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María Esther González Gil

Nutrición, inflamación y riesgo metabólico en niños y adolescentes europeos

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Esther María González Gil

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NUTRITION, INFLAMMATION AND METABOLIC RISK IN EUROPEAN CHILDREN AND ADOLESCENTS

Esther María González Gil

Tesis 2017

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Universidad de Zaragoza

A mi madre, el ejemplo de
superación y lucha de mi vida.

A mi familia.

A mi familia GENUD.

“De eso se trata, de coincidir con gente que te haga ver cosas que tu no ves. Que te enseñen a mirar con otros ojos.”

Mario Benedetti

NUTRICIÓN, INFLAMACIÓN Y RIESGO METABÓLICO EN NIÑOS Y ADOLESCENTES EUROPEOS

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La presente Tesis Doctoral se ha realizado por compendio de publicaciones. A continuación se enumera la lista de artículos incluidos:

I. **González-Gil EM,** Santabárbara J, Siani A, Ahrens W, Sioen I, Eiben G, Günther K, Iacoviello L, Molnar D, Risé P, Russo P, Tornaritis M, Veidebaum T, Galli C, Moreno LA. Whole-blood fatty acids and inflammation in European children: the IDEFICS Study. *Eur J Clin Nutr*. 2016 Jul;70(7):819-23. doi: 10.1038/ejcn.2015.219. Epub 2016 Jan 13.

II. **González-Gil EM,** Santabárbara J, Russo P, Ahrens W, Claessens M, Lissner L, Börnhorst C, Krogh V, Iacoviello L, Molnar D, Siani A, Tornaritis M, Veidebaum T, Moreno LA. Food intake and inflammation in European children: the IDEFICS study. *Eur J Nutr.* 2016 Dec;55(8):2459-2468. doi: 10.1007/s00394-015-1054-3

III. **González-Gil EM**, Tognon G, Lissner L, Intemann T, Pala V, Galli C, Wolters M, Siani A, Veidebaum T, Michels N, Molnar D, Kaprio J, Kourides Y, Fraterman A, Iacoviello L, Picó C, Fernández-Alvira JM, and Moreno Aznar LA on behalf of the IDEFICS Consortiu Prospective associations between dietary patterns and high sensitivity C-Reactive protein in European children: The IDEFICS study. *Eur J Nutr.* 2017 Mar 18. doi: 10.1007/s00394-017-1419-x. *[Epub ahead of print]*

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Ferrari, M, Palacios Le Blé, G , Siani, A, González-Gross, M, Gómez-Martínez, S, Marcos, A and Moreno Aznar,LA on behalf of the HELENA study Ideal cardiovascular health and inflammation in 1 European adolescents: the HELENA study. *(NMCD accepted)*

V. **González-Gil EM**, Gracia-Marco L, Santabárbara J, Molnar D, Amaro-Gahete FJ, Gottrand F, Arouca A, Kafatos A, Widhalm K, Manios Y, Siani A, González-Gross M, Gómez-Martínez S, Díaz L , Leclerq C, Jean Dallongeville J, Marcos A and Moreno Aznar LA on behalf of the HELENA study group. Inflammation and insulin resistance according to body composition in European adolescents: the HELENA study. *(Nutrición Hosp. accepted)*

VI. **González-Gil EM**, Cadenas-Sanchez C, Santabárbara J, Bueno-Lozano G, Iglesia I, González-Gross M, Molnar D, Gottrand F, De Henauw S, Kafatos A, Widhalm K, Manios Y, Siani A, Amaro-Gahete F, Rupérez AI, Cañada D, Censi L, Kersting M, Dallongeville J, Marcos A, Ortega F and Moreno LA on behalf of the HELENA study group. Inflammation in metabolically healthy and metabolically abnormal European adolescents: the HELENA study. *(Pediatric Obesity submitted)*

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RESUMEN

El estudio de los factores de riesgo cardio-metabólicos es importante para intentar prevenir enfermedades futuras. Estos factores de riesgo aparecen cada vez a edades más tempranas como en la adolescencia, o incluso en la infancia, y parecen estar asociados con algunos estilos de vida como la alimentación. Se ha observado que la la inflamación crónica de bajo grado se relaciona con factores de riesgo cardio-metabólicos. Por lo tanto, el estudio del estado inflamatorio en niños y adolescentes es necesario para evaluar esta relación desde sus orígenes y, de esta manera, poder entender sus mecanismos de aparición. Es por ello que el objetivo general de esta Tesis Doctoral es evaluar la relación entre la inflamación, valorada mediante una serie de marcadores inflamatorios, la ingesta y las alteraciones cardio-metabólicas asociadas con la obesidad en niños y adolescentes europeos. Esta memoria se ha realizado por compendio de publicaciones, incluyendo seis artículos.

