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ANÁLISIS DE LOS EFECTOS A LARGO PLAZO DE LA NUTRICIÓN PRECOZ SOBRE EL NEURODESARROLLO EN LOS NIÑOS MEDIANTE TÉCNICAS DE MINERÍA DE DATOS: BASE DE DATOS NUTRIMENTHE

TESIS DOCTORAL

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PARA OPTAR AL GRADO DE DOCTOR
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"If a machine is expected to be infallible, it cannot also be intelligent."
Alan Turing

FINANCIACIÓN

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TABLA DE CONTENIDOS

ABREVIATURAS

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5-MTHF - 5-metiltetrahidrofolato
ALA - Ácido a-linolénico
ARA - Ácido Araquidónico
CBCL - Child Behaviour Checklist
CHOP - Childhood Obesity Project
DHA - Ácido Docosahexaenoico
DMN - Red Neuronal por Defecto
EPA - Ácido Eicosapentaenoico
FO – Fish Oil (Aceite de pescado)
FPN - Red Neuronal Frontoparietal
HPG - Proyecto Genoma Humano
ICA - Análisis de Componentes Independientes
KDD - Knowledge Discovery in Databases
LA - Ácido linoleico
LC-PUFAs - Ácidos grasos poliinsaturados de cadena larga
NNB – NUTRIMENTHE Neuropsychological Battery
NUHEAL - Nutraceuticals for a Healthy Life
PCA - Análisis de Componentes Principales
SMN - Red Neuronal Sensoriomotriz
TEA - Trastornos del Espectro Autista
TDAH - Trastorno por Déficit de Atención e Hiperactividad
rs-fMRI - Resonancia Magnética Funcional en reposo

RESUMEN

RESUMEN

El estado nutricional y la alimentación materna durante la gestación van a jugar un papel fundamental en el neurodesarrollo de los niños. La deficiencia de nutrientes funcionales en periodos críticos del desarrollo, puede acarrear alteraciones del desarrollo estructural y funcional del cerebro de los niños con consecuencias en el neurodesarrollo a largo plazo.

El proyecto NUTRIMENTHE ha permitido obtener una ingente cantidad de datos que ha facilitado evaluar el *efecto de la dieta sobre el rendimiento mental de los niños* procedentes de siete países de la Unión Europea mediante la creación de la base global NUTRIMENTHE. La base NUTRIMENTHE está compuesta por tres cohortes: a) **Proyecto NUHEAL Follow-up** *(Nutraceuticals for a Healthy Life)* – España, Alemania y Hungría; b) **Proyecto CHOP** *(Childhood Obesity Project)* – Alemania, España, Italia, Polonia y Bélgica; y, c) **Generation R** – Países Bajos.

Los niños pertenecientes a estas cohortes fueron evaluados a los 7 y 9 años de edad, mediante la NUTRIMENTHE Neuropsychological Battery (NNB), batería de tests neuropsicológicos especialmente diseñada para este fin, capaz de cubrir todo el espectro funcional del cerebro a través de una exploración por diferentes dominios cerebrales *(percepción, motricidad, atención, memoria, lenguaje, funciones ejecutivas, velocidad de procesamiento)*. La NNB fue consensuada y traducida a los diferentes idiomas de los países implicados. Los problemas de desarrollo del comportamiento

también se evaluaron mediante el test Children's Behavior Checklist (CBCL) para medir los posibles problemas de comportamiento.

Además, los niños del Proyecto NUHEAL participaron en una evaluación funcional del cerebro a los 9.5 años mediante Resonancia Magnética Funcional en Reposo (rs-fMRI), que ha aportado datos de máximo interés.

Junto a los datos neuropsicológicos y de neuroimagen previamente mencionados, se obtuvieron datos correspondientes a potenciales variables confusoras, tales como: *edad materna, nivel educativo de la madre y del padre, tabaquismo durante el embarazo, edad gestacional, antropometría al nacimiento y lactancia materna durante al menos los tres primeros meses de vida,… así como datos evolutivos de las diferentes cohortes, desde el nacimiento hasta la edad de 9.5 años.*

El principal objetivo del presente estudio es el de aplicar técnicas de minería de datos y estadística avanzada a la base de datos global NUTRIMENTHE, y extraer conocimientos útiles y publicables en el ámbito de la nutrición clínica y desarrollo cerebral.

Después de preparar el conjunto de datos con técnicas de pre-procesamiento avanzadas, se aplicaron los procesos de descubrimiento de información *(KDD-Knowledge Discovery in Databases)* en sus dos vertientes principales*: Aprendizaje supervisado y No Supervisado.*

En el primer estudio, mediante el uso reglas de asociación sobre el conjunto de datos total de la base NUTRIMENTHE, se comprobó una relación causa-efecto entre el tabaquismo materno durante el embarazo y el desarrollo de **problemas externalizantes** de conducta en los niños en edad

escolar. No obstante, la lactancia materna durante los tres primeros meses de vida, así como el nivel educativo de los padres parecen contrarrestar los efectos nocivos del tabaquismo materno.

El segundo estudio se llevó a cabo con los datos obtenidos en el Proyecto NUHEAL; los resultados sugieren que la suplementación prenatal con FO se asoció a una mayor velocidad de procesamiento en las tareas del test CCTT-1 en los niños en edad escolar. Se comprueba que el índice AA/DHA materno en el momento del parto y la heterozigosis materna de la variante genética rs174556 de las FADS1 se asocian positivamente a la velocidad de procesamiento de los hijos a los 9 años. Por el contrario, las concentraciones de tHcy en el plasma materno en el momento del parto muestran un efecto negativo sobre la velocidad de procesamiento del niño a los 9 años.

En el tercer estudio, a los 9.5 años de edad, los niños cuyas madres recibieron suplementación prenatal con FO, mostraron una conectividad funcional débil en la red neuronal por defecto, y en las redes sensoriomotriz y frontoparietal comparadas con las muestran aquellos niños cuyas madres recibieron placebo o 5-metil-tetrahidrofolato (5-MTHF). Gracias a las técnicas de predicción y selección de características sobre la base de datos de NUHEAL, se pudo comprobar una asociación negativa entre conectividad funcional y las tareas de atención y velocidad de procesamiento en los hijos de madres suplementadas con 5-MTHF y/o placebo.

Conclusiones

1. Las técnicas de minería de datos resultan de vital importancia a la hora de descubrir conocimiento oculto en una base de datos de grandes

dimensiones, teniendo en cuenta que la adquisición de datos ha conllevado un gran esfuerzo, así como la cantidad de recursos destinados a dicha labor.

2. Gracias al uso de reglas de asociación, se justifica el hecho de que el tabaquismo materno durante la gestación puede influir en el desarrollo conductual de los niños en edad escolar, por lo que resulta de vital importancia fomentar la políticas anti-tabaco especialmente para concienciar a las mujeres en edad de concebir y embarazadas.

3. Los niños cuyas madres recibieron aceite de pescado (FO: 500 mg de DHA/día + 150 mg EPA/día) durante la gestación mostraron una mayor velocidad en la resolución de las tareas del CCTT1. El índice ácido araquidónico (ARA)/DHA materno al final de la gestación, y la heterocigosis de la variante genética rs174556 de la FADS1 ejercen un efecto beneficioso a largo plazo sobre la velocidad de procesamiento que muestran los niños a los 9 años de edad. El incremento de la Homocisteína total durante la gestación predice una menor velocidad de procesamiento cognitivo en los hijos a los 9 años de edad.

4. Gracias a la técnica de análisis de componentes independientes, la suplementación materna con FO aceite de pescado durante el embarazo parece tener efectos a largo plazo sobre sobre la conectividad funcional cerebral de los niños en edad escolar que se asocia a un mejor desarrollo del procesamiento cognitivo.

5. Se refuerza la hipótesis de la posibilidad de una programación nutricional precoz (*Early Nutrition Programming*) que determina efectos a muy largo plazo sobre el funcionamiento cerebral en los hijos.

INTRODUCCIÓN

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El mundo cuenta con una ingente cantidad de datos donde la extracción de conocimiento oculto se vuelve tediosa e insostenible usando sólo técnicas de estadística clásica. De este modo nace la ciencia de datos, campo disciplinario que aprovecha los avances computacionales para desarrollar propuestas algorítmicas en la resolución de problemas de cualquier índole (Figura 1).

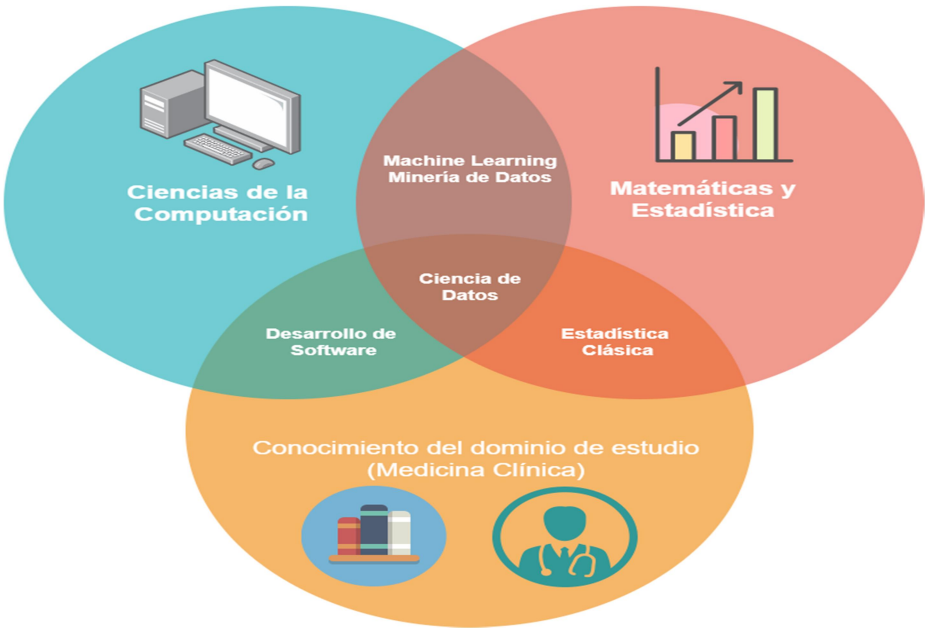

Figura 1. Proceso de ciencia de datos.

Gracias a la ciencia de datos se pueden realizar estudios de tal calibre como predecir la muerte cardiovascular en base al número de

latidos del corazón en intervalos de tiempo específicos [1], o ayudar a la detección precoz de células cancerígenas gracias al procesamiento de imágenes [2], entre muchas otras aplicaciones.

El Proyecto NUTRIMENTHE [3], financiado por el 7º Programa Marco de la Unión Europea, aborda cuestiones clave sobre el neurodesarrollo en la infancia y cómo la dieta puede desempeñar un papel fundamental sobre dominios cerebrales trascendentales en el desarrollo tales como la percepción, memoria, inteligencia, ansiedad, atención, funciones ejecutivas y comportamiento. Como resultado de la colaboración internacional realizada gracias al Proyecto NUTRIMENTHE, los investigadores responsables decidieron crear una nueva base de datos que recogiera información proveniente de 3 grandes estudios, a través de los cuales se pretende analizar el efecto de la dieta precoz sobre el crecimiento y neurodesarrollo de un millar de niños procedentes de siete países de la Unión Europea; las especiales características de esta base de datos donde se incluyen 2 proyectos de intervención nutricional (uno prenatal y otro postnatal) y 1 proyecto de cohorte epidemiológica, junto a la influencia cultural y de estilos de vida, hacen de la base NUTRIMENTHE una gran fuente de conocimiento sobre este tema, y constituye un reto de minería de datos merecedor de la máxima atención para este y futuros estudios.

La base de datos global NUTRIMENTHE permitirá abordar un proceso de descubrimiento de información (knowledge discovery in databases – KDD) y el correspondiente análisis inteligente de los datos, revelando patrones ocultos así como las posibles relaciones causa-efecto que se puedan detectar entre éstos. Para llegar a tal cometido, se pueden combinar técnicas tradicionales de extracción de conocimiento con propuestas recientes de la Inteligencia Artificial, dentro de la gran variedad de áreas y herramientas de minería de datos [4]. Este enfoque permitiría obtener nuevo conocimiento a partir de la base de datos que hasta la fecha solo ha sido tratada con técnicas estadísticas clásicas.

Se debe comenzar con el estudio y entendimiento del dominio del problema para identificar los posibles objetivos a analizar. Una vez definidos los objetivos, se procederá al pre-procesamiento de datos, siendo de vital importancia en el proceso KDD, ya que se centra en limpiar, transformar y eliminar ruido para la etapa posterior del análisis. El siguiente paso puede consistir en la selección de características que aporten más información útil descartando aquellas que sean redundantes. Una vez limpio el conjunto de datos, se procedería a la construcción de un modelo, paso clave en la minería de datos, proporcionando distintos enfoques a la hora de abordar un problema *(Aprendizaje Supervisado y No Supervisado).* El último paso sería la validación e interpretación del modelo de manera que puedan ser

entendidos por el público general. En la actualidad contamos con numerosas herramientas de libre distribución para la realización de procesos de minería de datos, siendo el lenguaje de programación R el predominante por su versatilidad y la ingente cantidad de librerías apoyada por una fuerte comunidad científica. En esta propuesta de proyecto de tesis doctoral se pretende aplicar técnicas de extracción de conocimiento, haciendo uso de técnicas de minería de datos, así como procedimientos de estadística clásica como avanzada para la base global NUTRIMENTHE, teniendo en cuenta las cohortes que la conforman. Para dicho cometido se hará uso del entorno de programación R, así como las herramientas óptimas disponibles para extraer conocimiento susceptible de ser publicado.

- **Impacto de factores socio-ambientales sobre el neurodesarrollo**

La interacción entre factores ambientales y sociales puede afectar el desarrollo conductual posterior de los niños, por lo que tiene importantes implicaciones para la salud pública y el desarrollo de políticas preventivas [5].

Factores de riego prenatales como el tabaquismo materno [6] y el consumo de alcohol [7] y/o estupefacientes [8] parecen tener efecto sobre el neurodesarrollo de los niños, pudiendo estar fuertemente

condicionado por otros factores como el nivel educativo de la madre, lactancia materna, así como el nivel socioeconómico de las familias [9].

Tabaquismo materno y conducta en los hijos en edad escolar

Según la Organización Mundial de la Salud (OMS), actualmente el continente europeo cuenta con la mayor proporción de fumadores de cigarrillos entre los adultos (28%) y una de las más altas entre los adolescentes, a pesar de todos los esfuerzos realizados por las entidades públicas y privadas para fomentar el abandono del hábito de fumar [10]. Si bien el consumo de tabaco predominaba anteriormente entre los hombres, la diferencia entre ambos géneros es actualmente muy pequeña (<5%) en países europeos como Dinamarca, Irlanda, Países Bajos, Noruega, Suecia y Reino Unido. Aproximadamente el 19% de las mujeres de 15 años o más fumaban tabaco en Europa según la OMS y, como motivo de preocupación, el cigarrillo representa un riesgo grave para la madre y el feto en desarrollo, siendo ahora reconocido como una causa importante de morbilidad y mortalidad infantil.

La exposición al humo del cigarrillo durante el embarazo puede perturbar el tracto respiratorio, el sistema cardiovascular y la morfología del cerebro del niño, provocando daños crónicos que pueden manifestarse posteriormente [11,12]. La mortalidad infantil perinatal, la muerte fetal, el parto prematuro, las anomalías

congénitas, el bajo peso al nacer y el lento desarrollo y crecimiento fetal son consecuencias comunes de la exposición al tabaquismo, al *vapeo* y la exposición pasiva al humo durante el embarazo [13–20].

Se han documentado perfiles neurocognitivos y conductuales perjudiciales de los niños en edad escolar nacidos de madres que fumaron durante el embarazo, lo que promueve la aparición temprana de síntomas psicopatológicos como el trastorno por déficit de atención con hiperactividad (TDAH) [21], comportamiento agresivo [22], trastornos del espectro autista (TEA) [23], riesgo de hipomanía[24], bajo rendimiento académico [25], así como deficiencias en el procesamiento del habla y el control de la atención [26,27]. Sin embargo, considerando la naturaleza observacional de los datos en esos estudios, parece que los resultados conductuales están vinculados de alguna manera a otros factores confusores como el género, la edad de los padres, la educación o los ingresos de los padres [28]. Además, los efectos de las características culturales y sociodemográficas de la población estudiada se consideran factores confusores [29]. Por el contrario, otros estudios apoyan la idea de que la asociación entre el tabaquismo materno durante el embarazo y el TDAH en los niños no está del todo probada [21].

- ## Desarrollo del cerebro fetal

La nutrición juega un papel fundamental durante los primeros 1000 días de vida (desde la concepción hasta los primeros 2 años) en el óptimo neuro-desarrollo de los niños. El cerebro humano no se puede considerar un órgano homogéneo, sino que está formado por numerosas áreas o regiones, donde cada una difiere de la otra considerando la trayectoria de desarrollo y las necesidades nutricionales. En este sentido, el neurodesarrollo fetal se considera un periodo crítico, donde deficiencias o deprivaciones de ciertos nutrientes podrían acarrear efectos duraderos o irreversibles del neurodesarrollo [30].

El comienzo de la tercera semana después de la concepción marca el inicio del período embrionario, etapa fundamental para el desarrollo del cerebro humano, dónde se forma el tubo neural (Figura 2).

Al final del período embrionario, se han establecido las estructuras básicas del cerebro y el sistema nervioso central y periférico. La producción de neuronas, o células cerebrales, comienza alrededor del día 42 después de la concepción y, en su mayoría, se completa en algún momento hacia la mitad del embarazo. A medida que se forman las neuronas, éstas se propagan a diferentes áreas del cerebro. Una vez que han alcanzado la ubicación correcta,

comienzan a formar conexiones con otras células neuronales, estableciendo redes neuronales rudimentarias.

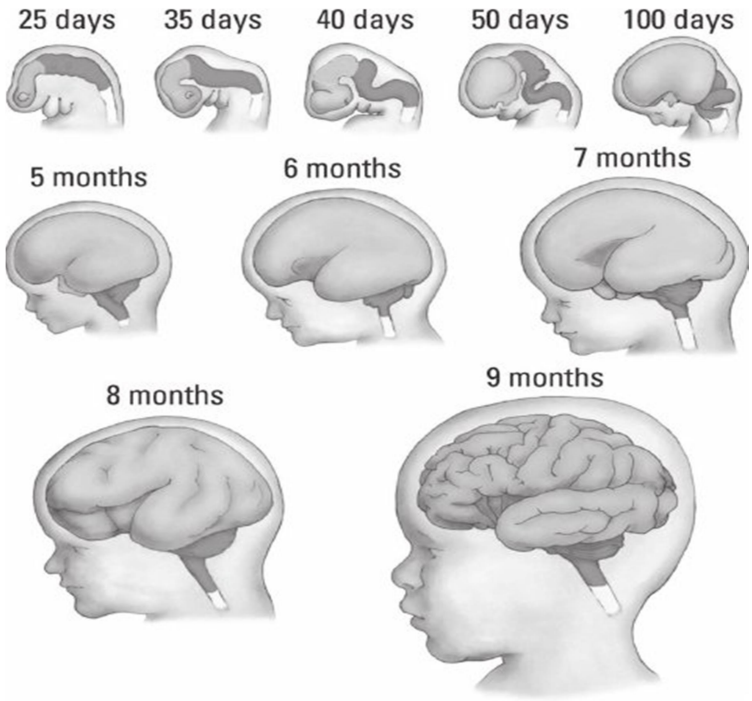

Figura 2. Desarrollo del cerebro en el periodo pre-natal [31].

El período fetal de desarrollo prenatal marca cambios importantes en el cerebro. Este período de desarrollo comienza durante la novena semana y dura hasta el nacimiento. Los primeros sistemas y estructuras corporales establecidos en la etapa embrionaria continúan desarrollándose. El tubo neural se desarrolla en el cerebro y la médula espinal y las neuronas continúan formándose hasta su posterior migración. Las sinapsis, o las conexiones entre neuronas, también comienzan su desarrollo. Alrededor de las 28 semanas, el

cerebro comienza a madurar más rápido, con una actividad que se parece mucho a la de un recién nacido dormido.

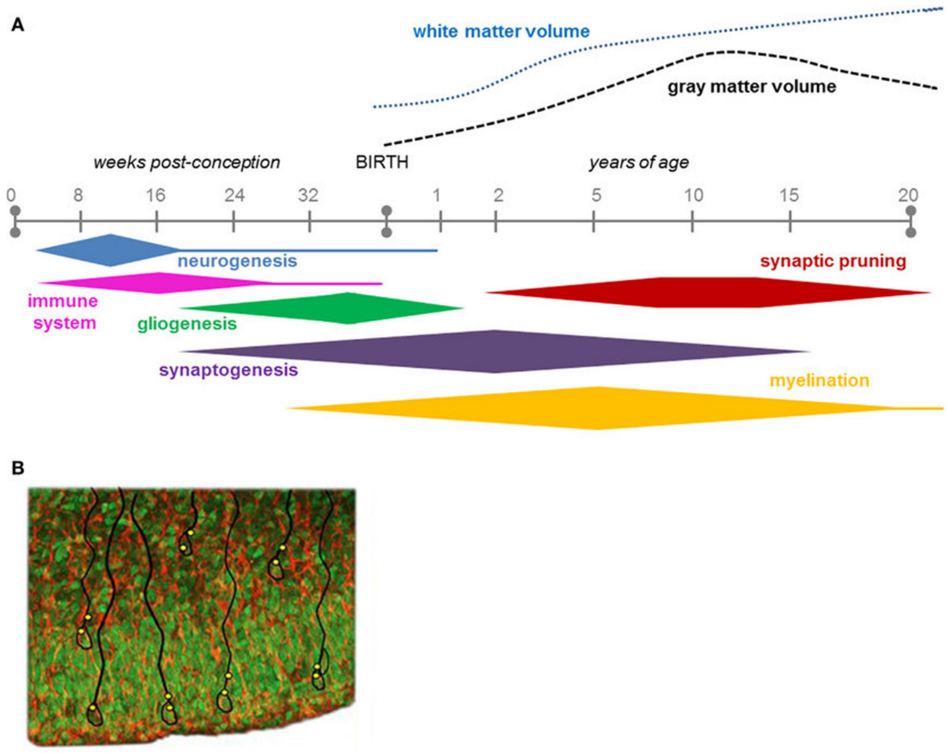

Figura 3. Evolución temporal del neurodesarrollo. En la figura 3-A se puede apreciar la evolución temporal de los procesos claves del neurodesarrollo en el ser humano, durante la gestación y hasta los 20 años. La mayor parte del proceso de neurogénesis está completa antes de nacer, mientras la sinaptogénesis continúa tras el nacimiento hasta la adolescencia. Considerando la figura 3-B, se puede apreciar la zona ventricular y subventricular con numerosas células proliferativas (verde; marca inmunológica) que poblará el córtex cerebral en desarrollo. Estas formará, los núcleos de las células gliales radiales (rojo/naranja; vimentin) alguna de las cuales se han marcado en negro [32].

Otro factor clave en el neurodesarrollo es la ganancia de peso cerebral. Durante el corto período de 9 meses, la célula "madre" inicial da lugar a más de 100 mil millones de células nerviosas, pesando el cerebro aproximadamente 400 g cuando nace el niño. Durante los

primeros 4 años de vida, el cerebro continúa creciendo, alcanzando el tamaño de 1200 gramos. Durante los próximos 10 a 15 años, el crecimiento del cerebro continúa, involucrando diferentes áreas cerebrales de una manera ligeramente diferente. Por ejemplo, el grosor de las diferentes regiones de la corteza cerebral cambia entre las edades de 5 y 18 años a diferentes ritmos, siendo las regiones importantes para el razonamiento, la planificación y la comunicación social las que maduran en último lugar [33].

Factores maternos, genéticos, nutricionales y ambientales pueden tener un efecto significativo sobre el desarrollo cerebral durante el periodo crítico de la vida fetal y a lo largo de los 2 primeros años de la vida.

- **Nutrición prenatal y neurodesarrollo**

Existen claras evidencias de que el factor ambiental más importante en el periodo prenatal y postnatal precoz determinante para el neurodesarrollo es la nutrición [34]. Un neurodesarrollo subóptimo implica un bajo nivel educativo y una trayectoria de una baja posición socioeconómica, que a su vez se asocia a pocas oportunidades en la vida. Una disponibilidad de nutrientes inadecuada durante la gestación se ha relacionado con enfermedades psiquiátricas, problemas de conducta, autismo y en

general alteraciones del desarrollo cognitivo. Estos efectos se asocian de forma clara a largo plazo con una reducción de la esperanza de vida y un aumento de la prevalencia de enfermedades como la depresión, esquizofrenia o el suicidio.

Nutrientes como los ácidos grasos poliinsaturados de cadena larga (LC-PUFAs), el ácido fólico y otras vitaminas del grupo B, proteínas, o minerales como el hierro son necesarios para el normal desarrollo del sistema nervioso fetal, y de forma específica para algunas regiones tan importantes como el hipocampo, cuerpo estriado, retina o la corteza cerebral, así como para establecer la arquitectura neural y los circuitos subyacentes (sinaptogénesis, poda neuronal y mielinización) que respaldan las principales funciones cerebrales [35–38].

El estado metabólico, la actividad de transporte placentario y el momento de liberación de los nutrientes influyen no solo en la cantidad sino también en la calidad de la entrega de nutrientes al feto.

✓ Ácidos grasos poliinsaturados de cadena larga (LC-PUFAs)

Según el número de átomos de carbono y la ausencia o presencia de dobles enlaces en su estructura química, los ácidos grasos se clasifican como: ácidos grasos saturados (AGS), ácidos grasos monoinsaturados (MUFAs), ácidos grasos poliinsaturados

(PUFAs) y ácidos grasos poliinsaturados de cadena larga (LC-PUFAs). Los ácidos grasos linoleico (LA, C18:2n-6) y α-linolénico (ALA, C18:3n-3) son esenciales, y por tanto, deben ser ingeridos a través de la dieta, y son precursores de los LC-PUFAs. La síntesis de LC-PUFAs se realiza mediante procesos de desaturación y elongación, gracias a las enzimas desaturasas (FADS1, FADS2, FADS3) y elongasas (ELOV2, ELOV5), las cuales son compartidas por ambas series de ácidos grasos poliinsaturados [31,39–42] (Figura 3).

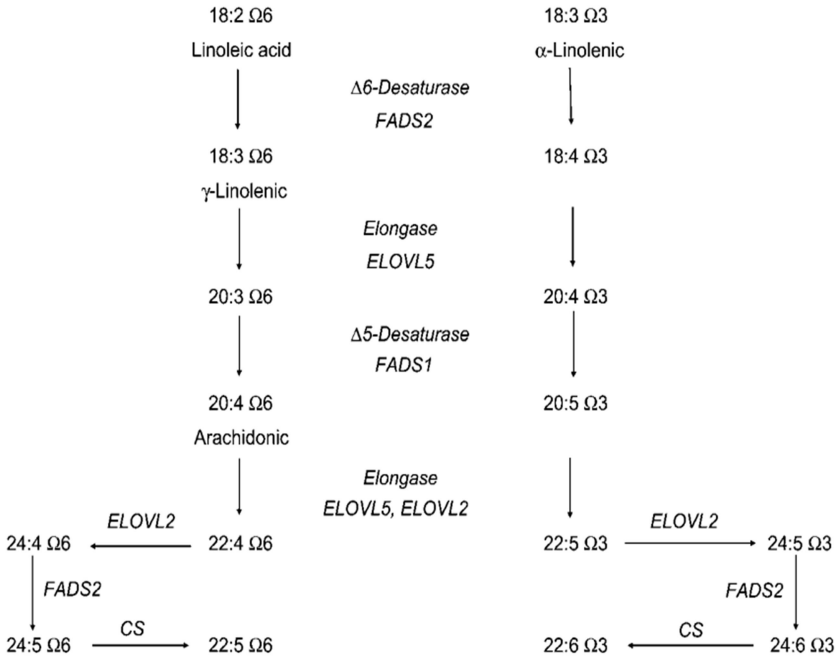

Figura 4. Ruta metabólica de los ácidos grasos esenciales [39].

Durante mucho tiempo se ha considerado que el aporte nutricional suficiente de PUFAs es beneficioso para la salud infantil. El aporte nutricional de PUFAs de las series ω-6 y ω-3 y sus derivados de

cadena larga como el ácido araquidónico (ARA) y en especial el ácido docosahexaenoico (DHA) se considera muy importante para varias funciones fisiológicas. Entre las principales funciones de los PUFAs se encuentran regular la fluidez de la membrana celular, así como actuar como precursores de eicosanoides y docosanoides, los cuales tienen un papel importante en los procesos inflamatorios [43]. Durante la vida fetal y la infancia temprana, el DHA es necesario para el desarrollo retiniano y neuronal adecuados [44], reflejado por la acumulación masiva de DHA en la retina y cerebro fetales durante el embarazo[45]. En años recientes, el interés de la investigación se ha volcado en la optimización del suministro fetal de DHA por la ingesta materna de suplementos de DHA durante el embarazo y la lactancia. Además de la importancia de la ingesta nutricional de AGPI, ha surgido evidencia de una variación interindividual considerable en la capacidad de cómo los ácidos grasos ω-6 y ω-3 se procesan endógenamente mediante la ruta de desaturación/elongación [46] De manera breve, el ácido linoleico y el ácido α-linolénico obtenidos de la dieta se convierten en ácidos grasos de cadena más larga gracias a la elongación de la cadena de carbonos del ácido graso y la inserción de enlaces dobles (es decir, desaturación) mediante esta vía, descrita por Sprecher [47].

