39 (0.42)	5 (0.05)	0.595	0.061
		5-MTHF	(0.66/0.34)	6(0.38)	9(0.56)	1(0.06)	0.5914	
		FO	(0.67/0.33)	12(0.41)	5(0.52)	2(0.07)	0.6714	
		FO+5MTHF	(0.88/0.12)	15(0.68)	6(0.57)	1(0.05)	0.5381	
		Placebo	(0.79/0.91)	16(0.62)	9(0.35)	1(0.03)	1	
rs174570	*FADS 2*	Total	C/T (0.90/0.10)	75(0.81)	18(0.19)	-	0.595	0.309
		5-MTHF	(0.88/0.12)	12(0.75)	4(0.25)	-	1	
		FO	(0.93/0.07)	25(0.86)	4(0.14)	-	-	
		FO+5MTHF	(0.91/0.09)	18(0.82)	4(0.18)	-	1	
		Placebo	(0.88/0.12)	20(0.77)	6(0.23)	-	1	
rs174575	*FADS 2*	Total	C/G (0.73/0.27)	47(0.51)	42(0.45)	4 (0.04)	0.195	0.038
		5-MTHF	(0.75/0.25)	8(0.5)	8(0.5)	-	0.5127	
		FO	(0.71/0.29)	14(0.48)	13(0.45)	2(0.07)	1	
		FO+5MTHF	(0.73/0.27)	11(0.50)	10(0.45)	1(0.05)	1	
		Placebo	(0.75/0.25)	14(0.52)	11(0.44)	1(0.04)	1	
rs174579	*FADS 2*	Total	C/T (0.81/0.19)	59(0.63)	33(0.35)	1 (0.01)	0.179	0.092
		5-MTHF	(0.81/0.19)	10(0.62)	6(0.38)	-	1	
		FO	(0.84/0.16)	21(0.72)	7(0.24)	1(0.03)	0.5185	
		FO+5MTHF	(0.77/0.23)	12(0.55)	10(0.45)	-	0.5381	
		Placebo	(0.81/0.19)	16(0.62)	10(0.8)	-	0.5449	
rs174602	*FADS 2*	Total	T/C (0.77/0.23)	53(0.57)	38(0.41)	2 (0.02)	0.143	0.753
		5-MTHF	(0.81/0.19)	11(0.69)	14(0.25)	1(0.06)	0.4344	
		FO	(0.76/0.24)	15(0.52)	14(0.48)	-		
		FO+5MTHF	(0.75/0.25)	19(0.55)	12(0.41)	1(0.04)	1	
		Placebo	(0.79/0.21)	15(0.58)	11(0.42)	-	0.5489	
rs498793	*FADS 2*	Total	C/T (0.55/0.45)	30(0.32)	42(0.45)	21(0.23)	0.406	0.329
		5-MTHF	(0.56/0.44)	5(0.31)	8(0.50)	3(0.19)	1	
		FO	(0.6/0.4)	10(0.34)	15(0.52)	4(0.14)	1	
		FO+5MTHF	(0.52/0.48)	6(0.27)	11(0.50)	5(0.23)	1	
		Placebo	(0.58/0.42)	11(0.42)	8(0.31)	7(0.27)	0.1023	
rs2727271	*FADS 2*	Total	A/T (0.89/0.11)	73(0.78)	20(0.22)	-	0.591	0.309
		5-MTHF	(0.84/0.16)	11(0.69)	5(0.31)	-	1	

SNP	Possible Functional Region	Supplementary Group	Alleles (Major/Minor) and Frequency	Number (%) of subjects with Genotype			P-value (Fisher exact test)	X^2 test
			0/1	0	1	2		
		FO	(0.84/0.16)	20(0.69)	9(0.31)	-	1	
		FO+5MTHF	(0.95/0.05)	20(0.91)	2(0.09)	-	1	
		Placebo	(0.92/0.08)	22(0.85)	4(0.15)	-	1	
		Total	T/C (0.69/0.31)	42(0.45)	45(0.48)	6(0.06)	0.229	
		5-MTHF	(0.72/0.28)	9(0.56)	5(0.31)	2(0.12)	0.5301	
rs174448	*FADS 3*	FO	(0.62/0.38)	11(0.38)	14(0.48)	4(0.14)	1	0.693
		FO+5MTHF	(0.64/0.36)	14(0.64)	8(0.36)	-	1	
		Placebo	(0.65/0.35)	8(0.31)	18(0.69)	-	0.0108	

0=Homozygous major , 1= Heterozygous, 2 = Homozygous minor
P: level of significance
Bold: *P values*<0.05

As is shown in **Table 11** LA concentration increased in children with *FADS2* (rs174575) minor homozygous genotypes compared to heterozygosis or major homozygous genotypes, if their mothers were supplemented with FO; on the contrary, children carrying the minor alleles of the FADS2 rs498793 polymorphism showed lower concentrations of LA in cheek-cell GPL. LA and AA concentrations increased in children with *FADS2* (rs174570) heterozygous genotypes in case of maternal 5-MTHF supplementation, but no other effects of the different polymorphisms or supplementation type were observed.

Those children whose mothers received FO supplementation during pregnancy, and having the minor alleles of the rs174556, rs174575 and rs498793 had a higher level of ALA in plasma phospholipids respect to those carrying the other polymorphisms. Furthermore, children carrying the heterozygous genotypes for rs174556, rs174575 and rs174602 and rs174579, whose mothers were supplemented with 5-MTHF, showed an increase of ALA concentrations in cheek-cell GPL.

On the other hand, the children with heterozygous for *FADS2* (rs174575, rs174579, rs174602, rs498793), *FADS3* (rs174448) polymorphisms, and when their mothers received 5-MTHF as supplement, EPA, DPA and DHA concentrations in cheek-cell GPL decreased, compared to those children with major homozygous; heterozygous *FADS1* (rs174556) or *FADS2* (rs174570, rs2727271) polymorphisms and mothers

supplemented with 5-MTHF during pregnancy, were followed by the same pattern, showing higher levels of EPA, DPA and DHA in children's cheek-cell GPL, compared to those having the major alleles. Nevertheless, the effect observed with the rs174448 (*FADS3*) in the 5-MTHF group were also present in the placebo group, increasing the EPA and DPA concentrations.

Children born to mothers who were supplemented with FO+5-MTHF had higher EPA concentrations in cheek-cell GPL when they had the major homozygous rs498793 and heterozygous rs2727271, similar to those born to mothers who received 5-MTHF; EPA concentrations were also higher when children had the major homozygous allele rs174570 and their mothers received FO+5-MTHF. Interestingly, when children were born of mothers who received FO+5-MTHF and they had the minor homozygous alleles of rs174556, rs174575 and rs174602, then the EPA concentrations in cheek cells were higher at 8 years old. Prenatal supplementation with FO+5MTHF in the mother determined higher concentrations of DHA, specially when children were heterozygous for *FADS1* (rs174556), *FADS2* (rs174575, rs174579, rs174602, rs498793, rs174570, rs174575) and rs174448 (*FADS3*).

No definitive pattern were found in the FA concentrations in those children born to mothers who received the placebo; in this case, the presence of the different *FADS* polymorphisms determined different results (EPA or DPA) or no differences (DHA).

Table 11. *Relationship between GPL-FAs concentrations in children's cheek cells and FADS gene cluster polymorphisms by group of supplementation.*

		Group of Supplementation																								
		5-MTHF							FO							FO + 5-MTHF							PLACEBO						
		0		1		2		p	0		1		2		p	0		1		2		p	0		1		2		p	p
Gene	SNP	N	M (SD)	N	M (SD)	N	M (SD)		N	M (SD)	N	M (SD)	N	M (SD)		N	M (SD)	N	M (SD)	N	M (SD)		N	M (SD)	N	M (SD)	N	M (SD)		
C18:2n-6 (LA)																														
FADS1	rs174556	15	15.99 (3.18)	22	15.80 (3.72)	3	19.79 (3.37)	0.833	29	15.72 (2.93)	38	17.05 (3.20)	6	20.33 (1.66)	0.257	36	16.57 (2.02)	13	16.00 (2.35)	3	16.19 (0.62)	0.208	46	15.23 (2.75)	22	19.16 (1.72)	2	13.58 (3.00)	0.089	**0.001**
	rs174570	28	15.73 (2.98)	12	15.86 (4.99)	-	-	**0.035**	63	16.92 (3.30)	10	15.98 (2.66)	-	-	0.428	42	16.26 (2.06)	10	17.05 (1.95)	-	-	0.837	54	16.12 (3.21)	16	17.40 (2.46)	-	-	0.226	0.564
	rs174575	21	15.51 (4.00)	19	16.07 (3.26)	-	-	3.888	38	15.89 (2.57)	29	17.24 (3.65)	6	20.33 (1.66)	**0.044**	28	16.23 (1.96)	21	16.67 (2.30)	3	16.19 (0.62)	0.189	38	15.51 (2.58)	30	17.76 (3.22)	2	13.58 (3.00)	0.502	**0.001**
FADS2	rs174579	27	15.61 (3.66)	13	16.11 (3.69)	-	-	0.972	57	16.61 (2.89)	13	16.58 (4.17)	3	21.08 (2.09)	0.194	29	16.32 (1.98)	23	16.52 (2.16)	-	-	0.674	44	15.51 (2.96)	26	17.94 (2.73)	-	-	0.656	**0.003**
	rs174602	28	15.54 (4.10)	9	15.75 (2.09)	3	17.99 (1.91)	0.083	38	16.10 (3.19)	35	17.54 (3.11)	-	-	0.873	27	16.06 (2.02)	22	16.86 (2.17)	3	16.19 (0.62)	0.241	38	16.39 (3.07)	32	16.45 (3.16)	-	-	0.864	0.115
	rs498793	8	16.34 (4.86)	18	16.26 (3.20)	14	14.84 (3.45)	0.381	22	17.35 (2.18)	41	16.63 (3.71)	10	16.22 (3.07)	**0.036**	12	15.47 (1.57)	27	16.99 (2.05)	13	16.07 (2.15)	0.549	26	16.97 (2.71)	24	26.35 (2.88)	20	15.78 (3.74)	0.281	0.108
	rs2727271	25	15.60 (3.13)	15	16.06 (4.44)	-	-	0.139	47	16.29 (3.47)	26	17.69 (2.51)	-	-	0.076	48	16.37 (2.10)	4	16.84 (1.39)	-	-	0.410	61	16.31 (3.17)	9	17.13 (2.49)	-	-	0.399	0.068
FADS3	rs174448	23	16.14 (3.35)	13	16.13 (5.06)	4	12.54 (2.79)	0.654	29	16.23 (2.63)	34	17.03 (3.47)	10	17.57 (3.88)	0.218	32	16.28 (1.90)	20	16.62 (2.29)	-	-	0.364	20	16.64 (4.03)	50	16.33 (2.66)	-	-	**0.023**	0.767
C20:4n-6 (AA)																														
FADS1	rs174556	15	2.51 (0.77)	22	2.64 (1.19)	3	2.30 (0.33)	0.075	29	3.05 (0.70)	38	2.82 (0.90)	6	2.18 0.70)	0.367	36	2.76 (0.97)	13	2.58 (0.83)	3	2.42 (1.06)	0.797	46	2.44 (0.87)	22	2.95 (0.94)	2	1.75 (0.55)	0.802	0.084

		Group of Supplementation																									
		5-MTHF							FO							FO + 5-MTHF							PLACEBO						
		0		1		2			0		1		2			0		1		2			0		1		2		
Gene	SNP	N	M (SD)	N	M (SD)	N	M (SD)	p	N	M (SD)	N	M (SD)	N	M (SD)	p	N	M (SD)	N	M (SD)	N	M (SD)	p	N	M (SD)	N	M (SD)	N	M (SD)	p	p
FADS2	rs174570	28	2.51 (0.80)	12	2.69 (1.40)	-	-	**0.022**	63	2.92 (0.85)	10	2.48 (0.62)	-	-	0.243	42	2.72 (0.99)	10	2.58 (0.64)	-	-	0.139	54	2.72 (0.89)	16	2.12 (0.89)	-	-	0.999	**0.029**
	rs174575	21	2.73 (1.15)	19	2.38 (0.78)	-	-	0.099	38	2.95 (0.73)	29	2.88 (0.94)	6	2.18 (0.70)	0.332	28	2.72 (1.03)	21	2.69 (0.81)	3	2.42 (1.06)	0.561	38	2.63 (0.77)	30	2.57 (1.08)	2	1.75 (0.55)	0.179	0.097
	rs174579	27	2.58 (1.07)	13	2.53 (0.86)	-	-	0.383	57	3.00 (0.81)	13	2.44 (0.73)	3	1.90 (0.56)	0.762	29	2.77 (1.03)	23	2.59 (0.79)	-	-	0.190	44	2.66 (0.91)	26	2.45 (0.92)	-	-	0.971	**0.026**
	rs174602	28	2.57 (1.05)	9	2.47 (1.00)	3	2.80 (0.64)	0.727	38	2.98 (0.91)	35	2.73 (0.73)	-	-	0.200	27	2.78 (1.00)	22	2.63 (0.85)	3	2.42 (1.06)	0.711	38	2.67 (0.81)	32	2.46 (1.03)	-	-	0.162	0.428
	rs498793	8	3.21 (1.43)	18	2.60 (0.82)	14	2.15 (0.75)	0.088	22	2.87 (0.64)	41	2.90 (0.94)	10	2.67 (0.81)	0.152	12	2.34 (0.71)	27	2.95 (1.06)	13	2.47 (0.68)	0.130	26	2.65 (0.92)	24	2.64 (0.96)	20	2.42 (0.88)	0.916	**0.025**
	rs2727271	25	2.55 (0.83)	15	2.60 (1.25)	-	-	0.085	47	2.77 (0.78)	26	3.02 (0.91)	-	-	0.383	48	2.68 (0.92)	4	2.82 (1.17)	-	-	0.555	61	2.55 (1.29)	9	2.77 (1.29)	-	-	0.097	0.146
FADS3	rs174448	23	2.79 (1.03)	13	2.13 (0.64)	4	2.68 (1.00)	0.102	29	3.14 (0.81)	34	2.63 (0.80)	10	2.81 (0.85)	0.978	32	2.71 (1.00)	20	2.66 (0.83)	-	-	0.360	20	2.40 (0.96)	50	2.65 (0.90)	-	-	0.745	0.260
C18:3n-3 (ALA)																													
FADS1	rs174556	15	0.35 (0.18)	22	0.40 (0.52)	3	0.95 (1.03)	**0.000**	29	0.28 (0.12)	38	0.30 (0.14)	6	0.60 (0.32)	**0.003**	36	0.31 (0.15)	13	0.24 (0.16)	3	0.31 (0.11)	0.824	46	0.29 (0.18)	22	0.39 (0.25)	2	0.14 (0.05)	0.104	**0.004**
	rs174570	28	0.42 (0.46)	12	0.42 (0.56)	-	-	0.417	63	0.33 (0.18)	10	0.24 (0.07)	-	-	**0.002**	42	0.29 (0.16)	10	0.31 (0.10)	-	-	0.093	54	0.30 (0.16)	16	0.40 (0.30)	-	-	**0.002**	0.530
	rs174575	21	0.32 (0.19)	19	0.53 (0.67)	-	-	**0.000**	38	0.28 (0.12)	29	0.31 (0.15)	6	0.60 (0.32)	**0.002**	28	0.32 (0.16)	21	0.26 (0.14)	3	0.31 (0.11)	0.651	38	0.26 (0.12)	30	0.41 (0.25)	2	0.14 (0.05)	**0.001**	**0.019**
FADS2	rs174579	27	0.31 (0.17)	13	0.66 (0.77)	-	-	**0.000**	57	0.31 (0.19)	13	0.29 (0.11)	3	0.47 (0.10)	0.077	29	0.32 (0.16)	23	0.27 (0.14)	-	-	0.442	44	0.28 (0.17)	26	0.39 (0.24)	-	-	**0.025**	0.074
	rs174602	28	0.37 (0.39)	9	0.54 (0.76)	3	0.55 (0.26)	**0.025**	38	0.30 (0.13)	35	0.34 (0.21)	-	-	**0.002**	27	0.32 (0.15)	22	0.27 (0.16)	3	0.31 (0.11)	0.827	38	0.29 (0.13)	32	0.36 (0.26)	-	-	**0.000**	0.377