La presente Tesis Doctoral se ha llevado a cabo teniendo en cuenta los resultados de dos grandes estudios europeos: el estudio IDEFICS (*Identification and Prevention of Dietary- and Lifestyle- induced Health Effects in Children and Infants*) y el estudio HELENA (*Healthy Lifestyle in Europe by Nutrition in Adolescence*). En el estudio IDEFICS se obtuvo información de más de 16.000 niños, con edades comprendidas entre 2 y 9 años, procedentes de ocho países europeos (Italia, Estonia, Chipre, Bélgica, Suecia, Alemania, Hungría y España). La medida incial se realizó durante el curso 2007-2008. Estos niños fueron re-evaluados dos años después del incio del estudio. Se seleccionaron sujetos de este estudio para valorar la asociación entre la dieta y la proteína C-reactiva de alta sensibilidad (PCR-hs), como marcador de inflamación. En el primer artículo se valoró la asociación entre los ácidos grasos, medidos en sangre total, y la inflamación. Los ácidos grasos son componentes de la dieta que se relacionan con el estado inflamatorio, especialmente en el caso de los ácidos grasos de cadena larga. Se observó que los acidos grasos omega-6 (suma de omega 6 y ácido linoleico) se asociaron con valores bajos de PCR-hs, en chicos, y con valores altos de PCR-hs en chicas (ácido araquidónico, suma de omega 6 altamente insaturados y relación

acido araquidónico/linoleico). En el segundo artículo se observó una asociación clara entre la frecuencia de consumo de algunos alimentos y la PCR-hs. Específicamente, la elevada frecuencia de consumo de vegetales se relacionaba inversamente con la inflamación mientras que otros tipos de alimentos, como las bebidas azucaradas o la mayonesa, se relacionaban directamente con la inflamación.

En el tercer artículo, tres tipos de patrones dietéticos fueron identificados y mantenidos a lo largo del seguimiento: el patrón "saludable", el patrón de "proteína animal y carbohidratos refinados" y el patrón "dulces y alimentos procesados". En el análisis transversal, realizado al final del seguimiento, se observó que aquellos niños incluidos en el patrón "dulces y alimentos procesados" mostraban una mayor probabilidad de tener la PCR-hs elevada, en comparación con aquellos asignados a un patrón "saludable". De igual manera, se observó que aquellos incluidos en un patrón de "dulces y alimentos procesados" mantenido en el tiempo, es decir, desde la valoración inicial hasta la medida de seguimiento, mostraban mayor probabilidad de tener valores más elevados de la PCR-hs, en comparación con los incluidos en un patrón saludable en las dos valoraciones.

En el estudio HELENA, realizado entre 2006 y 2007, se valoraron más de 3.000 adolescentes de 10 ciudades europeas: Atenas, Heraklion, Dortmund, Gante, Lille, Pecs, Roma, Estocolmo, Viena y Zaragoza. Las edades de los adolescentes participantes estaban entre 12,5 y 17,5 años. Con datos de este estudio, se valoraron las asociaciones entre el riesgo cardio-metabólico y la inflamación en la adolescencia. La *American Heart Asociation* (AHA) ha propuesto un índice de salud cardiovascular ideal (ISCI) que incluye cuatro comportamientos y tres factores saludables. Los criterios relacionados con los comportamientos son: no haber fumado, ser físicamente activo, tener un IMC normal y tener una alimentación saludable, mientras que los factores saludables incluidos son valores normales de: tensión arterial, colesterol total y glucosa. Mediante el uso de este índice se valoró la relación entre la salud cardiovascular y la inflamación, la cual fue medida mediante un índice inflamatorio y, a su vez, mediante los biomarcadores que componían el citado índice individualmente: PCR, el factor C3 y C4 del complemento, leptina y el recuento de glóbulos blancos. En este cuarto artículo, se observó que puntuaciones superiores del índice de salud

cardiovascular se relacionaban inversamente con los valores del índice inflamatorio y, además, con algunos de sus componentes individualmente.

En el quinto artículo, se observó que la composición corporal juega un papel importante en la relación entre la resistencia a la insulina y la inflamación, medida con varios marcadores inflamatorios. La asociación entre la resistencia a la insulina y el factor C3 del complemento fue especialmente relevante para aquellos adolescentes con mayores niveles de adiposidad. Finalmente, en el último artículo, se valoró la asociación entre la salud metabólica y varios marcadores inflamatorios seleccionados, teniendo en cuenta la presencia o no de sobrepeso/obesidad. La existencia de sobrepeso/obesidad y un estatus metabólico alterado se asocia con marcadores inflamatorios, siendo la PCR, C3 y C4 los marcadores más relacionados con esta condición. C3 y C4 se asociaron consistentemente con la salud cardio-metabólica.