Recientemente, los estudios de asociación genética han demostrado que los antecedentes genéticos son muy importantes

para la composición de PUFAs en los tejidos humanos. Así, los polimorfismos en los genes que codifican las desaturasas de ácidos grasos o FADS determinan la eficiencia con la cual los PUFAs se metabolizan endógenamente para producir los ácidos grasos poliinsaturados de cadena larga (LC-PUFAs). Los hallazgos recientes sobre interacción entre genes y nutrición sugieren que estos polimorfismos modulan el efecto de la ingesta nutricional de ácidos grasos sobre fenotipos complejos como la evolución cognitiva y el riesgo de asma en niños.

Los ácidos grasos son moléculas implicadas en una amplia gama de procesos biológicos que incluyen, entre otros, la señalización celular, la regulación de las respuestas inmunitarias y el control de la expresión de una variedad de genes [48]. Por tanto, son moléculas esenciales para el mantenimiento de la salud y el bienestar, y alteraciones en su composición pueden dar lugar a distintas disfunciones fisiológicas.

Debido a la inmadurez fisiológica del feto, los niveles de PUFA dependen casi exclusivamente del suministro materno. La ingesta dietética materna debería incluir una amplia gama de ácidos grasos, con diferentes efectos en el metabolismo materno y fetal. La mayoría de los estudios realizados durante el embarazo y la lactancia se centran en el papel estructural de los LC-PUFAS. Los LC-PUFA son esenciales para la síntesis de fosfolípidos de la membrana celular.

Además, los derivados de éstos desempeñan un papel crucial en la señalización y son necesarios para la función celular normal.

Varios estudios también han explorado el efecto de los PUFA en el desarrollo del tejido adiposo, pero los datos no son concluyentes. El desequilibrio entre la ingesta materna de PUFA n-6 y n-3, en lugar del consumo de PUFA n-6 o n-3 per se, durante el embarazo parece influir negativamente en el desarrollo del tejido adiposo fetal [49,50].

Los AGPI-CL son a su vez importantes mediadores de la expresión génica, mediante la activación de los receptores proliferadores peroxisomales (PPARs), controlando la expresión de genes involucrados tanto en el metabolismo de los lípidos y de la glucosa como en la adipogénesis [48,51]. Los PUFAs y sus metabolitos de cadena larga como el DHA y el AA, son esenciales para un desarrollo adecuado del cerebro, aunque Los ácidos grasos poliinsaturados de larga cadena (AGPI-CL) de la serie n-3, y especialmente el DHA, han demostrado ser críticos para el crecimiento y el neurodesarrollo, habiéndose comprobado cambios estructurales en el cerebro, en concreto una relación directa con el tamaño del núcleo caudado [52]. En el estudio ALSPAC se observado que una baja ingesta de AGPI-CL n-3 durante la gestación incrementa el riesgo de tener puntuaciones subóptimas del coeficiente de inteligencia (IQ) en la expresión verbal, alteraciones de la conducta pro-social, menor desarrollo psicomotor en la manipulación fina, problemas de comunicación y bajos niveles de desarrollo social [53]. Igualmente, se

ha demostrado que la incidencia de bajo peso para la edad de gestación disminuye con el incremento de la ingesta de pescado por la embarazada, lo que también se asocia a un mejor desarrollo visual. Varios estudios han sugerido beneficios de la suplementación materna de LC-PUFAs durante el embarazo sobre el desarrollo neurológico de los hijos. Los niños cuyas madres recibieron suplementos de LC-PUFAs desempeñaron significativamente mejor que el grupo de placebo con respecto al dominio de la comunicación cognitiva a las edades de 4 y 6 meses [54], coordinación de ojos y manos a los 2.5 años [55], y atención sostenida en 5 años [56]. Sin embargo, también hay estudios que no apreciaron ninguna asociación entre el suministro materno de LC-PUFA durante el embarazo y el desarrollo neurológico a las edades de 18 meses [57] y 5 [58] y 12 años [59].

✓ Ácido Fólico (5-MTHF)

Existe evidencia científica de que la suplementación materna durante las primeras 12 semanas de embarazo no solo previene los defectos del tubo neural [60], sino que también tienen un papel crucial en la apoptosis, la neurogénesis y el desarrollo óptimo del sistema nervioso [61,62].

La deficiencia cerebral de ácido fólico (5-MTHF) podría estar asociada con una alteración del transporte de folato, lo que podría conducir a la atrofia de las regiones fronto-temporales y la

desmielinización periventricular, como se ha observado en algunos estudios de neuroimagen [63][64][65].

Sin embargo, los efectos a largo plazo de la suplementación prenatal con ácido fólico más allá de las 12 semanas de gestación sobre el neurodesarrollo no están determinados, estando en discusión el tiempo de suplementación, la dosis y los potenciales efectos combinados al aportarlo junto a otros micronutrientes. De hecho, algunos estudios epidemiológicos [36] y un seguimiento previo del proyecto NUHEAL [66] han demostrado que la suplementación prenatal con ácido fólico mejora la atención sostenida en los niños a los 8 años de edad.

- ✓ **Impacto de Polimorfismos Genéticos en el neurodesarrollo**

El contenido de LC-PUFAs en los tejidos depende de dos factores, la ingesta dietética y los procesos metabólicos de desaturación y elongación (controlado por los polimorfismos genéticos) a partir de los precursores. En este sentido, se ha demostrado que las enzimas δ-5 y δ-6 desaturasas, codificadas en los genes FADS1 y FADS2, son esenciales para el metabolismo de ácidos grasos y pueden influir en la síntesis de los LC-PUFAs. Recientemente, se ha sugerido que las variantes genéticas de las FADS y ELOVs de las madres durante el embarazo, van a influir directamente sobre el

suministro de LC-PUFAs hacia el feto, pudiendo afectar su óptimo desarrollo cerebral [67].

El metabolismo de 1 carbono está mediado por diferentes nutrientes dadores de grupos metilo (ácido fólico, vitamina B12, vitamina B6, vitamina B2 o colina), donde dos ciclos principales se entrelazan: la biosíntesis del ADN y el ciclo de metilación, habiéndose comprobado la implicación de estos nutrientes en los procesos de metilación del ADN y de las histonas [68]. El ácido fólico participa en procesos biosintéticos y epigenéticos que facilitan la síntesis y metilación de ácidos nucleicos y proteínas. Se han identificado diferentes variaciones en los polimorfismos genéticos de la metiltetrahidrofolato reductasa (MTFHR), enzima implicada en la absorción de ácido fólico y su metabolismo [69], siendo el MTHFR 677 C/T el más importante en términos de prevalencia e impacto [70]; el genotipo 677 C/T se asocia a una reducción de la actividad enzimática del 60%, determinando un aumento de la homocisteína y reacciones de metilación (epigenéticas) [71]. El alelo materno MTHFR 677 T se ha descrito como un predictor independiente de un peor desarrollo neurológico infantil a los 24 meses de edad, mientras que los genotipos MTHFR 677 C/T del niño no se asociaron con el neurodesarrollo infantil [72]. Se ha sugerido que la variación genética materna en el metabolismo del ácido fólico durante el embarazo puede programar las trayectorias del desarrollo neurológico en los

hijos [72], en concreto, las variantes del gen materno MTHFR 677 T/T (rs1801133 C/T), CBS rs2234715 GT+TT y COMT AA (rs4680 G/A) se asociaron con un trastorno mental y con un mayor riesgo de autismo en los niños, coincidiendo con niveles más altos de homocysteína [73].

- **Velocidad de procesamiento y desarrollo cognitivo.**

La velocidad del procesamiento de la información es un proceso esencial para la función cognitiva de orden superior, incliuyendo funciones básicas como la memoria o las funciones ejecutivas [74]. Este proceso cognitivo se puede definir como el tiempo necesario para mover información de una neurona a la siguiente [75], o la rapidez con la que una persona puede realizar las operaciones mentales necesarias para completar una tarea [76]. En este caso, la velocidad de procesamiento está altamente relacionada con la mielinización intacta, crucial para la integración de información a través de redes neuronales distribuidas espacialmente. Además, la asociación entre la integridad de la materia blanca y la velocidad de procesamiento en las tareas cognitivas se ha establecido en la bibliografía [77,78].

- ## Nutrición y conectividad funcional

A pesar de la relevancia de la suplementación nutricional en la estructura del cerebro, se sabe poco sobre los efectos de la suplementación materna con LC-PUFAS y/o ácido fólico en la conectividad funcional del cerebro de los niños.

Las imágenes por resonancias magnéticas funcionales en reposo (rsfmri) se usan en el trazado cerebral para evaluar las interacciones regionales que ocurren cuando no se está realizando una tarea explícita. Esta técnica se basa en el hecho de que el flujo sanguíneo cerebral y la activación neuronal están acoplados; dicho de otro modo, cuando se activa un área del cerebro, también aumenta el flujo sanguíneo a esa región (BOLD – Blood-Oxygen-Level Dependant).

La conectividad funcional es la conexión entre regiones del cerebro que comparten propiedades funcionales. Se puede definir como la correlación temporal entre eventos neurofisiológicos espacialmente remotos, expresada como desviación de la independencia estadística a través de estos eventos en áreas y grupos neuronales distribuidos [79].

Las principales redes o áreas que nos podemos encontrar en un análisis de rs-fMRI son (Figura 5):

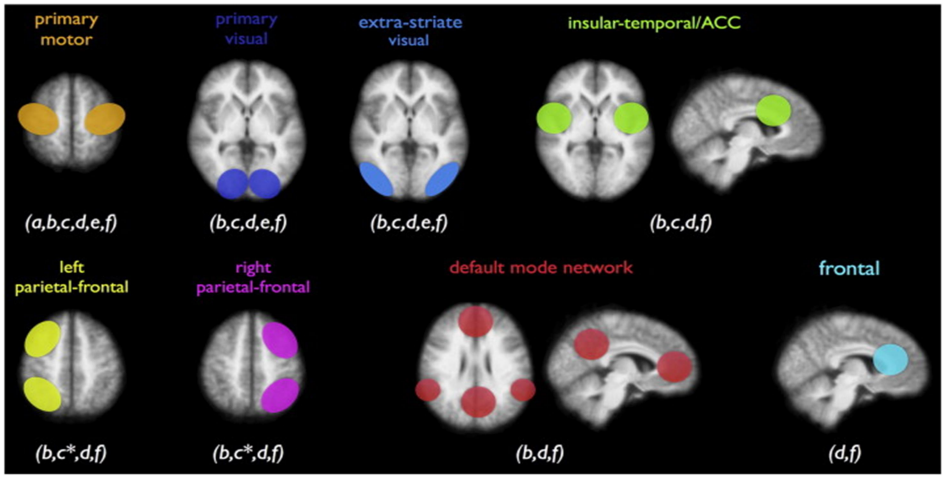

Figura 5. *Principales RSN identificadas por los siguientes estudios: (a) Biswal et al. (1995), (b) Beckamnn et al. (2005), (c) De Luca et al. (2006), d) Damoiseaux et al. (2006), (e) Salvador et al. (2005), and (f) Van den Heuvel et al. (2008).*

✓ Red Neuronal por Defecto *(Default Mode Network - DMN)*

La red de modo por defecto es una red de regiones del cerebro que están activas cuando un individuo está despierto y en reposo [80]. Se considera un sistema cerebral interconectado y anatómicamente definido que se activa preferentemente cuando las personas se concentran en tareas internas como soñar despierto, visualizar el futuro y recuperar recuerdos [81]. Se correlaciona negativamente con los sistemas cerebrales que se centran en señales visuales externas. Es una de las redes más estudiadas presentes durante el estado de reposo y es una de las redes más fáciles de visualizar [82].

- ✓ **Red Neuronal Sensoriomotora *(Sensoriomotor Network - SMN)***

La red sensorimotora es una de las más extensas, incluyendo numeroso procesos. El SMN incluye regiones somatosensoriales (post-central gyrus) y motoras (pre-central gyrus), extendiéndose a las áreas motoras suplementarias [83]. Numerosos trabajos han sugerido que esta red se activa durante las tareas motoras, como el golpeteo de los dedos [84], lo que indica que estas regiones pueden implicar un estado premeditado que prepara al cerebro para realizar y coordinar una tarea motora [83]. La disfunción en el SMN está implicada en varios trastornos neuropsiquiátricos.

- ✓ **Red Neuronal Frontoparietal (Frontoparietal Netwok - FPN)**

Es una red cerebral compuesta principalmente por la corteza prefrontal dorsolateral y la corteza parietal posterior [85], alrededor del surco intraparietal [86]. Dicha red esta involucrada en la atención sostenida, la resolución de problemas complejos y la memoria de trabajo [87].

HIPÓTESIS Y OBJETIVOS

HIPÓTESIS Y OBJETIVOS

La aplicación de procesos de minería de datos en el campo de la biomedicina está ocupando un papel central en el ámbito científico con múltiples propuestas algorítmicas y aplicaciones. La hipótesis de partida es el potencial de estos algoritmos para el procesamiento de datos imperfectos, el aprendizaje en sus dos vertientes (Supervisado y No supervisado) y la validación de los resultados obtenidos mediante análisis de estadística clásica y avanzada, presentando como aplicación práctica el análisis de la base de datos global de NUTRIMENTHE.

El objetivo general del presente proyecto de tesis es aplicar metodologías de procesamiento de datos en el ámbito de la nutrición y neurodesarrollo aplicados al proyecto NUTRIMENTHE. Para ello se hará uso de las técnicas más avanzadas de minería de datos aprovechando el hecho de que la información incluida en la base de datos es susceptible de ser convertida en conocimiento útil y de amplio interés para la comunidad científica.

Los análisis realizados han permitido la extracción de conocimiento oculto, aprovechando los enormes esfuerzos y recursos dedicados para obtener la ingente cantidad de datos de difícil manejo proporcionados por la base de datos NUTRIMENTHE, así como

las cohortes que lo conforman (NUHEAL, CHOP y Generation R). La minería de datos aporta un nuevo enfoque de análisis exploratorio, destacando los factores que más impacto tienen sobre las variables objetivo previamente seleccionadas, pudiendo estar ocultos al análisis estadístico convencional.

Los objetivos específicos propuestos son los siguientes:

1. Revisión bibliográfica de las técnicas de pre-procesamiento, selección de características, Subgroup Discovery y técnicas de minería de datos en general que deban ser utilizadas en el análisis inteligente de datos asociado a la base de datos NUTRIMENTHE.
2. Aplicación de técnicas de pre-procesamiento en general y selección de características en particular para determinar las variables más relevantes en la base de datos considerando diferentes hipótesis asociadas a los estudios de nutrición y neurodesarrollo infantil.
3. Diseño y aplicación de modelos de minería de datos para la predicción de variables de interés, la extracción de subgrupos (Subgroup Discovery) y la extracción de información y patrones ocultos mediante reglas de asociación.
4. Desarrollar estudios de investigación relacionados con los resultados previamente validados con análisis estadísticos.

4.1. Extracción de conocimiento de la Base Global NUTRIMENTHE. Análisis de los efectos del tabaquismo materno sobre el desarrollo de la conducta en los hijos a la edad escolar.

4.2. Análisis con modelos de efectos mixtos para analizar los efectos de la suplementación prenatal con aceite de pescado y/o 5-metiltetrahidrofolato (5-MTHF) sobre la velocidad de procesamiento.

4.3. Análisis de componentes independientes y técnicas exploratorias de minería de datos para determinar los efectos de la suplementación prenatal con aceite de pescado y/o 5-MTFH sobre la conectividad funcional del cerebro en situación de reposo *(resting state)* y su significado clínico en niños de 10 años de edad.

MATERIAL Y MÉTODOS

MATERIAL Y MÉTODOS

• Análisis de Minería de datos

El proceso general de extracción de conocimiento de una base de datos está expuesto en la figura 6.

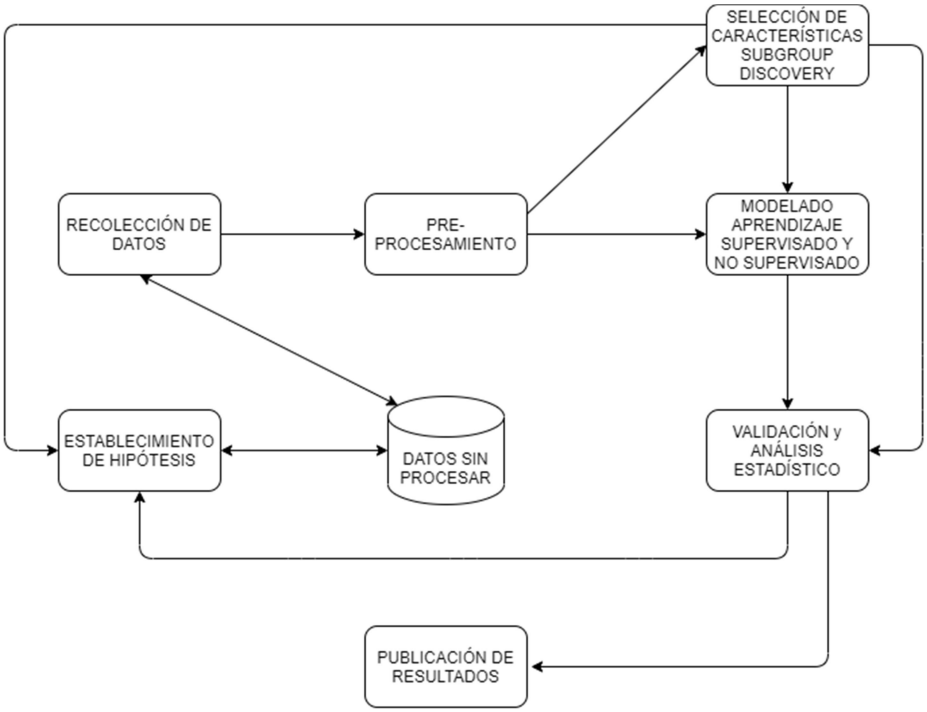

Figura 6. Proceso de extracción de conocimiento de una base de datos

El primer paso sería establecer las hipótesis de partida teniendo en cuenta los datos sin procesar que tenemos a nuestra disposición. El siguiente paso sería la recolección de datos para su posterior pre-procesamiento. Una vez que los datos estén limpios y estandarizados, se puede tomar dos caminos alternativos. Selección de características

junto a Subgroup discovery o crear modelos de predicción basados en aprendizaje supervisado o No supervisado. El siguiente paso (sea cual fuere el camino escogido anteriormente) es la validación de los resultados obtenidos usando las métricas pertinentes para cada algoritmo usado, así como técnicas de estadística clásica para validar nuestros resultados.

Adaptación del proceso de minería de datos a la base de datos NUTRIMENTHE.

1) **Establecimiento de hipótesis iniciales.** En nuestro caso sería el efecto de la dieta prenatal y otros factores disponibles en la NGDB en el neurodesarrollo de los niños.
2) **Recolección de datos**. La base de datos global de NUTRIMENTHE estará compuesta por 3 cohortes distintas (CHOP, NUHEAL y Generation R), incluyendo un total de un millar de niños con datos imperfectos, donde se han obtenido datos sobre el desarrollo cognitivo y de la conducta. Resulta de vital importancia analizar, recodificar y estandarizar las distintas bases de datos para permitir un análisis posterior fiable.
3) **Pre-procesamiento de datos:** Una vez fusionada la base de datos, será prioritario realizar un proceso de pre-procesamiento de datos, ya que las técnicas de minería de datos son sensibles a la calidad de la información sobre la que se pretende extraer conocimiento [88]. Los datos tomados en el proyecto de NUTRIMENTHE pueden

contener inconsistencias, errores, o de forma más genérica, no estar preparados para una etapa posterior de minería de datos. Gracias al pre-procesamiento de datos, se pueden adaptar los datos para cumplir las expectativas de entrada de cualquier algoritmo de aprendizaje. Entre los posibles técnicas que ofrece el pre-procesamiento de datos nos podemos encontrar con [88]:

- Limpieza y estandarización de datos.
- Imputación de valores perdidos si procede.
- Identificación de anomalías.
- Selección y generación de instancias.

4) **Selección de características**. Una de las tareas más complejas a la hora de aplicar técnicas clásicas de estadística para la base de NUTRIMENTHE es el número de variables con las que cuenta (aproximadamente dos mil, si consideramos todas y cada una de las variables antropomórficas en distintos intervalos de tiempo, así como datos provenientes de la cohorte NUHEAL>1000). Si consideramos cada una de ellas independientemente de las otras, solo podríamos conducir análisis parciales limitando la capacidades para verificar las hipótesis. La minería de datos proporciona mecanismos para reducir el número de variables concentrándose solo en aquellas que ofrezcan la misma o más cantidad de información útil [89],como es el caso de PCA, ICA, FS y por ello, hemos destacado esta técnica de forma independiente, debido a

la importancia que tendrá al abordar el análisis de esta base de datos.

- ✓ **Análisis de Componentes Independientes**

  Los Análisis de Componentes Independientes (ICA) se llevaron a cabo utilizando el Análisis Probabilístico de Componentes Independientes [90], similar a la implementación de MELODIC (Descomposición Lineal Exploratoria Multivariante en Componentes Independientes) Versión 3.14, parte de FSL. Los datos pre-procesados se blanquearon y proyectaron en un sub-espacio de 40 dimensiones mediante el análisis probabilístico de componentes principales. Los cuarenta componentes se comprobaron e identificaron visualmente. Los componentes que representan artefactos conocidos como movimiento, ruido de alta frecuencia o pulsación venosa [91,92] o aquellos que no se encuentran principalmente en la materia gris [93] fueron identificados y no tomados en cuenta en los siguientes análisis. Se descartaron catorce componentes por el motivo anterior; y las veintiséis redes de estado en reposo (RSN) restantes se etiquetaron basándose en la superposición con los atlas estructurales corticales y subcorticales de Harvard-Oxford disponibles en FSL.

5) **Obtención y verificación de hipótesis parciales**. En la mayoría de los casos la hipótesis general a probar resulta ser la más interesante, y a

la vez la más difícil de verificar. Sin embargo, podemos coincidir en el hecho de que a mayor número de variables, mayor es la complejidad a la hora definir la relación entre variables secundarias y variables objetivo definiendo quién es quién en la base de datos. La minería de datos proporciona técnicas como el Subgroup Discovery [94], diseñado específicamente para la extracción de grupos de interés asociadas a una clase, capaz de generar nuevas hipótesis describiendo las características del subgrupo dónde se hace cierta la nueva hipótesis. Éste nuevo enfoque permite rediseñar la hipótesis inicial para un proceso de verificación más riguroso. Por ejemplo, si queremos probar la hipótesis de que la suplementación materna de ácido fólico durante la gestación aumenta los efectos a largo plazo sobre la atención de los hijos, Subgroup Discovery nos proporcionaría los factores implicados en la confirmación de dicha hipótesis.

6) **Aprendizaje supervisado**, capaz de resolver problemas específicos teniendo un conjunto de variables explicativas y una variable objetivo [95]. Podemos encontrarnos con dos grandes variantes dependiendo del tipo de la variable objetivo.
   a. **Clasificación**, cuando la variable objetivo Y es discreta (número finito de valores) pudiendo dividir el conjunto de toda la población en clases en función de Y.
   b. **Regresión**, siendo la variable objetivo Y de tipo continuo.

7) **Aprendizaje no supervisado**, que no necesita información acerca de una variable objetivo, basándose sólo en las variables conocidas. Se abordará el uso de dos tipos de técnicas:
   a. **Clustering**, método capaz de seleccionar grupos de individuos acorde a unas características, que en conjunción con el Subgroup Discovery, podría proporcionarnos información relevante a la hora de caracterizar los grupos donde se cumpla una condición específica.
   b. **Reglas de asociación**, proceso por el cual se descubren relaciones entre variables de la forma causa-efecto [96]. En el caso del estudio de los datos de la base global NUTRIMENTHE, dicha idea podría proporcionarnos información acerca de la cadena causa-efecto para diagnosticar ciertas condiciones.
8) **Validación**. Una vez obtenidos los resultados en la etapa anterior, el siguiente paso sería comprobar la veracidad/calidad de dichos resultados, mediante validación cruzada. En esta etapa se selecciona aleatoriamente una parte del conjunto de datos llamado training, y el resto, llamado conjunto test, que se usaría para validar dicho modelo.

En la tabla 1 aparece un resumen de los paquetes disponibles R que mejores resultados nos dieron con la base de datos NUTRIMENTHE,

teniendo en cuenta siempre la fiabilidad y procedencia de le dichos paquetes (repositorio CRAN oficial de R).

Tabla 1. Algoritmos de pre-procesamiento y aprendizaje con mejor resultado para la base de datos NUTRIMENTHE.

FASE	TIPOS	PAQUETES R
PRE-PROCESAMIENTO DE DATOS	Limpieza de datos	caret[97]
	Imputación de valores perdidos	caret,MICE[98]
	Identificación de valores extremos	caret
	Selección de instancias	caret
SELECCIÓN DE CARACTERISTICAS	Selección de predictores	randomForest[99]
	Importancia relativa	relaimpo[100]
	Subconjunto de variables con máxima ganancia	WGCNA[101]
	Importancia absoluta	BORUTA[102]
SUBGROUP DISCOVERY	SDIGA	SDEFSR[103]
	MESDIF	SDEFSR
	NMEEF-SD	SDEFSR
	FuGePSD	SDEFSR
Aprendizaje Supervisado	RandomForest	randomForest
	Xgboost	caret
	CART	caret
Aprendizaje No Supervisado	Clustering	kmeans[104]
	Reglas de Asociación	arules[105]

- ## Proyecto NUTRIMENTHE

Los datos de este estudio provienen del Proyecto NUTRIMENTHE 7FP UE ("El efecto de la dieta en el rendimiento mental de los niños", GA n. ° 212652, www.nutrimenthe.eu), descrito anteriormente en [3]. Se ha conseguido establecer la Base de datos global NUTRIMENTHE (NGDB) uniendo los conjuntos de datos de tres Cohortes de

seguimiento europeas: el estudio *Nutraceuticals for a Healthy Life* (NUHEAL-Follow Up) [106] (Alemania, Hungría y España), el estudio *Childhood Obesity Project* (CHOP) [107] (Bélgica, Alemania, Italia, Polonia y España), y el estudio Generation R [108] (Países Bajos) . Después de combinar las variables comunes de los tres estudios, se formó una nueva base de datos con un total de un millar niños con datos imperfectos que fueron evaluados mediante un procedimiento neuropsicológico común a los 8 – 9.5 años de edad.

- ✓ **COHORTE NUHEAL Follow-UP (n=147)**

NUHEAL es un ensayo multicéntrico, randomizado, doble ciego, factorial 2x2 controlado por placebo, diseñado para evaluar los efectos de los n-3 LC-PUFA y la suplementación con 5-metil-tetrahidrofolato (5-MTHF) durante la segunda mitad del embarazo en desarrollo infantil. Las mujeres fueron reclutadas antes de las 20 semanas de gestación y fueron asignadas aleatoriamente a cuatro grupos diferentes. Desde la semana 20 hasta el parto, recibieron un suplemento diario consistente en Fish Oil (1) {FO: 500 mg de ácido docosahexanoico (DHA) + 150 mg de ácido eicosapentaenoico (EPA)}, (2) 400 µg de 5-MTHF, (3) ambos, o (4) Placebo, junto con vitaminas. y minerales siguiendo las recomendaciones europeas para

mujeres embarazadas. Para mas información sobre la cohorte, consúltese [106].

- ✓ **COHORTE CHOP (n=525)**

El Programa de Obesidad Infantil de la UE (CHOP) se dedicó a estudiar si la relación proteínas/grasas provista en la fórmula infantil y los alimentos complementarios tiene efectos duraderos sobre los riesgos de padecer obesidad. Dicho estudio permitió, por primera vez, realizar un ensayo de intervención multicéntrico en recién nacidos, para estudiar si la alimentación con fórmulas infantiles, que difieren en su nivel de proteínas de la leche, podrían estar asociados con en el riesgo posterior de obesidad infantil. El ensayo se llevó a cabo en cinco países (España, Alemania, Bélgica, Italia y Polonia) con diferentes ingestas habituales de proteínas (bajas y altas concentraciones) para probar la hipótesis de que una ingesta temprana de proteínas se asocia con el crecimiento infantil y el riesgo posterior de obesidad infantil.