		Group of Supplementation																									
		5-MTHF							FO							FO + 5-MTHF							PLACEBO						
		0		1		2		p	0		1		2		p	0		1		2		p	0		1		2		p	p
Gene	SNP	N	M (SD)	N	M (SD)	N	M (SD)		N	M (SD)	N	M (SD)	N	M (SD)		N	M (SD)	N	M (SD)	N	M (SD)		N	M (SD)	N	M (SD)	N	M (SD)		
	rs498793	8	0.48 (0.68)	18	0.48 (0.55)	14	0.31 (0.18)	**0.000**	22	0.33 (0.13)	41	0.29 (0.14)	10	0.39 (0.32)	**0.000**	12	0.24 (0.12)	27	0.31 (0.15)	13	0.33 (0.17)	0.524	26	0.34 (0.16)	24	0.33 (0.20)	20	0.28 (0.26)	0.081	0.874
	rs2727271	25	0.44 (0.48)	15	0.39 (0.50)	-	-	0.852	47	0.29 (0.13)	26	0.37 (0.23)	-	-	**0.001**	48	0.30 (0.15)	4	0.29 (0.10)	-	-	0.342	61	0.33 (0.21)	9	0.27 (0.16)	-	-	0.215	0.496
FADS3	rs174448	23	0.43 (0.50)	13	0.46 (0.54)	4	0.24 (0.19)	0.214	29	0.29 (0.14)	34	0.33 (0.21)	10	0.32 (0.13)	**0.037**	32	0.31 (0.16)	20	0.27 (0.14)	-	-	0.576	20	0.39 (0.30)	50	0.29 (0.14)	-	-	**0.000**	0.603
C20:5n-3 (EPA)																														
FADS1	rs174556	15	0.15 (0.07)	22	0.91 (3.48)	3	0.16 (0.06)	**0.000**	29	0.15 (0.07)	38	0.15 (0.09)	6	0.10 (0.11)	0.392	36	0.17 (0.17)	13	0.19 (0.10)	3	0.45 (0.48)	**0.001**	46	0.15 (0.11)	21	0.15 (0.06)	2	0.13 (0.01)	**0.011**	0.471
	rs174570	28	0.15 (0.07)	12	1.54 (4.71)	-	-	**0.000**	63	0.15 (0.09)	10	0.13 (0.06)	-	-	0.123	42	0.20 (0.21)	10	0.17 (0.07)	-	-	**0.001**	54	0.15 (0.10)	15	0.13 (0.06)	-	-	0.073	**0.051**
	rs174575	21	0.95 (3.57)	19	0.15 (0.07)	-	-	**0.000**	38	0.14 (0.07)	29	0.16 (0.09)	6	0.10 (0.11)	0.136	28	0.18 (0.19)	21	0.17 (0.09)	3	0.45 (0.48)	**0.000**	38	0.14 (0.07)	29	0.16 (0.12)	2	0.13 (0.01)	**0.009**	0.707
FADS2	rs174579	27	0.77 (3.15)	13	0.15 (0.07)	-	-	**0.000**	57	0.16 (0.08)	13	0.13 (0.07)	3	0.07 (0.05)	0.559	29	0.18 (0.19)	23	0.21 (0.19)	-	-	0.943	44	0.15 (0.11)	25	0.15 (0.06)	-	-	**0.010**	0.776
	rs174602	28	0.74 (3.09)	9	0.16 (0.07)	3	0.21 (0.06)	**0.000**	38	0.16 (0.09)	35	0.13 (0.07)	-	-	0.262	27	0.21 (0.20)	22	0.14 (0.07)	3	0.45 (0.48)	**0.000**	38	0.15 (0.07)	31	0.15 (0.11)	-	-	**0.019**	0.574
	rs498793	8	2.21 (5.76)	18	0.16 (0.08)	14	0.16 (0.07)	**0.000**	22	0.12 (0.06)	41	0.16 (0.09)	10	0.15 (0.10)	0.089	12	0.21 (0.26)	27	0.19 (0.14)	13	0.20 (0.22)	**0.024**	26	0.15 (0.16)	24	0.16 (0.06)	19	0.13 (0.08)	**0.002**	0.328
	rs2727271	25	0.15 (0.07)	15	1.27 (4.21)	-	-	**0.000**	47	0.14 (0.07)	26	0.17 (0.10)	-	-	0.080	48	0.18 (0.16)	4	0.38 (0.42)	-	-	**0.004**	60	0.15 (0.10)	9	0.13 (0.04)	-	-	**0.009**	**0.045**
FADS3	rs174448	23	0.88 (3.41)	13	0.16 (0.07)	4	0.11 (0.08)	**0.000**	29	0.17 (0.09)	34	0.14 (0.08)	10	0.11 (0.06)	0.282	32	0.17 (0.18)	20	0.23 (0.20)	-	-	0.663	19	0.16 (0.15)	50	0.14 (0.06)	-	-	**0.000**	0.451
C22:5n-3 (DPA)																														

		Group of Supplementation																									
		5-MTHF							FO							FO + 5-MTHF							PLACEBO						
		0		1		2		p	0		1		2		p	0		1		2		p	0		1		2		p	p
Gene	SNP	N	M (SD)	N	M (SD)	N	M (SD)		N	M (SD)	N	M (SD)	N	M (SD)		N	M (SD)	N	M (SD)	N	M (SD)		N	M (SD)	N	M (SD)	N	M (SD)		
FADS1	rs174556	15	0.22 (0.74)	22	0.92 (3.31)	3	0.27 (0.04)	**0.000**	29	0.21 (0.06)	38	0.22 (0.10)	6	0.18 (0.14)	**0.006**	36	0.22 (0.12)	13	0.25 (0.08)	3	0.16 (0.11)	0.265	46	0.19 (0.14)	22	0.24 (0.10)	2	0.16 (0.01)	0.065	0.404
	rs174570	28	0.21 (0.09)	12	1.53 (4.48)	-	-	**0.000**	63	0.22 (0.09)	10	0.18 (0.06)	-	-	0.224	42	0.23 (0.11)	10	0.21 (0.07)	-	-	0.053	54	0.22 (0.13)	16	0.18 (0.09)	-	-	**0.050**	0.065
	rs174575	21	0.97 (3.399	19	0.21 (0.09)	-	-	**0.000**	38	0.21 (0.07)	29	0.23 (0.10)	6	0.18 (0.14)	**0.017**	28	0.23 (0.13)	21	0.23 (0.07)	3	0.16 (0.11)	**0.040**	38	0.18 (0.09)	30	0.24 (0.16)	2	0.16 (0.01)	**0.002**	0.714 3
FADS2	rs174579	27	0.80 (2.99)	13	0.21 (0.10)	-	-	**0.000**	57	0.23 (0.09)	13	0.19 (0.08)	3	0.10 (0.04)	0.454	29	0.23 (0.13)	23	0.22 (0.08)	-	-	**0.029**	44	0.20 (0.14)	26	0.22 (0.09)	-	-	**0.034**	0.734 0
	rs174602	28	0.77 (2.94)	9	0.21 (0.09)	3	0.28 (0.06)	**0.000**	38	0.23 (0.09)	35	0.20 (0.09)	-	-	0.847	27	0.25 (0.12)	22	0.20 (0.08)	3	0.16 (0.11)	0.152	38	0.20 (0.10)	32	0.21 (0.15)	-	-	**0.015**	0.603
	rs498793	8	2.22 (5.47)	18	0.20 (0.08)	14	0.22 (0.09)	**0.000**	22	0.20 (0.06)	41	0.23 (0.09)	10	0.19 (0.10)	0.135	12	0.17 (0.08)	27	0.26 (0.12)	13	0.21 (0.07)	0.055	26	0.23 (0.10)	24	0.22 (0.17)	20	0.17 (0.09)	**0.006**	0.308
	rs2727271	25	0.21 (0.09)	15	1.27 (4.01)	-	-	**0.000**	47	0.20 (0.07)	26	0.25 (0.11)	-	-	**0.011**	48	0.23 (0.11)	4	0.18 (0.10)	-	-	0.901	61	0.21 (0.13)	9	0.19 (0.09)	-	-	0.200	**0.050**
FADS3	rs174448	23	0.90 (3.24)	13	0.22 (0.08)	4	0.19 (0.14)	**0.000**	29	0.23 (0.09)	34	0.21 (0.09)	10	0.17 (0.06)	0.453	32	0.22 (0.12)	20	0.23 (0.08)	-	-	0.052	20	0.23 (0.20)	50	0.20 (0.08)	-	-	**0.000**	0.446
C22:6n-3 (DHA)																														
FADS1	rs174556	15	0.59 (0.25)	22	1.47 (3.76)	3	0.57 (0.08)	**0.000**	29	0.61 (0.26)	38	0.63 (0.26)	6	0.38 (0.12)	0.161	36	0.70 (0.41)	13	0.79 (0.17)	3	0.53 (0.14)	**0.006**	46	0.58 (0.25)	22	0.53 (0.16)	2	0.46 (0.15)	0.088	**0.025**
	rs174570	28	0.62 (0.24)	12	2.13 (5.09)	-	-	**0.000**	63	0.62 (0.25)	10	0.52 (0.15)	-	-	0.094	42	0.70 (0.38)	10	0.74 (0.21)	-	-	**0.046**	54	0.56 (0.27)	16	0.55 (0.21)	-	-	0.205	0.654
FADS2	rs174575	21	1.46 (3.86)	19	0.63 (0.22)	-	-	**0.000**	38	0.57 (0.24)	29	0.672 (0.22)	6	0.38 (0.12)	0.240	28	0.68 (0.44)	21	0.77 (0.22)	3	0.53 (0.14)	**0.005**	38	0.60 (0.23)	30	0.52 (0.20)	2	0.46 (0.15)	0.699	0.705
	rs174579	27	1.31	13	0.59	-	-	**0.000**	57	0.60	13	0.65	3	0.46	0.236	29	0.70	23	0.72	-	-	**0.000**	44	0.58	26	0.53	-	-	0.592	0.214

		Group of Supplementation																									
		5-MTHF							FO							FO + 5-MTHF							PLACEBO						
		0		1		2		p	0		1		2		p	0		1		2		p	0		1		2		p	p
Gene	SNP	N	M (SD)	N	M (SD)	N	M (SD)		N	M (SD)	N	M (SD)	N	M (SD)		N	M (SD)	N	M (SD)	N	M (SD)		N	M (SD)	N	M (SD)	N	M (SD)		
			(3.40)		(0.21)					(0.24)		(0.26)		(0.07)			(0.44)		(0.20)					(0.23)		(0.21)				
	rs174602	28	1.25 (3.35)	9	0.74 (0.26)	3	0.39 (0.70)	**0.000**	38	0.66 (0.27)	35	0.55 (0.18)	-	-	**0.023**	27	0.71 (0.42)	22	0.73 (0.28)	3	0.53 (0.14)	0.065	38	0.61 (0.22)	32	0.51 (0.21)	-	-	0.684	0.443
	rs498793	8	2.74 (6.28)	18	0.68 (0.26)	14	0.63 (0.23)	**0.000**	22	0.56 (0.12)	41	0.61 (0.26)	10	0.67 (0.33)	**0.000**	12	0.57 (0.19)	27	0.75 (0.40)	13	0.75 (0.34)	**0.037**	26	0.56 (0.17)	24	0.57 (0.23)	20	0.55 (0.27)	0.109	0.574
	rs2727271	25	0.59 (0.24)	15	1.87 (4.54)	-	-	**0.000**	47	0.60 (0.25)	26	0.61 (0.21)	-	-	0.262	48	0.71 (0.36)	4	0.71 (0.36)	-	-	0.877	61	0.56 (0.23)	9	0.54 (0.18)	-	-	0.445	0.060
FADS3	rs174448	23	1.43 (3.68)	13	0.56 (0.18)	4	0.70 (0.33)	**0.000**	29	0.68 (0.27)	34	0.55 (0.22)	10	0.56 (0.82)	**0.003**	32	0.67 (0.43)	20	0.76 (0.18)	-	-	**0.000**	20	0.49 (0.20)	50	0.59 (0.22)	-	-	0.542	0.427

Random effect=time points (8, 9 and 9.5 years of age)
0=Homozygous major, 1= Heterozygous, 2 = Homozygous minor
N= Number of samples
M= Mean
SD = Standard Deviation
p = p-value from one way anova