En resumen, los resultados de esta Tesis Doctoral confirman la existencia de una asociación entre alimentación y PCR-hs desde la infancia y, a su vez, que los marcadores de riesgo cardio-metabólico están presentes desde la adolescencia y se asocian con distintos marcadores inflamatorios, en esta etapa de la vida. Estos resultados ponen de manifiesto la importancia del desarrollo de estrategias de prevención precoz, teniendo en cuenta la promoción de estilos de vida saludables, para evitar el desarrollo de los factores de riesgo cardio-metabólico y la aparición de un estado inflamatorio crónico de bajo grado, asociado a los mismos.

ABSTRACT

The study of the cardio-metabolic risk factors is important in order to prevent future diseases. These risk factors appear at an early age like in adolescence, or even in childhood, and they seem to be associated with some lifestyles like eating habits. It has been observed that the low-grade chronic inflammation is related to cardio-metabolic risk factors. Thus, the study of the inflammatory state in children and adolescents is necessary to evaluate this relationship from the origin and to understand the mechanisms that trigger this state. Therefore, the general objective of this Doctoral Thesis is to evaluate the association between inflammation, measured by a set of inflammatory markers, dietary intake and the cardio-metabolic alterations related with obesity, in European children and adolescents.

This Doctoral thesis has been performed with data from two large European studies: IDEFICS study (Identification and Prevention of Dietary- and Lifestyle-induced Health Effects in Children and Infants) and HELENA study (Healthy Lifestyle in Europe by Nutrition in Adolescence). In the IDEFICS study, information from more than 16.000 children aged between 2 to 9 years, from eight European countries (Italy, Estonia, Cyprus, Belgium, Sweden, Germany, Hungary and Spain) was obtained. These children were re-evaluated two years after the beginning of the study. Subjects from this study were selected to assess the association between the diet and the high sensitivity C-reactive protein (hs-CRP), as a marker of inflammation. In the first article, the association between whole blood fatty acids and inflammation was assessed. Fatty acids are dietary compounds related with the inflammatory state, especially the long chain fatty acids. It was observed that the omega-6 fatty acids (sum of omega 6 and linoleic acid) were associated with low concentrations of hs-CRP in boys, and with high concentrations of hs-CRP in girls (arachidonic acid, sum of highly unsaturated omega 6 and ratio arachidonic acid/linoleic acid).

In the second article, a clear association between the frequency of consumption of some food items and hs-CRP was observed. Specifically, the high frequency of consumption of vegetables was inversely related with inflammation while other type of food items, like sugar sweetened drinks or mayonnaise, were directly related.
In the third article, three dietary patterns were identified and maintained during the follow-up: the 'healthy' pattern, the 'animal protein and refined carbohydrates' and the 'sweet and processed' pattern. In the cross-sectional analysis performed at follow-up, children included in the 'sweet and processed' pattern had a higher probability of having high concentrations of hs-CRP, in comparison with those allocated to the 'healthy' pattern. Also, those children included in the 'sweet and processed' pattern over time, i.e. from the baseline measurement to follow-up, showed higher probability of having high concentrations of hs-CRP, in comparison to those included in a 'healthy' pattern in both measurements.

In the HELENA study, performed between 2006 and 2007, more than 3.000 adolescents from ten European cities (Athens, Heraklion, Dortmund, Ghent, Lille, Pecs, Rome, Stockholm, Vienna and Zaragoza), were measured. Age of participants ranged between 12.5 to 17.5. With data from this study, associations between cardio-metabolic risk and inflammation in adolescence were evaluated. The *American Heart Asociation* (AHA) proposed an index of ideal cardiovascular health (ICHI) including four health behaviours and three health factors. The criteria related with the health behaviours are: non-smoke, be physically active, having a normal BMI and eating a healthy diet while the health factors included are having normal values of: blood pressure, total cholesterol and glucose. This index was used to assess the relationship between cardiovascular health and inflammation, which was assessed with an inflammatory score, and with the components of the score: CRP, complement factor C3 and C4, leptin, and the white blood cell count, in the fourth article. It was observed that high values of the ICHI were inversely related with the values of the inflammatory score and, also, with some of the inflammatory components individually.

In the fifth article, it was observed that body composition plays an important role in the relationship between insulin resistance and inflammation, measured by a set of inflammatory markers. The association between insulin resistance and the complement factor C3 was especially relevant for those adolescents with higher levels of adiposity. Finally, in the last article, the association between metabolic health and selected biomarkers, taking into account the presence or not of overweight/ obesity was assessed. The existance of overweight/obesity and an altered metabolic status is associated with inflammatory markers, being the CRP, C3 and C4 the markers most related to this condition. C3 and C4 were associated consistently with cardio-metabolic health.