- ✓ **COHORTE Generation R (n=200)**

Generation R es un estudio de cohorte prospectivo desde la vida fetal hasta la edad adulta en una población urbana holandesa multiétnica [108]. El estudio está diseñado para identificar las causas ambientales

y genéticas tempranas del crecimiento, desarrollo y salud normales y anormales desde la vida fetal hasta la edad adulta. Eventualmente, los resultados del Estudio Generación R deben contribuir al desarrollo de estrategias para optimizar la salud y la atención médica de las mujeres embarazadas y los niños.

- **Estudio del Neurodesarrollo**

El desarrollo neurocognitivo de los niños se evaluó mediante la NUTRIMENTHE Neuropsycological Battery (NNB), una batería neuropsicológica integral desarrollada específicamente para el proyecto NUTRIMENTHE [109]. El NNB consta de quince pruebas cognitivas para evaluar siete dominios neuropsicológicos diferentes: velocidad de procesamiento, percepción, motricidad, memoria, atención, lenguaje y funciones ejecutivas, que se describen brevemente en la Tabla 2. [109]

Table 2. NUTRIMENTHE Neuropsychological Battery description

Domain	Function	Test
Memory	Visual Episodic Memory	Recall of Object Test (ROT)
	Verbal Memory	Rey Auditory Verbal Learning Test (RAVLT)
Attention	Sustained & Focused Attention	Continuous Performance Test (CPT)
	Spatial Attention	Pair Cancellation test (W-M)
Motor	Visio-Motor Coordination	Grooved Pegboard Test (GPT)
Perception	Visio-Perceptual Integration	Hooper Visual Organization Test (HVOT)
Language	Semantic Fluency	Categorical Fluency Test (F-A-S-Animals)
	Verbal Comprehension	Token test II (NEPSY-II)
Processing Speed	Processing Speed	Symbol Digit Modalities Test (SDMT)
Executive Functions	Impulsivity/Inhibition	Stroop Color & Word Test
	Update	Reversal Digits Subtest
		Matrix Analogies test - (K-ABC-II)
	Flexibility/Shifting	Children's Color Trail Test (CCTT)
	Decision Making	Hungry Donkey Task (HDT)

- **Estudio de la conducta**

El CBCL/6-18 (Child Behavior Checklist) [110] es una medida derivada empíricamente, altamente válida, basada en observaciones directas de los padres para proporcionar información sobre el comportamiento y los problemas emocionales de sus hijos, traduciendo los resultados a una puntuación.

El CBCL proporciona un índice (T-Score) del estado social y de comportamiento de los niños en relación con otros niños de la misma edad y sexo. Se obtienen once ítems provenientes del CBCL: tres

escalas totales (internalización, externalización y problemas totales) y ocho escalas individuales (quejas somáticas, abstinencia/depresión, ansiedad/depresión, rebeldía, agresividad, sociales, pensamiento y atención).

Se ha dicotomizado cada CBCL T-Score en dos clases: 1) Sujetos normales con un T-Score por debajo del percentil 60 para cualquier escala CBCL individual y por debajo del percentil 65 para la escala CBCL total. 2) Patología clínica para aquellos con una puntuación T igual o superior al percentil 60 para una escala de CBCL individual, e igual o superior al percentil 65 para la escala de CBCL total, como se detalla en [110].

- ## Análisis Bioquímicos (NUHEAL Follow-up)

✓ **Análisis de ácidos grasos (maternos y en el cordón umbilical)**

Para establecer las concentraciones de ácidos grasos en los fosfolípidos del plasma , se ha seguido el procedimiento descrito en [111] . Brevemente, la sangre se centrifugó a 3500 ppm x g durante 10 min a temperatura ambiente. A continuación, se extrajo el plasma y se almacenó a -80ºC. La extracción de lípidos del plasma se realizó según el método de Kolarovic y Fournier [112]. El análisis de los ácidos grasos a partir de fosfolípidos en plasma se realizó mediante

cromatografía gas-líquido de alta resolución. Las condiciones durante el análisis y los estándares utilizados están establecidos en [113]. Los resultados se expresaron como porcentajes en peso (% en peso) de todos los ácidos grasos cuantificados.

- ✓ **Análisis de ácido fólico**

El análisis de ácido fólico en plasma se llevó a cabo mediante un ensayo microbiológico utilizando una cepa de *Lactobacillus casei* resistente al cloranfenicol, como se describió anteriormente [114]. Los coeficientes de variación interensayo e intraensayo fueron inferiores al 11%.

- ✓ **Análisis de homocisteína**

Las concentraciones de homocisteína total (tHcy) se analizaron mediante inmunoensayo polarizado por fluorescencia con en el autoanalizador IMx [115]. La preparación de la muestra y las condiciones cromatográficas se realizaron como se describió anteriormente [116]. Las intensidades de fluorescencia se midieron con excitación a 385 nm y emisión a 515 nm.

- ✓ **Selección de SNP**

El genotipado de polimorfismos de un solo nucleótido (SNP) del grupo de genes FADS se realizó con el método iPLEX (Sequenom, San Diego, CA, EE. UU.) Mediante el método de espectrometría de masas

de ionización-tiempo de vuelo con desorción láser asistida por matriz (MALDI-TOF MS, Mass Array, Sequenom), de acuerdo con las instrucciones del fabricante. El control de calidad de genotipado estándar incluyó un 10% de muestras duplicadas y negativas. La tasa de discordancia de genotipado fue inferior al 0,3% La selección de SNP para los siguientes análisis se realizado calculando el Pairwise Linkage Disequilibrium.

- ✓ **MTHFR 677 C/T**

Se obtuvieron muestras de ADN a partir de la sangre materna y del cordón en el momento del parto. Dichas muestras se genotiparon para la variante 677 C/T de metilentetrahidrofolato reductasa (MTHFR) mediante PCR, con enzimas de restricción y separación de fragmentos de ADN por electroforesis como se describió previamente [115]. Se seleccionó MTHFR 677 C>T por su alta relevancia clínica en humanos debido a la asociación del genotipo TT con altas concentraciones de homocisteína en plasma [117].

- **Análisis de Neuroimágen (NUHEAL Follow-up)**

- ✓ **Adquisición y pre-procesamiento de imágenes (NUHEAL Follow-Up)**

Los datos de resonancia magnética funcional se recopilaron con un sistema 3T Philips Intera Achieva (Philips Medical Systems, Eindhoven, Países Bajos) equipado con una bobina de cabezal de matriz en fase de ocho canales. Se adquirió una secuencia de imágenes ecoplanares (EPI) ponderadas en T2* con los siguientes parámetros: tiempo de repetición (TR) = 2000 ms; tiempo de eco (TE) = 30 ms; ángulo de giro = 78°; campo de visión = 230 x 230 mm; número de slices = 23; dimensión del vóxel = 3 × 3 × 4 mm; espacio, 1 mm. Las imágenes estructurales también se obtuvieron como una secuencia de eco-gradiente turbo ponderada en T1 isotrópica en el plano sagital (TR = 8,3 ms; TE = 3,8 ms; ángulo de giro = 8 °; FOV = 240 x 240 mm; número de cortes = 160; grosor del corte = 1 mm; dimensión del vóxel = 1 × 1 × 1 mm). Antes del pre-procesamiento, el movimiento durante la adquisición se estimó utilizando la herramienta de realineación implementada en el software de mapeo paramétrico estadístico (SPM8) (Departamento de Neurología Cognitiva de Wellcome, Instituto de Neurología, Queen Square, Londres). Los sujetos con parámetros de movimiento superiores a 3 mm o 3 grados fueron excluidos del procesamiento y análisis posteriores.

El pre-procesamiento de rsfMRI se realizó con la biblioteca software (FMRIB) (v5.0.9, FSL, http://fsl.fmrib.ox.ac.uk/fsl/fslwiki). Los pasos previos al procesamiento incluyeron la eliminación de los diez primeros volúmenes, filtrado temporal de paso alto (120 s), corrección

de movimiento con la herramienta MCFLIRT, extracción del cerebro con BET (Smith, 200), suavizado espacial con un núcleo gaussiano de FWHM = 8 mm, registro en una plantilla estándar ponderada en T1 utilizando FSL-Linear Registration Tool (FLIRT) con 12 grados de libertad (DOF) y finalmente re-muestreo a una resolución de 4 mm.

• Variables confusoras

✓ *Base Global NUTRIMENTHE*

Se encontraron un conjunto de variables comunes entre las tres cohortes y se agregaron al presente análisis: País de origen, Cohorte, Edad materna (años) al parto, Edad gestacional al parto (semanas), Estado civil, Nivel educativo materno, Nivel educativo paterno, Sexo del niño, medidas antropométricas al nacer (peso (kg), altura (cm) y perímetro cefálico (cm)), y Lactancia materna al menos durante los primeros tres meses.

✓ **NUHEAL**

En conjunción con las variables previamente expuestas, la cohorte NUHEAL follow-up cuenta con más variables confusoras, como es el caso de los ácidos grasos, genotipado y resonancias magnéticas previamente establecidas.

• Análisis estadístico

Los resultados descriptivos se expresaron como medias ± desviación estándar para las variables continuas o porcentajes para las variables categóricas, después de evaluar la normalidad de éstas con el test de Kolmogorov-Smirnov o Shapiro-Wilk, dependiendo del tamaño de la muestra. Las diferencias en las características de la población entre los grupos de estudio se analizaron mediante el test t de Student o ANOVA para variables continuas o la prueba de Chi cuadrado para variables categóricas.

Para evaluar el *effect size* de la muestra, se ha hecho uso del coeficiente d de Cohen, siguiendo cuidadosamente la regla general, donde d≤0.2 se consideró como "pequeño", d≤0.5 como "mediano" y d ≥ 0,8 como tamaño de efecto "grande" [118].

Para analizar los efectos que podrían tener las variables confusoras, se realizaron modelos de regresión logística y de modelos mixtos en un procedimiento paso a paso para ajustar los posibles factores confusores que mostraban diferencias estadísticamente significativas (p <0,05) entre los grupos de estudio.

Para los datos de resonancias magnéticas, se implementó un método de regresión dual en FSL [119], seguido de pruebas t de dos grupos, para comparar mapas cerebrales entre los grupos de estudio. Se crearon mapas cerebrales estadísticos específicos del sujeto y se

colapsaron en un archivo 4D para cada RSN. En la primera etapa del método de regresión dual, los componentes espaciales generados durante el análisis ICA se regresaron con los RSN de cada sujeto para dar un conjunto de puntos de tiempo específicos del sujeto para cada componente. En la segunda etapa, esos puntos de tiempo se regresaron a los datos rs-fMRI para obtener mapas cerebrales espaciales específicos del sujeto para cada componente. Seguidamente, esos mapas cerebrales específicos del sujeto se usaron para comparar cada red cerebral entre grupos. Estas comparaciones se probaron a nivel de vóxels para determinar las diferencias entre los grupos mediante pruebas de permutación no paramétrica (5000 permutaciones) [120]. Para cada RSN, los mapas estadísticos resultantes tenían un umbral en $p < 0,05$.

Se realizaron correlaciones de Pearson y Spearman entre las puntuaciones de RSN y las pruebas de NNB, teniendo en cuenta la normalidad de las variables.

Tanto el análisis estadístico como los análisis de minería de datos se desarrollaron bajo el entorno y lenguaje de desarrollo R. Las ventajas principales que conlleva son: La inmensurable cantidad de paquetes de código abierto disponibles así como la rapidez de aplicar técnicas potentes de extracción de conocimiento.

PUBLICACIONES

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• Minería de datos aplicada

Partimos de la hipótesis de que la alimentación precoz pueda tener efecto en el neurodesarrollo y conducta de los niños de la NGDB. Para poder hacer uso de las técnicas de minería de datos, el conjunto de datos que tenemos a nuestra disposición tiene que cumplir con una serie de estándares de calidad. Por ejemplo, el problema de los valores extremos u *outliers* tiene fácil solución ya que los últimos algoritmos de modelado en inteligencia artificial se encuentran tan avanzados que son capaces de ajustar el error automáticamente. En el caso de selección de instancias, nos puede ayudar a maximizar el resultado objetivo que se desee, seleccionando el subconjunto de muestras que mejor satisfaga una hipótesis, aunque para datos clínicos lo más aconsejable es usar toda la muestra. El principal problema que nos encontramos en el pre-procesamiento de datos en la NGDB fue la gran cantidad de valores perdidos o incompletos; ya que los algoritmos de aprendizaje supervisado o no supervisado necesitan que los datos estén completos para una predicción fiable. El problema reside en que algunos casos, el

porcentaje de valores perdidos supera el 70 %, perdiendo el valor de esa variable por completo. Cuando la perdida de datos esta entre un 15 y un 30 %, mejor calidad tendrá el algoritmo de imputación de datos. El mejor algoritmo de imputación de valores perdidos en el caso de NUTRIMENTHE fue MICE (Multiple Imputations by Chained Equations), cuyo propio nombre indicia, se basa en la parametrizacion de las variables en polinomios de X grado, donde X tiende a ser el número de variables con las que cuenta nuestro conjunto de datos. La principal ventaja de este algoritmo de datos reside en su robustez y fiablidad, mientras que su principal desventaja es el tiempo de ejecución exponencial que depende del tamaño muestral (82 horas para una población de 872 individuos y 450 variables).

El siguiente paso fue hacer un análisis exploratorio (Aprendizaje NO Supervisado) poco convencional, usando las reglas de asociación como arma principal. Las reglas de asociación permiten de una forma simple, mostrar todas las relaciones causa y efecto intrínsecas en la base de datos. El único requisito metodológico es que la totalidad de las variables estén discretizadas. Se basa en métricas de soporte y confianza, relacionadas básicamente con las veces que se repite la regla o transacción en el conjunto de datos. No se encontró ninguna regla que relacionase la dieta prenatal con alguna variable de neurodesarrollo o conducta, en cambio la relación causa-efecto que

más aparecía estaba relacionada con problemas de conducta Externalizantes y el tabaquismo perinatal.

Smoking During Pregnancy=Yes --> CBCL_ExternalProblems=Borderline/Pathologic

{support = 0.95 , confidence=0.91, lift=0.99}

A partir de este resultado, se comprobó en la bibliografía la certeza de esta afirmación, y por consiguiente se desarrolló el manuscrito "**Maternal smoking during pregnancy and child behavior problems: A multicenter study from the NUTRIMENTHE EU Project**", que aparece en la sección Estudio 1.

El hecho de que no haya ninguna asociación directa entre alimentación precoz y neurodesarrollo para la base de datos global NUTRIMENTHE, se decidió seguir explorando la base de forma separada por cohortes. Se descubrió que la cohorte NUHEAL Follow-up contenía gran cantidad de información oculta susceptible de ser extraída con técnicas de minería de datos. Gracias otra vez a las reglas de asociación, se descubrieron las siguientes relaciones causa-efecto:

AA/DHA ratio = >p75 --> SDMT = >p75

{support = 0.94 , confidence=0.92, lift=0.98}

AA/DHA ratio = >p75 --> CCTT-1(sc) = >p25
{support = 0.91 , confidence=0.92, lift=0.98}

Se puede apreciar en las dos reglas que el ratio de concentraciones AA/DHA por encima del percentil 75 (previamente discretizado en los percentiles 25,50 y 75) estaba relacionado con ambas pruebas pertenecientes al dominio de velocidad de procesamiento (SDMT = Symbol Digit Modalities test y CCTT = Children Color Trails Test).

A partir de este resultado, se comprobó en la bibliografía la certeza de esta afirmación, y por consiguiente se desarrolló el manuscrito **"Long-chain polyunsaturated fatty acids, homocysteine at birth and fatty acid desaturase gene cluster polymorphisms are associated with children´s processing speed up to age 9 years."**, que aparece en la sección Estudio 2.

En relación, se partió desde la hipótesis inicial de que la suplementación prenatal con LC-PUFAS y/o 5-MTHF tendría un efecto sobre el conectoma cerebral de los niños; por lo que este estudio estará limitado al proyecto NUHEAL con un total de 57 niños finales que realizaron las sesiones de resonancia.

Después de realizarse los análisis de neuroimagen, implicando el análisis de componentes independientes, doble regresión, y re-agrupación de nuestros grupos en FO (FO o FO + 5-MTHF, n=33) y No-

FO (5-MTHF o placebo, n=24), se decidió estudiar las posibles relaciones vigentes entre las puntuaciones de las resonancias y el resto de variables que aparecen en la base de datos de NUHEAL. Aunque este estudio tenga solo 57 niños, contamos con un total de 1000 variables, incluyendo numerosas características basales, (al contrario que ocurrió con el proyecto NUTRIMENTHE), concentraciones de LC-PUFAS en la madre e hijo en diversos tiempos, parámetros antropomórficos, NNB, CBCL, etc..

En minería de datos, gracias a la selección de características, podemos depurar la base de datos seleccionando sólo los parámetros de interés que nos satisfagan. En el caso presente, se ha dividido la población en FO y NO-FO, y en cada grupo se ha seleccionado como variable objetivo las regiones del conectoma con diferencias significativas entre los dos grupos:

- DMN - Angular Gyrus
- SMN - Postcentral Gyrus, Precentral Gyrus (L&R) y Putamen
- FPN - Angular Gyrus

Una vez selecionadas las variables objetivo, se crearan modelos de aprendizaje (regresión-randomForest) incluyendo todas las variables predictoras disponibles en la base total de NUHEAL para intentar predecir el curso de la variable objetivo en cuestión. El hecho de haber elegir este algoritmo en concreto recae en su simplicidad y facilidad a la hora de entrenar y ajustar un modelo, como

consecuencia es de los algoritmos de aprendizaje más famosos y usados.

En la tabla 3, se reflejan los resultados de dichos modelos.

Tabla 3. Modelos randomForest para cada RSN significativa

RSN	Area	RMSE	MAE	R^2
Default Mode Network	Angular gyrus	12.14	121.20	0.62
Sensorimotor Network	Postcentral Gyrus	17.52	145.21	**0.87**
	Precentral Gyrus L	17.54	114.2	0.52
	Precentral Gyrus R	5.23	85.21	**0.91**
	Putamen	11.02	114.30	0.67
FrontoParietal Network	Angular Gyrus	7.52	170.44	**0.89**

RSN = Resting state Netowk, RMSE = Root mean squarred error, MAE = Mean absolute error, R^2=R-squared, BOLD = very high R^2

Tanto el Postcentral y Precentral R Gyrus pertenecientes a la red SMN, asi como el Angular gyrus, de la red FPN tuvieron como resultado una R^2 bastante elevada, indicando que las variables explicativas o predictoras son capaces de describir en más de un 90 % las variables objetivo previamente mencionadas.

Se ha usado la función Importance, que viene con el paquete randomForest de R para calcular las 5 características que más se asocian a cada variable objetivo y descubrir, de entre el conjunto

ingente de datos que proporciona NUHEAL, los factores que mejor influyen sobre las puntuaciones de las RSN.

Las variables que más influyen sobre la activación del Postcentral Gyrus (SMN), Precentral R Gyrus (SMN) y Angular gyrus (FPN) se pueden en apreciar en la tabla 4.

Se ha descubierto que el CT hits (Cancellation Test, perteneciente a la NNB - Dominio de atención) tiene un alto índice de participación en la activación de las tres RSN, así como el SDMT (perteneciente a la NNB - Dominio de velocidad de procesamiento) se asociaba fuertemente también al Angular Gyrus de la FPN.

Gracias a estos resultados, se consiguió descubrir que la activación del conectoma humano se correlacionaba negativamente con el rendimiento en atención y velocidad de procesamiento, y prueba de ella es artículo **"Effects of Maternal Fish Oil and/or 5-MethylTetrahydrofolate Supplementation during Pregnancy on Offspring Brain Resting-State at 10 Years Old: A Follow-Up Study from the NUHEAL Randomized Controlled Trial"**, publicado en Septiembre del 2020.

Tabla 4. Selección de características para RSN significativa.

Postcentral Gyrus (SMN)		**Precentral R Gyrus (SMN)**		**Angular Gyrus (FPN)**	
Variables Importantes	**%IncMSE**	**Variables Importantes**	**%IncMSE**	**Variables Importantes**	**%IncMSE**
CT Hits (NNB)	55.32	CT Hits (NNB)	49.52	SDMT hits (NNB)	35.32
Índice n6/n3 (semana 30 de gestación)	7.45	IMC materno (40 sem)	8.25	CT hits (NNB)	31.51
Ácido araquidónico (cordón umbilical)	2.55	K-ABC test	3.99	Homocigoto menor SNP rs174556	4.32
Educación de la Madre	1.26	APGAR 5'	1.22	Ácido α-linolénico (cordón umbilical)	3.52
Perímetro cefálico (12 meses)	0.98	IMC al nacimiento	0.95	País de Orígen - Alemania	0.75

%IncMSE = porcentaje que incrementa el error cuadrático medio si quitamos la variable del modelo

ESTUDIO 1

"Maternal smoking during pregnancy and child behavior problems: A multicenter study from the NUTRIMENTHE EU Project."

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Maternal smoking during pregnancy and child behavior problems: A multicenter study from the NUTRIMENTHE EU Project.

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ABSTRACT

Background

Maternal smoking may alter child development harming different organs and systems such lungs, cardiovascular system or brain structure and function; these effects may increase the risk to develop chronic non-communicable diseases later in life, including mental disorders. In the present study, a possible association between maternal smoking during pregnancy and later behavioral problems in the offspring at school age (7 – 8 years old) was investigated.

Methods

A total of 818 children who participated in the NUTRIMENTHE project were included in the present observational study. Data comes from three well-established Europeans cohorts (CHOP, Generation R and NUHEAL), including information from six countries: Germany, The Netherlands, Poland, Italy, Belgium and Spain. Mothers were divided in two groups: perinatal Smokers (n=167) and Non-Smokers (n=651). All children were assessed at age 7 - 8 with the Child Behaviour Checklist (CBCL).

Results

Children born to non-smokers mothers shown consistently lower T score (range from 49 to 49.5) than those born to the smoking mothers group (range from 51 to 54) in Total, Internalizing and Externalizing continuous CBCL scales. After categorizing outcomes in Normal and Borderline/Clinical pathology, and including potential confounders (cohort, country of origin, parental education, and breastfeeding at three months) in the adjusted analyses, only externalizing problems remained statistically significant (OR: 2.03; 95% CI: 1.23-3.11, p-value=0.012).

Conclusions

These results support previously reported effects about maternal smoking during pregnancy and offspring behaviour; in general, children born to non-smokers mothers showed fewer behavioural problems during childhood. However, country of origin, parental educational level and breastfeeding at 3 months seems to modify the effect of maternal smoking during perinatal period on children behavioral development. Tobacco control efforts and reduce smoking rates, has the potential to prevention and mitigation of perinatal risk factors-related childhood behavioral problems, as well as the associated long-lasting health consequences.

Keywords: Maternal smoking, pregnancy, CBCL, NUTRIMENTHE, Children Behavior.

BACKGROUND

Interaction between genetic, environmental and social factors may affect later behavioral development in children, which has major implications for public health and policy development. According to the World Health Organization (WHO), Europe has currently the highest proportion of cigarette smokers among adults (28%) and one of the highest among adolescents, despite all the efforts made by public and private entities to encourage smoking cessation [1]. While tobacco use was previously predominant among men, difference between both genders is currently very small (<5%) in European countries such as Denmark, Ireland, the Netherlands, Norway, Sweden and United Kingdom. Some 19% of women aged 15 and above smoked tobacco in the WHO European Region [1] and, as a matter of concern, cigarette poses serious risk to the mother and her developing fetus, being now recognized as an important cause of child morbidity and mortality. Cigarette smoke exposure during pregnancy can perturb child's respiratory tract, cardiovascular system and brain morphology, leading to chronic damage that can manifest later in life [2, 3]. Perinatal infant mortality, stillbirth, preterm birth, congenital anomalies, lower birth weight and slow fetal development and growth, are common consequences of exposure to maternal smoking, use of smokeless tobacco during pregnancy and of second-hand smoke exposure [4–11].

Detrimental neurocognitive and behavioral profiles of school-aged children born to mothers who smoked during pregnancy have been documented, promoting early appearance of psychopathological symptoms such as attention deficit hyperactivity disorder (ADHD) [12], aggressive behavior [13], autism spectrum disorders (ASD) [14], risk of hypomania [15], low academic achievements [16], as well as speech-processing and attention control deficits [17, 18]. However, considering the observational nature of data in those studies, it appears that behavioral outcomes are linked somehow to other confounding factors such as gender, parental age, parental education or parental income [19]. Moreover, effects of cultural and sociodemographic characteristics of the population studied are considered confounding factors [20]. Conversely, other studies support the idea that association between maternal smoking during pregnancy and offspring ADHD reflects unmeasured confounding [12].

The goal of the present study is to investigate a plausible association between maternal smoking during pregnancy and behavioral profile of children at 7 - 8 years old, evaluated

through the Childhood Behavior Checklist (CBCL). The analysis was performed involving mother-child pairs from six different European countries, taking into account potential effects of various confounding factors collected through the NUTRIMENTHE research project

METHODS

Study setting

This is a descriptive cross-sectional study which data comes from the NUTRIMENTHE 7FP EU Project, previously described in [21]. Within NUTRIMENTHE EU Project ("*The Effect of Diet on the Mental Performance of Children*", GA no. 212652, www.nutrimenthe.eu) framework, the NUTRIMENTHE Global Database (NGDB) was established joining the data sets from three different follow-up European cohorts, the Childhood Obesity Project (CHOP) study (Belgium, Germany, Italy, Poland and Spain), the Generation R study (The Netherlands) and the Nutraceuticals for a Healthy Life (NUHEAL) study (Germany, Hungary and Spain). Description of participants of these cohorts has been published elsewhere [22–24]. After combining the common variables from the three studies, a new cohort was formed with a total of 1050 children who were assessed using a common neuropsychological procedure; all data sets from these children were included in the NGDB [20].

Participants

Some 818 healthy children from six European countries whose mothers fully completed the Child Behavior Checklist questionnaire were included in the present analysis: 129 Spanish and German children from the "Nutraceuticals for a Healthier Life (NUHEAL)" [24]; 509 children from Germany, Spain, Poland, Italy and Belgium participating in the EU Childhood Obesity Programme (CHOP) [22]; and 180 children from the Generation R epidemiological study in The Netherlands [23]. Due to significant drop-out rate, participants from Hungary were excluded from further analysis.

Study variable and sample size

Maternal smoking was self-reported and harmonized to derive a dichotomous variable as 'Yes' and 'No' for maternal smoking during pregnancy, encompassing a period extended before, during and after pregnancy. From the 818 children, some 651 were born to mothers who did

not smoke during pregnancy and 167 children whose mothers were smokers. For the sample size calculation, r package "pwr" was used. Considering a standard deviation of 0.5, significance level of 0.05 and a desired power of 80%, we need a minimum sample size of 63 for each group.

Outcomes

The Child Behavior Checklist (CBCL/6-18) [26] is an empirically derived measure, highly reliable and valid test, based on direct observations from parents to provide information about behavior and emotional problems of their children, and translating the results into a score.

The CBCL provides an index (T-score) of the children's social and behavioral status relative to other children of the same age and gender. Each CBCL T-Score were categorized in two classes: 1) Normal subjects with a T-Score below percentile 60 for any single CBCL scale and below percentile 65 for total CBCL scale. 2) Borderline/Clinical pathology for those with a T-Score equal or above percentile 60 for single CBCL scale, and equal or above percentile 65 for total CBCL scale, as detailed elsewhere [27]. Eleven items are obtained from the CBCL checklist: three *total scales* (Internalizing, Externalizing and Total problems) and eight *single scales* (Somatic Complaints, Withdrawn/Depressed, Anxiety/Depression, Rule Breaking, Aggressive Behaviors, Social (child dependent, lonely, jealous, clumsy, among others), Thought, and Attention Problems). CHOP children were evaluated at 8 years, those from Generation R at 7 years and those participating in the NUHEAL study at 7·5 years [20].

Confounding variables

A set of common variables were found between the three cohorts and added to the present analysis: Country of origin, Cohort , Maternal Age (years) at delivery, Gestational Age at delivery (weeks), Marital Status, Maternal Educational level, Paternal Educational level, Child gender, anthropometrical measures at birth (weight (kg), height (cm) and head circumference (cm)) ,and Breastfeeding at least for the first three months.

Statistical analysis

Descriptive results were expressed as means ± standard deviation (SD) or percentages, after assessing normal distribution of variables with Kolmogorov–Smirnov test. Differences in

144 population characteristics considering maternal smoking habit as study groups were analyzed
145 by Student's t-test for continuous variables, or Chi square test for categorical variables.

146 In order to study differences in behavioral performance among children born to mothers who
147 smoked against those born to non-smokers mothers, both descriptive and one-way ANOVA
148 analysis were conducted using smoking during pregnancy (Yes or No) as the independent
149 factor, and CBCL T scores as continuous dependent variables. To evaluate size effect of
150 maternal smoking during pregnancy on each CBCL T score, Cohen's *d* coefficient analysis was
151 performed, following carefully the general "rule of thumb",where $d \leq 0.2$ was considered as
152 "small", $d \leq 0.5$ as "medium" and $d \geq 0.8$ as "large" effect size [28, 29].