Table 12. *Associations between polyunsaturated fatty acids concentrations in glycerophospholipids from children cheek cell samples and selected SNPs of FADS gene cluster polymorphism adjusted by different confounders.*

	CONS	rs174556	Rs174570	rs174575	Rs174579	rs174602	rs498793	rs2727271	rs174448	Sex	MA	BMI20	BMI30	S20	S30	PWD	GS	P value	P value adjusted
C18:2n-6 (LA)																			
OR	19.13	1.53	0.36	-0.50	0.33	1.02	-0.63	-0.82	-1.06	-1.20	0.15	-0.49	0.18	1.40	-0.27	0.00	0.09		
CI low	13.98	0.06	-0.90	-1.94	-1.73	0.05	-1.19	-3.06	-1.76	-1.19	0.06	-0.94	-0.23	0.31	-1.73	-0.01	-0.24	**<0.0001**	-
CI up	24.27	3.08	1.64	0.93	2.41	2.01	-0.08	1.42	-0.36	-0.42	0.24	-0.03	0.60	3.13	1.17	0.04	0.43		
P	**0.00**	**0.04**	0.57	0.49	0.74	**0.03**	**0.02**	0.47	**0.00**	**0.00**	**0.00**	**0.03**	0.39	0.10	0.71	0.88	0.59		
C20:4n-6 (AA)																			
OR	4.82	-0.44	0.80	-0.55	0.62	0.06	-0.09	0.79	0.11	-0.45	0.01	-0.18	0.14	-0.13	-0.41	-0.01	-0.01		
CI low	3.24	-0.90	-1.19	-0.99	-0.01	-0.23	-0.26	0.10	-0.10	-0.69	-0.01	-0.32	0.01	-0.66	-0.86	-0.10	-0.10	**<0.0001**	-
CI up	6.24	0.1	-0.40	-0.11	1.25	0.36	0.07	1.48	0.32	-0.22	0.04	-0.04	0.27	0.39	0.03	0.10	0.1		
P	**0.00**	**0.05**	**0.00**	**0.01**	**0.05**	0.67	0.28	**0.02**	0.31	**0.00**	0.33	**0.00**	**0.02**	0.60	0.07	0.26	0.59		
C18:3n-3 (ALA)																			
OR	0.82	0.10	0.10	0.08	-0.10	-0.01	-0.02	-0.12	-0.05	0.06	0.03	-0.01	-0.01	0.12	-0.05	0.00	-0.17		
CI low	0.42	-0.01	0.01	-0.02	-0.26	-0.08	-0.06	-0.29	-0.11	-0.12	-0.04	-0.05	-0.05	-0.01	-0.16	-0.01	-0.05	**<0.0001**	-
CI up	1.20	0.01	0.20	0.19	0.05	0.06	0.02	0.05	-0.04	-0.05	0.1	0.02	0.03	0.25	0.05	-0.01	-0.1		
P	**0.00**	0.07	**0.03**	0.14	0.19	0.79	0.31	0.17	**0.03**	**0.03**	0.39	0.49	0.59	0.07	0.33	0.63	0.19		
C20:5n-3 (EPA)																			

	CONS	rs174556	Rs174570	rs174575	Rs174579	rs174602	rs498793	rs2727271	rs174448	Sex	MA	BMI20	BMI30	S20	S30	PWD	GS	P value	P value adjusted
OR	0.25	-0.03	-0.04	-0.04	0.06	0.00	0.05	0.09	-0.01	-0.02	0.00	-0.01	0.01	0.04	-0.03	0.00	0.00		
CI low	0.05	-0.09	-0.09	-0.06	-0.01	-0.03	-0.01	0.04	-0.04	-0.05	-0.01	-0.03	-0.05	-0.06	-0.08	-0.00	-0.01	0.2810	**0.0437**
CI up	0.45	0.01	0.01	0.04	0.14	0.03	0.02	0.17	0.01	0.00	0.01	0.03	0.02	0.06	0.02	0.01	0.01		
P	**0.01**	0.19	0.06	0.83	0.13	0.99	0.67	**0.04**	0.34	0.09	0.66	0.11	0.19	0.91	0.27	0.97	0.99		
C22:5n-3 (DPA)																			
OR	0.44	-0.03	-0.03	-0.00	0.03	-0.01	-0.08	0.05	-0.01	-0.03	0.04	-0.01	0.08	-0.03	-0.03	-0.00	-0.04		
CI low	0.27	-0.08	-0.07	-0.04	-0.03	-0.05	-0.02	-0.02	-0.03	-0.06	0.00	-0.02	-0.06	-0.08	-0.08	-0.00	-0.01	**<0.0001**	-
CI up	0.61	0.01	0.01	0.04	0.10	0.01	0.01	0.1	-0.01	-0.01	0.01	0.01	0.02	0.02	0.01	0.00	0.07		
P	**0.00**	0.20	0.08	0.98	0.32	0.26	0.41	0.16	0.32	**0.00**	**0.02**	0.06	0.27	0.26	0.16	0.24	0.51		
C22:6n-3 (DHA)																			
OR	0.67	-0.24	-0.14	-0.09	0.34	-0.03	0.06	035	0.02	-0.04	0.04	-0.02	0.01	-0.07	0.01	0.00	-0.02		
CI low	0.15	-0.39	-0.26	-0.24	0.13	-0.13	0.05	0.12	-0.04	-0.11	-0.05	-0.11	-0.06	-0.02	-0.24	-0.00	-0.06	0.0899	0.2068
CI up	1.18	-0.09	-0.01	0.04	0.55	0.06	0.11	0.57	0.09	0.03	0.1	0.03	0.01	0.06	0.09	0.00	0.08		
P	**0.01**	**0.00**	**0.03**	0.17	**0.00**	0.51	**0.03**	**0.00**	0.47	0.25	0.41	0.31	0.40	0.39	0.98	0.34	0.13		

CONS=Constant Value; GS=Group of supplementation; MA=Maternal age; GA=Gestational age; BMI20=Body Mass Index at week 20 of pregnancy; BMI30=Body Mass Index at week 30 of pregnancy; S20=Smoking at week 20 of pregnancy; S30=Smoking at week 30 of pregnancy; PWD=Placental weight at delivery; OR=Odds ratio; CI=Confidence Interval

We then analysed the effects of SNPs and different cofounders on the FA concentrations in GPL from cheek cells (**Table 12**). LA concentrations were influenced by rs174556, rs174602, rs498793 and rs174448 genotypes, the child's gender, maternal age and BMI 20 ($p<0.0001$). ALA was influenced by rs174570, rs174448 and gender. In the case of AA, this FA was influenced by all the SNP studied, except rs174602, rs498793 and rs174448. EPA shows a liaison with rs2727271, meanwhile DPA shows no correlation with any SNPs, but does with gender and maternal age. Different SNPs influenced the DHA concentrations, but the model showed no statistical significance.

Fatty acids from cheek cells (by country of origin)

To examine the associations between diet and cheek-cell GPL-FA composition, and how these associations vary in relation to age, sex, country of origin 130 pairs of mothers and children were included in these analyses. 17 children have been to excluded because they did not complete the FFQ and diet diaries properly. The baseline characteristics depending on the country of origin are shown in **Table 13**. Maternal age at delivery was higher in Spain than in Germany women (P=0.0015). Maternal body mass index (BMI) at week 20 and 30 of pregnancy was lower in Spanish women than in German ones. The Spanish women smoked more than the German ones, both at week 20 of pregnancy (16.83% vs. 0%) and in week 30 (21.78% vs. 0%). There were no significant differences with respect to sex, gestational age or placental weight.

The mean values of GPL-FAs in the cheek cells samples are shown in **Table 14**. The percentages resulted significantly different between both countries in water, proteins, folate, AA and DHA. To notice, that in general the cheek cells GPL-FAs concentrations were higher in German children than in Spanish children, in the three time points evaluated, except for Myristic, Palmitic and Steraic acid.

Table 13. *Main general characteristics by country of origin of the children included in the present study.*

Variables	Spain (n=101)	Germany (n=29)	*P*
Group of supplementation			**0.018**
FO	22 (21.78)	5 (17.24)	
5-MTHF	27 (26.73)	10 (34.48)	
Placebo	24 (23.76)	6 (20.69)	
FO+5-MTHF	28 (27.72)	8 (27.59)	
Sex			0.761
boy*	49 (48.51)	15 (41.72)	
girl*	52 (51.49)	14 (48.28)	
Maternal age at delivery#	33.67 ± 2.98	30.56 ± 4.70	**0.0015**
Gestational age#	39.90 ± 1.65	39.60 ± 2.67	0.0525
Maternal BMI week 20#	24.31 ± 3.54	25.35 ± 2.84	**0.0217**
Maternal BMI week 30#	26.41 ± 3.84	27.53 ± 2.98	**0.0278**
Smoking week 20			
no*	84 (83.17)	29 (100)	
yes*	17 (16.83)	0 (0)	
Smoking week 30			
no*	79 (78.22)	29 (100)	
yes*	22 (21.78)	0 (0)	
Placenta weight#	547.31 ± 85.36	539.90 ± 121.33	0.4816

** n (%)*
#:Values expressed mean ± standard deviation
P: level of significance
Bold: P values<0.05

Table 14. *Fatty acid levels in glycerophospholipids measured in cheek cells from NUHEAL children at 8, 9 and 9.5 years old.*

Fatty acids (%)	Country	8 years		*P*	9 years		*P*	9.5 years		*P*
		Mean	SD		Mean	SD		Mean	SD	
Myristic acid (C14:0)	Germany	1. 16	0.56	0.257	0.89	0.53	0.055	1.23	0.66	0.364
	Spain	1.00	0.76		1.11	0.58		1.20	0.95	
Palmitic acid (C16:0)	Germany	16.72	1.50	**<0.001**	16.74	3.39	**<0.001**	16.14	2.08	**<0.001**
	Spain	20.83	2.92		20.52	3.67		20.91	3.92	
Palmitoleic acid (C16:1n-7)	Germany	6.89	1.26	**<0.001**	5.77	1.54	0.131	7.06	1.51	**<0.001**
	Spain	5.23	1.50		5.32	1.42		5.02	1.60	
Margaric acid (C17:0)	Germany	0.84	0.17	**0.015**	0.82	0.19	0.737	0.79	0.14	0.717
	Spain	0.75	0.20		0.80	0.22		0.80	0.19	
Stearic acid (C18:0)	Germany	13.28	1.40	**<0.001**	13.58	2.26	**<0.001**	12.64	1.66	**<0.001**
	Spain	23.75	7.06		21.63	5.97		23.78	8.52	
Oleic acid (C18:1n-9)	Germany	29.28	1.81	**<0.001**	29.23	4.69	**<0.001**	30.11	4.96	**<0.001**
	Spain	22.80	4.98		23.49	4.46		21.77	4.69	
Vaccenic acid (C18:1n-7)	Germany	4.91	0.52	0.314	3.90	0.83	**0.008**	4.13	0.63	**<0.001**
	Spain	4.36	3.25		3.42	0.91		3.15	0.91	
Linoleic acid (C18:2n-6)	Germany	18.85	1.93	**<0.001**	18.17	3.03	**<0.001**	18.50	2.10	**<0.001**
	Spain	15.56	3.50		15.36	2.75		14.81	3.96	
Gamma-linolenic acid (C18:3n-6)	Germany	0.12	0.04	0.059	0.16	0.05	**<0.001**	0.15	0.04	0.087

	Spain	0.10	0.05		0.12	0.04		0.13	0.05	
Alpha-linolenic acid (C18:3n-3)	Germany	0.47	0.35	**<0.001**	0.42	0.26	**0.040**	0.39	0.16	**<0.001**
	Spain	0.27	0.14		0.30	0.29		0.26	0.13	
	Germany	0.52	0.16		0.52	0.10		0.49	0.12	
Arachidic acid (C20:0)	Spain	0.44	0.09	**<0.001**	0.35	0.07	**<0.001**	0.43	0.11	**0.034**
	Germany	0.24	0.07		0.27	0.08		0.21	0.04	
Eicosenoic acid (C20:1n-9)	Spain	0.22	0.09	0.169	0.23	0.06	**0.007**	0.22	0.09	0.599
	Germany	1.32	0.33		1.17	0.39		1.21	0.37	
Dihomo-gamma-linolenic acid (C20:3n-6)	Spain	0.96	0.32	**<0.001**	0.99	0.31	**0.011**	0.95	0.31	**<0.001**
	Germany	3.63	0.86		2.93	0.91		3.00	0.72	
Arachidonic acid (AA) (C20:4n-6)	Spain	2.58	0.80	**<0.001**	2.54	0.78	**0.026**	2.29	0.86	**<0.001**
	Germany	0.36	0.19		0.50	0.18		0.34	0.12	
Behenic acid (C22:0)	Spain	0.19	0.07	**<0.001**	0.26	0.08	**<0.001**	0.31	0.12	0.377
	Germany	0.15	0.08		0.65	2.80		0.24	0.06	
Eicosapentaenoic acid (C20:5n-3)	Spain	0.11	0.11	0.084	0.18	0.12	0.121	0.14	0.07	**<0.001**
	Germany	0.16	0.18		0.46	0.33		0.09	0.03	
Docosapentaenoic acid (C22:5n-6)	Spain	0.08	0.07	**<0.001**	0.09	0.06	**<0.001**	0.23	0.23	**0.002**
	Germany	0.27	0.10		0.71	2.66		0.35	0.08	
Docosapentaenoic acid (C22:5n-3)	Spain	0.17	0.07	**<0.001**	0.19	0.09	0.072	0.18	0.07	**<0.001**
	Germany	0.67	0.23		1.07	3.05		0.59	0.18	
Docosahexaenoic acid (C22:6n-3)	Spain	0.59	0.25	0.127	0.65	0.30	0.213	0.61	0.27	0.531

Values expressed mean standard deviation of the % of each fatty acid measured in cheek cells
P: level of significance
Bold: *P values*<0.05

PCA was first used to explore the data structure; indeed, it is a very useful technique, which emphasizes variation and brings out strong patterns in a dataset. In the PCA score plot on GPL-FA data from children 8 years old (**Figure 14a**), the country of origin (Germany or Spain) was driving the data. As well, when we built up a cross-validate score plot for PLS-DA model on fatty acids data (R2X=0.546, R2Y=0.673, Q2=0.513) (**Figure 14b**), the country of origin again was responsible of the separation. The two first components of the model cover 51% of the variance. The features most influential for the model showed in **Figure 14b** can be selected on the basis of variable importance in the projection (VIP) scores. VIP plot is coloured according to VIP values. Stearic acid, Palmitic acid and DPA turned out to be the most influential fatty acids for the model.