In summary, results from this Doctoral Thesis confirm the presence of an association between food intake and hs-CRP since childhood and, also, that cardio-metabolic risk markers are present since adolescence and are associated with different inflammatory biomarkers, at this stage of life. These results highlight the importance of the development of strategies of early prevention, taking into account the promotion of healthy lifestyles, in order to avoid the development of cardio-metabolic risk factors and the onset of a low grade chronic inflammation, associated to them.

INTRODUCCIÓN

INTRODUCTION

Hace décadas, en la literatura científica, la inflamación era considerada únicamente como un mecanismo de defensa a corto plazo, caracterizado por una respuesta del organismo frente a agentes externos o propios (1). Esta respuesta adaptativa es un componente crucial de reparación de los tejidos, compuesto, además, por un conjunto de señales complejas de células y órganos que ayudan a la recuperación de la homeostasis. Sin embargo, actualmente existen cada vez más datos que asocian la inflamación con alteración en los procesos metabólicos del organismo. Este estado, mantenido en el tiempo, se relaciona con enfermedades metabólicas y, especialmente, con aquellas asociadas con el riesgo de presentar enfermedades cardiovasculares (2-4). La obesidad, específicamente, el exceso de adiposidad, puede ser uno de los principales desencadenantes de esta inflamación crónica (5) que puede estar presente, incluso, en niños y adolescentes.

LA INFLAMACIÓN

En presencia de una ingesta elevada de alimentos, el estado metabólico se puede desequilibrar originando un exceso de adiposidad y la aparición de los problemas de salud asociados. A su vez, la capacidad de luchar contra las infecciones ha dado lugar a la selección de respuestas inmunes intensas, especialmente tras periodos de epidemias y pandemias. La autorregulación de esta respuesta inmune o inflamatoria involucra mecanismos que producen citoquinas inflamatorias, cascadas de señalización inflamatoria, receptores para mediadores inflamatorios y activación de células reguladoras.

Desde el punto de vista evolutivo, existe una íntima relación entre los sistemas de respuesta inmune y metabólica. El tejido adiposo, el hígado y el sistema hematopoyético han mantenido su herencia evolutiva ya que, en organismos primitivos, eran lugares de coordinación de las respuestas a los patógenos y del estado metabólico (4). Por tanto, sería posible imaginar una situación en la que rutas comunes o superpuestas regularían las funciones metabólicas e inmunes a través de moléculas clave y sistemas de señalización. Es por ello que un exceso nutricional y/o una baja actividad física puedan originar un estado inflamatorio alterado y/o comorbilidades metabólicas, a través de las rutas metabólicas comunes.

Mientras que siempre se ha observado la existencia de enfermedades inflamatorias, sólo en las últimas décadas se ha descrito la condición de inflamación crónica de bajo grado, particularmente en relación con la obesidad, el síndrome metabólico y las enfermedades cardiovasculares.

MECANISMOS DE LA RESPUESTA INFLAMATORIA

La inflamación es un tipo de inmunidad inespecífica. La presencia de estructuras extrañas ponen en marcha mecanismos de reconocimiento de inmunidad innata que inician la respuesta inflamatoria. En la inflamación aguda, la infiltración del tejido por leucocitos inflamatorios requiere la producción de mediadores en el foco de inflamación. Las primeras células en llegar al foco inflamatorio son los neutrófilos, a los que siguen los leucocitos mononucleares (macrófagos o linfocitos), que llegan a ser dominantes en el caso de que la inflamación se haga crónica. Estos neutrófilos, eliminan partículas extrañas mediante fagocitosis. Asimismo, entre las células que infiltran posteriormente el tejido, figuran los macrófagos, que proceden de la maduración de los monocitos circulantes, y que son también especialistas en fagocitosis. Estos macrófagos producen un considerable número de citoquinas (denominadas monoquinas). Entre estas monoquinas, se encuentran el factor alfa de necrosis tumoral (TNF-α), la interleukina-1 (IL-1) y la IL-6, actuando como pirógenos endógenos. Sin embargo, cuando la inflamación es crónica, los neutrófilos, predominantes en la inflamación aguda, son progresivamente reemplazados por células

mononucleares (macrófagos y linfocitos), aumentando la producción de moléculas pro-inflamatorias que pueden aumentar el riesgo metabólico (6).

A su vez, el conjunto de estas respuestas de inmunidad específica pueden inducir inflamación como mecanismo efector; como por ejemplo, la activación de la vía clásica del complemento, como consecuencia de la formación de inmunocomplejos. El sistema del complemento, que está compuesto por proteínas plasmáticas, también puede activar su vía clásica, en presencia de la PCR. La PCR es una proteína plasmática sintetizada en el hígado, principalmente por la IL-6, la cual está a su vez regulada por otras citoquinas inflamatorias como la IL-1 y la TNF-α. Adicionalmente, también se puede producir localmente, por los linfocitos y los monocitos, en las lesiones ateroescleróticas. La PCR comparte propiedades funcionales con las inmunoglobulinas. Es por esta analogía con los anticuerpos, que contribuye a la defensa contra las infecciones y al estado inflamatorio (7).