153 To analyze effects of maternal smoking during pregnancy on each CBCL scale, logistic
154 regression models were then performed in a step by step procedure to adjust for those
155 potential confounders which showed statistically significant differences ($p<0.05$) between
156 smokers and non-smokers mother groups. Statistical models were as follows:1) unadjusted; 2)
157 adjusted by cohort and country of origin (Model 1); 3) Model 1 plus maternal and paternal
158 educational level (Model 2); 4) Model 2 plus breastfeeding at least during three months (Model
159 3).Statistical analysis were conducted using statistical software R version 3.4 (The R Project for
160 Statistical Computing, https://www.r-project.org/).

161 **Ethical consideration**

162 The three studies were performed in line with the Declaration of Helsinki II Principles [25], and
163 the procedures were authorized by the Ethical Committees of all centers involved in each
164 country. All families were properly informed about the types of procedures and signed a written
165 consent prior to their inclusion in the study.

166 **RESULTS**

167 **Study participants**

168 Our observational study included 818 mother-children pairs who fully answered CBCL
169 questionnaires. Baseline characteristics of subjects involved are detailed in **Table 1**. Total
170 number of smoking mothers was 167 (20.42%), whereas number in the non-smoking group
171 was 651 (79.58%). There were no statistically significant differences ($p>0.05$) between groups

regarding maternal age at pregnancy, gestational age at delivery and child gender, neither in newborn weight, length or head circumference at birth. Nevertheless, there were statistically significant differences regarding breastfeeding at three months: women who had never smoked were more likely to continue maternal breastfeeding than smoker mothers ($p<0.0001$). The majority of the current study population were married or living with a partner, regardless of smoking status ($p=0.08$). Concerning maternal educational level, non-smokers group had a predominance of high (higher education) and medium (secondary education) educational level, whereas smokers group had between medium and low education (primary or no education) status ($p<0.0001$). Considering paternal education, a high proportion of the fathers had medium or low educational level in the smoking group, while educational level was almost equivalent through the three educational categories in the non-smoking group ($p<0.0001$). Statistically significant differences between groups regarding cohort and country of origin were detected ($p<0.0001$).

Table 1. General characteristics of the population participating in the study.

		Non Smokers Mothers (n=651)	Smokers Mothers (n=167)	P
Country of origin	Spain	205 (31.49)	86 (51.49)	**<0.0001**
	Germany	76 (11.67)	15 (8.98)	
	Italy	73 (11.21)	24 (14.37)	
	The Netherlands	179 (27.50)	1 (0.60)	

	Poland	67 (10.29)	34 (20.36)	
	Belgium	51 (7.83)	7 (4.19)	
Cohort	NUHEAL	106 (16.28)	23 (13.77)	**<0.0001**
	CHOP	366 (56.22)	143 (85.63)	
	Generation R	179 (27.50)	1 (0.60)	
Maternal Age (years) at delivery		31.46±4.80	30.41±5.70	0.28
Gestational Age at delivery (weeks)		39.81±1.54	39.65±1.37	0.43
Marital Status	Married/Living together	545 (94.45)	112 (90.32)	0.08
	Single/No partner	32 (5.55)	12 (9.68)	
Maternal Educational level	High	317 (48.99)	31 (18.56)	**<0.0001**
	Medium	284 (43.89)	93 (55.69)	
	Low	46 (7.11)	43 (25.74)	
Paternal Educational level	High	229 (37.36)	21 (8.40)	**<0.0001**
	Medium	207 (33.77)	85 (29.11)	
	Low	177 (28.87)	59 (25.00)	
Child gender	Girl	342 (52.53)	78 (46.71)	0.15
	Boy	309 (47.47)	89 (53.29)	
Newborn weight at birth (kg)		3.45±0.35	3.31±0.32	0.32
Newborn length at birth (cm)		51.02±2.82	50.54±2.55	0.51
Newborn HC at birth (cm)		34.72±1.50	34.52±2.01	0.12
Breastfeeding from birth (at least during 3 months)	Yes	314 (52.42)	26 (19.85)	**<0.0001**
	No	285 (47.58)	105 (80.15)	

Data are expressed as n (%) or media ±SD. HC: head circumference. P from t and Chi-square test Bold = Significance. CHOP: Childhood Obesity Project; NUHEAL: Nutraceuticals for a Healthy Life.

Effects of smoking during pregnancy on CBCL outcomes in children of 7.5 and 8 years.

Analysis of CBCL T scores in children born to smoking mothers *vs.* non-smoking mothers are shown in **Table 2.** Children born to non-smoking mothers shown consistently lower CBCL T scores (between 49 and 49.5) than children born to smoking mothers (between 51 and 54), which generally correlates with fewer behavioural problems.

There were significant differences ($p<0.05$) in almost all CBCL scales, except for Thought problems ($p=0.16$, $d=0.13$) and Social problems ($p=0.066$, $d=0.17$), where no differences were found between groups. The majority of scales showed a medium-small effect size between smoking and non-smoking groups on CBCL scale outcomes (between 0.21 and 0.54).

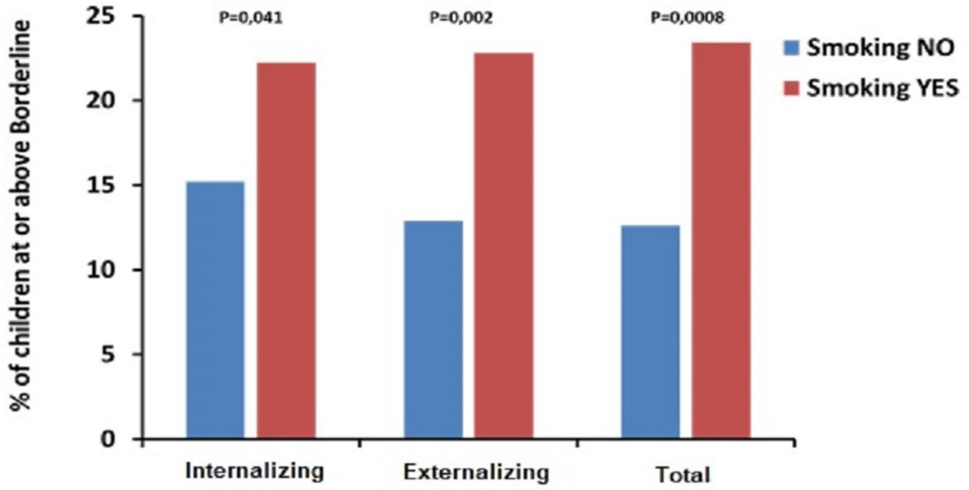
Table 2. Differences on CBCL T scores between children's born to smokers and non-smokers mothers.

CBCL Scale	Non Smokers Mothers	Smokers Mothers	d	P
Anxious/Depressed	49.31±9.93	52.65±9.76	0.34	**<0.0001**
Withdrawn/Depressed	49.55±9.62	51.60±11.07	0.21	**<0.01**
Somatic complaints	49.49±9.72	51.93±10.85	0.24	**<0.001**
Social problems	49.50±10.02	51.14±9.24	0.17	0.066
Thought problems	49.61±9.59	50.86±10.67	0.13	0.16
Attention problems	48.90±9.51	54.20±10.59	0.54	**<0.0001**
Rule breaking behavior	49.40±9.86	51.65±10.24	0.23	**<0.01**
Aggressive behavior	49.32±9.65	52.42±10.40	0.35	**<0.0001**
Internalizing Problems	49.39±9.96	52.26±9.70	0.31	**<0.0001**
Externalizing Problems	49.28±9.66	52.63±10.43	0.35	**<0.0001**
Total Problems	49.14±9.64	53.10±10.28	0.41	**<0.0001**

Results are expressed as Mean±SD; P = p value from one way ANOVA; d from Cohen's effect size. Bold = Significance.

Next, differences in the percentage of children categorized at or above Borderline/Clinical Range for the CBCL Scales regarding Internalizing, Externalizing and Total problems were studied. As shown in **Figure 1,** children born to smoking mothers shown these kind of behavioral disturbances more frequently compared to children born to mothers who never smoked ($p<0.05$).

Figure 1. Percentage of children at or above the borderline/clinical range for CBCL scales in internalising, externalising or total problems according to maternal smoking status during pregnancy. P = p-value from Chi-square test.



Associations between CBCL T scores scales and potential confounders

Subsequently, strength of association between CBCL T scores and mothers smoking status was examined, via multiple logistic regression analysis (**Table 3**). Statistical significant differences were found in Internalizing, Externalizing and Total Problems (p=0.041, p=0.004, p=0.001, respectively), as well as in the Thought and Attention Problems and Somatic complaints scales (p=0.029, p=0.017, p=0.003, respectively) in the unadjusted model. After adjusting by cohort and country of origin (Model 1), effect of maternal smoking during pregnancy remained significant on Thought, Attention and Externalizing Problems (p=0.029, p=0.017, p=0.013, respectively). The inclusion of maternal and paternal educational level as confounders in the Model 2, results in a loss of significance in relation to Thought Problems, but kept it for Attention and Externalizing Problems (p=0.029, p=0.021, respectively). In Model 3, which comprised also breastfeeding at least during 3 months as confounding factor, significant differences were detected for to Thought Problems (p=0.024) and Externalizing Problems scale (p=0.012). A trend of significance in Total Problems outcome, although marginal, was also detected in this Model 3 (p=0.057).

Table 3. Effects of maternal smoking on categorical CBCL outcomes considering potential confounders.

CBCL Scale	Unadjusted model (n=818)		MODEL 1 (n=818)		MODEL 2 (n=777)		MODEL
	OR (CI 95%)	P	OR (CI 95%)	P	OR (CI 95%)	P	OR (CI 9
Anxious/Depressed	1.29 (0.75-2.13)	0.331	0.81 (0.39-1.32)	0.068	1.03 (0.49-1.71)	0.799	1.17 (0.
Withdrawn/Depressed	1.48 (0.81-2.59)	0.182	1.28 (0.66-2.12)	0.411	0.98 (0.51-1.97)	0.332	1.21 (0.
Somatic complaints	**1.52 (0.84-5.65)**	**0.003**	1.05 (0.52-2.12)	0.311	1.37 (0.68-2.61)	0.271	1.31 (0.
Social problems	0.86 (0.44-1.57)	0.629	0.79 (0.39-1.42)	0.611	0.71 (0.32-1.11)	0.621	0.83 (0.
Thought problems	**1.98 (1.04-3.72)**	**0.034**	**2.08 (0.99-3.61)**	**0.029**	1.65 (0.88-3.05)	0.098	**2.37 (1.**
Attention problems	**2.81 (1.41-4.56)**	**0.001**	**1.79 (1.13-2.87)**	**0.017**	**1.81 (0.99-3.01)**	**0.029**	1.35 (0.
Rule breaking behavior	1.17 (0.66-2.01)	0.577	1.27 (0.74-2.02)	0.302	1.25 (0.67-1.98)	0.598	0.98 (0.
Aggressive behavior	1.66 (0.91-2.93)	0.08	1.56 (0.79-3.01)	0.112	1.47 (0.69-2.89)	0.198	1.77 (0.
Internalizing Problems	**1.56 (1.01-2.37)**	**0.041**	1.42 (0.71-1.65)	0.501	1.55 (0.82-1.96)	0.311	1.41 (0.
Externalizing Problems	**1.87 (1.20-2.86)**	**0.004**	**1.82 (1.02-2.94)**	**0.013**	**1.86 (1.01-2.69)**	**0.021**	**2.03 (1.**
Total Problems	**2.01 (1.3-3.09)**	**0.001**	1.49 (0.81-2.02)	0.069	1.59 (1.02-2.29)	0.071	1.77 (0.

Logistic regression analysis of CBCL scores according to maternal smoking status during pregnancy, adjusted by: Model 1: Country of origin and
1+ Maternal and Paternal Educational Level; Model 3: Model 2+ Breastfeeding at least for 3 months. OR = Odds Ratio, CI= Confidence Interval, P

DISCUSSION

The main goal of this observational study was to investigate a potential association between maternal smoking and behavioral profile of European children at 7 - 8 years old, assessed through CBCL questionnaires in a multicenter cohort, involving mother-children pairs from the NUTRIMENTHE project. Overall, results show that children born to smoking mothers have higher risk of Externalizing behavioral problems after adjusting for confounding variables (cohort and country of origin, maternal and paternal educational level, and breastfeeding at least during three months) than children born to non-smoker mothers. Childhood internalizing/externalizing problems seems to be predictive of later negative adolescent and adult behavioral, emotional, cognitive, and physical health outcomes [30]. Internalizing problems refer to inwardly focused negative behaviors such as anxiety, depression, and somatic symptoms, while externalizing problems refer to outwardly focused negative behaviors such as hyperactivity, aggression, disruptive conduct, and substance use [31, 32].

It is also worth to mention that parental education and breastfeeding from birth at least for three months seems to modulate effects of maternal smoking on Thought and Attention problems respectively, in children at 7 - 8 years of age. It is well established the protective role of breastfeeding on children internalizing, externalizing behavioral problems and low intelligence in childhood [33].

Our results are in agreement with other previously published studies [34-36], which found behavioral problems in children exposed to maternal smoking during pregnancy. Conduct and behavioral disorders have been found frequently associated with prenatal tobacco exposure, in children as young as 18–24 months, and in older children (6–16 years old) [37]. However, other researchers have reported no causal association between maternal smoking during pregnancy and ADHD, using twin studies, in vitro fertilization cohorts or genetic epidemiology approaches [38, 39], which support the idea that association between maternal smoking during pregnancy and offspring ADHD is not due to causal intrauterine effects, but reflects unmeasured confounding [12]. Other authors suggest that effect of smoking on behavior is small and less important than found in previous studies, and influence may be due to time-stable familial factors, such as environmental and genetic factors [40]. On the other hand, other researchers

have found changes in superior frontal and parietal cortices and smaller global brain volumes in children aged 6 to 8 years who were prenatally exposed to tobacco, even after adjustment for socioeconomic confounding factors [41].

Nevertheless, there is a wide consensus about the pernicious effects of tobacco smoke during pregnancy, which occurs in the context of a set of socioeconomic circumstances that place the child at increased developmental risk, but may not be causally related to the children's behavior [35].

The statistical association of maternal smoking with children behavioral problems could be strongly confounded by sociodemographic characteristics. It has been previously reported that mothers who smoke during their pregnancies are on average of lower income status and have less education [42]. In the current study, the smoker group mothers also shows between medium and low educational level, while non-smoking mothers have higher level of education. Moreover, country of origin has been previously reported as a factor that should be considered an important confounder in studies measuring neuropsychological performance [20] having in mind always the strong relationship that prevail with behavior outcomes.

On the other hand, systematic review of genetically informative studies, suggest that minimal evidence exist to support a causal effect on offspring ADHD or conduct problems of maternal smoking during pregnancy. Moreover, much evidence suggest associations reflect familial confounding and passive gene-environment correlation [38].

Some limitations of the study have to be acknowledged: there are no available data regarding paternal smoking status. Maternal smoking habit was based only on questionnaires. No data were available regarding mothers' smoking duration, nor number of cigarettes smoked per day. This may imply a risk of misclassification, and excludes the possibility of assessing a possible dose-response relationship. On the other hand, smoking habit period encompass an extended period (before, during and after pregnancy), thereby limiting conclusions to the whole perinatal time. Some researchers have pointed out that children exposed prenatally to tobacco were more likely to exhibit abnormal behaviors compared to children exposed only during postnatal period [43], thus emphasizing that gestation is a critical period in the development of behavioral disorders. Another limitation of the current study is that no data about possible maternal psychological problems were available, which could affect maternal perception of their children,

nor the influence of familiar social background. In that sense, depression and psychopathological problems are more commons in mothers who smoked during pregnancy than non-smoker mothers [44], and mothers who smoke during pregnancy are on average of lower educational level and income status [42]. Regarding Generation R cohort, it was previously reported [35] a higher percentage of smoking mothers (21.7%) in this population, while participants of the Generation R cohort in the current study show a lower percentage of smoking mothers. However, sensitivity analyses excluding children from Generation R study were performed, and results obtained did not change current study conclusions.

In summary, our results point out to a higher risk of Borderline/Clinical pathological Externalizing Problems in children at 7 - 8 years of age, born to smoking mothers, even after adjusting for confounding variables. In addition, the effect of smoking during pregnancy considering Thought and Attention Problems scales, seems to be conditioned by parental educational level and breastfeeding at three months respectively. Other confounding factors could not be discarded to influence the effects of tobacco smoking in child neurodevelopment.

Therefore, it is important to reinforce tobacco control efforts and reduce smoking rates, including pregnant women and households with young children, as effects of tobacco smoke in child growth has been reported. Understanding this phenomenon has the potential to facilitate recognition, prevention, and mitigation of perinatal risk factors-related childhood behavioral problems, as well as the associated long-lasting negative health consequences.

DECLARATIONS

Ethics approval and consent to participate

All studies involved in the current analysis (“Nutraceuticals for a Healthier Life (NUHEAL)” [24], EU Childhood Obesity Programme (CHOP) [22]; and the Generation R epidemiological study [23]), were performed in line with the Declaration of Helsinki II Principles [25], and the procedures were authorized by the Ethical Committees of all centers involved in each country. All families were properly informed about the types of procedures and signed a written consent prior to their inclusion in the study.

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated and/or analysed during the current study are not publicly available due to nature of the information that could compromise research participant privacy/consent, but are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

CC designed, coordinated the study and reviewed the manuscript. HA, MGB and JAGS analyzed data and written the draft. All the remaining co-authors contributed deeply in data acquisition, results interpretation, review and final manuscript editing. All authors read and approved the final manuscript.

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ESTUDIO 2

"Long-chain polyunsaturated fatty acids, homocysteine at birth and fatty acid desaturase gene cluster polymorphisms are associated with children´s processing speed up to age 9 years."

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DRAFT submitted - Under review

Long-chain polyunsaturated fatty acids, homocysteine at birth and fatty acid desaturase gene cluster polymorphisms are associated with children´s processing speed up to age 9 years.

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Abstract: Both pre- and early postnatal supplementation with docosahexaenoic acid (DHA), arachidonic acid (AA) and folate have been related to many neural processes, but its long-term effects on later neural function remain unclear. We evaluated the long-term effects of maternal prenatal supplementation with fish-oil (FO), 5-methyltetrahydrofolate (5-MTHF), placebo or FO+5-MTHF, as well as the role of fatty acid desaturase (*FADS*) gene cluster polymorphisms, on their offspring's processing speed at later school age. This study was conducted in 143 NUHEAL children at 7.5 and 9 years old. Processing speed tasks were assessed using *Symbol Digit Modalities Test (SDMT), Children Color Trails Test (CCTT)* and *Stroop Color and Word Test (SCWT)*. Long-chain polyunsaturated fatty acids, folate and total homocysteine (tHcy) levels were determined at delivery from maternal and cord blood samples. *FADS* and methylenetetrahydrofolate reductase (*MTHFR*) 677C>T genetic polymorphisms were analyzed. Multivariate linear and mixed effects logistic regression models were performed There were significant differences in processing speed performance among children at different ages (p<0.001). However, type of prenatal supplementation had no effect on processing speed in children up to 9 years. Secondary exploratory analyses indicated that children born to mothers with higher AA/DHA ratio at delivery (p<0.001) and heterozygotes for *FADS1* rs174556 (p<0.05) showed better performance in processing speed at 9 years. Negative associations between processing speed scores and maternal tHcy levels at delivery were found. Our findings suggest speed processing development in children up to 9 years could be related to maternal factors, including AA/DHA and tHcy levels, and their genetic background, mainly *FADS* polymorphism. These considerations support that maternal prenatal supplementation should be quantitatively adequate and individualized to obtain better brain development and mental performance in the offspring.

Keywords: Long-chain polyunsaturated fatty acids, folate, prenatal supplementation, processing speed, neurodevelopment, *FADS* gene, children.

1. Introduction

The speed of information processing is an essential basis for higher order cognitive function, including memory or executive functions [1]. This cognitive process can be defined as the time required to move information from one neuron to the next [2], or how quickly a person can perform the mental operations needed to complete a task [3]. Then, processing speed is highly related to intact myelination, which is important for the integration of informa tion across spatially distributed neural networks. Moreover, the association between white matter integrity and processing speed in cognitive tasks has been consistently established [4,5].

Long-chain polyunsaturated fatty acids (LC-PUFAs) and folic acid play an important role in brain development, particularly during fetal and early postnatal life [6,7]. Interestingly, their effects on neurodevelopment depend of the timing of

occurrence and brain needs for particular nutrients at that time [8]. For instance, essential fatty acids (FA) deficiencies during the first year of life lead to severe impairments in synapse formation and myelination [9], which may have negative effects on processing speed tasks later in life [10].

LC-PUFAs, particularly docosahexaenoic acid (DHA, 22:6 *n*-3) and arachidonic acid (AA, 20:4 *n*-6) are incorporated into the brain in relatively large amounts during the pre- and postnatal growth spurt [11-13]. However, due to a limited capacity in the fetus and neonate for PUFAs elongation and desaturation, tissue deposition of DHA and AA strongly depends on the pre-formed LC-PUFAs supply via the placenta and postnatal diet [14]. On the other side, PUFAs composition is also clearly related to polymorphisms in the fatty acid desaturase (*FADS*) gene cluster [15]. There is evidence that dietary LC-PUFAs supply in early life may modulate information processing [12,16], cognitive and visual development [17,18], as well as early mental and motor skills development [6,19]. Recently, higher maternal DHA status has been also related to better performance in language and short-term memory in the offspring [20]. Moreover, early availability of *n*-6 PUFAs, mainly AA, during pre- and postnatal periods has been positively associated to cognitive performance and mental function in later childhood [21-23].

In addition, folate intake before and during pregnancy is also essential for normal brain development, differentiation and cognitive performance [24-27]. Thus, maternal folate deficiency causes structural brain abnormalities during fetal development and poor childhood cognitive ability [26], while maternal folate supplementation in pregnancy improves neurological development and may reduce the prevalence of autism spectrum disorders in their offspring [24,28].

Having in mind that LC-PUFAs and folate play key roles in synaptogenesis, synapse maturation and myelination, it seems clear that dietary intake of both nutrients may have effects on processing speed [2]. Long-term effects of nutritional interventions on processing speed have been classically evaluated using perceptual speed tasks [29], but further studies evaluating specific neuropsychological domains are still missing [6,30]. Therefore, our main objective was to evaluate the potential role of LC-PUFAs and total homocysteine (functional maker of folate status) at delivery, as well as maternal *FADS* polymorphisms, on children´s processing speed at 7.5 and 9 years.

2. Materials and Methods

2.1. Study Design and Subjects

This is a follow-up study of the NUHEAL (*Nutraceuticals for a Healthier Life*) trial, registered at www.ClinicalTrials.gov, Identifier NTC01180933. Detailed study design, subject recruitment, and population characteristics have been described elsewhere [31,32]. Briefly, NUHEAL project is a multicenter, randomized, double-blind,

placebo-controlled trial in healthy pregnant women from Munich, Pécs and Granada. Pregnant women were assigned by blockwise randomization to receive either a modified fish-oil (FO) preparation [500 mg DHA + 150 mg eicosapentaenoic acid (EPA)/day], 5-methyl-tetrahydrofolate (5-MTHF) (400 µg/day), a combination of both supplements (FO+5-MTHF), or placebo, from gestational week 20 until delivery. Detailed information on sociodemographic data and course of pregnancy together with maternal blood samples were collected at 20 and 30 weeks of pregnancy and at delivery; additionally, cord blood samples were also obtained.

Of 270 women participating in NUHEAL study until giving birth, 152 mothers agreed to participate in the follow-up for their offspring at 7.5 and 9 years of age. Processing speed tasks were entirely performed in 143 NUHEAL children, from which38 were born to mothers who received FO, 29 whose mothers were supplemented with 5-MTHF, 36 born to mothers supplemented with FO+5-MTHF, and 40 children whose mothers received placebo during pregnancy **(Figure 1)**.

The follow-up study protocols were approved by the Ethical Committees from all centers involved in the study. Written informed consent was obtained from parents of all participating children at study entry and at each follow-up.

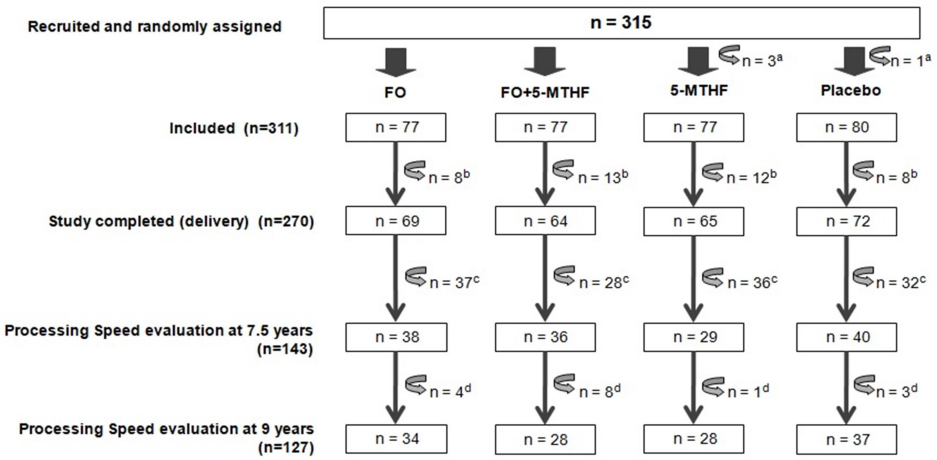

Figure 1. Flowchart of NUHEAL participants up to 9 years. FO: fish-oil; 5-MTHF: 5-methyltetrahydrofolate. [a] 4 participants who did not meet the inclusion criteria : 2 women weighed >92 kg, 1 of whom used commercial FO preparations; 2 women regularly consumed FO preparations. [b] 41 participants did not complete the study: noncompliance (n=2), relocation (n=1), aversion to or bad taste of the supplement (n=9), loss of contact (n=2) and unknown reasons (n=27). [b] 133 participants lost to follow up at 7.5 years: relocation (n=3), loss of contact (n=74), infants born prematurely (n=4), congenital left-side anophthalmus (n=1), craniosynostosis (n=1), left-side deafness (n=1), unwillingness to continue (n=50). [d] Processing speed tasks were not entirely performed in 16 participants at 9 years

151 *2.2. Neuropsychological assessment*

152 NUTRIMENTHE Neuropsychological Battery (NNB) was used to evaluate the
153 whole spectrum of neuropsychological functioning in children aged 7.5-9 years,
154 including processing speed tasks such as *Symbol Digit Modalities Test* ***(SDMT)***, *Children*
155 *Color Trails Test* ***(part 1) (CCTT-1)*** and *Stroop Color and Word Test* ***(SCWT)*** [33].

156 The *Symbol Digit Modalities Test* ***(SDMT)*** was used to assess
157 information-processing speed and attention [34]. This test requires individuals to
158 write the correct number under the corresponding symbol according to a key code
159 specified on the top of the page, which links different meaningless geometric symbols
160 with numbers 1 through 9. The participant is given 90 s to complete the task. Number
161 of correctly identified symbols (hits) is recorded as score.

162 The *Children Color Trails Test* ***(CCTT)*** is an individually administered
163 neuropsychological instrument which consists of two parts used to evaluate sustained
164 visual attention, sequencing, psychomotor speed (part-1), and cognitive flexibility
165 (part-2). This test requires the connection of one set of encircled numbers (1-25) in
166 ascending order. Current study used only the part 1 of this test, which even numbers
167 are printed in a yellow background while odd numbers are printed in a pink
168 background. The final score is the time (in seconds) taken to complete part-1 of the
169 ***CCTT*** [35].

170 Finally, Golden´s version of the *Stroop Color and Word Test* ***(SCWT)*** was used to
171 evaluate cognitive flexibility, selective attention, cognitive inhibition and information
172 processing speed [36]. There are three components to this task. First, participants are
173 asked to read aloud color words (blue, green and red) printed in black ink. Second, the
174 child is asked to say the colors of "XXXX" printed in blue, green or red. Finally, the
175 child is asked to name the ink color of color words (blue, green or red) printed in
176 incongruent colors as quickly and accurately as possible in 45 seconds time. As
177 consequence, this test produces three direct scores: *the word-reading (WR)* score, *the*
178 *color-naming (CN)* score, and *the color-word (CW)* score, respectively [37]. The increase
179 in time taken to perform the *CW* test compared with the *WR* and *CN* tests is called
180 color-word interference effect or Stroop effect, which is considered as the main
181 dependent variable for *SCWT* test [38]. *CW* score is associated to attention
182 development, and for this reason has not been taken into account in the current study.

183 *2.3. Fatty acid analyses in maternal and umbilical cord plasma phospholipids*

184 Procedures of analysis for FA determinations in plasma phospholipids have been
185 described in detail elsewhere [31,32]. Briefly, blood was centrifuged at 3500x*g* for 10
186 min at room temperature within 2 h. Plasma was thereafter removed and stored at
187 -80ºC. Lipid extraction from plasma was performed according to the method of
188 Kolarovic and Fournier [39]. Analysis of FA methyl esters from plasma phospholipids
189 was performed by high-resolution capillary gas-liquid chromatography. Conditions

during the analysis and standards used were described elsewhere [40]. Results were expressed as weight percentages (wt %) of all quantified FA.