We observed once more in the score plot of PCA model on an entire data set (all time points included) (**Figure 15a**) that the country of origin drives the distribution. The first two components cover approximately 47% of variance. **Figure 15b** shows the clear difference between the two countries (R2X 0.514, R2Y 0.698, Q2 0.657), (the model uses two time points, 8 and 9.5 years of age) and in the VIP plot we observe that Stearic acid and Palmitic acid turned out to be the most influential fatty acids for the model.

Considering fatty acids in glycerophospholipids present in the cheek cells obtained from Spanish children only, in relation to the time points studied (R2X 0.199, R2Y 0.555, Q2 0.326), **Figure 16a** shows the evolution of the fatty acid profile between 8 and 9.5 years. Weobserved that Vaccenic acid and Behenic were the most relevant fatty acids in the GPL fraction influencing the distribution of the profile obtained in the PLS-DA model. **Figure 16b** shows the evaluation of FAs profile over the time. It was built considering the GPL-FAs obtained from cheek cells, taking into account the German children only, with time points as class variables (model uses two time points, 8 and 9.5 years of age; R2X 0.28, R2Y 0.799, Q2 0.573). As in the other cases, it is interesting that at the coefficient plot for the same model, the most influential fatty acids of those determined were both Vaccenic acid and EPA. Therefore, the model shows that Spanish and German children at 8 and 9.5 years of age have differences between them as far as the fatty acid composition is concerned.

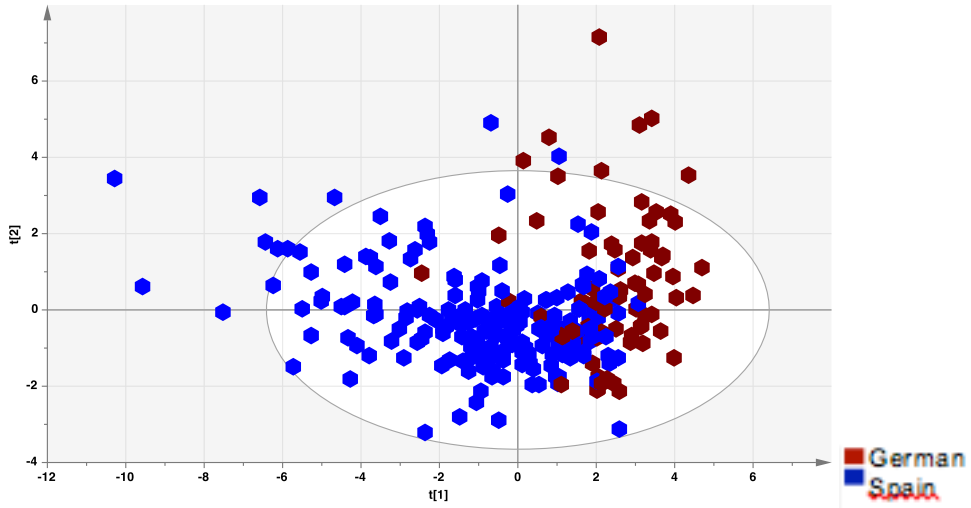


Figure 14a. *PCA score plot on fatty acids in glycerophospholipids from NUHEAL children's cheek cells at 8y.*

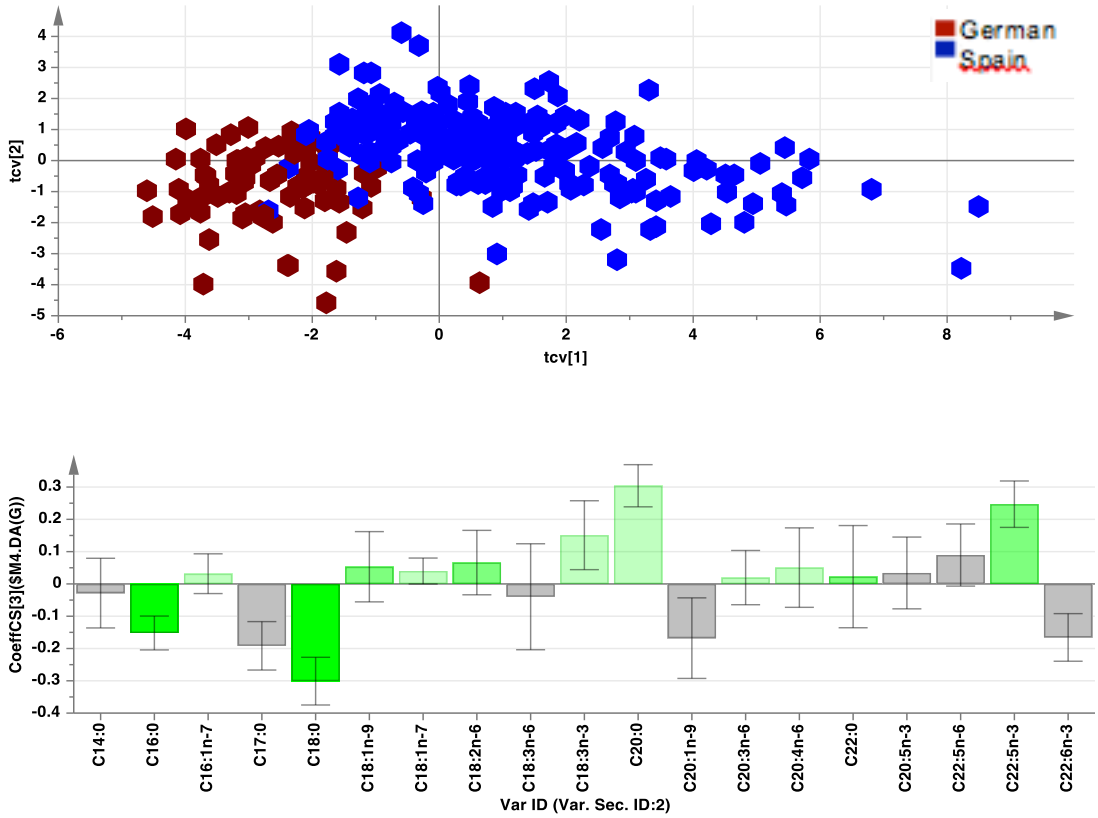


Figure 14b. *Cross-validated score plot of a PLS-DA model and Coefficient plot for the same model according to their VIP values (Fatty acids in glycerophospholipids obtained from German and Spanish children's cheek cells at 8 and 9.5y).*

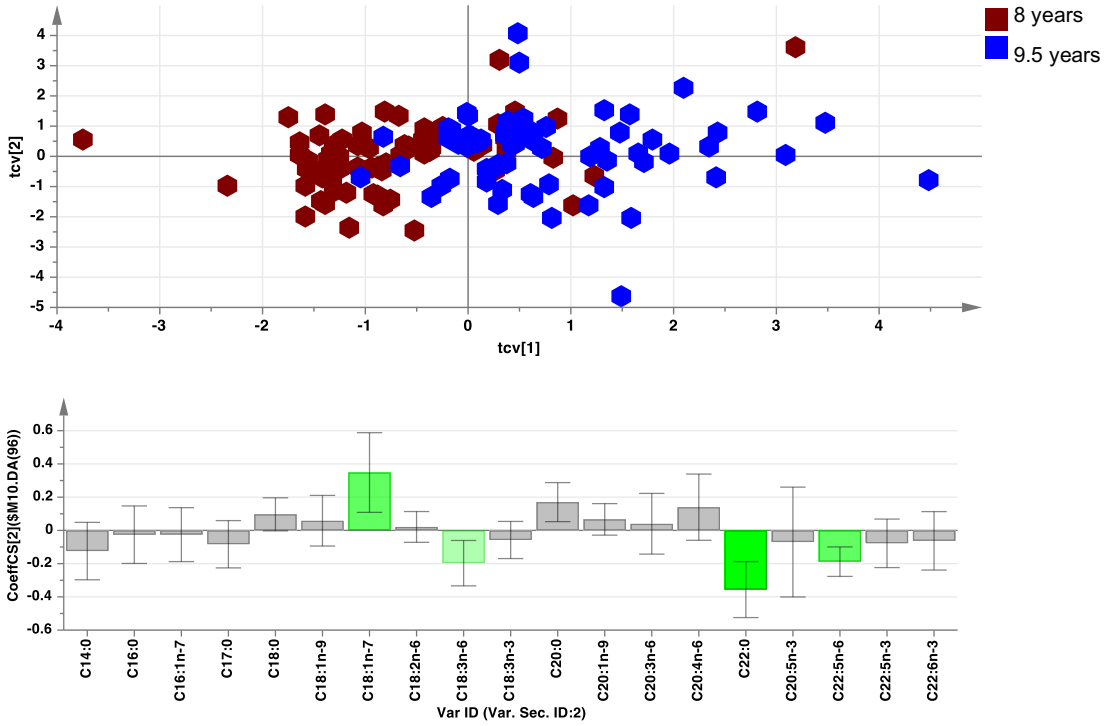


Figure 15a. *PCA score plot on fatty acids in glycerophospholipids from NUHEAL children's cheek cells at 8y, 9y and 9.5y;* **b.** *Cross-validated score plot of a PLS-DA model and Coefficient plot for the same model according to their VIP values (Fatty acids in glycerophospholipids obtained from German and Spanish children's cheek cells at 8y and 9.5y).*

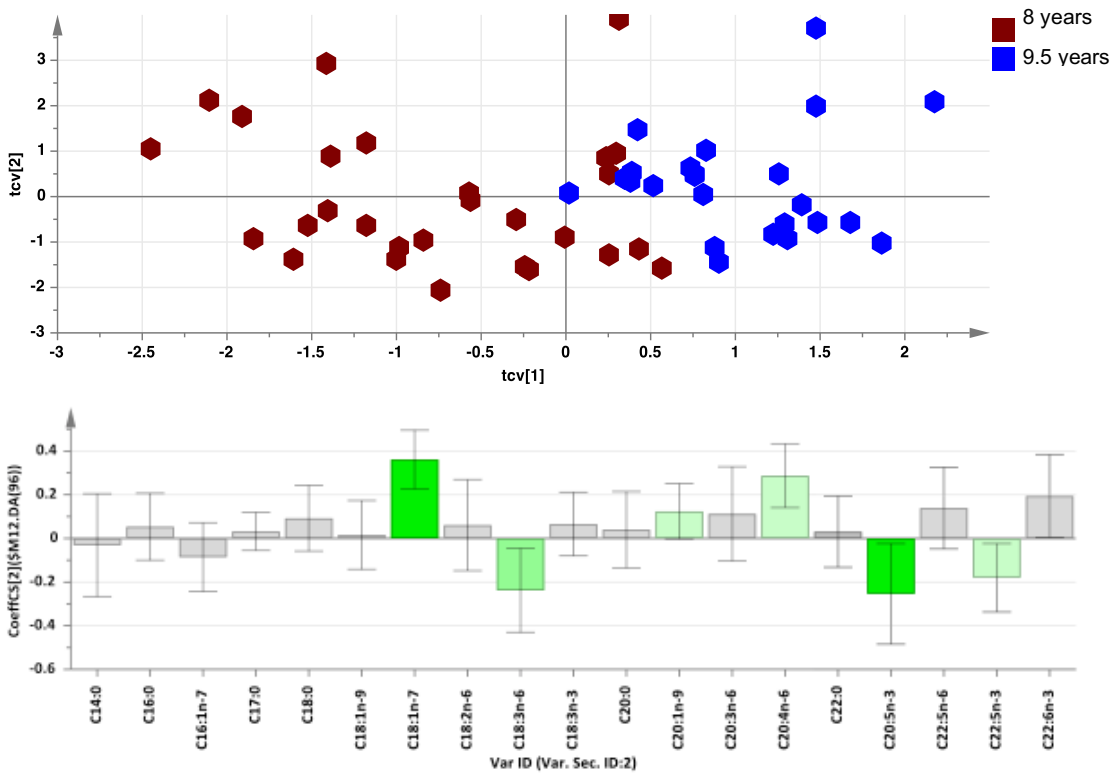


Figure 16a. *Cross-validated score plot of a PLS-DA model and Coefficient plot for the same model according to their VIP values (Fatty acids in glycerophospholipids measured in NUHEAL Spanish children's cheek cells at 8y and 9.5y);* ***b.*** *Cross-validated score plot of a PLS-DA model and Coefficient plot for the same model according to their VIP values (Fatty acids in glycerophospholipids measured in NUHEAL German children's cheek cells at 8 and 9.5y).*

Dietary intake

Daily dietary intakes of the NUHEAL children aged from 7.5 to 8 years are shown in **Table 15**. The averages of the two countries (Spain and Germany) are significantly different for water (P=0.0023), proteins (P<0.001), folate (P=0.0066), AA (P<0.001) and DHA (P=0.002). Higher intake of proteins and folate and were confirmed in the Spanish children, and the intake of water, AA and DHA resulted higher in German ones.

Table 15. *Daily dietary intakes of the NUHEAL children aged from 7.5 to 8.*

Variables	Spain		Germany		*P*
	Mean	SD	Mean	SD	
Energy (Kcal)	1856.66	336.15	1765.01	356.14	0.2317
Energy (KJ)	7726.71	1392.16	7384.81	1489.16	0.2765
Water (g)	1324.39	373.61	1585.01	316.21	**0.0023**
Protein (g)	71.26	15.50	53.39	9.62	**0.0000**
Lipids (g)	72.47	16.48	65.42	15.10	0.0620
Carbohydrates (g)	222.00	50.40	235.44	66.52	0.3963
Fiber (g)	14.81	4.62	13.37	2.99	0.2350
Folate (g)	191.80	55.22	159.58	74.65	**0.0066**
AA (g)	0.05	0.03	0.21	0.07	**0.0000**
EPA (g)	0.07	0.21	0.04	0.05	0.4686
DHA (g)	0.07	0.08	0.15	0.12	**0.0002**
PUFAs-n3 (g)	1.33	0.62	1.08	0.31	0.0637
PUFAs-n6 (g)	7.49	2.71	6.97	1.97	0.4947

Values expressed mean standard deviation cells*; P: level of significance;*
Bold: *P values*<0.05

Associations between PUFA concentrations in GPL from all cheek-cell samples obtained and the selected confounders and dietary intake are shown in **Table 16**.