MARCADORES INFLAMATORIOS

De entre todos los marcadores inflamatorios disponibles para valorar la inflamación, la PCR es el marcador utilizado con más frecuencia, tanto para investigaciones clínicas como para estudios epidemiológicos (8). Sin embargo, la PCR no siempre se asocia con aterosclerosis medida con técnicas de imagen (9). Existen otros parámetros que también se emplean para evaluar el estado inflamatorio ya que el metabolismo de varios de estos biomarcadores pueden compartir rutas metabólicas o verse también alterados. Por ejemplo, la síntesis hepática de la PCR, así como de los factores C3 y C4 del complemento, depende de citoquinas pro inflamatorias liberadas por el tejido adiposo, como TNF-α, IL-1 o la IL-6 (10). Así pues, la elevación de éstas, produce una cascada metabólica que deriva en la elevación de otros biomarcadores. También hay otras moléculas que se pueden ver alteradas en condiciones inflamatorias como las moléculas de adhesión, elevadas durante el estado inflamatorio (11). En la Figura 1 se observan las monoquinas más relevantes secretados por el tejido adiposo.

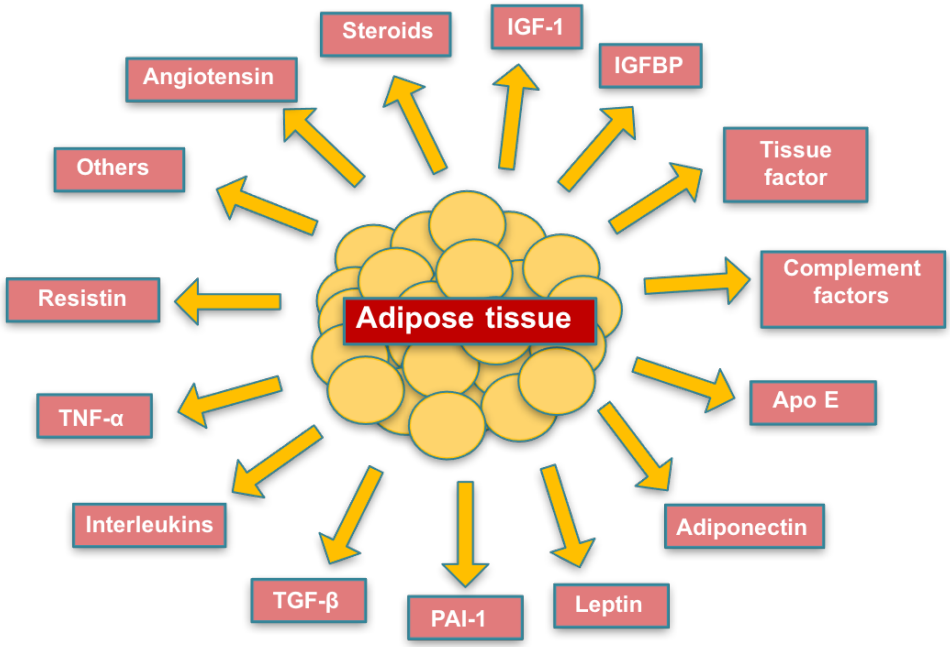

Figura 1. *Adipoquinas producidas en el tejido adiposo blanco. Adaptado de Frühbeck G, et al. (12)*

VALORACIÓN DE LA INGESTA

Hay distintos tipos de métodos de valoración de la ingesta dietética. Entre ellos, por cuestiones de relevancia y practicidad, los más usados son el cuestionario de frecuencia de consumo de alimentos (CFCA) y el recuerdo de 24-horas. Aunque existen otros métodos, como el registro de alimentos de tres o más días o la historia dietética, sólo se revisan aquí los utilizados en esta memoria.

El CFCA es el método más utilizado habitualmente, ya que tiene un bajo coste, es sencillo de completar y ayuda a estimar la ingesta en largos periodos de tiempo. Además, suelen ser auto administrados, lo que facilita mucho su aplicación en estudios epidemiológicos. EL CFCA es útil para clasificar a los individuos según su ingesta de alimentos, grupo de alimentos o nutrientes y se usa habitualmente para valorar la asociación entre dieta y riesgo de enfermedad (13, 14). Sin embargo, este método también tiene sus desventajas, ya que no es muy preciso, al ser difícil la cuantificación de las porciones y al existir detalles de la ingesta que no se consideran, como el tipo de cocinado.