2.4. Folate analysis

Analysis of plasma folate was carried out by microbiological assay using a chloramphenicol resistant strain of *Lactobacillus casei*, as previously described [41]. Inter and intra-assay coefficients of variation were below of 11%.

2.5. Total homocysteine

Total homocysteine (tHcy) concentrations were assayed by fluorescence polarized immunoassay on the IMx autoanalyser [42]. Sample preparation and chromatographic conditions were performance as described previously [43]. The fluorescence intensities were measured with excitation at 385 nm and emission at 515 nm.

2.6. SNP selection and genotyping

Genotyping of single-nucleotide polymorphisms (SNPs) from *FADS* gene cluster was performed with the iPLEX method (Sequenom, San Diego, CA, USA) by means of matrix-assisted laser desorption ionization-time of 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. Genotyping discordance rate was below 0.3%. SNP selection for following analyses is showed in **Supplemental Methods (Supplemental Figure 1 and Supplemental Tables 1-3).**

2.7. MTHFR 677 C/T polymorphism

Genomic DNA was prepared from maternal and umbilical cord blood samples obtained at delivery. DNA samples were genotyped for the methylenetetrahydrofolate reductase (*MTHFR*) 677 C/T variant by polymerase chain reaction (PCR), restriction enzyme digestion, and DNA fragment separation by electrophoresis as described previously [42]. *MTHFR* 677 C>T was selected for its high clinical relevance in humans due to the association of TT genotype with high plasma homocysteine concentrations [44].

2.8. Statistical Analysis

Using standard approaches, power for the current study was calculated, setting α value as 0.05 and β value as 0.2. Different intervention groups (FO, 5-MTHF and FO+5-MTHF) were grouped and compared with placebo group for processing speed tasks, including ***SDMT***, ***CCTT*** and ***SCWT***, in children at 7.5 years old. A statistical power of 80% was obtained for selected tasks in aforementioned population **(Supplemental Table 4)**. "PowerEQTL v 0.1.3" (R software) was also used to calculate

the statistical power for our genetic study; except for *FADS2* rs174570 and *FADS3* rs2727271, a statistical power of 90% was obtained for analyzed SNPs (**Supplemental Figure 2 and Supplemental Table 5)**.

A descriptive analysis of quantitative variables were performed using summary measures *(mean, standard deviation, standard error of the mean, centiles, median, interquartile or amplitude ranges),* meanwhile frequency distribution was used for qualitative variables. Comparisons among different groups of treatment (FO, 5-MTHF, both or placebo) were made using one-way ANOVA for continuous variables or χ^2 test for categorical variables. In order to verify the underlying hypothesis of one-way ANOVA (variance's homogeneity and normality), Box-Cox transformation was computed when considered necessary. When one-way ANOVA resulted significant, Bonferroni test post-hoc was applied.

A multivariate multiple linear regression model was adjusted in order to analyze independent effects of different covariates and confounders, including maternal age, maternal education level, maternal smoking, maternal BMI, mode of delivery, gestational age and child´s sex. Those confounders with a significant level of $p<0.05$ were included in the final models for the multivariate analyses. Variables of processing speed obtained from ***SDMT***, ***CCTT-1*** and ***SCWT*** tests were considered as dependent variables. Due to hierarchical structure resulted from processing speed evaluation in children at 7.5 and 9 years, a multivariate linear model was computed using mixed-effects logistic regression models, where children were considered as level one and each of two time points measures were fixed as second level. Time (7.5 years and 9 years) was used as fixed effects factor. Logistic regression mixed model was used when dependent variables were dichotomized (below or above third quartile), in which case the measure of the effect was the suitable odds ratio with corresponding confidence interval. Interactions between groups of prenatal supplementation and confounders were analyzed in each multivariate analysis performed, but significant differences were not observed **(Supplemental Tables 5 and 6)**.

All statistical analyses were performed using the statistical package STATA 12.1 (Stata Corp, College Station, TX). *P* values <0.05 were considered as statistically significant

3. Results

3.1. Background and baseline characteristics of the NUHEAL study participants

The baseline characteristics of the mothers whose children were evaluated at 7.5 and 9 years of age are shown in **Table 1.** No difference between prenatal supplementation groups were observed in those descriptive variables analyzed prior or during pregnancy, including maternal age, BMI at 20 and 30 weeks of pregnancy, hematocrit at 30 weeks of pregnancy, parity, smoking during pregnancy, gravidity

risk at 20 weeks of pregnancy, high maternal education, family status or gestational age at delivery. Moreover, we analyzed maternal biochemical parameters at delivery. As expected, plasma levels of folate were significantly higher in those mothers who received 5-MTHF or FO+5-MTHF supplementation during pregnancy (p<0.001). However, type of prenatal supplementation had no effect on maternal AA/DHA ratio and tHcy levels.

Table 1. General characteristics of the studied population

	FO (n=38)	5-MTHF (n=29)	FO+5-MTHF (n=36)	Placebo (n=40)	*p*
Maternal age (years)[1]	28.81 ± 5.25	30.70 ± 5.68	29.56 ± 4.32	30.71 ± 3.90	NS
BMI (kg/m2)[1]					
20 weeks	26.03 ± 3.62	24.92 ± 2.45	25.28 ± 2.77	24.74 ± 2.28	NS
30 weeks	28.52 ± 3.91	26.87 ± 2.43	27.23 ± 2.86	26.91 ± 2.29	NS
Hematocrit (%) at 30 weeks[1]	33.85 ± 3.73	32.61 ± 5.14	33.37 ± 2.58	33.13 ± 2.75	NS
Parity [n (%)]					NS
0	23 (60.5%)	23 (79.3%)	28 (93.3%)	28 (70%)	
≥1	3 (7.9%)	3 (12.5%)	2 (5.5%)	4 (10%)	
Smoking in pregnancy [Yes= n (%)]	7 (18.4%)	5 (17.2%)	7 (19.4%)	4 (10%)	NS
Gravidity risk at 20 weeks [n (%)]					NS
No risk factors	6 (15.8%)	6 (20.7%)	8 (22.2%)	11 (27.5%)	
≥1 risk factors	18 (47.4%)	18 (62.1%)	22 (61.1%)	21 (52.5%)	
High Maternal education [n (%)]	8 (21.05%)	8 (27.58%)	14 (38.89%)	10 (25%)	NS
Family Status Pregnancy [n (%)]					NS
Single	8 (21.1%)	8 (27.4%)	1 (2.7%)	10 (25%)	
Partnership	23 (60.5%)	23 (44.8%)	29 (80.6%)	29 (72.5%)	
Gestational Age (weeks)[1]	38.90 ± 1.51	38.75 ± 1.62	38.73 ± 2.06	39.43 ± 1.43	NS
AA/DHA[1,2]	0.90 ± 0.38	1.13 ± 0.54	0.97 ± 0.42	1.14 ± 0.63	NS
Plasma Folate (μg/L)[1,2]	6.17 ± 4.33	12.10 ± 5.55	13.46 ± 5.78	6.06 ± 0.82	**<0.001**
tHcy (μmol/L)[1,2]	7.08 ± 2.83	6.29 ± 2.81	6.93 ± 3.09	6.78 ± 2.39	NS

[1]: Values expressed as mean ± standard deviation; [2]: Values obtained from mothers at delivery. 5-MTHF: 5-methyltetrahydrofolate; AA: arachidonic acid; DHA: docosahexaenoic acid; FO: fish-oil; tHcy: total homocysteine. n=number of cases; p: level of significance from one-way ANOVA for continuous variables or χ^2 test for categorical variables. Noted that there are missing values for some descriptive variables.

3.2. Processing speed task of the NUHEAL children at 7.5 and 9 years old

As shown in **Table 2**, no significant statistical differences were found among the type of prenatal supplementation in all analyzed processing speed tasks at 7.5 years, except for ***CCTT-1*** test. In fact, children born to mothers who were supplemented with 5-MTHF during pregnancy showed a decrease in the timing to solve ***CCTT-1*** *(p*= 0.017*)*. Moreover, there were no differences between type of prenatal supplementation

and processing speed tasks at 9 years old. In general, we observed that children aged 9 years had better processing speed than those aged 7.5 years in terms of higher scores and less time to solve ***CCTT-1*** test.

Table 2. Descriptive analysis of processing speed evaluation in the NUHEAL children at 7.5 and 9 years

	FO		5-MTHF		FO+5-MTHF		Placebo		*p*
Children at 7.5 y	**n**	**Mean ± SD**	**n**	**Mean ± SD**	**n**	**Mean ± SD**	**n**	**Mean ± SD**	
SDMT – hits	36	23.56 ± 7.62	28	22.61 ± 6.87	34	22.38 ± 5.27	41	24.66 ± 6.38	0.204
CCTT-1 (sc)	36	100.44 ± 39.27	28	87.85 ± 30.05	34	96.56 ± 34.48	41	99.88 ± 50.77	**0.017**
STROOP Test – hits -1 (Word-reading)	37	59.49 ± 17.72	28	61.82 ± 11.66	34	57.68 ± 16.34	41	59.34 ± 16.41	0.155
STROOP Test – hits -2 (Color-naming)	37	38.97 ± 7.77	28	42.14 ± 7.26	34	42.18 ± 9.36	41	41.95 ± 8.41	0.523
Children at 9 y									
SDMT – hits	33	31.42 ± 6.66	26	30.00 ± 7.16	28	30.25 ± 7.73	37	33.76 ± 8.60	0.500
CCTT-1 (sc)	33	57.60 ± 17.13	28	67.99 ± 23.16	28	73.61 ± 24.73	37	66.58 ± 25.99	0.112
STROOP Test – hits -1 (Word-Reading)	34	70.53 ± 12.69	28	71.54 ± 10.29	28	72.32 ± 8.71	37	73.62 ± 9.65	0.187
STROOP Test – hits -2 (Color-Naming)	34	48.26 ± 7.58	28	49.32 ± 9.49	28	50.61 ± 8.57	37	51.41 ± 7.94	0.627

5-MTHF: 5-methyltetrahydrofolate; CCTT-1: Children Color Trails Test; FO: fish-oil;SDMT: Symbol Digit Modalities Test. n= number of cases; *p*: level of significance obtained for $\chi 2$ test; Bold: $p < 0.05$.

3.3. Prenatal predictors of processing speed in children up to 9 years

3.3.1. Symbol Digit Modalities Test ***(SDMT)*** Hits

Type of prenatal supplementation had no long-term effects on ***SDMT*** hits in children at 7.5 and 9 years, after adjusting for *maternal blood parameters, mother´s genetic polymorphisms and lifestyle variables in pregnancy* **(Table 3)**. However, we observed an age-dependent increase in ***SDMT*** hits; in fact, children at 9 years showed an increase of 10.72 points in the mean of hits [(95% CI: 7.98-13.47); $p < 0.001$] compared to children at 7.5 years. Moreover, each unit of increase in maternal AA/DHA ratio at delivery predicted an increase of 8.80 points in the mean of ***SDMT*** hits [(95% CI: 3.61-13.99); $p = 0.001$]. Conversely, higher maternal weight gain between 20 and 30 weeks of pregnancy (dBMI) [(95% CI:-1.25 - -0.02); $p = 0.043$] and maternal tHcy [(95% CI: -1.02 - -0.002); $p = 0.049$] were associated with a decrease of 0.63 and 0.52 points in the mean of ***SDMT*** hits, respectively.

Further logistic regression analysis, characterizing those children aged 9 years having hits above P75, showed that maternal AA/DHA ratio at delivery was the best predictor to obtain higher number of ***SMDT*** hits [OR: 30.46 (95% CI: 3.68-252.0); *p*= 0.002] **(Table 6)**. This probability is also increased in those children whose mothers had a high educational level (*p*= 0.013) or were heterozygote for *FADS1* rs174556 (*p*= 0.014). However, maternal tHcy at delivery reduced the odds of placing children aged 9 years above the P75, with a probability of 0.71 to obtain less hits per each unit (μmol/L) of increase of tHcy [OR: 0.71 (95% CI: 0.56-0.89); *p*= 0.003].

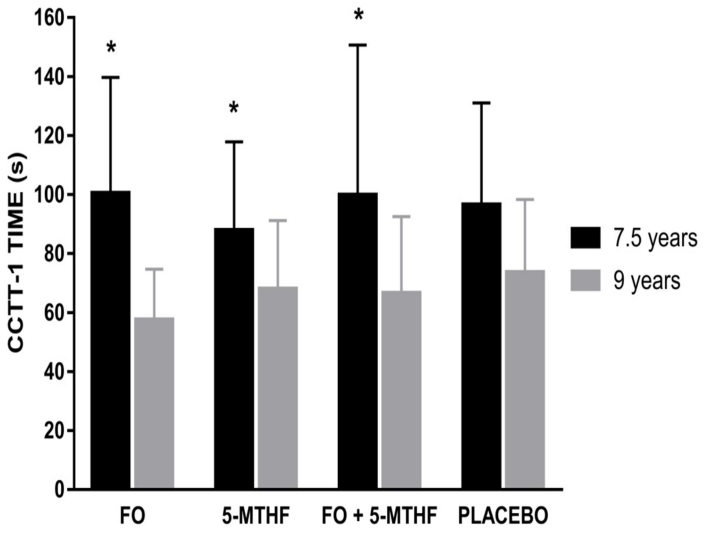

Figure 2. Effects of prenatal supplementation on *CCTT-1* test in children at 7.5 and 9 years. Level of significance was obtained from ONE-WAY ANOVA. * Significant differences at 7.5 years and 9 years were observed in time spent (s) to solve the ***CCTT*-1** test between children whose mothers were supplemented with FO (*p*= 0.0001), 5-MTHF (*p*= 0.0113) or both treatments (FO+5MTHF) (*p*= 0.038) during pregnancy .5-MTHF: 5-Methyltetrahydrofolate; CCTT-1: Children´s Color Trails Test; FO: fish-oil.

328 **Table 3.** Effect of selected cofounders on the Symbol Digit Modalities Test ***(SDMT)*** hits in the
329 NUHEAL children

Variables	Categories	*b*	CI (95%)		*p*
			LCL	UCL	
Prenatal Supplementation	FO	1.60	-2.45	5.64	0.439
	5-MTHF	-3.02	-7.06	1.03	0.144
	FO+5-MTHF	-0.08	-4.52	4.69	0.972
	Placebo	0	------	------	------
Time point	7.5y	0	------	------	------
	9y	10.72	7.98	13.47	**<0.001**
Study Group * Time point	FO * 9y	-2.40	-6.67	1.86	0.269
	5-MTHF * 9y	-2.28	-6.57	2.01	0.299
	FO+5-MTHF * 9y	-1.50	-5.82	2.82	0.497
	Placebo * 9y	0	------	------	------
Maternal Age		0.22	-0.04	0.48	0.100
Maternal Education Level	None + Primary	0	------	------	------
	Secondary or >	1.47	-1.02	3.96	0.248
Smoking	No	0	------	------	------
	Yes	2.08	-1.01	5.18	0.188
dBMI (kg/m2)		-0.63	-1.25	-0.02	**0.043**
Mode of delivery	Spontaneous	0	------	------	------
	Forceps	1.73	-2.55	6.02	0.427
	Vacuum	-2.65	-6.85	1.56	0.217
	Cesarean section	0.17	-2.43	2.77	0.898
Gestational Age		0.44	-0.27	1.16	0.228
Sex	Girls	0	------	------	------
	Boys	-1.00	3.21	1.21	0.373
AA/DHA ratio[1,2]		8.80	3.61	13.99	**0.001**
FADS1 rs174556 polymorphism		1.24	-0.51	3.01	0.165
Plasma Folate (μg/L)[1]		0.11	-0.16	0.37	0.439
tHcy (μmol/L)[1]		-0.52	-1.02	-0.002	**0.049**
*MTHF*R 677 C/T polymorphism		-0.47	-2.10	1.16	0.574

330 1: values obtained from mothers at delivery; 2:fatty acids measured as % of total fatty acids; 5-MTHF:
331 5-Methyltetrahydrofolate; AA: Arachidonic acid; b: Regression coefficient; CI: Confidence Interval;
332 dBMI: difference of body mass index between 20 and 30 weeks of pregnancy; DHA: Docosahexaenoic
333 acid; FADS: Fatty acid desaturase; FO: Fish-oil; LCL: lower confidence limit; MTHFR:
334 Methyl-tetrahydrofolate reductase; tHcy: total homocysteine; UCL; upper confidence limit. p=level of
335 significance obtained from multivariate multiple linear regression model; Bold: $p<0.05$

3.3.2. Children Color Trails Test ***(CCTT-1)***

No statistically significant differences in elapsed time for ***CCTT-1*** at 7.5 and 9 years were found among groups of prenatal supplementation after adjustment for selected confounders *(maternal blood parameters, maternal FADS1 polymorphism and pregnancy and lifestyle characteristics).* Independently of prenatal supplementation, children aged 9 years showed a decrease in the timing spent to solve the task compared to their results at the previous examination at age 7.5 years (p <0.001) **(Table 4)**.

After considering the interaction between age and prenatal supplementation, we observed that decrease in time elapsed was higher in those children whose mothers were supplemented with FO during pregnancy (p= 0.0001), 5-MTHF (p= 0.0113) or FO+5-MTHF (p= 0.038) **(Figure 2)**, but not in the placebo group.

As shown in **Table 4**, other selected cofounders had also a significant effect on the time required to complete ***CCTT-1*** test. In fact, vacuum delivery determined an increase in the time elapsed of 24.77 s for the task (p= 0.010) compared to those children whose mothers had an uncomplicated spontaneous delivery. Moreover, smoking during pregnancy had also a negative effect on the child´s ***CCTT-1*** hits, increasing the time of solving this task in 23.43 s (p= 0.001). Finally, we observed that maternal AA/DHA ratio at delivery was a significant factor determining less timing to solve the ***CCTT-1*** task of their offspring up to 9 years (-27.87 s, p= 0.019). Positive association between maternal AA/DHA ratio and ***CCTT-1*** was also determined after logistic regression analysis, and characterizing those children aged 9 years with elapsed time of solving the ***CCTT-1*** below the P25 [OR: 18.53 (95% CI: 2.13-160.9); p= 0.008] **(Table 6)**. Shorter solving times were also observed in those children born to mothers who were heterozygote for *FADS1* rs174556 [OR: 2.75 (95% CI: 1.02-7.39; p= 0.045].

Table 4. Associations between interest cofounders and Children Color Trails Test ***(CCTT-1)*** Hits in the NUHEAL children at school age

Variables	Categories	*b*	CI (95%)		*p*
			LCL	UCL	
Prenatal Supplementation	FO	-12.69	-31.22	5.84	0.180
	5-MTHF	-8.43	-27.01	10.14	0.373
	FO+5-MTHF	-15.25	-36.34	5.83	0.156
	Placebo	0	------	------	------
Time point	7.5y	0	------	------	------
	9y	-34.12	-46.92	-21.10	**<0.001**
Study Group * Time point	FO * 9y	-13.57	-33.42	6.28	0.180
	5-MTHF * 9y	16.41	-3.55	36.38	0.107
	FO+5-MTHF * 9y	15.28	-5.04	35.61	0.141
	Placebo * 9y	0	------	------	------
Maternal Age		-0.80	-1.97	0.38	0.183
Maternal Education Level	None + Primary	0	------	------	------
	Secondary or >	-7.92	-19.22	3.38	0.170
Smoking	No	0	------	------	------
	Yes	23.43	9.43	37.44	**0.001**
dBMI (kg/m2)		-0.62	-3.41	2.18	0.666
Mode of delivery	Spontaneous	0	------	------	------
	Forceps	-5.91	-25.07	13.24	0.545
	Vacuum	24.77	6.01	43.53	**0.010**
	Cesarean section	2.66	-9.15	14.47	0.659
Gestational Age		3.22	-0.05	6.49	0.054
Sex	Girls	0	------	------	------
	Boys	6.06	-3.98	16.10	0.237
AA/DHA ratio[1,2]		-27.87	-51.13	-4.61	**0.019**
FADS1 rs174556 polymorphism		-7.15	-15.15	0.86	0.080
Plasma Folate (µg/L)[1]		-0.26	-1.47	0.95	0.674
tHcy (µmol/L)[1]		-1.06	-2.39	1.27	0.371
*MTHF*R 677 C/T polymorphism		0.37	-7.04	7.78	0.922

1: values obtained from mothers at delivery; 2: fatty acids measured as % of total fatty acids; 5-MTHF: 5-Methyltetrahydrofolate; AA: Arachidonic acid; b: Regression coefficient; CI: Confidence Interval; dBMI: difference of body mass index between 20 and 30 weeks of pregnancy; DHA: Docosahexaenoic acid; FADS: Fatty acid desaturase; FO: Fish-oil; LCL: lower confidence limit; MTHFR: Methyl-tetrahydrofolate reductase; tHcy; total homocysteine; UCL; upper confidence limit. p= level of significance obtained from multivariate multiple linear regression model; Bold: p <0.05

3.3.3. Stroop Color and Word Test ***(SCWT)***

We analyzed the influence of prenatal supplementation and other selected cofounders on the information processing speed using Stroop Color and Word test and its obtained scores: Word-Reading (*WRST*) and Color-Naming (*CNST*).

Similarly to initial descriptive evaluation, further adjusted analysis for selected cofounders did not show statistically significant differences between different types of prenatal supplementation in the number of *WRST* hits obtained by children at 7.5 and 9 years **(Table 5)**. However, independently of maternal supplementation during pregnancy, children at 9 years showed an increase of 15.37 points in the number of hits for *WRST* (p <0.001) respect to children at 7.5 years. In relation to other cofounders considered in the model, boys showed 4.93 less hits than girls solving the task (p= 0.019). Moreover, we also observed that each increment of one unit (μmol/L) of maternal tHcy at delivery predicted that their offspring had 1.09 less hits by average solving the task (p= 0.025). Further logistic regression analysis, characterizing those children aged 9 years which hits of solving the *WRST* test below the P50, determined that maternal tHcy at delivery had a negative influence on this test [OR: 0.69 (95% CI: 0.51-0.93); p= 0.015] **(Table 6).**

When analyzing the effects of both prenatal supplementation and child´s age on *CNST*, similar results to those mentioned above were found. In fact, cofounder adjustment analysis did not show statistical differences between different groups of prenatal supplementation in the number of hits obtained by NUHEAL children at 7.5 and 9 years. Once again, there was an increase of *CNST* hits at 9 years compared to 7.5 years (p <0.001), but this effect was independent of type of supplementation during pregnancy **(Table 5)**. Interestingly, we observed that each increment of one unit of AA/DHA ratio in maternal blood at delivery determined that their offspring had 6.55 more hits by average solving the *CNST* (p= 0.034) **(Table 5)**. After logistic regression analysis, we observed that maternal age positively influenced the likelihood for children aged 9 years to be above the P75 to solve *CNST* test (p=0.039), but maternal tHcy level at delivery reduced this probability (p=0.005) **(Table 6)**.

414 **Table 5.** Analysis of the influence of selected cofounders on the Word-Reading Stroop Test
415 *(WRST)* and Color-Naming Stroop Test *(CNST)* in the NUHEAL children

		Word-Reading Stroop Test (WRST)-hits				*Color-Naming Stroop Test (CNST)-hits*			
Variables	**Categories**	***b***	**CI (95%)**		***p***	***b***	**CI (95%)**		***p***
			LCL	**UCL**			**LCL**	**UCL**	
Prenatal Supplementation	FO	4.02	-3.25	11.30	0.278	0.85	-3.91	5.62	0.726
	5-MTHF	1.09	-6.16	8.35	0.768	-1.97	-6.74	2.81	0.419
	FO+5-MTHF	-1.33	-9.70	7.01	0.754	2.46	-2.97	7.90	0.374
	Placebo	0	------	------	------	0	------	------	------
Time point	7.5y	0	------	------	------	0	------	------	------
	9y	15.37	11.20	19.55	**<0.001**	10.00	6.80	13.21	**<0.001**
Study Group * Time point	FO * 9y	-3.09	-9.55	3.37	0.348	-1.02	-5.95	3.92	0.686
	5-MTHF * 9y	-6.35	-12.80	0.11	0.054	-1.89	-6.86	3.06	0.453
	FO+5-MTHF * 9y	-2.21	-8.82	4.39	0.511	-2.52	-7.57	2.53	0.328
	Placebo * 9y	0	------	------	------	0	------	------	------
Maternal Age		-0.30	-0.85	0.24	0.279	0.046	-0.26	0.35	0.769
Maternal Education Level	None + Primary	0	------	------	------	0	------	------	------
	Secondary or >	2.32	-2.33	6.98	0.328	-0.44	-3.37	2.50	0.768
Smoking	No	0	------	------	------	0	------	------	------
	Yes	5.58	-0.20	11.35	0.058	1.47	-2.17	5.11	0.430
dBMI (kg/m2)		-0.46	-1.54	0.73	0.485	-0.57	-1.29	0.16	0.126
Mode of delivery	Spontaneous	0	------	------	------	0	------	------	------
	Forceps	2.00	-5.96	9.98	0.621	3.12	-1.87	8.11	0.221
	Vacuum	1.85	-5.86	9.55	0.639	-0.771	-5.65	4.10	0.756
	Cesarean section	-0.18	-5.04	4.66	0.939	1.91	-1.15	4.98	0.222
Gestational Age		-0.28	-1.62	1.06	0.683	0.32	-0.53	1.17	0.466
Sex	Girls	0	------	------	------	0	------	------	------
	Boys	-4.93	-9.06	-0.81	**0.019**	-1.86	-4.48	0.74	0.161
AA/DHA ratio[1,2]		8.15	-1.47	17.78	0.097	6.55	0.50	12.60	**0.034**
FADS1 rs174556 polymorphism		-1.47	-4.78	1.83	0.382	-1.07	-3.15	1.01	0.315
Plasma Folate (μg/L)[1]		0.24	-0.26	0.74	0.343	0.28	-0.03	0.60	0.079
tHcy (μmol/L)[1]		-1.09	-2.04	-0.14	**0.025**	-0.47	-1.07	0.14	0.129
*MTHF*R 677 C/T polymorphism		2.61	-0.43	5.65	0.093	-0.39	-2.32	1.53	0.688

416 [1]: values obtained from mothers at delivery; [2]:fatty acids measured as % of total fatty acids; 5-MTHF:
417 5-Methyltetrahydrofolate; AA: Arachidonic acid; *b*: Regression coefficient; CI: Confidence Interval; dBMI:

difference of body mass index between 20 and 30 weeks of pregnancy; DHA: Docosahexaenoic acid; *FADS*: Fatty acid desaturase; FO: Fish-oil; LCL: lower confidence limit; *MTHFR*: Methyl-tetrahydrofolate reductase; tHcy; total homocysteine; UCL; upper confidence limit. *p*= level of significance obtained from multivariate multiple linear regression model; Bold: $p < 0.05$

Table 6. Logistic regression analysis after characterizing children aged 9 years of age below P75, P50 or P25 for Symbol Digit Modalities Test ***(SDMT)***, Children Color Trails Test ***(CCTT-1)*** and ***STROOP*** Test

	Maternal AA/DHA	***FADS1* rs174556**		**Maternal tHcy**	**Maternal High Education**	**Maternal age**
		1	**2**			
***SDMT* - hits p75**						
Contrast	30.46	3.31	1.43	0.71	3.63	1.04
95% CI	(3.68, 252.0)	(1.28,	(0.31, 6.55)	(0.56, 0.89)	(1.32, 9.98)	(0.94, 1.14)
p-value	**0.002**	8.56)	0.64	**0.003**	**0.013**	0.446
		0.014				
***CCTT-1* (sc) p25**						
Contrast	18.53	2.75	4.95	1.03	2.03	1.03
95% CI	(2.13, 160.9)	(1.02,	(0.91,	(0.85, 1.26)	(0.71, 5.77)	(0.93, 1.14)
p-value	**0.008**	7.39)	27.07)	0.733	0.184	0.587
		0.045	0.065			
***STROOP* Test – hits 1**						
***(Word-reading)* p50**	3.73	0.63	1.67	0.69	1.47	0.95
Contrast	(0.31, 44.28)	(0.19,	(0.22,	(0.51, 0.93)	(0.44, 4.85)	(0.84, 1.08)
95% CI	0.297	2.03)	12.85)	**0.015**	0.529	0.440
p-value		0.439	0.624			
***STROOP* Test – hits 2**						
***(Color-naming)* p75**	21.70	0.28	0.67	0.51	0.69	1.20
Contrast	(0.88,	(0.07,	(0.07, 6.48)	(0.32, 0.81)	(0.17, 2.76)	(1.01, 1.42)
95% CI	534.97)	1.16)	0.733	**0.005**	0.602	**0.039**
p-value	0.06	0.08				

1= Heterozygous, 2 = Homozygous minor; AA: arachidonic acid; CCTT-1: Children Color Trails Test; DHA: Docosahexaenoic acid; *FADS*: Fatty Acid Desaturase; SDMT: Symbol Digit Modalities Test; tHcy: total homocysteine. *p*= level of significance obtained from logistic regression mixed model; Bold: $p<0.05$

4. Discussion

This study was performed to analyze the long-term effects of prenatal supplementation, as well as maternal *FADS* and *MTHFR* genetic polymorphisms, on processing speed in healthy school-age children. Our results suggest that neither FO

nor folate prenatal supplementation predicted high processing speed scores at school-age children. However, our secondary exploratory analyses seem to indicate that maternal AA/DHA ratio and *FADS1* rs174556 SNPs, were positively associated with later processing speed in their offspring up to 9 years, while tHcy concentrations in maternal plasma at delivery showed a negatively effect on child processing speed.