It was confirmed that diet clearly influences the % of FAs present in the children's cheek cells; specially, AA daily intake showed a high impact on the percentage of GPL-FAs found in the children's cheek cells. The intake of lipids and fibre decreases the level of AA. The intake of folate and EPA increases the level of AA in GPLs of cheek

cells. EPA increases the level of EPA and DPA. Finally, DHA decreases the concentration of DPA.

Table 16. *Associations between polyunsaturated fatty acid concentrations in glycerophospholipids from all cheek cells samples obtained, the selected confounders and the dietary intake.*

	Linoleic acid (LA) (C18:2n-6)			Arachidonic acid (AA) (C20:4n-6)			Alpha-Linolenic (ALA) (C18:3n-3)			Eicosapentaenoic acid (EPA) (C20:5n-3)			Docosapentaenoic acid (DPA) (C22:5n-3)			Docosahexaenoic acid (DHA) (C22:6n-3)		
	Beta	CI (95 %)	p-value	Beta	CI (95 %)	p-value	Beta	CI (95 %)	p-value	Beta	CI (95 %)	p-value	Beta	CI (95 %)	p-value	Beta	CI (95 %)	p-value
Country	-3.19	(-5.36, -1.01)	**0.004**	-0.14	(-0.77, 0.49)	0.667	-0.29	(-0.44, -0.15)	**0.000**	0.01	(-0.07, 0.08)	0.867	-0.07	(-0.12, 0.01)	**0.027**	0.09	(-0.14, 0.32)	0.458
Group of supplementation	0.03	(-0.28, 0.33)	0.873	-0.05	(-0.13, 0.04)	0.315	-0.02	(-0.04, 0.01)	0.139	-0.00	(-0.01, 0.01)	0.545	-0.01	(-0.01, 0.00)	0.110	-0.02	(-0.05, 0.01)	0.219
Maternal age	0.02	(-0.06, 0.10)	0.617	-0.01	(-0.03, 0.01)	0.446	-0.00	(-0.01, 0.00)	0.161	0.00	(-0.00, 0.01)	0.088	0.00	(0.00, 0.00)	**0.043**	0.01	(0.00, 0.02)	**0.043**
Gestational Age	0.13	(-0.00, 0.26)	0.057	0.00	(-0.04, 0.04)	0.897	0.00	(-0.01, 0.01)	0.591	-0.00	(-0.01, 0.00)	0.123	-0.00	(-0.01, 0.00)	0.158	-0.01	(-0.02, 0.01)	0.452
BMI at week 20	-0.22	(-0.67, 0.21)	0.314	0.04	(-0.09, 0.16)	0.565	-0.01	(-0.03, 0.03)	0.763	-0.01	(-0.02, 0.01)	0.432	-0.00	(-0.01, 0.01)	0.970	0.02	(-0.02, 0.07)	0.317
BMI at week 30	0.06	(-0.34, 0.46)	0.775	-0.03	(-0.15, 0.09)	0.630	-0.01	(-0.03, 0.02)	0.688	0.01	(-0.01, 0.02)	0.436	-0.00	(-0.01, 0.01)	0.878	-0.02	(-0.07, 0.02)	0.261
Smoking at week 20	1.07	(-0.76, 2.90)	0.251	0.44	(-0.09, 0.97)	0.102	0.06	(-0.07, 0.18)	0.364	-0.03	(-0.10, 0.03)	0.344	-0.04	(-0.09, 0.01)	0.133	-0.16	(-0.35, 0.03)	0.106
Smoking at week 30	0.54	(-1.21, 2.30)	0.546	-0.44	(-0.94, 0.07)	0.092	-0.04	(-0.16, 0.08)	0.479	´0.00	(-0.06, 0.06)	0.939	0.02	(-0.03, 0.06)	0.470	0.11	(-0.08, 0.29)	0.259
Placental wight	0.00	(-0.00, 0.00)	0.784	-0.00	(-0.00, 0.00)	0.183	-0.00	(-0.00, 0.00)	0.915	0.00	(-0.00, 0.00)	0.583	0.00	(-0.00, 0.00)	0.695	0.00	(-0.00, 0.00)	**0.049**
Sex	-0.65	(-1.39, 0.09)	0.085	-0.39	(-0.61, -0.18)	**0.000**	-0.02	(-0.07, 0.03)	0.411	-0.01	(-0.04, 0.01)	0.378	-0.01	(-0.03, 0.01)	0.356	0.02	(-0.06, 0.10)	0.568
Energy (Kcal)	0.01	(-0.03, 0.05)	0.714	0.01	(-0.00, 0.02)	0.192	-0.00	(-0.00, 0.00)	0.657	-0.00	(-0.00, 0.00)	0.599	0.00	(-0.00, 0.00)	0.621	-0.00	(-0.01, 0.00)	0.610
Energy (KJ)	-0.00	(-0.01, -0.00)	**0.015**	0.00	(0.00, 0.00)	**0.011**	-0.00	(-0.00, 0.00)	0.456	-0.00	(-0.00, 0.00)	0.991	0.00	(0.00, 0.00)	**0.038**	0.00	(-0.00, 0.00)	0.358
Water	0.00	(-0.00, 0.00)	0.068	0.00	(0.00, 0.00)	**0.030**	-0.00	(-0.00, 0.00)	0.385	0.00	(-0.00, 0.00)	0.527	0.00	(-0.00, 0.00)	0.725	-0.00	(-0.00, 0.00)	0.707
Proteins	0.05	(-0.12, 0.23)	0.552	-0.05	(-0.10, 0.00)	0.071	0.00	(-0.01, 0.02)	0.544	0.00	(-0.00, 0.01)	0.496	-0.00	(-0.01, 0.00)	0.332	0.01	(-0.01, 0.02)	0.607
Lipids	0.06	(-0.32, 0.43)	0.775	-0.12	(-0.23, -0.01)	**0.029**	0.01	(-0.02, 0.04)	0.440	0.00	(-0.01, 0.02)	0.686	-0.01	(-0.02, 0.01)	0.290	0.00	(-0.04, 0.04)	0.895
Carbohydrates	0.02	(-0.14, 1.19)	0.800	-0.05	(-0.10, 0.00)	0.058	0.00	(-0.01, 0.02)	0.535	0.00	(-0.00, 0.01)	0.575	-0.00	(0.01, 0.00)	0.319	0.00	(-0.02, 0.02)	0.795
Fiber	-0.04	(-0.22, 0.13)	0.634	-0.07	(-0.12, -0.02)	**0.004**	0.01	(-0.01, 0.02)	0.322	0.00	(-0.01, 0.01)	0.641	-0.00	(-0.01, 0.00)	0.373	0.00	(-0.02, 0.02)	0.899
Folate	-0.01	(-0.02, 0.00)	0.321	0.00	(0.00, 0.01)	**0.027**	0.00	(-0.00, 0.00)	0.476	0.00	(-0.00, 0.00)	0.390	0.00	(-0.00, 0.00)	0.149	0.00	(-0.00, 0.00)	0.242
AA	-1.03	(-11.47, 9.41)	0.847	3.77	(0.75, 6.78)	**0.014**	-0.29	(-1.00, 0.42)	0.428	0.22	(-0.15, 0.58)	0.246	0.25	(-0.02, 0.53)	0.073	0.50	(-0.60, 1.60)	0.369
EPA	9.17	(-1.82, 20.16)	0.102	2.81	(-0.35, 5.98)	0.081	0.47	(-0.28, 1.22)	0.215	0.54	(0.15, 0.92)	**0.006**	0.41	(0.13, 0.70)	**0.005**	1.00	(-0.13, 2.12)	0.082

	Linoleic acid (LA) (C18:2n-6)			Arachidonic acid (AA) (C20:4n-6)			Alpha-Linolenic (ALA) (C18:3n-3)			Eicosapentaenoic acid (EPA) (C20:5n-3)			Docosapentaenoic acid (DPA) (C22:5n-3)			Docosahexaenoic acid (DHA) (C22:6n-3)		
	Beta	CI (95 %)	p-value	Beta	CI (95 %)	p-value	Beta	CI (95 %)	p-value	Beta	CI (95 %)	p-value	Beta	CI (95 %)	p-value	Beta	CI (95 %)	p-value
DHA	-0.64	(-8.80, 7.53)	0.879	-1.90	(-4.24, 0.46)	0.114	-0.40	(-0.96, 0.16)	0.158	-0.25	(-0.53, 0.04)	0.091	-0.22	(-0.43, -0.00)	**0.050**	-0.07	(-0.90, 0.76)	0.873
PUFA n3	-0.77	(-2.38, 0.85)	0.351	0.50	(0.04, 0.97)	0.035	-0.04	(-0.15, 0.07)	0.480	-0.03	(-0.09, 0.03)	0.269	0.01	(-0.04, 0.05)	0.811	0.07	(-0.10, 0.24)	0.438
PUFA n6	0.07	(-0.16, 0.30)	0.558	-0.01	(-0.08, 0.06)	0.769	-0.00	(-0.02, 0.01)	0.587	0.01	(-0-00, 0.01)	0.231	-0.00	(-0.01, 0.01)	0.742	0.00	(-0.02, 0.03)	0.917
_cons	17.82	(9.13, 26.51)	0.000	3.27	(0.76, 5.78)	0.011	1.14	(0.55, 1.73)	0.000	0.18	(-0.13, 0.48)	0.259	0.37	(0.14, 0.60)	**0.002**	0.13	(-0.78, 1.05)	0.773

Beta: Regression coefficient β from linear regression of the fatty acid (outcome) on the confounders and dietary intake
P: level of significance
Bold: P values<0.05

1.3. Discussion

This is the first study to demonstrate the long-term effects of maternal supplementation with DHA and/or 5-MTHF during pregnancy on PUFA status and metabolism in the offspring; furthermore, this study shows that there is an interaction effect between maternal supplementation during pregnancy and the offspring's *FADS* polymorphisms in the children's PUFA concentrations in cheek-cell GPL.

The results obtained in this study demonstrate a long-term effect of maternal supplementation with FO and/or 5-MTHF during pregnancy on fatty acid status in their offspring up to 9.5 years. Supplementation with FO+5-MTHF during pregnancy determined an increase of children's Behenic acid and DPA concentrations in cheek-cell GPL at 8 years old. Furthermore, prenatal supplementation with FO determined higher oleic acid concentrations in GPL in the offspring at 9 years old, compared to those children born to mothers from 5-MTHF or FO+5-MTHF groups.

We demonstrated previously a significant increase of PUFAs in plasma phospholipids and erythrocyte membranes after supplementation of pregnant women with FO or FO+5-MTHF from 20 weeks of pregnancy till delivery; these changes determined clear associations between maternal fatty acid concentrations in phospholipids during pregnancy and those levels found in their offspring (umbilical cord) [(9)]. However, the major interest of our findings is the long-term influence of maternal supplementation on their offspring's FA concentrations in cheek- cell GPL.

It has been demonstrated that maternal nutritional condition and FA composition of the diet during pregnancy and/or lactation are critical factors that are strongly associated with normal fetal and postnatal development and also seem to influence the modifications in fetal programming, altering the individual risk for developing metabolic diseases throughout life. Essential FAs and LC-PUFAs are central nutrients required for the synthesis of structural lipids, and fundamental to fetal and postnatal development and normal cell function [(1)]. Nevertheless, an excess of certain FAs may harm the availability of others, producing adverse consequences to the fetus and newborn. The current findings suggest a very long-term programming effect on the pathways involved in fatty acids metabolism, which independently from other factors,

may determine the final concentrations in GPL-FA status 9 years after, reflecting unknown long-lasting effects. The data obtained in this study indicates the possible impact of an individual effects of n-3 and n-6 PUFAs on the global lipid metabolism or changes in the balance between n-3 and n-6 in maternal diet on their offspring long-term lipid metabolism. Moreover, an interaction between folic acid and FO is shown, which seems to determine different patterns on the functioning of metabolic pathways.

Analysis of FAs in various body compartments such as plasma, erythrocytes and adipose tissue, have been demonstrated to be useful to obtain information on FA status. These approaches are invasive and not well accepted in population studies, particularly in those including infants and young children as participants. In this study we measured FAs in cheek cells as a reference of FA status. Cheek cells' GPL-FA have also been proposed as biological marker, but are rarely used in clinical studies due to limitations in sample quality and quantity; however, buccal mucosal cells phospholipids are feasible for use as a non-invasive marker for long-chain polyunsaturated fatty acid status in children. [28]

The second aim of this study is to evaluate whether *FADS1*, *FADS2* and *FADS3* genotype polymorphisms may modify PUFAs concentrations in school-age children's cheek cells. We know that FADS gene variants account for up to 28.5 % of the variability in the PUFA and LC-PUFA levels in human tissues [7, 148]). Therefore, blood and tissue levels of the essential fatty acids LA and ALA and of their biologically active LC-PUFA derivatives are influenced not only by diet, but to a large extent also by genetic variation. The importance of our study lies in the fact that this is the first one that has found very long-term effects of nutritional prenatal supplementation and associations of the genetic variants in the *FADS1*, *FADS2* and *FADS3* gene cluster precisely with the cheek cells GPL-FAs in an independent population-based study. From the results obtained, it is demonstrated that there is a clear effect of programming of the lipidic metabolism of children that extends up to 9.5 years of age and where the interaction of 5-MTHF supplementation, with or without FO, with genetic polymorphisms, determines that children have an increase in concentrations of ω-3 LC-PUFA. However, supplementation only with FO, upon interaction with *FADS* polymorphisms causes an increase in the concentration of ALA (precursor of ω-3 LC-

PUFA). This pattern of interaction is not seen in infants born to mothers supplemented with placebo. Similar findings were also seen in other studies [149], where child *FADS* genotypes influenced amounts of cord plasma LC-PUFA and FA ratios and demonstrated a specific effect of the minor allele for it on FA metabolism.

From this study we can deduce that also with the supplementation (FO and/or 5-MTHF), major allele carriers are more frequent for *FADS2* and *FADS3*. In contrast, in the placebo group, the minor allele carriers are more frequent for *FADS1*. Several authors had already demonstrated the association between variants in the *FADS1* and *FADS2* and *FADS3* genes and blood levels of PUFAs [30-36]. It is interesting to mention that in previous studies it has been shown that elevated ALA concentrations in adipose tissue are associated with a lower prevalence of the metabolic syndrome [150]. It could be interesting to find out if concentrations of this FA in cheek cell samples are also related to this illness or other kinds of diseases.