El recuerdo de 24-horas, se usa para describir la ingesta dietética media de un grupo, ya que las medias son robustas y no se ven afectadas por la variación intra-persona (15). El sujeto debe recordar e informar de todas las bebidas y alimentos consumidos durante las últimas 24 horas o durante el día previo. Habitualmente se obtienen recuerdos de días distintos, con semanas de diferencia, para poder tener una visión global de la ingesta. El recuerdo se realiza mediante una entrevista, con un investigador con formación específica en alimentación (15). Entre las ventajas, se encuentra que es un cuestionario detallado y que, al referirse al día anterior, es bastante posible que el entrevistado recuerde su ingesta. Sin embargo, esto también puede ser una limitación, si la persona no la recuerda. Asimismo, otra limitación es que la ingesta de un día no se debe usar para estimar la adecuación de la dieta. Otra de las principales limitaciones es que el recuerdo de 24-horas difícilmente refleja los alimentos consumidos de manera poco frecuente. Por esta razón, se debe completar su información con la de cuestionarios que identifiquen el consumo de los alimentos ocasionales.

ALIMENTACIÓN, PATRONES DIETÉTICOS E INFLAMACIÓN

NUTRIENTES

El consumo de determinados alimentos y nutrientes de la dieta, así como los hábitos o patrones dietéticos, juegan un papel importante en el desencadenamiento de los mecanismos inflamatorios (16-18). Se han descrito distintos nutrientes asociados con la inflamación, aunque los que tienen un papel más importante son los ácidos grasos poliinsaturados (19). La respuesta inflamatoria puede estar modulada por algunos ácidos grasos, a través de distintos mecanismos, como la disminución de la transcripción de citoquinas pro inflamatorias y la expresión superficial vascular de las moléculas de adhesión endotelial leucocitaria (20). Sin embargo, no todos los ácidos grasos juegan el mismo papel en el proceso inflamatorio. El consumo de ácidos grasos poli-insaturados parece que reduce la inflamación (18, 21, 22). Especialmente, los ácidos grasos omega 3 de cadena larga: ácido docosahexaenoico y ácido eicosapentaenoico parece que reducen la producción de mediadores inflamatorios y la expresión de moléculas de adhesión, que son precursores de resolvinas (23, 24). Éstos ácidos grasos también se han relacionado

independientemente con menores concentraciones de PCR (25). Por el contrario, el ácido graso omega 6 de cadena larga más relevante, el ácido araquidónico (AA), puede tener propiedades pro inflamatorias mediante la generación de mediadores lipídicos que causan inflamación venosa y disfunción plaquetaria y endotelial (26). Hay que tener en cuenta que existe una variación individual considerable en las concentraciones de ácidos grasos, que dependen de la ingesta dietética, su absorción y su metabolismo.

ALIMENTOS

El consumo de algunos alimentos, en particular aquellos con características específicas, también pueden estar relacionados con la inflamación (19, 27, 28). Por ejemplo, los alimentos integrales, suelen ser ricos en compuestos bio-activos, con propiedades antiinflamatorias (29). También, los vegetales y frutas, son alimentos de baja densidad energética con alto contenido en agua, fibra, componentes antioxidantes y compuestos fitoquímicos antiinflamatorios (16). Por el contrario, parece que el consumo de determinados alimentos, como la carne roja y algunos alimentos con alto índice glucémico, pueden contribuir al estrés oxidativo y al estado inflamatorio crónico (30-32). La mayoría de los estudios hasta la fecha, se han centrado en el efecto de la ingesta de nutrientes, más que en el consumo de alimentos.

PATRONES DIETÉTICOS

Los patrones dietéticos se perfilan como el mejor método de valoración del comportamiento alimentario ya que consideran la dieta en su conjunto, teniendo en cuenta también las posibles interacciones entre los alimentos, y no solo alimentos o componentes específicos. Además, los patrones dietéticos, pueden dar una mayor perspectiva de los comportamientos dietéticos de las poblaciones estudiadas (33) y parecen ser útiles para relacionar patrones específicos con enfermedades crónicas (34). Algunos estudios ya han valorado la posible relación entre los patrones dietéticos y la inflamación (35, 36). Parece ser que hay una clara asociación positiva entre los patrones dietéticos no saludables y la inflamación ya que aquellos caracterizados por altas ingestas de carne roja y carnes procesadas, dulces, postres, alimentos fritos, y granos refinados están positivamente relacionados con un aumento de marcadores inflamatorios,

moléculas de adhesión y promotores aterogénicos (36, 37). En cambio, patrones caracterizados por elevada ingesta de frutas y verduras, están inversamente asociadas con el estado inflamatorio (38-40).

La alimentación parecen tener también un papel importante en el estado inflamatorio en niños y adolescentes. En cuanto a la relación entre alimentos e inflamación, se han observado los beneficios de las frutas y verduras como alimentos antiinflamatorios en adolescentes (41). También en niños se ha observado que el consumo de verduras y granos se relacionaba negativamente con la PCR, independientemente de la composición corporal. (42). Sin embargo, no hay estudios que relacionen los ácidos grasos en sangre total o los patrones dietéticos con los marcadores inflamatorios en la edad pediátrica. Es por ello que el estudio de los factores modificables, como la dieta, es importante para prevenir y reducir el riesgo a padecer enfermedades en la edad adulta.