Prenatal folic acid supplementation has been related to better neurodevelopment in offspring, in terms of reducing the risk of behavioral problems [45], language delay [46], inattention [24], hyperactivity and peer problems [47,48]. However, their potential effects on cognitive and mental performance during development are inconsistent, partly due to the very limited number of studies published [49]. Folic acid acts as methyl donor in the metabolic conversion of homocysteine (Hcy) to methionine [50]. As consequence, folate deficit, alone or in combination with others B-vitamins, attenuates this metabolic pathway, which subsequently increases total Hcy levels. There is evidence that higher maternal Hcy (≥ 8.3 μmol/L) may not only negatively influence placental development, birth weight and pregnancy outcomes [51], but also are related to cytotoxic- and oxidative stress-dependent endothelial cell impairment and apoptosis of placental trophoblast [52,53]. Interestingly, our results suggest a negative predictive role of maternal tHcy on child´s cognitive function. In fact, children born to mothers with high tHcy levels during pregnancy showed a decrease in ***SDMT*** and ***SCWT*** hits, as well as lower likelihood to be in the upper percentiles of the ***WRST*** and ***CNST***. Because tHcy concentrations are considered as functional indicator of folate status, use of supplements containing folic acid during pregnancy must be considered to avoid congenital malformations during fetal development [54], while at the same time enhancing neurodevelopment and healthy outcomes at childhood stage.

On the other hand, it is also clear that fetal LC-PUFAs, including DHA and AA levels, are strongly related to maternal LC-PUFAs status during pregnancy, playing a major role for an optimal brain development [20,55]. In this regard, DHA is related to synaptogenesis, nerve growth factor expression and neuronal differentiation [56]. Furthermore, AA is involved in several synaptic signaling pathways [57], synthesis of eicosanoids, prostaglandins and leukotrienes [11,12], growth-related early gene expression and cell growth [58]. However, there is no consistent evidence for long-term beneficial effects of *n*-3 or *n*-6 LC-PUFAs supplementation during pregnancy or lactation on child´s neurodevelopment [6,17,31,59-61]. As a consequence, there is growing interest to analyze the long-term effects of AA/DHA ratio on child neurodevelopment, which reflects both its endogenous synthesis and exogenous supply. Moreover, because both FAs compete for the same enzymatic pathways to convert them into biologically active eicosanoids, AA/DHA ratio is strongly influenced by the prevalence of genetic predisposition for *FADS* and elongase genes [56]. Recently, higher DHA/AA ratio and higher DHA concentrations in cord blood have been considered favorable for infant visual, cognitive and motor development in Arctic Inuit exposed to high intakes of seafood and *n*-3 LC-PUFAs [62]. According to these findings, we did not find clear associations between type of

prenatal supplementation and processing speed development, except for beneficial effects of prenatal FO supplementation on ***CCTT-1*** elapsed time at 9 years. However, higher maternal AA/DHA ratio seems to be a positive and long-term modulator of processing speed (mainly on ***SDMT***, ***CCTT-1*** and ***CNST***) in the offspring, indicating the importance not only of DHA, but also its equilibrium with AA. Interestingly, at 9 years, children whose mothers were heterozygotes for *FADS1* rs174556 performed better the processing speed tasks respect to those born to mothers with homozygous major alleles. Our findings are also consistent with PUFAs´ role on myelination and white matter integrity shown in animal studies [63-65]. In this regard, DHA may increase processing speed by changing the physical-chemical and structural properties of membrane [66]. Moreover, Peters *et al.* [67] not only demonstrated that erythrocyte membrane PUFAs concentrations in young adults seem to be robustly related to white matter integrity, but also showed that these associations were mostly related to AA levels. So, as the connection between white matter integrity and processing speed in cognitive tasks is consistently established, our results show positive and strong long-term effects of perinatal LC-PUFAs, in terms of adequate AA/DHA ratio and *FADS1* polymorphism, on cognitive development, suggesting an increase of white matter volume and better integrity.

The major strength of the present study is the long-term follow-up, from pregnancy to 9years, allowing us to obtain better evaluation of long-term effects of prenatal supplementation with FO, 5-MTHF or FO+5-MTHF on child´s cognitive abilities. Moreover, the NUHEAL study was conducted in three different countries (Spain, Germany, and Hungary) with distinct eating habits; due to country of origin has been eliminated as confounder, our data reinforces the idea that long-term effects observed on processing speed are independent of the women's diet. In this regard, we highlight the influence of the cultural level of the mothers on the processing speed of their children, which increases the need to take into account the different socio-environmental factors during early life that may influence on later speed processing capacities. Finally, neuropsychological tests used to evaluate processing speed were administered by different technicians in each country, although all of them received a common training to reduce the examiner and cultural influences on the results.

Our results have some limitations. First, the number of children who belong to each study group was homogeneous but relatively low. However, after combining the data from children born to mothers who were supplemented during pregnancy (n=103), the effects of supplement combinations were significant with respect to children whose mothers received placebo. Secondly, the effects of FO, 5-MTHF or FO+5-MTHF supplementation were not evaluated at different time-points of administration. Moreover, our study has been conducted only in a selected age range (7.5-9 years). Thus, future studies will be necessary to evaluate whether our findings can be extended to other times of administration or at different ages during development. Finally, conversion of homocysteine to methionine is largely based on

both folate and vitamin B12 levels, which act as substrate and cofactor, respectively. However, the status of vitamin B12 level in our study is largely unknown.

Findings obtained in this study should be interpreted with caution. NUHEAL women belonging to FO and FO+5-MTHF groups received fish oil preparation, alone or in combination with folate, at a dose of 500 mg DHA+150 mg EPA from week 20 until delivery, which are higher than current international recommendations (200-300 mg of DHA/day) [12,68]. In addition, study participants followed their usual eating patterns, including PUFAs rich food. Therefore, it is of utmost importance to determine the timing, necessary duration and dosage of DHA+EPA supplementation (in equilibrium to AA) during pregnancy, for obtaining the best cognitive development in the offspring. Interestingly, the mixed supplementation including FO and 5-MTHF had no effect on processing speed up to age 9 years. Thus, we propose that maternal supplementation based on folate, DHA and EPA should be individualized, taking into account diet, habits, folate status and maternal *FADS1* genetic variant rs174556 G/A, and perhaps not together at the same time during pregnancy.

5. Conclusions

In summary, herewith we report that maternal AA/DHA ratio at delivery and mothers who were heterozygote for *FADS1* genetic variant rs174556 seem to have positive long-term effects on processing speed in the offspring up to 9 years. Processing speed tasks, in terms of less time to solve ***CCTT-1*** task time, was also better in the offspring of mothers who received prenatal FO supplementation. Our results also suggest that the increase of tHcy levels can be considered as an important factor at perinatal time predicting worse speed processing development at 9 years. Thus, maternal LC-PUFAs and folate requirements should be within normal ranges till the end of pregnancy to avoid undesirable long-term effects. Finally, we suggest that data obtained from this study might be useful for designing new studies to assess the effects of increased folic acid, DHA and AA consumption at certain times during pregnancy and early life to improve cognition and mental performance in children.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: title, Table S1: title, Video S1: title.

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Supplemental Methods

Long-chain polyunsaturated fatty acids, homocysteine at birth and fatty acid desaturase gene cluster polymorphisms are associated with children´s processing speed up to age 9 years.

SNP selection and genotyping

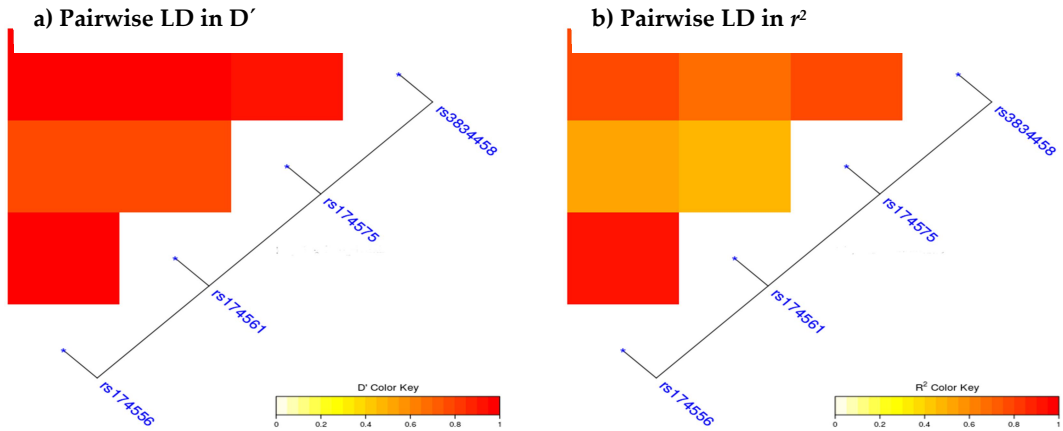
Genotyping of SNPs (single-nucleotide polymorphisms) from *FADS* gene cluster was performed with the iPLEX method (Sequenom, San Diego, CA, USA) (see main text for more information). Initially, seventeen SNPs from the *FADS1* (rs174548, rs174556 and rs174561), *FADS2* (rs174570, rs174574, rs174575, rs174576, rs174578, rs174579, rs174602, rs498793, rs968567, rs2727271 and rs3834458) and *FADS3* (rs174448, rs174449 and rs174455) gene cluster from the NUHEAL mothers were genotyped **.**Specific SNPs, including rs174556, rs174561, rs3834458, rs174548, rs174574 and rs174578, were selected due to its relation with FA levels [1,2], while 11 SNPs remain were included according to its localization in *FADS* gene cluster on chromosome 11. Based on the linkage disequilibrium data from Lewontin´s D` (D`>0.9) and pairwise-squared correlations r^2 *(r^2>0.7)*, 4 SNPs out of the initial 17 SNPs (*FADS1* rs174556 and rs174561, *FADS2* rs174575 and rs3834458) were considered for further analyses **(Supplemental Figure 1)**. Hardy-Weinberg *(H-W)* equilibrium for the genotypes was calculated using the statistical software R (Version 3.2.2, package "genetics"). Deviations from the H-W equilibrium were analyzed by Fisher's exact test **(Supplemental Table 1)**, where *FADS1* rs174561 was discarded due to its low significance (p= 0.016*)*. To test the linkage disequilibrium, Lewontin's D' and pairwise squared correlations r^2 were also performed **(Supplemental Table 3)**. Finally, we have selected the *FADS1* rs174556 as a representative of the block and were included in our following analysis.

Supplemental Table 1. Characteristics of the 4 selected variants in *FADS 1/2* gene from the initial 17 SNPs analyzed in the NUHEAL population

SNP	Supplementation group	Alleles (Major/Minor) and Frequency 0/1	Number (%) of subjects with Genotype			*p*
			0	1	2	
FADS1 **rs174556**		G/A (0.74/0.26)	80 (0.57)	47 (0.34)	13 (0.09)	0.127
	FO	G/A (0.67/0.33)	22(0.41)	13(0.52)	4(0.07)	0.671
	5-MTHF	G/A (0.66/0.34)	12 (0.38)	12 (0.56)	5 (0.06)	0.591
	FO+5-MTFH	G/A (0.73/0.27)	22 (0.68)	10 (0.27)	-	0.538
	Placebo	G/A (0.79/0.21)	24(0.62)	12(0.35)	4 (0.04)	1
FADS1 **rs174561**		A/G (0.76/0.24)	81 (0.61)	38 (0.29)	13 (0.10)	0.016
	FO	A/G (0.68/0.32)	22 (0.43)	12 (0.50)	4 (0.07)	0.671
	5-MTHF	A/G (0.67/0.13)	12(0.40)	10 (0.53)	5 (0.07)	0.616
	FO+5-MTFH	A/G (0.75/0.25)	23 (0.68)	7 (0.32)	0	1
	Placebo	A/G (0.80/0.20)	24 (0.64)	9(0.32)	4 (0.04)	1
FADS2 **rs174575**		C/G (0.71/0.29)	74 (0.52)	53 (0.37)	15 (0.11)	0.230
	FO	C/G (0.71/0.29)	21 (0.48)	14 (0.45)	5 (0.07)	0.274
	5-MTHF	C/G (0.75/0.25)	12 (0.45)	14 (0.45)	3(0.10)	1
	FO+5-MTFH	C/G (0.73/0.27)	20 (0.50)	11 (0.45)	3 (0.05)	0.385
	Placebo	C/G (0.75/0.25)	21 (0.54)	14 (0.42)	4 (0.04)	0.448
FADS2 **rs3834458**		T/Z (0.68/0.32)	68 (0.49	55 (0.39)	17 (0.12)	0.249
	FO	T/Z (0.64/0.34)	18 (0.38)	15(0.55)	2 (0.07)	0.413
	5-MTHF	T/Z (0.66/0.34)	11 (0.38)	13 (0.56)	5 (0.06)	0.591
	FO+5-MTFH	T/Z (0.75/0.25)	19 (0.55)	2 (0.41)	2 (0.05)	1
	Placebo	T/Z (0.73/0.27)	20 (0.50)	15 (0.46)	5 (0.04)	0.631

0= Homozygous major; 1= Heterozygous; 2=Homozygous minor; 5-MTHF: 5-Methyltetrahydrofolate*; FADS*: Fatty Acid Desaturase; FO: Fish-oil; *p*: level of significance obtained from Hardy-Weinberg Equilibrium Test; Bold: $p < 0.05$

Supplemental Figure 1. Pairwise LD measured in D′ **(a)** and r^2 **(b)** for selected maternal *FADS* SNPs



Supplemental Table 2. Power calculation obtained for processing speed evaluation in NUHEAL children

Speed processing tasks at 7.5 years	Pooled Intervention group (FO+5MTHF+MIX) Mean (SD)	Placebo group Mean (SD)	z
SDMT–hits	22.87(6.22)	24.66(6.38)	0.80
CCTT-1 (sc)	86.12(41.12)	99.88(50.77)	0.80
STROOP Test–hits-1 (Word-Reading)	60.13(14.85)	59.34(16.41)	0.80
STROOP Test–hits-2 (Color-Naming)	41.30(8.12)	41.95(8.41)	0.80

Values expressed as mean (standard deviation); 5-MTHF: 5-Methyltetrahydrofolate; ***CCTT***: Children Color Trails test; FO: Fish-oil; MIX: FO + 5-MTHF; ***SDMT***: Symbol Digit Modalities test; z= power calculation

Interestingly, power calculation for genetic study was also calculated using the package "powerEQTL v0.1.3" (R software). For this purpose, we considered an

one-way unbalanced ANOVA with different ranges of sample size (50, 100, our-case 143, 200, 250 and 300), α value of 0.05 and 17 analyzed SNPs in *FADS* 1/2/3 gene cluster. As show in **and Supplemental Table 2 and 3**, a statistical power of 90% was obtained for analyzed SNPs, except for rs174570 and rs2727271.

Supplemental Table 3. Power calculation obtained for genetic study in NUHEAL children

SNPs	Possible Functional Region	Alleles Frequency (Major/Minor)	Power Calculation
rs174548	*FADS1*	G/C (0.73/0.27)	>0.90
rs174556	*FADS1*	G/A (0.74/0.26)	>0.90
rs174561	*FADS1*	A/G (0.75/0.25)	>0.90
rs174570	*FADS2*	C/T (0.90/0.10)	>0.80
rs174574	*FADS2*	C/A (0.68/0.32)	>0.90
rs174575	*FADS2*	C/G (0.73/0.27)	>0.90
rs174576	*FADS2*	C/A (0.68/0.32)	>0.90
rs174578	*FADS2*	T/A (0.67/0.33)	>0.90
rs174579	*FADS2*	C/T (0.81/0.19)	>0.90
rs174602	*FADS2*	T/C (0.77/0.23)	>0.90
rs498793	*FADS2*	C/T (0.55/0.45)	>0.90
rs968567	*FADS2*	C/T (0.83/0.17)	>0.90
rs2727271	*FADS2*	A/T (0.89/0.11)	>0.80
rs3834458	*FADS2*	T/Z (0.70/0.30)	>0.90
rs174448	*FADS3*	T/C (0.69/0.31)	>0.90
rs174449	*FADS3*	T/C (0.69/0.31)	>0.90
rs174455	*FADS3*	T/C (0.68/0.32)	>0.90

FADS: Fatty Acid Desaturase; SNP: Single-Nucleotide polymorphism.

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ESTUDIO 3

"Effects of Maternal Fish Oil and/or 5-Methyl-Tetrahydrofolate Supplementation during Pregnancy on Offspring Brain Resting-State at 10 Years Old: A Follow-up Study from the NUHEAL Randomized Controlled Trial"

Hatim Azaryah, Juan Verdejo-Román, Cristina Martin-Pérez, José Antonio García-Santos, Cristina Martínez-Zaldívar, Francisco J. Torres-Espínola, Daniel Campos, Berthold Koletzko, Miguel Pérez-García , Andrés Catena y Cristina Campoy. Nutrients 2020, 12, 2701; doi:10.3390/nu12092701. IF: 4.546

Effects of Maternal Fish Oil and/or 5-Methyl-Tetrahydrofolate Supplementation during Pregnancy on Offspring Brain Resting-State at 10 Years Old: A Follow-up Study from the NUHEAL Randomized Controlled Trial

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Abstract: Recent studies have shown that maternal supplementation with folate and long-chain polyunsaturated fatty acids (LC-PUFAs) during pregnancy may affect children's brain development. We aimed at examining the potential long-term effect of maternal supplementation with fish oil (FO) and/or 5-methyl-tetrahydrofolate (5-MTHF) on the brain functionality of offspring at the age of 9.5–10 years. The current study was conducted as a follow-up of the Spanish participants belonging to the Nutraceuticals for a Healthier Life (NUHEAL) project; 57 children were divided into groups according to mother's supplementation and assessed through functional magnetic resonance imaging (fMRI) scanning and neurodevelopment testing. Independent component analysis and double regression methods were implemented to investigate plausible associations. Children born to mothers supplemented with FO (FO and FO + 5-MTHF groups, n = 33) showed weaker functional connectivity in the default mode (DM) (angular gyrus), the sensorimotor (SM) (motor and somatosensory cortices) and the fronto-parietal (FP) (angular gyrus) networks compared to the No-FO group (placebo and 5-MTHF groups, n = 24) ($P_{FWE} < 0.05$). Furthermore, no differences were found regarding the neuropsychological tests, except for a trend of better results in an object recall (memory) test. Considering the No-FO group, the aforementioned networks were associated negatively with attention and speed-processing functions. Mother´s FO supplementation during pregnancy seems to be able to shape resting-state network functioning in their children at school age and appears to produce long-term effects on children´s cognitive processing.

Keywords: fish oil; folate; pregnancy; brain function; resting-state functional neuroimaging; neurodevelopment; children

1. Introduction

The key role of nutrition during the "first 1000 days" (from conception to 2 years after birth) on optimal brain development in later life is well established. Rather than a homogenous organ, the human brain is formed by distinct regions and processes, each of which differs in its developmental trajectory and nutritional needs. In this line, fetal life has been established as a "critical" timeframe where deficiencies of some nutrients may lead to long-lasting or irreversible effects for later neurodevelopment in offspring [1].

Maternal intake during pregnancy of certain nutrients, including long-chain polyunsaturated fatty acids (LC-PUFAs), folic acid, and other B-group vitamins, is needed for fetal nervous system development, but also for the normal development of specific brain regions (i.e., the hippocampus, striatum, retina or cortex) and to establish the neural architecture and its underlying circuitry (synaptogenesis, neuronal pruning, and myelination) that supports major brain functions [2–5].

In fact, the links between LC-PUFAs (contained in fish oil) and the function of these networks underpinning cognition in children are well established. DHA

supplementation has been associated with cortical circuit maturation in children [6] and increased pre-frontal activation during sustained attention [7]. Low DHA concentrations have been associated with anomalies such as reduced indices in cortical integrity in the anterior cingulate (which implies a slower reaction time during sustained attention) [8], and increased risk for developing affective or bipolar disorders [9]. Regarding folic acid, there are consistent indications that prenatal supplements during the first 12 weeks of pregnancy not only prevent neural tube defects [10], but also affect apoptosis, neurogenesis, and overall nervous system development [11,12]. Cerebral 5-methyl-tetrahydrofolate (5-MTHF) deficiency could be associated with disturbed folate transportation or increased turnover from the central nervous system, which could lead to atrophy of fronto-temporal regions and periventricular demyelination, as can be observed in some neuroimaging studies [13].

Resting-state functional magnetic resonance imaging (rsfMRI) has been identified as a useful tool to explore brain function in the absence of task-demanding stimuli [14]. The rsfMRI allows for identification of the intrinsic brain networks implicated in primary (i.e., sensorimotor or visual networks) and high-order functions (i.e., fronto-parietal or default mode networks), such as executive functions or attention networks [15]. Despite the relevance of nutritional supplementation on brain structure, little is known about the effects of these nutrients on brain functioning in humans. A previous study has shown associations between PUFAs and the functional connectivity in areas within the default mode network, and the visual and limbic systems in elderly adults [16].

Recently, in a previous publication from the Nutraceuticals for a Healthier Life (NUHEAL) project, a long-term early nutrition programming effect of maternal folate and omega-3 fatty acid supplementation on offspring metabolism has been suggested [17]. To the best of our knowledge, no other study has explored the early nutrition programming effects on brain resting-state in school children at 10 years old. Due to the lack of research in this field, in the present study, we hypothesized that maternal prenatal supplementation with fish oil (FO) and/or 5-methyl-tetrahydrofolate (5-MTHF) might have a long-term effect on the brain resting-state networks of offspring at the age of 9.5–10 years. Furthermore, we will also explore the association between functional connectivity and children's mental processing.

2. Materials and Methods

2.1. Study Design

The current study was conducted as a follow-up of the Spanish children enrolled in the NUHEAL double-blind randomized trial www.ClinicalTrials.gov Identifier NCT01180933). A detailed study design, subject recruitment, and population characteristics have been described elsewhere [18]. Briefly, NUHEAL is a multicenter, randomized, double-blind, 2x2 factorial placebo-controlled trial designed to assess the effects of n-3 LC-PUFAs and 5-MTHF–supplementation during the second half of pregnancy on infant development. Healthy pregnant women were recruited before the 20th weeks of gestation and were randomly assigned to four different groups.

From week 20 to delivery, they received a daily supplement consisting of fish oil (FO: 500 mg of docosahexaenoic acid (DHA) + 150 mg eicosapentaenoic acid (EPA)), 400 µg 5-MTHF, both, or placebo, together with vitamins and minerals following European recommendations for pregnant women. All children were born at term and with birth weight appropriate for gestational age. At the age of 9 years, a total of 154 NUHEAL children were assessed by the NUTRIMENTHE neuropsychological battery (NNB) [19], and 85 of them also underwent an MR-scanning session at the age of 9.5–10 years.

The study was approved by the Ethics Committees for human research of the Universidad de Granada (Grant agreement number: 212652) and conducted in accordance with the Helsinki Declaration for human research studies in 2013 [20]. Written informed consent forms were obtained from all participants at the beginning of the study and before the magnetic resonance session.

2.2. Sample Size

A total of 85 Spanish children underwent an MR-scanning session at the age of 9.5–10 years. Twenty-eight of the datasets were discarded due to excessive motion, previous head injuries, claustrophobia and implanted ferromagnetic objects; therefore, a total of 57 children had valid data for all NNB and resting-state functional magnetic resonance imaging (rsfMRI) and were included in the analyses. From these children, 19 were born to mothers who received FO, 10 whose mothers were supplemented with 5-MTHF, 14 whose mothers received placebo and 14 were born to mothers supplemented with FO + 5-MTHF during pregnancy (Figure 1). All participants met the inclusion and exclusion criteria established for this study.

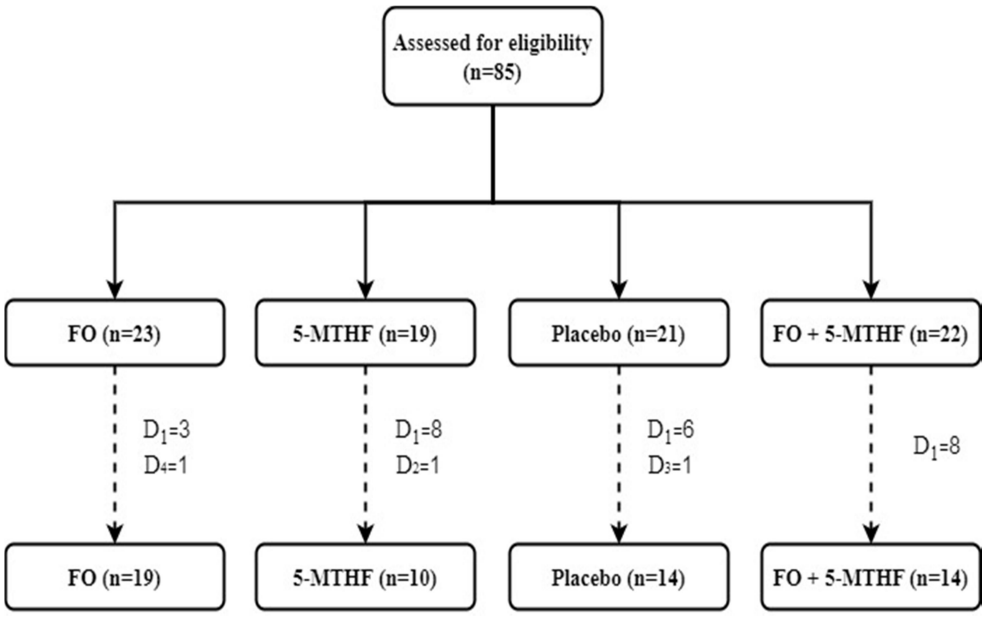

Figure 1. Participant flowchart. FO= fish oil, 5-MTHF = 5-methyl-tetrahydrofolate. Discards, D_1 = excessive motion, D_2 = previous head injuries, D_3 = claustrophobia, D_4 = implanted ferromagnetic object.

To achieve a statistical power of 80%, a medium-to-large effect size (0.7–0.9) between study groups, and considering a significance level as 0.05, the minimum sample size required is 20 per group [21]. Due to a high number of discards, future clustering of our originals groups will be made to reach the minimum sample size required for a robust and reliable neuroimaging analysis.

2.3. Resting-State fMRI Experimental Procedure (Primary Outcome)

Before starting the fMRI acquisition, children were trained by a master's degree psychologist to lie down on the patient table, keep calm and close their eyes during the scanner session. They were instructed to think about nothing in particular, but not to fall asleep during the resting-state acquisition. Resting-state brain images were acquired for a total time of 6 min.

2.3.1. Independent Component Analyses

Independent component analyses (ICA) was carried out using the probabilistic independent component analysis [22] as implemented in MELODIC (Multivariate Exploratory Linear Decomposition into Independent Components) version 3.14, part of FSL (FMRIB Software Library). Pre-processed data were whitened and projected onto a 40-dimensional subspace using probabilistic principal component analysis. The forty components were visually checked and identified. Components that represent well-known artifacts as motion, high-frequency noise, or venous pulsation [23,24] or those not located mainly in the gray matter [25] were identified and not taken into account in the following analyses. Fourteen components were discarded because of the previous reason; and the remaining twenty-six resting-state networks (RSNs) were labeled based on the overlap with the Harvard–Oxford cortical and subcortical structural atlases available in FSL.

2.3.2. Imaging Data Acquisition and Pre-Processing

fMRI data were collected with a 3T Philips Intera Achieva System (Philips Medical Systems, Eindhoven, The Netherlands) equipped with an eight-channel phased-array head coil. A T2*-weighted echo-planar imaging (EPI) sequence was acquired with the following parameters: repetition time (TR) = 2000 ms; echo time (TE) = 30 ms; flip angle = 78°; field of view = 230 × 230 mm; number of slices = 23; voxel dimension = 3 × 3 × 4 mm; gap, 1 mm. Structural images were also obtained as an isotropic T1-weighted turbo-gradient-echo sequence in the sagittal plane (TR = 8.3 ms; TE = 3.8 ms; flip angle = 8°; FOV (Field Of View) = 240 × 240 mm; number of slices = 160; slice thickness = 1 mm; voxel dimension = 1 × 1 × 1 mm). Prior to specific resting-state preprocessing, motion during acquisition was estimated using the realign tool implemented in statistical parametric mapping (SPM8) software (Wellcome Department of Cognitive Neurology, Institute of Neurology, Queen Square, London). Subjects with motion parameters exceeding 3 mm or 3 degrees were excluded from the subsequent processing and analyses.