It seems that maternal FO supplementation determines a decrease in the *FAD* activity and thus an increase in precursors of ω-3 and ω-6 FAs. 5-MTHF supplementation during pregnancy stimulates desaturation and elongation of ω-3 FAs in the offspring, increasing LC-PUFAs concentrations. It well known that folic acid improves the remethylation of homocysteine, which leads to the formation of methionine. Some authors [151] suggest that methionine stimulates phosphatidylethanolamine methylation, thereby altering the ratio of phosphatidylcholine to phosphatidylethanolamine in liver microsomes. This in turn increases the activity of δ 5 and δ 6-acyl-coenzyme A desaturases, and consequently, increases LC-PUFAs concentrations.

It should also be noted that AA only was influenced for a unique SNP of *FADS2*. This means that in a natural way the *FADS* do not have much effect on AA. Some authors report, after their investigations, that children with atopic eczema had lower levels of several n ω-6 PUFA members, such as DGLA and AA [152]. Therefore, some kinds of illnesses maybe influenced by the interaction between FADS polymorphisms and levels of FA in the organism.

Recently, in the NUHEAL study it has been demonstrated long-term effects of supplementation during pregnancy with FO and/or 5-MTHF on offspring

neurodevelopment, showing differences in Processing Speed [153] and Attention at 9 years [12]. On the basis of the results discussed so far, we should seriously consider the repercussions on neurodevelopment in these children when they have a different lipidic metabolism.

Other authors have been discussing about the beneficial effects of LC-PUFAs on other health outcomes, depending on our individual *FADS1* genotypes [152] . We further hypothesised that these effects could also be linked to *FADS2* and FAs status. It would be very interesting to perform further studies to evaluate this hypothesis in different fields of children's health.

Finally, we believe it is important to mention that these results have an added value, and the fact that, so far, to the best of our knowledge, no study has made such a long-term follow-up as our current study.

To achieve the fourth aim of this study we used data to evaluate the cheek cells GPL-FA composition from children of the NUHEAL cohort, which are considered as biomarkers of FA status, taking into account the diet and the country of origin.

It is very well known that PUFAs are classified into two principal families, the n-6 (or ω-6) and the n-3 (or ω-3), according to the position of the terminal double bond. The parent FAs of these families, linoleic acid (LA) and α-linolenic acid (ALA), cannot be synthesized by humans and so, they must be provided by the diet and are therefore are defined as EFA. LA and ALA serve as substrates for the synthesis of other important fatty acids and are in turn precursors of AA, DHA and EPA.

Several authors [154, 155] have demonstrated that in Western diets, PUFAs comprise up to 20% of dietary fat. In most cases, LA and ALA contribute more than 95% of dietary PUFA intake. LA is the primary PUFA in quantity terms; in fact, over the last 40 years, LA intake increased markedly in these countries because the raise of cooking oils and margarines popularity. Although ALA intake has changed rather slightly over this time, consumption of both PUFAs exceeds minimal requirements needed to prevent essential FA deficiency. The increased LA intake has changed the ratio of n-6/n-3 PUFAs in the diet. The marked increase of this ratio is estimated today at 5 to 20 in Western countries. In contrast to the consumption of LA, dietary intake of LC-PUFAs is

markedly lower. Intakes of AA are typically in the range of 50 to 500 mg/day. EPA and DHA are found in marine foods, especially in oily fish; one oily fish meal can provide 1.5 to 3.5 g of n-3 LC-PUFAs. In the absence of oily fish consumption, intakes of n-3 LC-PUFAs are very low, at approximately less than 100 mg/day.

Most pregnant women in Europe do not reach the recommendations of LC-PUFA intake. New dietary recommendations for pregnant women include the intake of 2 pieces of oily fish per week to achieve the LC-PUFA needs for an optimal foetal growth and better pregnancy outcomes [(156)]. In this study we found some long-term effects of prenatal supplementation with FO and/or 5-MTHF on the children FA status; in fact, statistical differences were found between the four supplemented groups in the FA profile in cheek cells in childhood. On the other hand, nutritional status during early life does not seem to have any long-lasting impact on children's FA status, and the major factor influencing the FAs concentrations are diet, country of origin and FADS polymorphisms.

In this study, the intake of n-3 LC-PUFAs were significantly lower than n-6 LC-PUFAs intake. During the first six months of life, total dietary fat should account for 40-60% of total energy (E), falling to 35% E between six months and two years, and 30-35 % E subsequently. An intake <25% E has a negative impact on growth. The requirements of essential fatty acids are high in infants and decrease at later ages. The European Society of Gastroenterology, Hepatology and Paediatric Nutrition (ESPGHAN) recommends for children under six months a similar intake to the composition of breast milk. For later ages the Food and Agriculture Organization (FAO) (2010) estimates are commended PUFA intake <15% E (n-6 Total <10% E), linoleic acid 3-4.5% E of α-linolenic acid 0,4- 0.6% E and docosahexaenoic acid (DHA) 10 to 12 mg/kg. Dietary Reference Intakes (DRIs) for macronutrients of Institute of Medicine of the National Academies of the United States and Canada (2002) recommends a LA intake for boys (9-13 years) of 12 g/day and for girls of 10 g/day, and an ALA intake for boys of 1.2 g/day and for girls of 1 g/day. The NUHEAL children daily intake of PUFAs-n6 was of 7.49g (Spanish) and 7.24g (German); and the daily intake of PUFAs-n3 was of 1.35g (Spanish) and 1.04g (German). Therefore, for both countries the DRIs of PUFAs-n6 are slightly deficient, more in Germany than in Spain (taking into account the LA intake as

reference). Nevertheless, for the PUFAs-n3 intake (taking into account ALA as reference), Spain is slightly superior to the recommendation [11].

As stated before, it was possible to discriminate the age of Spanish and German children according the FA composition. For the Spanish children, the most influential compounds within the model were (in order of decreasing coefficient) Behenic acid, Vaccenic acid, GLA and DPA, since their concentration levels fluctuated in a wider range than the others. Two of them (Vaccenic acid and GLA) were also relevant when the German children were studied; although for this group, the influence of Eicosenoic acid, AA, EPA and DHA was also remarkable, being Eicosanoic acid and EPA those with higher influence.

CHAPTER 2

Evaluation of the relationship between children FAs status, mothers eating behaviour and children behaviourall problems at 8 years of age.

This section discusses the relationship between the status of the fatty acid profile from cheek cell samples from school-age children and behavioural problems that they may exhibit.

In addition, we aims to establish to what extent there is an influence of the feeding behaviour and psychological characteristics of mothers on the behaviour, both internalizing and externalizing problems, in their children at eight years of age.

3.1. Statistical analyses

A non-parametric test (U Mann-Whitney Test) was performed using SPSS 20.0.

SPSS version 20.0 was also used to check the difference between the study groups. ANOVA was performed, with subsequent post-hoc corrections using the Bonferroni test. We also conducted a study using binary logistic regression steps forward, categorizing discrete variables, to determine the influence not only of eating behaviour and maternal psychological state on the behaviour of their children at age 8, but also to evaluate the influence of other factors such as maternal age, sex, maternal weight, educational level, socioeconomic status, etc. A minimum level of significance was considered at $P < 0.05$.

We achieved a statistical power of 80 % to detect 1% (difference between any of the groups of supplementation) if 100 pairs of mother and children were studied. For all estimations an error level of 0.05 is assumed.

3.2. Results

154 Spanish children were re-call for a new visit at 8 years of age. From them, to demonstrate potential associations between fatty acid status and behavioural problems in children, 132 Spanish and German ones participants in NUHEAL Study performed CBCL tests [89], and cheek cells were sampled at 8 years to obtain the FA profile [116].

To establish to what extent the mother's eating behaviour may influence the behaviour of their children at eight years old, we used the Spanish population (111 children), and only 94 cases were included in these analyses. Only this number of pairs of mother and children had all the data required for the statistical analyses.

Relationship between behaviour and fatty acids

Table 17 shows the Pearson correlations between the concentrations of the most relevant FAs (ALA, LA, EPA, DPA and DHA) from check cell samples of school-age children at 8 years old and the total scores obtained from their CBCL for the following measured subsets:

- Anxious/Depressed
- Withdrawn/Depressed
- Somatic Complaints
- Social Problems
- Thought Problems
- Attention Problems
- Rule-Breaking Behaviour
- Aggressive Behaviour
- Internalizing Problems
- Externalizing Problems
- Total Problems
- Play and Sporting Competence

The majority correlated negatively. We found that a higher concentration of ALA, a higher prevalence of play and sporting problems; a higher concentration of EPA, higher prevalence of thought problems, aggressive behaviour and play and sporting problems; a higher concentration of DPA, higher prevalence of play and sporting problems; and, a higher level of DHA, higher prevalence of play and sporting problems.

Table 17. *Children's behaviour depending in the concentration of the fatty acids in cheek cell glycerophospholipids.*

	C18:3n-3 (ALA)	C18:2n-6 (LA)	C20:5n-3 (EPA)	C22:5n-3 (DPA)	C22:6n-3 (DHA)
Anxious/Depressed	- 0.158	- 0.147	- 0.110	- 0.179	- 0.114
Withdrawn/Depressed	- 0.068	- 0.055	- 0.055	- 0.124	- 0.031
Somatic Complaints	- 0.194	- 0.192	0.020	- 0.107	- 0.018
Social Problems	- 0.211	- 0.101	- 0.096	- 0.163	- 0.078
Thought Problems	- 0.025	- 0.118	0.064	- 0.066	- 0.007
Attention Problems	- 0.155	- 0.156	- 0.045	- 0.201	- 0.112
Rule-Breaking Behaviour	- 0.163	- 0.102	- 0.070	- 0.130	- 0.125
Aggressive Behaviour	- 0.151	- 0.100	0.001	- 0.113	- 0.124
Internalizing Problems	- 0.177	- 0.166	- 0.064	- 0.171	- 0.074
Externalizing Problems	- 0.165	- 0.108	- 0.019	- 0.125	- 0.133
Total Problems	- 0.214	-0.182	-0.053	-0.198	-0.121
Play and Sporting Competence	0.037	-0.104	0.078	0.034	0.065

The effect of the mother's eating behaviour and psychological problems on their children

The scoring for the different scales, DT, BD and P, according to the group of supplementation can be seen in **Table 18**.

Comparison of the results of the CBCL concerning the development of the conduct and the existence of internalizing and/or externalizing problems in children of NUHEAL Study at 8 years of age, showed no significant differences among the 4 study groups.

To analyse the possible influence of eating behaviour and maternal psychological status on the behaviour of their children, the scoring obtained from CBCL was dichotomised using for this the score of 60 and categorizing the cases above and below this score in pathological and non-pathological, respectively (**Table 19**).

The results revealed (**Table 20**) that 32.26% of the children studied had internalizing problems and 25.81% had externalizing problems.

Table 18. *Scoring of mother's EDI-2 Scales classification depending on their supplementation group.*

						95% CI			
		N	Mean	SD	SE	LL	UL	Min	Max
DT	FO	27	4.74	6.17	1.19	2.30	7.18	0.00	21.00
	5-MTHF	19	4.16	3.91	0.90	2.28	6.04	0.00	14.00
	Placebo	27	3.11	3.29	0.63	1.81	4.41	0.00	14.00
	FO+5-MTHF	21	3.86	4.04	0.88	2.02	5.70	0.00	14.00
	Total	94	3.96	4.53	0.47	3.03	4.89	0.00	21.00
BD	FO	27	6.70	7.34	1.41	3.80	9.61	0.00	25.00
	5-MTHF	19	6.79	7.23	1.66	3.30	10.28	0.00	25.00
	Placebo	27	3.56	3.89	0.75	2.02	5.09	0.00	16.00
	FO+5-MTHF	21	5.24	5.18	1.13	2.88	7.59	0.00	18.00
	Total	94	5.49	6.09	0.63	4.24	6.74	0.00	25.00
P	FO	27	2.89	2.93	0.57	1.73	4.05	0.00	11.00
	5-MTHF	19	2.00	1.53	0.35	1.26	2.74	0.00	5.00
	Placebo	27	2.52	2.77	0.53	1.42	3.61	0.00	10.00
	FO+5-MTHF	21	1.81	1.75	0.38	1.01	2.61	0.00	5.00
	Total	94	2.36	2.41	0.25	1.87	2.86	0.00	11.00

SD: Standard deviation; SE: Standard error; LL: Lower limit; UL: Upper limit
DT: Drive for thinness; BD: Body dissatisfaction; P: Perfectionism

Table 19. *Number of mothers with pathological eating disorders by group of supplementation.*

	FO	5-MTHF	Placebo	FO+5-MTHF
Non pathological	23	19	27	21
Pathological	4	0	0	0
Total	27	19	27	21

Table 20. *Pathological and non-pathological behaviour of children.*

	Pathologic Cases	Non Pathologic Cases
Internalizing problems	30 (32.26%)	57 (61.29%)
Externalizing problems	24 (25.81%)	63 (77.01%)

The binary logistic regression showed that mothers classified as pathological on the scale of Drive for thinness (DT) have 1,138 times more likely to be determinants of internalizing problems of their children. (**Table 21**).

Table 21. *Maternal drive for thinness influences on their children internalizing problems.*

	B	E.T.	Wald	Degrees of freedom	Significance	Odds ratio
EDI-2 Value DT Scale	0,130	0,054	5,755	1	**0,016**	1,138

3.3. Discussion

Several studies have provided evidence for the concept of foetal 'programming' [157]. One of the environmental factors that have been found to contribute to foetal and later human development is maternal nutrition during pregnancy [158]. Moreover, nutrition during pregnancy has been linked to foetal brain development and subsequent offspring behaviour. Associations between maternal dietary patterns and offspring behaviour is less well known about [159].

Poor nutritional status during pregnancy has been found to predispose to antisocial personality disorder in adulthood [160], whilst poor nutrition in early childhood is associated with increased aggressive and conduct disordered behaviour in childhood and adolescence [161]. Poor nutrition is hypothesized to negatively impact brain structure and function which in turn predisposes to risk factors for antisocial behaviour [162, 163]. Brain abnormalities have been found to characterize not just adult offenders [164], but also conduct disordered children and children with callous-unemotional traits [165, 166]. As such, poor nutrition is a plausible risk factor for the development of antisocial and aggressive behaviour.