INFLAMACIÓN, OBESIDAD Y RIESGO CARDIO-METABÓLICO

OBESIDAD

En individuos con obesidad, la función endocrina se encuentra afectada y los adipocitos y macrófagos del tejido adiposo contribuyen a la producción de citoquinas pro inflamatorias y otras moléculas relacionadas con el metabolismo inflamatorio (43). En personas con obesidad, la función inmune también se puede ver comprometida, pudiendo a su vez derivar en un estado inflamatorio crónico, a través de respuestas inmunes adaptativas e innatas (44). Además del tejido adiposo, existen otros tejidos críticos relacionados con la inflamación crónica, particularmente el tejido hepático. El estado inflamatorio crónico, asociado a la obesidad, está también presente en niños y adolescentes (45).

ENFERMEDAD CARDIOVASCULAR

El estado inflamatorio crónico, se asocia con las enfermedades cardiovasculares (46). La evidencia sugiere que la inflamación tiene un rol importante en el origen y en el desarrollo de la aterosclerosis (47), especialmente en sus estados iniciales,

ya que desencadena la formación de la placa de ateroma y su evolución a placa compleja (46). La aterosclerosis puede originar, en el futuro, la aparición de enfermedades cardiovasculares como las enfermedades coronarias. Por otro lado, la placa aterosclerótica, puede contribuir a su vez a la cascada de producción de más componentes pro inflamatorios que liberan compuestos pro-trombóticos y pro coagulantes que pueden precipitar la formación de trombos (3). En cuanto a las enfermedades cardiovasculares, la aterosclerosis tiene su origen en la infancia (46) y se relaciona con factores de riesgo de aparición precoz (48), aunque los síntomas puedan aparecer varios años después (49). Otros marcadores también se han asociado con el riesgo cardiovascular, incluso en la infancia (50, 51). La salud cardiovascular durante la adolescencia es clave para evitar el desarrollo de futuras enfermedades cardiovasculares (52). La *American Heart Asociation* (AHA) ha propuesto un índice de salud cardiovascular ideal (ISCI) que incluye cuatro comportamientos, y tres factores saludables (53). Los criterios relacionados con los comportamientos son: no haber fumado, ser físicamente activo, tener un IMC normal y llevar una alimentación saludable, mientras que los factores saludables incluidos son valores normales de: tensión arterial, colesterol total y glucosa. En una muestra de adolescentes estadounidenses se ha observado una baja prevalencia del ISCI, siendo los marcadores más difíciles de cumplir, la actividad física y la dieta (54). Además, en otro estudio en población adolescente, el índice de salud cardiovascular ideal se relacionaba inversamente con la intima-media a nivel de la aorta y directamente con la elasticidad aórtica demostrando la importancia de este índice para la prevención precoz de eventos cardiovasculares futuros (55).

RESISTENCIA A LA INSULINA

Además de la contribución al desarrollo de las enfermedades cardiovasculares, la inflamación parece tener un papel importante en la patogénesis de la resistencia a la insulina (56). En sujetos con obesidad, la respuesta inflamatoria puede dar lugar a señales alteradas en las rutas metabólicas que inhiben directamente los receptores de insulina (57). Algunos estudios han observado una asociación entre inflamación e incidencia de diabetes y riesgo de desarrollo de la misma (58, 59).

Además del estado inflamatorio alterado, en estos niños, se pueden observar complicaciones metabólicas asociadas a la inflamación, como resistencia a la insulina (60) y al riesgo de aparición de enfermedades cardiovasculares (61, 62). Esto puede ser debido al cada vez más temprano desarrollo de la obesidad, que conlleva la disfunción del tejido adiposo anteriormente mencionado, pudiendo derivar en un estado inflamatorio crónico.

No siempre la inflamación crónica se asocia con el riesgo cardio-metabólico. Algunos estudios en adolescentes no encontraron una clara asociación entre inflamación crónica y resistencia a la insulina (63, 64), mientras otros sí lo observaron (65). Por lo tanto, se necesitan más estudios en estas poblaciones para confirmar los resultados.

SALUD METABÓLICA

Se ha observado que algunos individuos con obesidad con un perfil metabólico sano (ObMS), presentan un bajo riesgo de enfermedades cardiovasculares (66). Una revisión de la caracterización de estos individuos adultos con obesidad metabólicamente sana ha concluido que bajas concentraciones de marcadores inflamatorios, baja deposición de tejido adiposo y sensibilidad insulínica preservada, contribuyen a este fenotipo de obesidad (67). Sin embargo, no hay ningún estudio hasta la fecha que valore la relación entre inflamación y la ObMS en poblaciones de niños y adolescentes.