Resting-state functional imaging pre-processing was performed using Functional MRI of the Brain (FMRIB) Software Library (v5.0.9, FSL,

http://fsl.fmrib.ox.ac.uk/fsl/fslwiki). Pre-processing steps included the removal of the ten first volumes, high-pass temporal filtering (120s), motion correction using the MCFLIRT tool [26], brain extraction using BET (Smith, 200), spatial smoothing using a Gaussian kernel of FWHM (Full Width at Half Maximum) = 8 mm, registration to a T1-weighted standard template using the FSL linear registration tool (FLIRT) with 12 degrees of freedom (DOF) and finally resampling to a 4 mm resolution.

2.4. Neuropsychological Assessment (Secondary Outcome)

Children´s neurocognitive development was assessed using a NUTRIMENTHE neuropsychological battery (NNB), a comprehensive neuropsychological battery specifically developed for the NUTRIMENTHE project [19,27,28]. For the present study, NNB was used to evaluate long-term effects of prenatal supplementation on the whole spectrum of neuropsychological functioning in children. The NNB consists of fifteen cognitive tests to assess seven different neuropsychological domains: processing speed, perception, motor, memory, attention, language, and executive functions, which are described briefly in Table 1 (a full description was already published elsewhere [19]).

Table 1. NUTRIMENTHE neuropsychological battery description [19,28,29].

Domain	Function	Test
Memory	Visual Episodic Memory	Recall of Objects Test (ROT)
	Verbal Memory	Rey Auditory Verbal Learning Test (RAVLT)
Attention	Sustained and Focused Attention	Continuous Performance Test (CPT)
	Spatial Attention	Pair Cancellation Test (W-M)
Motor	Visio-Motor Coordination	Grooved Pegboard Test (GPT)
Perception	Visio-Perceptual Integration	Hooper Visual Organization Test (HVOT)
Language	Semantic Fluency	Categorical Fluency Test (F-A-S-Animals)
	Verbal Comprehension	Token Test II (NEPSY-II)
Processing Speed	Processing Speed	Symbol Digit Modalities Test (SDMT)
Executive Functions	Impulsivity/Inhibition	Stroop Color and Word Test
	Update	Reversal Digits Subtest
		Matrix Analogies Test-(K-ABC-II)
	Flexibility/Shifting	Children's Color Trail Test (CCTT)
	Decision Making	Hungry Donkey Task (HDT)

2.5. Statistical Analyses

2.5.1. Resting-State fMRI

A dual regression method, described elsewhere [30], was implemented in FSL, followed by two-group t-tests, to compare brain maps across groups. Briefly, subject-specific statistical brain maps were created and collapsed in a 4D file for each resting-state network. In the first stage of the dual regression method, the spatial components generated during the ICA analysis were regressed into each subject's resting-state data to give a set of subject-specific time courses for each component. In the second stage, those time courses were regressed into the resting-state data to obtain subject-specific spatial brain maps for each component. Then, those subject-specific brain maps were used to compare each brain network between groups. These comparisons were tested voxel-wise for differences between groups using nonparametric permutation testing (5000 permutations) [31]. For each RSN, the resulting statistical maps had a threshold at $p < 0.05$ (threshold-free cluster enhancement-corrected for family-wise errors).

2.5.2. Neuropsychological Outcomes

The normality of variables was assessed for both RSN scores and neurodevelopment outcomes with Shapiro–Wilk's method. NNB outcomes were represented as mean (standard errors), and significant differences were assessed through Student t and Kruskal–Wallis tests. Pearson and Spearman correlations between RSN scores and NNB tests were carried out, taking into account the normality of variables.

3. Results

The general characteristics of the study population obtained at the study entry before the fMRI session are shown in Table 2.

Table 2. Sociodemographic characteristics of the study population by study group.

	FO (*n* = 19)	**5-MTHF (*n* = 10)**	**Placebo (*n* = 14)**	**FO + 5-MTHF (*n* = 14)**
Mothers' characteristics				
Age, years	29.68 (4.73)	34.15 (5.77)	31.86 (3.25)	30.68 (4.81)
BMI (20 w), kg/m^2	25.96 (3.67)	24.87 (2.13)	25.63 (2.47)	26.40 (2.89)
BMI (30 w), kg/m^2	28.47 (4.19)	26.64 (2.07)	27.50 (2.50)	28.46 (2.59)
BMI (delivery), kg/m^2	29.98 (4.58)	27.81 (2.05)	29.08 (3.08)	29.39 (2.86)
Weight gain, kg	10.64 (3.65)	7.67 (3.31)	8.86 (3.16)	7.25 (4.61)
Relative weight gain, %	15.45 (5.14)	12.08 (6.30)	13.49 (5.15)	10.99 (7.33)
Education *				
Mother	8 (42.1)	2 (50.00)	4 (50.00)	4 (44.45)
Father	11 (57.9)	2 (50.00)	4 (50.00)	5 (55.55)
Parity, *n* (%)				
0	13 (68.42)	5 (50.0)	7 (50.0)	6 (42.86)
≥1	6 (31.58)	5 (50.0)	7 (50.0)	8 (57.14)
Smoking (20 w), *n* (%)	5 (26.32)	1 (10.0)	0 (0.0)	4 (28.57)

Gravidity risk at 20 w, *n* (%)				
No risk factors	3 (15.79)	3 (30.0)	5 (38.46)	3 (21.43)
≥1 risk factor	16 (84.21)	7 (70.0)	8 (61.54)	11 (78.57)
Delivery risk				
No risk factors	6 (31.58)	4 (40.0)	7 (53.85)	5 (35.71)
≥1 risk factor	13 (68.42)	6 (60.0)	6 (46.15)	9 (64.29)
Children's characteristics				
Age, years	9.67 (0.26)	9.76 (0.20)	9.75 (0.19)	9.69 (0.22)
BMI, kg/m^2	19.45 (4.22)	17.64 (2.60)	17.76 (2.87)	19.08 (2.87)
Sex, *n* (%)				
Female	8 (42.11)	7 (70.0)	3 (21.43)	6 (42.86)
Male	11 (57.89)	3 (30.0)	11 (78.57)	8 (57.14)

FO: fish oil; 5-MTHF: 5-methyl-tetrahydrofolate; BMI: body mass index. * Attained general qualification level for university entrance or university degree. For qualitative variables (frequency (percentage)); for quantitative variables (mean (standard deviation)).

3.1. Resting-State fMRI Results

To assess the effect of prenatal FO supplementation, participants were clustered into two groups: (i) 33 children from the FO group (FO or FO + 5-MTHF), and (ii) 24 children from the No-FO group (5-MTHF alone or placebo). Three resting-state networks showed significant differences between the study groups. Specifically, children from No-FO group showed strong functional connectivity in the default mode (DM) (angular gyrus), the sensorimotor (SM) (motor and somatosensory cortices) and the fronto-parietal (FP) (angular gyrus) networks compared to the FO group ($P_{FWE} < 0.05$) (see Figure 2 and Table 3).

Alternatively, to assess the effect of folate supplementation during pregnancy, participants were divided into the following: (i) 24 children born to mothers supplemented with 5-MTHF or FO + 5-MTHF and (ii) 33 children whose mothers were supplemented during pregnancy only with FO or placebo. We found no significant differences in brain networks during resting-state between children supplemented with or without 5-MTHF.

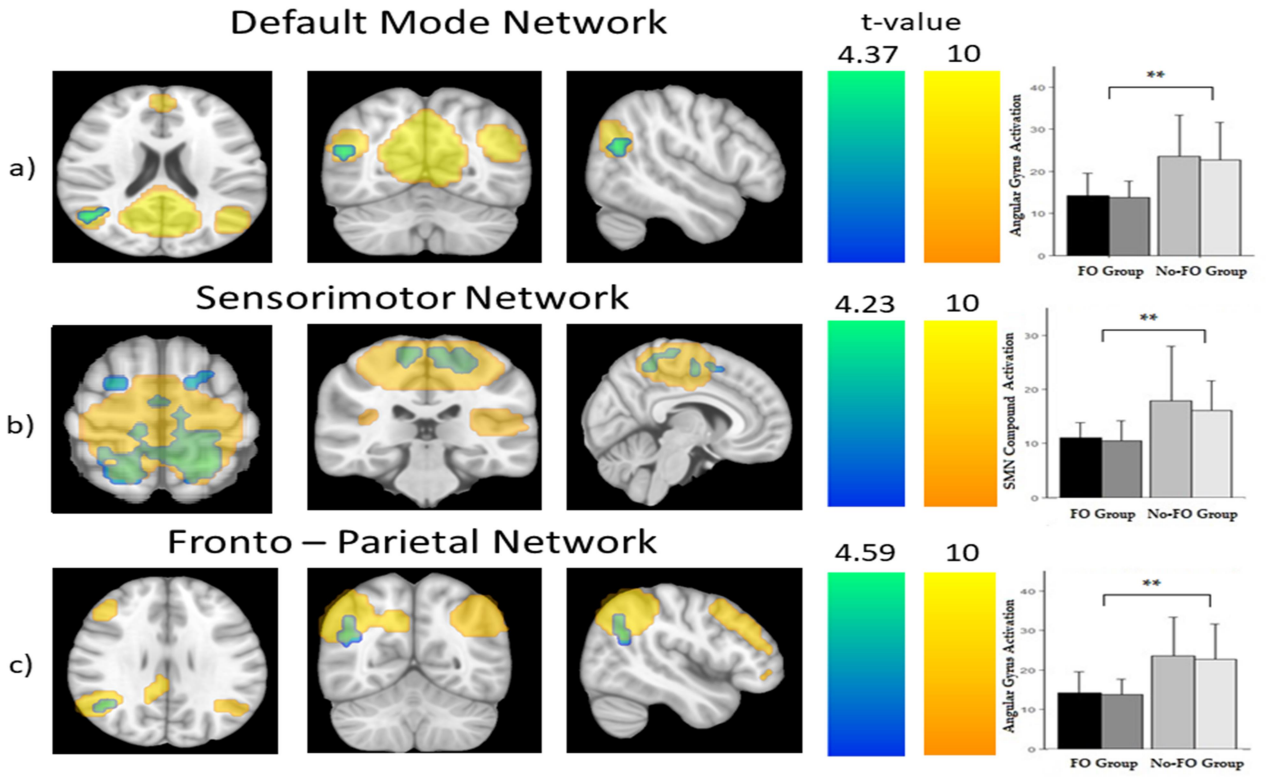

Figure 2. Children's resting-state functional magnetic resonance imaging (fMRI). Voxel-wise significant differences betwee maternal supplementation or not with FO during pregnancy. Color bars represent t-value. Warm colors represent the whole brain colors represented over the networks show the brain areas with significant differences between groups. (a) Default mode network, si gyrus; (b) sensorimotor network, significant areas: postcentral gyrus, precentral (left and right) gyrus and putamen; (c) fronto-pariet area: angular gyrus. (FO: fish oil, 5-MTHF : 5-methyl-tetrahydrofolate). ** PFWE < 0.05.

Table 3. Brain regions showing differences between groups ($P_{FWE} < 0.05$).

	Side	MNI Coordinates			Volume (mm^3)	t-Value
		X	Y	Z		
Default Mode Network						
Angular Gyrus	R	50	−62	20	176	4.37
Sensorimotor Network						
Postcentral Gyrus	L	−26	−50	60	2792	3.83
Precentral Gyrus	L	−18	2	44	760	4.17
Precentral Gyrus	R	22	−2	68	672	3.40
Putamen	L	−22	10	12	336	4.23
Fronto-Parietal Network						
Angular Gyrus	R	46	−58	28	296	4.58

MNI: Montreal Neurological Institute; X Y and Z: Axis; R: Right, L: Left.

3.2. Neuropsychological Outcomes and RSN Scores

No significant differences were found between the FO and the No-FO groups regarding the neuropsychological test results (see Table 4), except for a trend of children belonging to the FO group performing better in the Recall of Objects (ROT) test (memory domain) ($p = 0.065$).

In Table 5, correlations established between RSN scores and neuropsychological results are shown, considering the FO or No-FO groups. No significant results were found between the default mode network (DMN) and the NNB scores. The No-FO group showed a negative association between the angular gyrus within the fronto-parietal network (FPN) regarding SDMT hits (speed-processing domain) and CT hits (attention domain) (See Figure 3). In addition, the No-FO group presented a negative correlation between the precentral (right) and postcentral gyrus within the SMN (sensorimotor network) and CT hits (attention domain) (See Figure 3).

Table 4. Hits, timing and scores obtained by school children in the neuropsychological assessment depending on their mothers' fish oil supplementation during pregnancy.

NNB	FO Group (FO and FO + 5-MTHF) n = 33	No-FO Group (5-MTHF + Placebo) n = 24	p-Value
SDMT Hits	24.74 (0.96)	24.38 (1.38)	0.812
Grooved DH	45.03 (2.51)	40.25 (2.41)	0.162
Grooved NDH	54.77 (4.87)	45.17 (3.42)	0.101
HVOT Hits	16.06 (0.59)	16.62 (0.61)	0.512
CT Hits	74.12 (2.7)	72.33 (2.76)	0.654
CPT BL7 OMI	9.35 (1.43)	9.25 (1.39)	0.962
ROT Immediate Hits	5.81 (0.4)	4.83 (0.34)	0.065
ROT Delayed Hits	0.34 (0.15)	0.25 (0.09)	0.589
RAVLT Hits Trial 1	4.61 (0.25)	4.5 (0.42)	0.795
RAVLT Hits Trial 1–5	10.29 (0.35)	9.71 (0.49)	0.344
RAVLT Delayed Trial	13.13 (0.3)	12.83 (0.39)	0.562
Animals Total Hits	12.38 (0.63)	12.5 (0.65)	0.875
Token Test Total Hits	20.78 (0.37)	21.58 (0.58)	0.255
Stroop Interference	−1.57 (0.87)	−1.47 (1.18)	0.514
Reversal Digits, Hits	9.12 (0.41)	9.46 (0.63)	0.652
K-ABC-II	12.75 (0.46)	13.46 (0.88)	0.451
CCTT Part 1(s)	107.94 (5.8)	108.71 (10.13)	0.352
HDT Total Hits	0.81 (3.96)	0.27 (3.45)	0.114

NNB: NUTRIMENTHE neuropsychological battery; SDMT, Symbol Digit Modalities Test (total hits); DH, dominant hand; NDH, non-dominant hand; HVOT, Hooper Visual Organization Test (total hits); CT, Cancellation Test (total hits); CPT BL7, continuous performance test (total hits), Block7; OMI, omissions; ROT, Recall of Objects test (immediate and delayed recalled pictures); RAVLT, Rey Auditory Verbal Learning Test (recalled words in trial 1, trial 1–5 and delayed (hits)); K-ABC-II, Matrix Analogies Test (total hits); CCTT, Children's Color Trail Test (time, part 1(s)); HDT, Hungry Donkey Task (total score); (s), seconds. FO : fish oil; 5-MTHF : 5-methyl-tetrahydrofolate. Data are expressed by mean (standard errors). *P*-value from Student t-test or Kruskal–Wallis test depending on normality.

Table 5. Correlations between resting-state network (RSN) scores and neuropsychological battery (NNB) tests for each supplen

NNB	Default Mode Network		Sensorimotor Network								Fro
	Angular Gyrus		Postcentral Gyrus		Precentral Gyrus (L)		Precentral Gyrus (R)		Putamen		
	FO	No-FO	FO	No-FO	FO	No-FO	FO	No-FO	FO	No-FO	
SDMT hits s	0.055	−0.110	0.180	−0.245	0.130	−0.300	−0.005	−0.225	0.147	−0.211	−
Grooved DH s	−0.144	0.046	−0.244	0.210	−0.114	0.411	0.160	0.350	0.044	0.250	−
Grooved NDH s	0.043	−0.079	−0.349	0.082	−0.189	0.240	−0.197	0.183	0.066	0.161	−
HVOT Hits s	−0.044	0.092	0.221	−0.033	−0.077	−0.236	−0.224	−0.001	−0.154	−0.056	0
CT Hits s	−0.147	−0.332	0.040	−0.489 *	0.109	−0.228	−0.26	−0.491 *	−0.042	−0.164	−
CPT BL7 OMI s	0.106	−0.137	0.201	−0.044	0.214	0.063	0.355	−0.05	0.144	−0.221	0
ROT Immediate Hits s	0.219	−0.138	0.237	−0.231	−0.114	−0.274	−0.067	−0.282	−0.224	0.026	−
ROT Delayed Hits s	0.084	−0.108	−0.077	−0.152	0.470	−0.026	0.095	−0.219	0.180	−0.167	0
RAVLT Hits Trial−1 p	−0.285	−0.206	−0.076	−0.136	0.007	−0.108	−0.025	−0.159	−0.379	0.162	0
RAVLT Hits Trial-1-5 s	−0.166	−0.031	0.084	−0.042	−0.111	−0.021	0.425	−0.095	−0.497	0.175	0
RAVLT Delayed Trial s	0.145	−0.233	−0.043	0.138	0.022	0.228	−0.198	0.155	−0.121	0.357	0
Animals Total Hits s	0.072	−0.209	−0.271	−0.206	0.112	0.151	−0.503	−0.147	−0.019	0.439	−
Token Test Total Hits p	−0.037	−0.190	0.163	−0.153	−0.312	−0.222	−0.161	−0.069	−0.045	0.04	−
Stroop Interference p	0.267	−0.253	0.369	−0.315	0.042	−0.394	0.017	−0.200	0.120	−0.247	0
Reversal Digits, Hits p	−0.221	0.139	−0.081	−0.038	−0.108	−0.188	−0.149	−0.118	−0.165	0.011	−
K-ABC-II s	0.124	−0.276	0.124	−0.221	−0.035	−0.187	−0.147	−0.302	0.201	−0.160	−
CCTT Part 1(sec) p	0.087	0.119	0.101	0.155	−0.214	0.003	0.054	0.339	0.292	0.005	0
HDT Total Hits s	−0.041	0.084	0.259	0.232	0.017	0.122	0.478	0.258	0.214	0.283	−

NNB: NUTRIMENTHE neuropsychological battery; SDMT, Symbol Digit Modalities Test(total hits); DH, dominant hand; NDH hand; HVOT, Hooper Visual Organization Test (total hits); CT, Cancellation Test (total hits); CPT BL7, continuous performanc Block7; OMI, omissions; ROT, Recall of Objects test (immediate and delayed recalled pictures); RAVLT, Rey Auditory Verb (recalled words in trial 1, trial 1–5 and delayed (hits)); K-ABC-II, Matrix Analogies Test (total hits); CCTT, Children's Color Tra 1 (s)); HDT, Hungry Donkey Task (total score); (s), seconds; FO = fish oil. Data are expressed as r correlation coefficient from p Spearman, * $p < 0.05$.

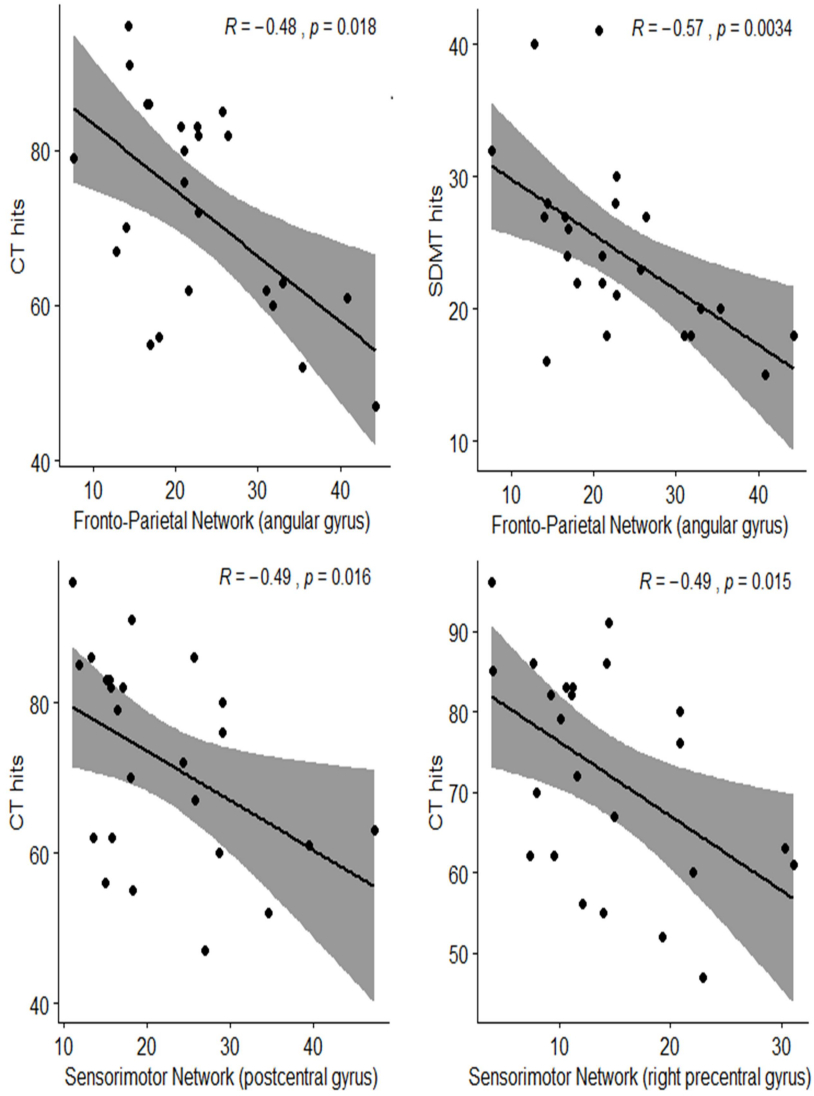

Figure 3. Spearman correlations between NNB and RSN scores; R = correlation coefficient; *p* = *p*-value; CT = Cancellation Test (total hits) from the attention domain; SDMT = Symbol Digit Modalities Test (total hits) from the speed-processing domain.

4. Discussion

This is the first study to examine the long-term effect of maternal supplementation with FO and/or 5-MTHF on the RSNs of the offspring at school age. We found that FO, but not 5-MTHF, supplementation during the second half of pregnancy is associated with decreased functional connectivity of children's brain networks at 9.5–10 years of age. Specifically, the default mode, the sensorimotor and the fronto-parietal networks displayed weaker functional connectivity in children born to mothers supplemented with FO or FO + 5-MTHF. Furthermore, after

correlating the resting-state scores and NNB tests, we found that children born to mothers who did not take FO supplements performed poorly regarding speed processing and attention tests.

Weak functional connectivity does not necessarily indicate poor cognitive neurodevelopment; in fact, some studies have shown for instance that the variance in IQ levels within a heterogeneous population was mostly explained by the distributed communication efficiency of brain networks built using moderately weak, long-distance connections, with only a smaller contribution of stronger connections [32]. In this study, children exposed to prenatal FO did not show any functional disadvantage but tended to have better memory. Another example is the weaker brain resting-state of bilingual subjects compared to monolingual ones [33,34], where the strength of resting-state functional connectivity correlated inversely with behavioral performance.

Considering the main brain area differences found in the present study, the angular gyrus is a cross-modal region which might act as a "connector hub" for the global processing of information [35], but also as a shifting area between internal (DMN) and external/salient (FPN) information [36]. These functions are competing but complementary [37] and could mask a high intrinsic relationship between functional connectivity and neurodevelopment. Indeed, the children born to mothers not supplemented with FO showed that the angular gyrus activity within the FPN was negatively related to the performance in attention and processing speed, suggesting an FO-related conjoint alteration between the most relevant brain network related to cognition (FPN) and the performance in important cognitive processes.

Regarding several alterations in the sensorimotor network (SMN), both somatosensory (postcentral) and motor (putamen and precentral gyrus) areas are involved in pre-mediated state of readiness to perform/coordinate a motor task [38], and their alterations in healthy subjects might lead to a maladaptive coordination of movements and motor learning, as proposed by previous animal studies [38]. However, this brain network´s function is not limited to motor execution. Indeed, it also mediates action execution programs [39], and its activity is known to be more related to attention to external stimuli in comparison with internal attentional processes [40]. Those children born to mothers not supplemented with FO showed a negative correlation between SMN and the performance in neuropsychological tasks related to attention; these results support the previously studied association between attentional and speed-processing processes and the SMN function [40]. It is well established that motor performance, visual-motor coordination [41] and attention [3] start to develop during gestation, especially at the second and third trimester of pregnancy; thus, these domains may be affected by nutritional interventions during this critical period of neurodevelopment, noting that omega-3 polyunsaturated fatty acids are essential due to their role in neurogenesis, fluidity and membrane fatty acid composition [42].

To date, clinical literature focused on neurodevelopmental effects of key nutrients is broadly based on nutrient deficiencies or deprivation, but optimal nutrient doses and potential long-term effects at different developmental ages remain unclear [1,43]. Some studies suggest that prenatal or postnatal dietary interventions based on

LC-PUFAs could have long-term effects on late child neurodevelopment, mainly on sustained attention, language, and processing speed [44–47]. For instance, children whose mothers received fish oil supplementation performed significantly better than the placebo group regarding the communication domain at the ages of 4 and 6 months [48], eye and hand coordination at 2.5 years [49], and sustained attention at 5 years [44]. However, there is no conclusive RCT evidencing long-term benefits of LC-PUFA supplementation during early life on neurodevelopment in healthy term infants [50–52], reflecting differences in terms of timing of supplementation, doses, and combination with other micronutrients. In relation to folic acid, some epidemiological studies [53] and a previous EEG/ERP (Electroencephalography/Event-Related Potential) research from the NUHEAL project [54] have reported that prenatal folate supplementation improves neurodevelopmental performance (mainly in attention system) in offspring at school age. However, there is no clear or robust evidence to support the use of multivitamin-containing folic acid supplementation during pregnancy on mental performance later in life [55,56]. Interestingly, these contradictory results come from the evaluation of mental performance as a global measure or an inappropriate timeframe for targeted neuropsychological domains, which could mask specific effects of a nutritional intervention during "critical periods" of brain development on long-term neurodevelopment. We must keep in mind that our No-FO group comprehends not only the 5-MTHF subgroup, but also the placebo subgroup, and the results of this study are directed to enforce the protective role of maternal LC-PUFA supplementation on children's brain development and function.

The main strengths of this study are in relation to its long term follow-up and monitoring, brain imaging to assess functional connectivity, and the use of an extensive neuropsychological batteries of tests to measure different brain domains. This novel approach has facilitated the study of associations between early nutrition, brain networks, and mental performance. The results provided in this manuscript provide more insight into the impact of FO supplementation during pregnancy on children's neurodevelopment and brain functioning. A large number of validated techniques have been used to assess our hypothesis, and the data come from a well-established cohort.

The main limitation of this study is the relatively small sample size in combination with multiple outcomes. Due to a large number of participants being excluded from the study (32.9%), mainly due to excessive movement during the rsfMRI session, we were required to cluster our four initial groups into two. In this case, we were able to guarantee 80% of statistical power (minimum required n = 20 for each group) and to detect a medium-to-large effect size (0.7–0.9). Furthermore, to avoid false positives regarding our neuroimaging analysis, the TFCE (threshold-free cluster enhancement) is one of the most reliable approaches, which, in our case, involves 5000 permutations to control for the family-wise error rate. –We believe that large differences between study groups are considerably rare and unlikely. All subjects eligible for this study are within the normal range of cognitive capabilities with no previous neuropathology diagnosed.. Nevertheless, the results obtained here can be interpreted as preliminary data and cannot be considered generalizable.

Further research in this area is needed to optimize the recommendations regarding LC-PUFAs and folic acid supplements during pregnancy.

5. Conclusions

This study further elucidates how maternal FO supplementation during pregnancy may be able to shape the resting-state network functioning of children at school age and as a consequence to produce effects on children´s cognitive processing; these results reinforce the idea of an early nutrition programming effect on brain functioning during childhood.

Author Contributions All authors helped in the interpretation of results and contributed to manuscript preparation. H.A. wrote the first draft. C.C. and B.K. designed the study and were coordinators of the study at University of Granada and Ludwig-Maximilians-Universität Munich, respectively. C.C. also wrote part of the manuscript and B.K. supervised it; C.M.-Z., F.J.T.-E. and D.C. performed the children examinations; M.P.-G. designed the NNB and reviewed the manuscript; H.A., J.V.-R., C.M.-P., A.C. and J.A.G.-S. performed the data analysis and interpretation.