On the other hand, as I mentioned in the introduction, n-3 fats have been replaced in many modern diets by saturated and artificial fats and to some extent by n-6 fats. The latter are also essential to health, but an appropriate balance is required, and relative

deficiencies of n-3 appear to underlie a wide range of physical and mental health conditions that pose increasing problems in developed countries.

The NUHEAL Study, as the previous studies mentioned above, demonstrates the association between FA levels in cheek cells of school-age children and their behaviour. Further research is needed to continue to improve our knowledge about this issue.

On the other hand, an investigation about the influence of eating behaviour and maternal psychological status in the behaviour of their children has been conducted. Various studies, such as K. Lyons-Ruth in 2005 [92], demonstrate this relationship.

The influence of mothers who received supplementation during pregnancy as not, in this case, significance. However, recent studies have shown that folic acid deficiency during pregnancy is associated with a higher incidence of disruption of behavioural development, and higher incidence of internalizing and externalizing problems in children at 4 years old, and an intake suitable to protect against these disorders [167].

However, research indicates that internalizing problems are the most common patterns of psychological distress found in childhood. In fact, in our study it appears more in children with such problems than in those with externalizing problems [92] (30 children versus 24).

Despite the many studies conducted, it appears that compared with prevention efforts in other health problems, the work done for the prevention of eating disorders is still quite limited [168]. The results indicate a clear influence of the eating behaviour and psychopathology of mothers on the development of internalizing problems in their children.

It might be speculated whether the presence of such problems at 8 years of age may lead, over time, to typical alterations of eating behaviour in these children's characteristics, closing the cycle again. This hypothesis is strengthened since internalizing problems includes problems of withdrawal/anxiety, which in turn lead to socialization problems characteristic of people with anorexia nervosa [169].

These results suggest the need for further studies on children with internalizing problems whose mothers are characterized by presenting problems of eating behaviour and to monitor the incidence of anorexia nervosa or symptoms that lead to this disorder.

Eating behaviour and maternal psychological status appear to be decisive in the development of behavioural problems in their children. This conclusion also leads us to suggest of the need for further studies to corroborate these results.

CHAPTER 3

Relationship between several head circumference and brain structure.

This section aims to explore the relationship between several head circumference measurements from birth and distributions of grey-matter volume and sub-cortical volume in later childhood.

5.1. Statistical analyses

Descriptive statistics were computed for sociodemographic data, pregnancy outcomes, anthropometric variables, cognitive abilities (MPI score), brain volumes, and cortical surface area. Standard deviation scores (SDS) were computed for the anthropometric measures taking as reference the whole sample of the NUHEAL Project (n=316). Student's t-tests were carried out to compare total GMV, WMV, TIV, and TBSA measurements between sexes.

We used an analysis of covariance (ANCOVA) approach to evaluate early supplementation (four groups: FO, 5-MTHF, FO+5-MTHF, and placebo) effects on head circumference measurements and cognitive abilities (MPI score), as well as on total and regional brain volumes and inner cortical surface area. Dependent variables were submitted to a 2 (FO) x 2 (5-MTHF) between subjects ANCOVA. The covariates were sex, age in months, laterality, and family socio-economic status. For the regional brain surface analysis, we computed the following lobe areas for each brain hemisphere: frontal, parietal, limbic, occipital, temporal, and sub-lobar.

Statistical analyses on the relationship between head circumference measurements, cognitive abilities, and MRI data were carried out using a three-level approach: global, regional, and sub-cortical. For the global analysis, we computed partial correlations to quantify the relationships between head circumference measurements with total GMV, WMV, TIV, and TBSA. We also computed four multiple regression models with head circumference as the variable of interest, and total GMV, WMV, TIV, and TBSA as the dependents. For both analyses, sex, age in months, height, weight, laterality, and family

socioeconomic status effects were partialled out. We performed consecutive analysis including head circumference at birth, 4 years, and 10 years. For these regression models, sequential-Bonferroni correction (p-level≤0.05) was applied. All these analysis were done using IBM SPSS v.20 (IBM Inc). Regional-level analysis aimed at uncovering whether head circumference measurements explained variations at regional volumes and surface areas. We used multiple linear regression involving voxel volumes as the dependents and the same set of predictors and confounding factors listed for the global analysis. We also corrected for TIV to control for overall head size effects for regional volumes. For this analysis, we used an individual voxel threshold p<0.005 and a minimum cluster size of 341 voxels to correct for multiple comparisons. This yielded a corrected p-value of 0.05, as determined by a Monte Carlo simulation implemented in REST Alphasim software (available at www.restfmri.net), with parameters: 1000 runs, voxel p-value=0.005, full width at half maximum (FWHM)=8mm, connectivity radius=7.10, with mask. We also used multiple linear regression involving the surface areas as the dependents and the same set of predictors and confounding factors listed for the global analysis. Sequential-Bonferroni correction (p-level≤0.05) was applied to statistical decision on cortical surface areas. Results were labelled following according to the automatic anatomical labelling of Tzourio-Moyer and colleagues [170]. Finally, partial correlations were also computed to quantify relationships between head circumference measurements with sub-cortical bilateral volumes, controlled for sex, age, height, weight, laterality, and family socio-economic status..

5.2. Results

The final cohort was composed of 74 Caucasian children (40 (54%) males and 68 (92%) right-handed), aged 9.69±0.22 years. **Table 22** shows the mothers' sociodemographic data and intelligence quotient, as well as pregnancy outcome.

The number of children for each supplemented group was: 23 (FO), 14 (5-MTHF), 22 (Placebo), and 15 (FO+5-MTHF). Growth curve of head circumference, weight, and height from birth to age 10 years are presented in **Table 23**.

Table 22. *Socio-demographic data and intelligence quotient of the 74 participating mothers, as well as pregnancy outcome.*

Variables	N (%)	Mean	SD	Min	Max
Mother's age at delivery (years)		30.7	5.0	18.8	41.5
Family socio-economic status					
High	7 (9.46)				
Medium	56 (75.68)				
Low	11 (14.86)				
Mother's IQ		91.2	14.24	68	136
Educational level					
Elementary	48 (67.6)				
Secondary or higher	23 (32.4)				
Apgar at birth		8.8	1.1	4	10
Apgar at 5 minutes		9.7	0.7	7	10
Breastfeeding length					
None	14 (18.9)				
1-4 months	41 (55.4)				
>4 months	19 (25.7)				
Placenta weight (g)		538.0	116.7	300	890

M, Mean; SD, Standard deviation; Min, Minimum; Max, Maximum; IQ, Intelligence quotient.

Head circumference (mean±standard deviation, SD) increased from 34.9±1.4 cm at birth to 51.0±1.2 cm and 54.0±1.6 cm at 4 and 10 years of age, respectively. At the age of 6.5 years, participants averaged around the standard mean of 100 on the MPI scale (mean±SD, 110.4±8.7). None of the participants had a MPI score equal to or below 70, which would be indicative of a significant delay or deficit in intellectual development (Minimum: 86; Maximum: 137). At the age of 10 years, total GMV was (mean±SD) 692.2±51.2 cm3. Mean (unadjusted) TBSA, GMV, WMV, as well as TIV for all children are shown in **Table 24**. There were significantly greater values of brain surface area and total brain volumes in male than in female participants ($p<0.005$).

Table 23. *Growth trajectories of head circumference, weight, and height from the 74 children at birth, and ages 4 and 10 years.*

	Birth			4 years			10 years		
	M (SD)	Mdn	Min-Max	M (SD)	Mdn	Min-Max	M (SD)	Mdn	Min-Max
Age	39.4 (1.7)	39.6	35-42.9	4.1 (0.2)	3.9	3.9-4.6	9.7 (0.2)	9.7	9.2-10.1
HC (cm)	34.9 (1.4)	34.7	32-41	51.0 (1.2)	51.0	48.9-54.5	54.0 (1.6)	54.1	50.1-59.0
Weight (kg)	3.3 (0.4)	3.3	2.1-4.2	17.6 (2.1)	17.5	13.7-25.9	38.2 (10.1)	36.3	23.2-76.2
Height (cm)	50.9 (1.7)	51	47-56	103.7 (11.5)	103.4	95.8-114.7	141.1 (7.8)	140	124.6-159

M, Mean; SD, Standard deviation; Mdn, Median; Min, Minimum; Max, Maximum; HC, Head circumference.
Age at birth refers to gestational age in weeks.

Table 24. *Unadjusted mean inner brain surface area and global brain volumes at age 10 in the 74 children.*

	Total			Males			Females		
	M	SD	Min-Max	M	SD	Min-Max	M	SD	Min-Max
TBSA (cm^2)	1408	138.9	1074-1838	1461.3	137.9	1135.8-1837.6*	1345.6	112.8	1073.5-1647.3
TIV (cm^3)	1391.5	106.4	1171.4-1718.8	1439.2	99.8	1249.7-1718.8*	1335.3	85.3	1171.4-1551.3
GMV (cm^3)	692.2	51.2	577.3-856.6	715.7	47.6	622.7-856.6*	664.6	41.1	577.3-756.0
WMV (cm^3)	462.8	39.7	381.3-571.6	479.9	37.6	408.1-571.6*	442.7	32.2	381.3-540.6

M, Mean; SD, Standard deviation; Min, Minimum; Max, Maximum; TBSA, Total brain surface area; TIV, Total intracranial volume; GMV, Grey matter volume; WMV, White matter volume.
**p<0.005*

Prenatal supplementation effects on head circumference, cognitive abilities measurements and neuro-imaging results

Early supplementation had no significant effects on any head circumference measurement at birth, 4 years, or 10 years ($p>0.13$). Also, there was no effect on the MPI score at 7 years ($p>0.58$). The ANCOVAs for the total, regional, and sub-cortical brain volumes, as well as total inner brain surface area, did not yield any significant difference between the groups (all $p>0.20$)either.

We observed a significant difference between groups in the left parietal inner surface area, $F(1,66)=6.02$, $p<0.02$. Specifically, this surface area was larger for the 5-MTHF supplemented group (180.10 cm2) than remaining groups (FO=170.73 cm2, Placebo=170.49 cm2, and FO+5-MTHF=170.46 cm2). No differences were observed for the right parietal or for the remaining lobes.

Relationships among global neuro-imaging results, head circumference, and cognitive abilities measurements

We examined relationships between head circumference measurements with brain volumes and brain surface area computing partial Pearson correlation coefficients. When age, sex, height and weight at 10 years, laterality, and family socio-economic status were partialled out, all head circumference measurements significantly correlated with GMV, WMV, TIV, and TBSA (**Table 25a**). Head circumference at 4 years shown highest correlations with total GMV ($rp=0.65$), total WMV ($rp=0.62$), TIV ($rp=0.65$), and TBSA ($rp=0.55$) (all p-values <0.05).

Multiple regression models were performed to establish relevance of head circumference measurements predicting total brain volumes and TBSA, after controlling for age, sex, height and weight at 10 years, laterality, and family socio-economic status. Head circumferences at birth, 4 and 10 years significantly predicted more than 50% of variance of the TBSA (adj-$R^2=0.59$), and more than 70% of variance of GMV, WMV, and TIV (adj-$R^2=0.77$; 0.72; 0.78, respectively) (**Table 25b**).

We examined relationships between head circumference measurements with MPI score computing partial Pearson correlation coefficients. Head circumference at birth was not related to MPI scores ($p>0.42$). When age, sex, height and weight at 10 years, laterality, and family socio-economic status were partialled out, head circumference at 4 and 10 years correlated with MPI scores ($r=0.38$, $p<0.001$; $r=0.44$, $p<0.001$, respectively). Additionally, larger GMV, WMV, and total inner brain surface area were associated to higher MPI scores ($r=0.39$, $p=0.001$; $r=0.42$, $p<0.001$; $r=0.37$, $p=0.002$).

Table 28. ***a)*** *Partial linear correlations between the total inner brain surface area, gray matter volume, white matter volume, and total brain volume and the head circumference from birth, and ages 4 and 10 years (after sex, age, height, weight, laterality, and family status have been partialled out).* ***b)*** *Multiple regression models predicting the total brain surface area, grey matter volume, white matter volume, and total brain volume from head circumference measures at birth, and ages 4 and 10 years (controlled for sex, age, height, weight, laterality, and family status).*

a)	HC	r_p	b)	HC	β	adj-R^2	F
	Birth	0.43*		Birth	0.31†		
TBSA	4 years	0.55*	**TBSA**	4 years	0.34†	0.59	36.7†
	10 years	0.52*		10 years	0.28†		
	Birth	0.40*		Birth	0.27†		
GMV	4 years	0.65*	**GMV**	4 years	0.41†	0.77	84.5†
	10 years	0.56*		10 years	0.36†		
	Birth	0.44*		Birth	0.31†		
WMV	4 years	0.62*	**WMV**	4 years	0.40†	0.72	63.6†
	10 years	0.57*		10 years	0.30†		
	Birth	0.43*		Birth	0.30†		
TIV	4 years	0.65*	**TIV**	4 years	0.39†	0.78	89.1†
	10 years	0.58*		10 years	0.36†		

Note. HC, Head circumference; TBSA, Total brain surface area; GMV, Grey matter volume; WMV, White matter volume; TIV, Total intracranial volume; β, Standardized coefficient; adj, Adjusted.
**p-value <0.05*
†corrected p-value ≤0.05

Relationships among regional neuro-imaging results, head circumference, and cognitive abilities measurements

Multiple regression models were performed to establish relevance of head circumference measurements predicting voxel volumes/surface areas. We did not find

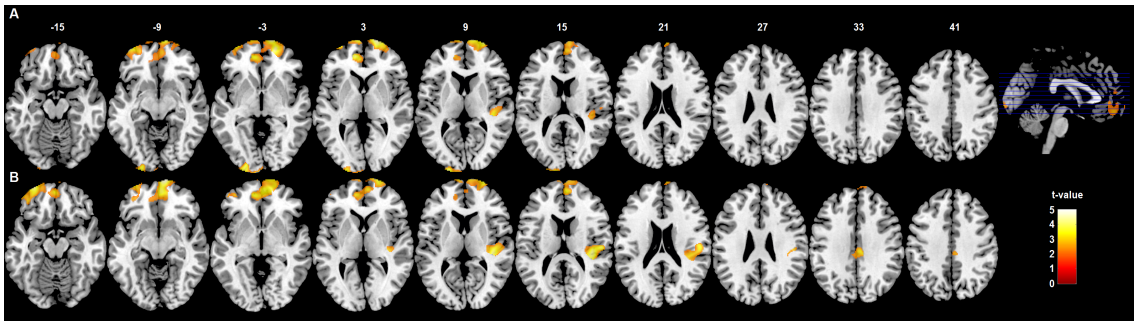
results supporting a relationship between head circumference measurements at birth and local GMV in later childhood. Head circumference at 4 years was strongly associated to GMV in later childhood (**Table 26**, **Figure 17**) of bilateral prefrontal cortex, right temporal, and bilateral occipital areas. Volumes of these significant GM clusters were associated to MPI scores (r=0.27, p=0.03), and, as expected, with head circumference at 4 years (r=0.80, p<0.001). Association between head circumference measurements at 10 years with GMV at the same time was restricted to three clusters in frontal, temporal and limbic areas (**Table 29**, **Figure 17**). Volumes of these significant grey matter clusters were associated to MPI scores (r=0.26, p=0.03), and, as expected, to head circumference at 10 years (r=0.77, p<0.001).

Table 26. *Brain areas showing significant positive association between grey matter volumes at 10 years and head circumference measurements at 4 years.*

Area	Label	k	T	X	Y	Z
Frontal Right	Frontal Superior	1176	4.98	17	72	0
	Frontal Superior Medial	681				
	Frontal Medial Orbital	515				
	Frontal Middle	328	4.00	-32	64	2
Frontal Left	Frontal Superior	193				
	Frontal Medial Orbital	469	3.60	-36	60	-8
	Cingulate Anterior	404				
	Frontal Superior Medial	425				
Right Temporal	Heschl's Gyrus	301	3.74	41	-27	9
	Temporal Superior	102				
	Rolandic Operculum	77				
Left Occipital	Occipital Middle	403	4.36	-23	-102	11
	Occipital Inferior	116				
	Calcarine	116				
	Occipital Superior	107				

x, y, and z are in the MNI space.
All the displayed results were significant with a corrected p-value ≤0.05.

Figure 17. *Relationships between regional grey matter volumes and head circumference.*



At 10 years, grey matter regions that showed significant positive correlations with head circumference measurements at 4 years (A) and 10 years (B) in 74 healthy children. No significant correlations were found at birth. The grey matter volume values in the figure were extracted from the significant clusters after sex, age, height, weight, laterality, total intracranial volume, and family socio-economic status of each subject were regressed out. Significant areas (corrected p-values≤0.05) are overlaid on MNI average brain axial sections (numbers indicate z coordinate). The bar on the yellowish/reddish colour scale indicates the range of t-values (lighter colours correspond to higher values).

Table 30. *Brain areas showing significant positive association between grey matter volumes and head circumference measurements at 10 years.*

Area	Label	k	T	X	Y	Z
Right Temporal	Rolandic Operculum	677	4.12	41	-26	12
	Heschl's Gyrus	413				
	Insula	286				
	Temporal Superior	249				
Righ Frontal	Frontal Medial Orbital	1008	4.50	11	72	-7
	Frontal Superior Medial	805				
Left Frontal	Frontal Medial Orbital	619	4.39	-33	61	-13
	Frontal Superior Medial	396				
	Frontal Middle	326				
Limbic	Cingulate Anterior	272				
	Cingulate Middle R	263	3.61	6	-26	35
	Cingulate Middle L	91				

L, Left; R, Right. x, y, and z are in the MNI space.
All the displayed results were significant with a corrected p-value ≤0.05.

Partial correlations results indicated that head circumference measurements at 4 were significantly associated to local inner brain surface areas, especially at bilateral frontal and temporal locations as cingulate gyrus, superior frontal gyrus, precentral gyrus, and superior and inferior temporal gyri (**Table 31**).

Table 31. *Partial correlations between local inner brain surface areas and head circumference at birth and 4 years, after age, sex, height and weight at 10 years, laterality, and family socio-economic status have been partialled out. Only significant correlations are displayed.*

Hemisphere	Gyrus/area	Birth	4 years
L	Angular	0.50	0.43
	Cingulate	0.43	0.53
	Insula		0.40
	Middle Temporal		0.52
	PreCentral	0.49	0.45
	PostCentral		0.43
	Superior Frontal		0.41
	Superior Temporal		0.47
R	Anterior Orbital		0.42
	Cingulate		0.52
	Insula		0.39
	Postcentral	0.42	0.54
	Posterior Orbital		0.40
	Precentral	0.51	0.48
	Superior Frontal	0.40	0.42
	Supramarginal		0.52
	Middle Temporal	0.40	0.40
	Transverse Frontal		0.49
	Transverse Temporal		0.47
	Pars Triangularis		0.43
	Superior Temporal		0.40

L, Left; R, Right.
All the displayed results were significant with a corrected p-value ≤0.05.

Relationships among sub-cortical neuro-imaging results, head circumference, and cognitive abilities measurements

Partial correlations coefficients indicated that head circumference measurements at birth, 4 and 10 years were significantly associated to bilateral volume of putamen and thalamus ($p<0.05$). Head circumference measurements at 4 and 10 years were significantly associated to bilateral volume of caudate nucleus and globus pallidus ($p<0.05$) (**Table 32**). MPI scores were associated to bilateral volumes of caudate nucleus, globus pallidus, putamen, and thalamus ($p<0.05$).

Table 32. *Partial correlations between bilateral sub-cortical structure volumes and head circumference measurements (at birth, 4 and 10 years), and the cognitive abilities index, after sex, age, height, weight, laterality, and family socio-economic status have been partialled out.*

HC	Hippocampus	Caudate nucleus	Putamen	Accumbens	Amygdala	Globus Pallidus	Thalamus	Brainstem
Birth	0.215	0.169	0.346*	0.096	0.217	0.173	0.273*	0.209
4 years	0.126	0.392*	0.489*	0.188	-0.015	0.317*	0.343*	0.141
10 years	0.133	0.263*	0.409*	0.191	-0.064	0.301*	0.311*	0.069
MPI	0.223	0.271*	0.477*	0.099	-0.170	0.257*	0.355*	0.083

HC, Head circumference; MPI, Mental Processing Index from the Kaufman Assessment Battery for Children – Second Edition [135].
**p-value <0.05*

5.3. Discussion

Head circumference during infancy and childhood reflects both biological and environmental factors, including prenatal nutritional supplementation. Thus, the measurement of the head circumference is an easy, quick, and inexpensive approach that may be valuable for assessing neurodevelopment. So far, no study had examined the relationship between head circumference and regional GMVs in children exposed to high LC-PUFAs and folate during their foetal lives. Some cross-sectional studies have suggested existence of a significant positive association between head circumference and total GMV [67-69]. Its accuracy as index of brain size would depend on age [67, 69]. and the successful control for several confounding factors, including prenatal exposures. However, such cross-sectional studies do not indicate the potential relevance of successive head circumference measurements – beginning at birth – and the role of prenatal nutrition to predict brain size in late childhood. Our results suggest that head circumference at 4 years of age is the most relevant measure for predicting total and regional brain volumes in later childhood. Neither LC-PUFA nor folate prenatal exposition would appear to have a significant role for predicting total and regional brain volumes at late childhood.

During foetal and early postnatal time periods, the brain is particularly vulnerable and plastic [70]. Thus, brain development trajectories up to reaching an adult's brain size

would be deeply determined not only by inherited influences, but also by gestational conditions and environmental postnatal factors. Anthropometric measurements at birth, including head size, presumably represent differential prenatal exposures, whereas measurements performed during childhood presumably represent prenatal as well as postnatal exposures. In our results, head size at birth had a significant weight for predicting total brain volumes and TBSA, but was not related to any regional GMVs at late childhood. On the other hand, measurements of head size during childhood had a significant weight for predicting total brain volumes and TBSA, and were also predictive of specific frontal, temporal and occipital GMVs in later childhood. Thus, it might be assumed that postnatal factors have the most relevant role in structural maturation of certain cortical areas from birth to late childhood. These postnatal factors imply a genetic endowment that influences the child's growth and maturation processes and a phenotype depending on environmental circumstances, such as health status and nutrition, throughout the growth years.

The long-term signature effect of prenatal micronutrients (by means of either deficits or high exposition) on postnatal brain structure is still unclear and more MRI studies are needed. We found that early supplementary nutrition might have an impact on brain development as measured by brain surface area. Specifically, 5-MTHF early supplementation seems to increase the size of surface area in parietal cortex. This lobe has been involved in sensory processing and visuomotor control [(168)], attention processing [(169)], touch, and grasping [(171)], and other superior cognitive functions (as numeric representation [(172)], or working memory [(173)]. This increased surface area is coherent to attentional processing advantages observed in the children of the 5-MTHF supplemented mothers [(82)].

Postnatal neurodevelopment during the first 5 years of life is one of the most active stages of brain development [(174)]. This period also coincides with the surface of many developmental neuropsychiatric disorders, including autism spectrum disorders and attention deficit/hyperactivity disorder [(175)]. It is generally accepted that there are developmental windows that allow the brain to be moulded by experience in infancy and childhood [(176)]. This early period is extremely relevant for neurodevelopment and we found that, effectively, head circumference at 4 years is highly correlated with MRI

data at 10 years. It was also strongly associated to total GMV, WMV, and TBSA; regional GMVs in frontal, temporal, and occipital areas; and to the cognitive abilities index. Head circumference at 4 years was also positively related to the sub-cortical structure volumes (caudate nucleus, globus pallidus, putamen, and thalamus) in later childhood.

Since the 19th century, several diverse ways for estimating brain size from cranial vault capacity have been tested [177]. Even though new advances in radiology and imaging have led to more accurate estimations of brain volume and its relationship to head size, no study to date has characterized the age-dependent relationships between head circumference and regional cortical and sub-cortical volumes. Here, for the first time, we examined the associations between head circumference measurements at birth and childhood (4 and 10 years) with later regional cortical volumes, as well as with sub-cortical structures volume and cognitive abilities. We demonstrate that age-dependent relationships between head size and brain volumes are regionally specific, with prenatal supplementation having a minor effect on parietal surface are

7. CONCLUSIONS

7. Conclusions

1. FO and/or 5-MTHF supplementation during pregnancy have long-term effects on the GPL-FA profiles in children's cheek cells at school age; a programming effect of fatty acids metabolic pathways is suggested, which may cause differences in children's FA status.
2. Children's *FADS1* and *FADS2* gene cluster polymorphisms influence PUFA concentrations in children's cheek cells. The majority of the associations were established between *FADS1* and *FADS2* SNPs with LA and ALA.
3. The interaction between early life nutrition and children's *FADS1*, *FADS2* and *FADS3* genotype, demonstrate long-term effects on GPL concentrations of the oral mucosa in children, suggesting an early programming of the metabolic pathways involved in the status of these FAs, driven by the genetic background.
4. Differences amongst the FA profiles from check-cell samples analysed in the NUHEAL children are strongly related to the country of origin, and therefore diet, lifestyle, etc.
5. Dietary intake also appears to influence the fatty acid concentrations present in the cheek cells of the studied children, the daily intake of AA especially shows a high impact on the percentage of fatty acids found in the cheek cells of the children.
6. There is a relationship between certain characteristics of maternal eating disorders, such as the "obsession with thinness", and the subsequent development of internalizing behaviour problems in their children at 8 years of age. This relationship determines that those mothers, who have problems related to the obsession with being thin, increase the likelihood of their children having 1,138 times more risk of internalizing behaviour problems compared to those children whose mothers do not show this obsession.
7. We have verified that the measurement of the cephalic perimeter at 4 years of age serve as a marker of cognitive development at 10 years of age. In addition, this anthropometric measurement is very useful from birth to 4 years, as this period is considered the most sensitive for brain growth. Certain postnatal factors, such as diet or LC-PUFAs status, may have a more significant impact on the structural maturation

of certain cortical areas and sub-cortical nuclei, independent of the effects determined by prenatal supplementation.

Conclusiones

1. Los suplementos de FO y/o 5-MTHF durante el embarazo tienen efectos a largo plazo sobre el perfil de GPL-FA en las células de la mejilla de los niños en edad escolar. Se sugiere un efecto de programación de las vías metabólicas de los ácidos grasos, lo que puede causar diferencias en el estado de los ácidos grasos en los niños.
2. Los polimorfismos de los genotipos *FADS1* y *FADS2* influyen en las concentraciones de PUFAs en las células de la mejilla de los niños. La mayoría de las asociaciones se establecieron entre *FADS1* y *FADS2* SNP con LA y ALA.
3. La nutrición temprana, junto al genotipo de las *FADS1*, *FADS2* y *FADS3*, tienen efectos a largo plazo en las concentraciones de los GPL de la mucosa bucal, lo que sugiere una programación temprana de las vías metabólicas implicadas en el estado de dichos FA, impulsada por loa antecedentes genéticos
4. Las diferencias encontradas en el perfil de ácidos grasos de las muestras de células de la mejilla analizadas en los niños del estudio NUHEAL están fuertemente relacionadas con el país de origen, lo que sugiere el efecto de la dieta, los estilos de vida, etc.
5. La ingesta dietética también parece influir sobre las concentraciones de ácidos grasos presentes en las células de la mejilla de los niños estudiados, especialmente la ingesta diaria de AA muestra un alto impacto en el porcentaje de ácidos grasos encontrado en las células de la mejilla de los niños.
6. Existe una relación entre ciertas características de los trastornos alimentarios maternos, como la "obsesión por la delgadez", y el posterior desarrollo de problemas internalizantes de conducta en sus hijos a los 8 años de edad. Esta relación determina que aquellas madres que tienen problemas relacionados con la obsesión por ser delgadas, aumentan la probabilidad de que sus hijos tengan un riesgo 1.138 veces mayor de problemas internalizantes de conducta en comparación con aquellos niños cuyas madres no muestran esta obsesión.

7. Hemos verificado que la medición del perímetro cefálico a los 4 años sirve como marcador del desarrollo cognitivo a los 10 años de edad. Además, esta medición antropométrica es muy útil desde el nacimiento hasta los 4 años, ya que este período se considera como el más sensible del crecimiento cerebral. Determinados factores posnatales, como la alimentación o el estado de LC-PUFAs, podrían tener un impacto más relevante sobre la maduración estructural de ciertas áreas corticales y núcleos subcorticales, y de forma independiente a los efectos determinados por la suplementación prenatal.

8. REFERENCES

8. References

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