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Abbreviations

5-MTHF	5-methyl-tetrahydrofolate
CCTT	Children's Colors Trail Test
CPTBL7	Continuous Performance Test Block7
CT	Cancellation Test
DHA	Docosahexanoic acid
DH	Dominant Hand
DMN	Default Mode Network
EPA	Eicosapentaenoic acid
FO	Fish Oil
FPN	Fronto-Parietal Network
HDT	Hungry Donkey Task
HVOT	Hooper Visual Organization Test

K-ABC-II	Matrix Analogies Test
LC-PUFAs	long-chain polyunsaturated fatty acids
NDH	Non Dominant Hand
NNB	NUTRIMENTHE Neuropsychological Battery
NUHEAL	NUtraceuticals for a HEALthier life
RAVLT	Rey Auditory Verbal Learning Test
ROT	Recall of Object Test
rsfMRI	Resting-state functional magnetic resonance imaging
RSN	Resting-State Network
SDMT	Symbol Digit Modalities Test
SMN	SensoriMotor Network

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DISCUSIÓN

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Las técnicas de minería de datos han resultado de vital importancia a la hora de extraer conocimiento útil de la base de datos global de NUTRIMENTHE, teniendo en cuenta las complejidades intrínsecas asociadas, como es el caso de la imperfección de datos y el gran número de variables con las que se cuenta. En el caso de los **estudios 1 y 2**, gracias a las reglas de asociación, se ha conseguido extraer de una manera simple y sin apenas esfuerzo, la relación vigente entre el tabaquismo materno durante el embarazo, y los problemas conductuales externalizantes en sus hijos en edad escolar. Así mismo, se ha comprobado la asociación entre el índice AA/DHA en la madre en el momento del parto y la velocidad de procesamiento en los niños. Utilizando sólo técnicas de estadística clásica dicha tarea habría sido ardua, tediosa y difícil de demostrar teniendo en cuenta la hipótesis inicial del estudio (suplementación y con FO y/o 5-MTHF y neurodesarrollo).

Considerando los resultados obtenidos en el **estudio 3**, la técnica de minería de datos ICA (Análisis de Componentes Independientes), ha permitido descubrir las diferencias de conectividad cerebral entre los grupos de estudio mediante análisis complejos de neuroimágenes, mientras que las técnicas de

aprendizaje supervisado (randomForest) y selección de características (caret) ha facilitado demostrar la asociación entre la conectividad funcional cerebral y la expresión clínica del neurodesarrollo en los niños, tras el descarte de un gran número variables potenciales que realmente proporcionaban poca o nula información.

A continuación se justifican por separado los resultados obtenidos en cada uno de los estudios.

ESTUDIO 1 : "Maternal smoking during pregnancy and child behavior problems: A multicenter study from the NUTRIMENTHE EU Project."

Este estudio observacional realizado en niños de entre 7 y 8 años, procedentes de 3 cohortes diferentes y de 6 países europeos, se ha llevado a cabo con el objetivo de investigar una posible asociación entre el tabaquismo materno durante la gestación, y el perfil conductual de los hijos en edad escolar evaluados mediante el CBCL. Se obtuvieron datos de las parejas de madre-hijo participantes en el proyecto Europeo NUTRIMENTHE. En general, los resultados muestran que los niños nacidos de madres fumadoras tienen un mayor riesgo de **externalizar problemas de comportamiento** *respecto a* los niños

nacidos de madres no fumadoras, incluso después de ajustar por variables de confusión *(cohorte y país de origen, nivel educativo materno y paterno y lactancia materna al menos durante tres meses)*. Los problemas de internalización / externalización en la niñez parecen predecir los resultados negativos posteriores de salud conductual, emocional, cognitiva y física de adolescentes y adultos [121].

Los problemas de internalización se refieren a conductas negativas enfocadas hacia el interior, como ansiedad, depresión y síntomas somáticos, mientras que los problemas de externalización se refieren a conductas negativas enfocadas hacia el exterior, como hiperactividad, agresión, conducta disruptiva y uso de sustancias psicotrópicas [122,123].

También vale la pena mencionar que la educación de los padres y la lactancia materna parecen modular los efectos del tabaquismo materno en los problemas de pensamiento y atención, respectivamente. Está bien establecido el papel protector de la lactancia materna frente a los problemas de internalización y externalización del comportamiento y a una alteración del desarrollo cognitivo en la infancia [124].

Los resultados obtenidos concuerdan con otros estudios publicados previamente [125–127], dónde se han comprobado problemas de comportamiento en niños expuestos al tabaquismo materno durante la gestación. Se han encontrado trastornos de conducta y del comportamiento asociados con frecuencia a la exposición prenatal al tabaco, en niños de tan solo 18-24 meses y en niños mayores (6-16 años) [128]. Sin embargo, otros investigadores no han encontrado una asociación causal entre el tabaquismo materno durante el embarazo y el TDAH, utilizando estudios de gemelos, cohortes de fertilización in vitro o enfoques de epidemiología genética [129,130], que apoyan la idea de que la asociación entre el tabaquismo materno durante el embarazo y el TDAH de la descendencia no se debe a efectos prenatales causales, sino que refleja una confusión no medida [21]. Otros autores sugieren que el efecto del tabaquismo sobre el comportamiento es pequeño y menos importante que el encontrado en estudios previos, y la influencia puede deberse a factores familiares estables en el tiempo, tales como factores ambientales y genéticos [40]. Por otro lado, otros investigadores han encontrado cambios en las cortezas frontal y parietal superiores

y volúmenes cerebrales globales más pequeños en niños de 6 a 8 años que estuvieron expuestos prenatalmente al tabaco, incluso después de ajustar por factores confusores socioeconómicos [131].

No obstante, existe un amplio consenso acerca de los efectos perniciosos del humo del tabaco durante el embarazo, que se produce en el contexto de un conjunto de circunstancias socioeconómicas que colocan al niño en un mayor riesgo de desarrollo, pero que pueden no estar relacionadas causalmente con el comportamiento del niño [126].

La asociación del tabaquismo materno con los problemas de comportamiento de los niños podría verse fuertemente afectada por las características sociodemográficas. Se ha visto anteriormente que las madres fumadoras durante el embarazo tienen, en promedio, un nivel de ingresos más bajo y menos educación [132]. En el presente estudio, las madres del grupo de fumadoras también presentan niveles educativos entre medio y bajo, mientras que las madres no fumadoras tienen un nivel educativo superior. Además, el país de origen se ha sugerido previamente como un factor confusor importante en los estudios que miden el rendimiento neuropsicológico [29], teniendo

siempre en cuenta la fuerte relación que prevalece con los resultados conductuales.

Una de las limitaciones del presente estudio es la carencia de datos disponibles sobre el tabaquismo paterno. El tabaquismo materno se basó únicamente en cuestionarios, no se disponía de datos sobre la duración del tabaquismo de las madres ni sobre el número de cigarrillos fumados al día. Esto puede implicar un riesgo de *bias* y excluye la posibilidad de evaluar una posible relación dosis-respuesta. Por otro lado, el período de tabaquismo abarca un período prolongado (antes, durante y después del embarazo), lo que determina que las conclusiones se refieran a los probables efectos del tabaco tanto durante el periodo prenatal como tras el parto. Algunos investigadores han señalado que los niños expuestos prenatalmente al tabaco eran más propensos a exhibir comportamientos anormales en comparación con los niños expuestos solo durante el período posnatal [133], enfatizando así que la gestación es un período crítico en el desarrollo de trastornos conductuales. Otra limitación del presente estudio es la carencia de datos sobre posibles problemas psicológicos maternos, que pudieran afectar la percepción materna de sus

hijos, ni la influencia del entorno social familiar. En ese sentido, la depresión y los problemas psicopatológicos fueron más comunes en las madres que fumaron durante el embarazo que en las madres no fumadoras [134].

Los resultados obtenidos en el presente estudio demuestran la necesidad de incrementar los esfuerzos de control del tabaco y reducir las tasas de tabaquismo en mujeres jóvenes y en edad de procrear, en embarazadas y en los hogares con niños pequeños. La comprensión de este fenómeno tiene el potencial de facilitar el reconocimiento, la prevención y la mitigación de los problemas de conducta infantil relacionados con los factores de riesgo en la vida precoz, así como las consecuencias negativas para la salud a largo plazo.

ESTUDIO 2: "Long-chain polyunsaturated fatty acids, homocysteine at birth and fatty acid desaturase gene cluster polymorphisms are associated with children´s processing speed up to age 9 years."

Este estudio se realizó para analizar los efectos a largo plazo de la suplementación prenatal con FO y/o 5-MTHF, así como el impacto de los polimorfismos genéticos maternos de las

FADS y MTHFR, sobre la velocidad de procesamiento en niños sanos en edad escolar. Nuestros resultados sugieren que ni la suplementación prenatal con FO ni con 5-MTFH predijeron puntuaciones altas de velocidad de procesamiento en niños en edad escolar. Sin embargo, los análisis exploratorios secundarios indican que la proporción de AA/DHA maternos y la heterocigosis de FADS1 en el SNP rs174556 se asocian positivamente con el desarrollo de la velocidad de procesamiento de los niños hasta los 9 años. Sin embargo, las concentraciones de tHcy en el plasma materno en el momento del parto mostraron un efecto negativo sobre la velocidad de procesamiento del niño en edad escolar.

La suplementación prenatal con ácido fólico se ha relacionado con un mejor desarrollo neurológico en la descendencia, en términos de reducción del riesgo de problemas de comportamiento [135], retraso del lenguaje [136], falta de atención [36], hiperactividad y problemas con los compañeros [137]. Sin embargo, los efectos del ácido fólico potenciales sobre el rendimiento cognitivo y mental durante el desarrollo son inconsistentes, en parte debido al número muy

limitado de estudios publicados [138]. El ácido fólico actúa como donante de grupos metilo en la conversión metabólica de homocisteína (Hcy) a metionina [138]. Como consecuencia, el déficit de folatos, sólo o en combinación con otras vitaminas B, atenúa esta vía metabólica, aumentando posteriormente los niveles totales de Hcy. Existe evidencia científica de que una tHcy materna más alta (≥ 8,3 µmol/L) puede no sólo influir negativamente en el desarrollo placentario, el peso al nacimiento y los resultados del embarazo [139], sino que también se relaciona con el deterioro de las células endoteliales dependientes del estrés oxidativo, citotóxico y de la apoptosis del trofoblasto placentario [140,141]. Curiosamente, nuestros resultados sugieren un papel predictor negativo de la tHcy materna en la función cognitiva del niño. De hecho, los niños nacidos de madres con altos niveles de tHcy durante el embarazo muestran una disminución en los aciertos en el SDMT y SCWT, así como una menor probabilidad de estar en los percentiles superiores de WRST y CNST. Debido a que las concentraciones de tHcy se consideran un indicador funcional del estado de folatos, se recomienda el uso de suplementos que contienen ácido fólico durante el primer trimestre de la

gestación para evitar malformaciones congénitas durante el desarrollo fetal [142], y mantener un adecuado estado nutricional de esta vitamina durante todo el embarazo para la optimizar el desarrollo neurológico.

Por otro lado, también está claro que las concentraciones de LC-PUFAs fetales, incluidos los niveles de DHA y AA, están fuertemente relacionados con el estado materno de los LC-PUFAs durante el embarazo, desempeñando un papel importante en el desarrollo cerebral [143] [144]. En este sentido, el DHA está relacionado con la sinaptogénesis, la expresión del factor de crecimiento nervioso y la diferenciación neuronal [145]. Además, el AA está involucrado en varias vías de señalización sináptica [146], síntesis de eicosanoides, prostaglandinas y leucotrienos, y expresión génica temprana relacionada con el desarrollo antropométrico y crecimiento celular [147]. Sin embargo, no existe evidencia consistente de los efectos beneficiosos a largo plazo de la suplementación con CL-PUFAs n-3 o n-6 durante el embarazo o la lactancia en el desarrollo neurológico de los niños [3,55,148]. Como consecuencia, existe un interés creciente por analizar los efectos a largo plazo de la relación AA/DHA sobre el desarrollo

neurológico infantil, que refleja tanto su síntesis endógena como su suministro exógeno [144]. Además, debido a que ambos ácidos grasos compiten por las mismas vías enzimáticas, la proporción AA/DHA está fuertemente influida por la prevalencia de polimorfismos genéticos de las FADS y de elongasas (ELOV1, ELOV2, ELOV3) [145]. Recientemente, una proporción más alta del índice DHA/AA y concentraciones más altas de DHA en la sangre del cordón umbilical se han asociado a un mejor desarrollo visual, cognitivo y motor del bebé en los inuit árticos expuestos a ingestas elevadas de mariscos y LC-PUFAs n-3 [149]. De acuerdo con estos hallazgos, no encontramos asociaciones claras entre el tipo de suplementación prenatal y el desarrollo de la velocidad de procesamiento, excepto por los efectos beneficiosos de la suplementación con FO sobre el tiempo transcurrido de CCTT-1 a los 9 años. Sin embargo, un índice AA/DHA materno más elevado parece ser un modulador positivo a largo plazo de la velocidad de procesamiento (principalmente en SDMT, CCTT-1) en la descendencia, lo que indica la importancia no solo del DHA, sino también de su equilibrio con el AA. Curiosamente, a los 9 años, los niños cuyas madres eran heterocigotas para FADS1 rs174556 realizaron mejor

las tareas de velocidad de procesamiento con respecto a los nacidos de madres con alelos mayores homocigotos. Nuestros hallazgos también son consistentes con el papel de los LC-PUFAs en la mielinización y la integridad de la materia blanca mostrado en estudios con animales [150–152]. En este sentido, el DHA puede aumentar la velocidad de procesamiento al cambiar las propiedades físico-químicas y estructurales de la membrana [153]. Además, Peters et al. [154] no solo demostró que las concentraciones de PUFAs en la membrana de los eritrocitos en adultos jóvenes parecen estar fuertemente relacionadas con la integridad de la sustancia blanca, sino que también mostró que estas asociaciones estaban relacionadas principalmente con los niveles de AA. Así pues, como la conexión entre la integridad de la materia blanca y la velocidad de procesamiento en las tareas cognitivas se establece de manera consistente, nuestros resultados muestran efectos positivos y fuertes a largo plazo de los LC-PUFAs perinatales, (adecuado índice AA/DHA) y los polimorfismos FADS1, sobre el desarrollo cognitivo, lo que sugiere un aumento del volumen de materia blanca y una mejor integridad.

La mayor fortaleza del presente estudio es el seguimiento a largo plazo, desde el embarazo hasta los 9 años, lo que nos permite obtener una mejor evaluación de los efectos a largo plazo de la suplementación prenatal con FO, 5-MTHF o FO+5-MTHF en las capacidades cognitivas del niño. Además, el estudio NUHEAL se realizó en tres países diferentes (España, Alemania y Hungría) con hábitos alimentarios distintos; debido a que el país de origen se ha eliminado como factor de confusión, nuestros datos refuerzan la idea de que los efectos a largo plazo observados en la velocidad de procesamiento son independientes de la dieta de la mujer. En este sentido, destacamos la influencia del nivel cultural de las madres en la velocidad de procesamiento de sus hijos, lo que aumenta la necesidad de tener en cuenta los diferentes factores socioambientales durante la vida temprana que pueden influir en la velocidad de procesamiento posterior. Finalmente, las pruebas neuropsicológicas utilizadas para evaluar la velocidad de procesamiento fueron administradas por diferentes técnicos en cada país, aunque todos recibieron una formación común para reducir la influencia de los examinadores y las diferencias culturales en los resultados.

Los resultados obtenidos tienen algunas limitaciones. Primero, el número de niños que pertenecen a cada grupo de estudio fue homogéneo pero relativamente bajo. Sin embargo, después de combinar los datos de los niños nacidos de madres que recibieron suplementos durante el embarazo (n = 103), los efectos de las combinaciones de suplementos fueron significativos con respecto a los niños cuyas madres recibieron placebo. En segundo lugar, los efectos de la suplementación con FO, 5-MTHF o FO+5-MTHF no se evaluaron en diferentes puntos temporales de administración. Además, nuestro estudio se ha realizado solo en un rango de edad seleccionado (7,5 a 9 años). Por lo tanto, serán necesarios estudios futuros para evaluar si nuestros hallazgos se pueden extender a otros momentos de administración o en diferentes edades durante el desarrollo. Finalmente, la conversión de homocisteína en metionina se basa en gran medida en los niveles de ácido fólico y vitamina B12, que actúan como sustrato y cofactor, respectivamente.

Los resultados obtenidos en este estudio deben interpretarse con precaución. Los participantes del estudio

siguieron sus patrones de alimentación habituales, incluidos los alimentos ricos en PUFAs. Por tanto, es de suma importancia determinar el momento, la duración necesaria y la dosificación de la suplementación de DHA + EPA (en equilibrio con AA) durante el embarazo, y la suplementación de las embarazadas con ácido fólico, para obtener el mejor desarrollo cognitivo de la descendencia. Curiosamente, la suplementación mixta que incluye FO y 5-MTHF no tuvo ningún efecto sobre la velocidad de procesamiento hasta los 9 años, indicando un efecto de interacción de estos dos nutrientes. Por lo tanto, proponemos que la suplementación materna basada en ácido fólico, DHA y EPA debe individualizarse, teniendo en cuenta la dieta, los hábitos, el estado de folato y la variante genética materna FADS1 rs174556 G/A, y tal vez se deban recomendar en distintos momentos de la gestación.

ESTUDIO 3: "Effects of Maternal Fish Oil and/or 5-Methyl-Tetrahydrofolate Supplementation during Pregnancy on Offspring Brain Resting-State at 10 Years Old: A Follow-up Study from the NUHEAL Randomized Controlled Trial"

Este es el primer estudio que examina el efecto a largo plazo de la suplementación materna con FO y/o 5-MTHF en las RSN de los niños en edad escolar. Encontramos que la suplementación con FO, pero no con 5-MTHF, durante la segunda mitad del embarazo se asocia con una menor conectividad funcional de las redes cerebrales de los niños entre los 9,5 y los 10 años de edad. Específicamente, las redes DMN, SMN y FPN mostraron una conectividad funcional más débil en los niños nacidos de madres suplementadas con FO o FO+5-MTHF. Además, después de correlacionar las RSN con las pruebas de NNB, encontramos que en el grupo NO-FO, los niños tuvieron un rendimiento peor en cuanto a pruebas de atención y velocidad de procesamiento.

El hecho de tener una conectividad funcional débil no necesariamente indica un desarrollo neurológico cognitivo deficiente, de hecho, algunos estudios han demostrado, por ejemplo, que la variación en los niveles del cociente intelectual dentro de una población heterogénea se explica principalmente por la eficiencia de la comunicación distribuida de las redes cerebrales usando conexiones moderadamente débiles de larga distancia, con solo una contribución menor de

conexiones más fuertes [155]. En este estudio, los niños expuestos a FO prenatal no mostraron ninguna desventaja funcional pero tendieron a tener mejor memoria. Otro ejemplo de conectividad funcional débil está en los sujetos bilingües en comparación con los monolingües [156,157], donde la fuerza de la conectividad funcional se correlacionó inversamente con el rendimiento conductual.

Teniendo en cuenta las principales diferencias del área del cerebro encontradas en el presente estudio, el *angular gyrus* es una región intermodal que podría actuar como un "centro conector" para el procesamiento global de la información [158], pero también como un área de intercambio entre el DMN y el FPN [159]. Estas funciones son competitivas pero complementarias [160] y podrían enmascarar una relación intrínseca entre la conectividad funcional y el neurodesarrollo. De hecho, los niños nacidos de madres no suplementadas con FO mostraron que la actividad del *angular gyrus* dentro del FPN se relacionó negativamente con el rendimiento en atención y velocidad de procesamiento, lo que sugiere una alteración conjunta relacionada con FO entre la red cerebral más relevante asociada a la cognición (FPN) y el desempeño en

importantes procesos de la inteligencia. Con respecto a varias alteraciones en la red sensoriomotora (SMN), tanto las áreas somatosensoriales (poscentral) como las motoras (*putamen y precentral gyrus*) están involucradas en un estado pre-mediado de disposición para realizar y/o coordinar una tarea motora [83], y sus alteraciones pueden conducir a una descoordinación de movimientos y dificultad de aprendizaje, tal y como lo proponen estudios previos en animales [83]. Sin embargo, la función de esta red cerebral no se limita a la ejecución motora. De hecho, también está presente en los procesos ejecutivos [161], y se sabe que su actividad está más relacionada con la atención a los estímulos externos en comparación con los procesos de atención internos [40]. Los hijos de madres no suplementadas con FO presentaron correlación negativa entre SMN y el desempeño en tareas neuropsicológicas relacionadas con la atención; estos resultados apoyan la asociación previamente estudiada entre los procesos de atención y de procesamiento de velocidad y la función SMN [162].

Se sabe que el motor, la coordinación visomotora [163] y la atención [164] comienzan a desarrollarse durante la gestación, especialmente en el segundo y tercer trimestre del

embarazo; por lo tanto, estos dominios pueden verse afectados por intervenciones nutricionales durante este período crítico de neurodesarrollo, teniendo en cuenta que los ácidos grasos poliinsaturados omega-3 son esenciales debido a su papel en la neurogénesis, la fluidez y la composición de ácidos grasos de la membrana [165]. Algunos estudios sugieren que las intervenciones dietéticas prenatales o posnatales basadas en LC-PUFAs podrían tener efectos a largo plazo en el desarrollo neurológico tardío del niño, principalmente en la atención sostenida, el lenguaje y la velocidad de procesamiento [56,75,166,167]. Sin embargo, no existe ningún estudio concluyente que demuestre los beneficios a largo plazo de la suplementación con LC-PUFAs durante las primeras etapas de la vida sobre el desarrollo neurológico en nutrientes en niños sanos.

En relación al ácido fólico, algunos estudios epidemiológicos [64] y una investigación previa de EEG/ERP del proyecto NUHEAL [66] han sugerido que la suplementación prenatal con folato mejora el rendimiento del neurodesarrollo (principalmente en el sistema de atención) en los niños en edad escolar. Sin embargo, al igual que pasaba con los LC-PUFAs, no existe evidencia clara o sólida que respalde el uso de

suplementos de ácido fólico durante el embarazo y un mejor neurodesarrollo en los niños [168,169]. Debemos tener en cuenta que el grupo No-FO comprende no solo el subgrupo 5-MTHF, sino también el subgrupo placebo, y los resultados de este estudio están dirigidos a reforzar el papel protector de la suplementación materna con LC-PUFAs en el desarrollo y función cerebral de los niños.

Las principales fortalezas de este estudio en el seguimiento a largo plazo, rs-fMRI para evaluar la conectividad funcional y el uso de la NNB para medir diferentes dominios cerebrales. Este nuevo enfoque ha facilitado el estudio de las asociaciones entre la nutrición prenatal, conectoma y el rendimiento cognitivo. Los resultados proporcionados en este manuscrito brindan más información sobre el impacto de la suplementación con LC-PUFAS durante el embarazo en el neurodesarrollo y el funcionamiento cerebral de los niños. Se ha utilizado una gran cantidad de técnicas validadas para evaluar nuestra hipótesis y los datos provienen de una cohorte bien establecida.

La principal limitación de este estudio es el tamaño de la muestra relativamente pequeño en combinación con múltiples resultados. Debido a que un gran número de participantes

fueron excluidos del estudio (32,9%), principalmente debido a un exceso de movimiento durante la sesión de rs-fMRI, se requirió agrupar nuestros cuatro grupos iniciales en dos. En este caso, pudimos garantizar el 80% de la potencia estadística (mínimo requerido n=20 para cada grupo) y para detectar un effect size de 0.7 a 0.9. Además, para evitar falsos positivos con respecto a al análisis de neuroimagen realizado, el TFCE es uno de los enfoques más fiables, que, en nuestro caso, implica 5000 permutaciones para controlar la tasa de error.

Los resultados obtenidos en el presente estudio pueden interpretarse como datos preliminares y no se deben generalizar.

CONCLUSIONES

CONCLUSIONES

1. Las técnicas de minería de datos resultan de vital importancia a la hora de descubrir conocimiento oculto en una base de datos de grandes dimensiones, teniendo en cuenta que la adquisición de datos ha conllevado un gran esfuerzo, así como la cantidad de recursos destinados a dicha labor.
2. Gracias al uso de reglas de asociación, se justifica el hecho de que el tabaquismo materno durante la gestación puede influir en el desarrollo conductual de los niños en edad escolar, incluso considerando otros factores confusores, como es el caso de país de origen, educación de los padres y lactancia materna. Por lo tanto, resulta de vital importancia fomentar la políticas anti-tabaco especialmente para concienciar a las mujeres en edad de concebir y embarazadas.
3. Los niños cuyas madres recibieron aceite de pescado (FO: 500 mg de DHA/día + 150 mg EPA/día) durante la gestación mostraron una mayor velocidad de

procesamiento en la resolución de las tareas del CCTT1. El índice AA/DHA materno al final de la gestación, y la heterocigosis de la variante genética rs174556 de la FADS1 ejercen un efecto beneficioso a largo plazo sobre la velocidad de procesamiento que muestran los niños a los 9 años de edad. El incremento de la Homocisteína total durante la gestación predice una menor velocidad de procesamiento cognitivo en los hijos a los 9 años de edad.

4. Gracias a la técnica de análisis de componentes independientes, la suplementación materna con aceite de pescado durante el embarazo parece tener efectos a largo plazo sobre sobre la conectividad funcional cerebral de los niños en edad escolar, que se asocia a un mejor desarrollo del procesamiento cognitivo.
5. Se refuerza la hipótesis de la posibilidad de una programación nutricional precoz (*Early Nutrition Programming*) que determina efectos a muy largo plazo sobre el funcionamiento cerebral en los hijos.

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ANEXO

Publicaciones asociadas a esta tesis doctoral

- **Hatim Azaryah**, José Antonio García-Santos, Francisco J. Torres-Espínola, Berthold Koletzko, Miguel Pérez-García and Cristina Campoy. ***"Maternal smoking during pregnancy and child behavior problems: A multicenter study from the NUTRIMENTHE EU Project."*** *Submitted - Under Review*
- Cristina Campoy, **Hatim Azaryah** , Francisco José Torres-Espínola, Cristina Martínez-Zaldívar , José Antonio García-Santos, Berthold Koletzko and Miguel Pérez-García. ***"Long-chain polyunsaturated fatty acids, homocysteine at birth and fatty acid desaturase gene cluster polymorphisms are associated with children´s processing speed up to age 9 years.",*** *Submitted - Under Review*
- **Hatim Azaryah**, Juan Verdejo-Román, Cristina Martin-Pérez, José Antonio García-Santos, Cristina Martínez-Zaldívar, Francisco J. Torres-Espínola, Daniel Campos, Berthold Koletzko, Miguel Pérez-García , Andrés Catena and Cristina Campoy. ***"Effects of Maternal Fish Oil and/or 5-Methyl-Tetrahydrofolate Supplementation during Pregnancy on Offspring Brain Resting-State at 10 Years Old: A Follow-up Study from the NUHEAL Randomized Controlled Trial",*** *Nutrients 2020, 12, 2701; doi:10.3390/nu12092701. IF: 4.546*

Otras publicaciones relacionadas con la Tesis doctoral

- Cristina Martínez-Zaldívar, **Hatim Azaryah**, José A. García-Santos, Hans Demmelmair, Signe Altmäe, Eva Reischl, Peter Rzehak, Berthold Koletzko, Cristina Campoy and the NUHEAL team. **"*Early nutrition in combination with polymorphisms in fatty acid desaturase gen cluster modulate fatty acid composition of***

cheek cells' glycerophospholipids in school-age children". *British Journal of Nutrition*, 2019**.**

Otras colaboraciones en publicaciones durante el periodo de Doctorado

- Daniel Campos, Mireia Escudero-Marín , Camila M. Snitman , Francisco José Torres- Espínola , **Hatim Azaryah**, Andrés Catena ,Cristina Campoy, "The Nutritional Profile of Food Advertising for School- Aged Children Via Television. A Longitudinal Approach". Nutrients, Under Review 2020
- Daniel Campos, Francisco J. Torres-Espínola, **Hatim Azaryah**, Cristina Martínez-Zaldívar, Mireia Escudero Martin, Tamas Decsi, Berthold Koletzko, Cristina Campoy. ***"Associations between maternal anthropometry and offspring growth during childhood",*** *Clinical Nutrition* ,2018.
- Tomás Cerdó, Alicia Ruiz, Ruy Jáuregui, **Hatim Azaryah,** Francisco José Torres-Espínola, Luz García-Valdés, M. Teresa Segura, Antonio Suárez, Cristina Campoy. ***"Maternal obesity is associated with gut microbial metabolic potential in offspring during infancy",*** Journal of physiology and biochemistry, 2017.

Comunicaciones presentadas a Congresos

- Tomás Cerdó, Alicia Ruiz, Francisco José Torres-Espínola,Antonio Suarez y Cristina Campoy **Hatim Azaryah,** "Modelo Predictivo Del Desarrollo Cognitivo Infantil En Función De La Microbiota Intestinal, 66 Congreso de la AEP Junio 2018, Zaragoza
- Daniel Campos, Francisco Ortega Porcel, **Hatim Azaryah**, Cristina Campoy. **"Timing of adiposity rebound and Behaviour Problems in early Childhood"** International Symposium «ACTIVE BRAINS FOR ALL», Junio 2017, Granada