JUSTIFICACIÓN

JUSTIFICATION

Las enfermedades crónicas relacionadas con la nutrición, entre las que destacan por su frecuencia las enfermedades cardiovasculares, representan la principal causa de mortalidad y morbilidad en la mayoría de los países del mundo. Es durante la infancia y la adolescencia cuando se establecen y asientan los estilos de vida, siendo esta época cuando pueden aparecer los factores de riesgo de futuras enfermedades cardio-metabólicas. Entre estos estilos de vida, se encuentra la alimentación, relacionada en adultos con el estado inflamatorio crónico de bajo grado, cuyo origen es cada vez más temprano (Figura 2).

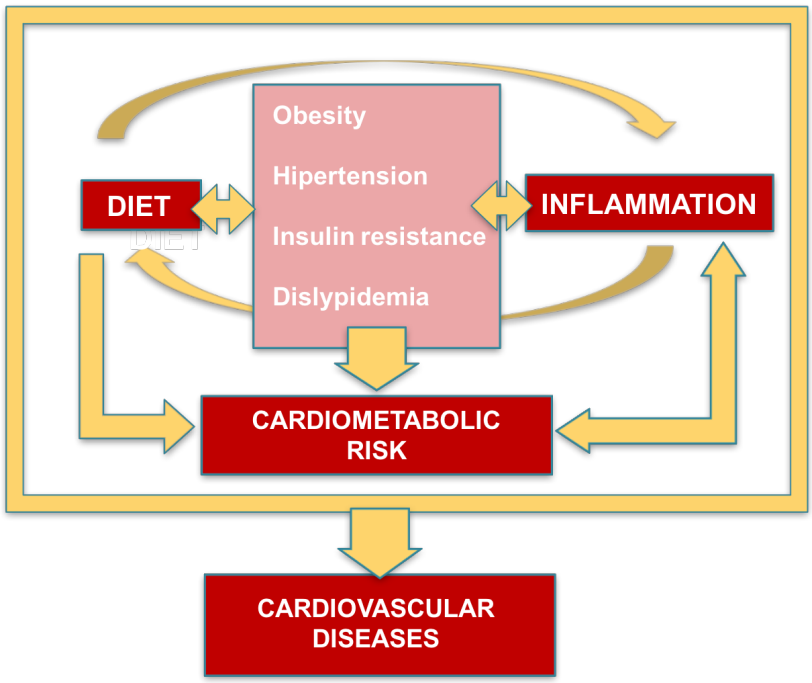

***Figura 2**. Marco conceptual de la relación entre dieta, inflamación y riesgo cardio-metabólico en cuanto a la aparición de enfermedades cardiovasculares.*

Actualmente, no hay estudios en población pediátrica que valoren esta asociación. Es por ello, que el estudio de distintos componentes de la dieta, como los ácidos grasos, **(artículo I),** los alimentos **(artículo II)** y los patrones dietéticos **(artículo III)** parece una buena estrategia para poder valorar, de manera específica y global, si estas asociaciones están presentes desde la infancia.

Asimismo, la relación de la inflamación con la obesidad, y otros factores de riesgo cardio-metabólico, ha sido estudiada en adultos y en poblaciones juveniles. El estudio de esta relación en adolescentes parece una oportuna manera de valorar el riesgo de desarrollar enfermedades futuras. Entre otros, la inflamación se ha relacionado con los factores de riesgo cardiovascular **(artículo IV),** la resistencia a la insulina **(artículo V)** y la salud metabólica **(artículo VI).** Sin embargo, actualmente hay pocos estudios que evalúen la relación entre la inflamación y estos factores de riesgo cardio-metabólico en adolescentes.

Por lo tanto, el estudio y la valoración de estas relaciones entre la dieta, como factor modificable de los estilos de vida, el riesgo metabólico y la inflamación, en las poblaciones infanto-juveniles, pueden ayudar al desarrollo de estrategias de prevención para reducir la probabilidad de desarrollar enfermedades cardiovasculares en la edad adulta.

OBJETIVOS

El **objetivo general** de la presente Tesis Doctoral es evaluar la relación entre la inflamación, valorada mediante una serie de marcadores inflamatorios, las alteraciones cardio-metabólicas asociadas con la obesidad y la ingesta, en niños y adolescentes europeos.
Los objetivos específicos de los seis artículos de la tesis son los siguientes:

ARTICULO I: *Whole-blood fatty acids and inflammation in European Children: the IDEFICS study.*
Objetivo: Valorar la relación entre los ácidos grasos medidos en sangre total y la concentración de PCR-hs en niños europeos.

ARTICULO II: *Food intake and inflammation in European children: the IDEFICS study.*
Objetivo: Valorar la asociación entre frecuencia de consumo de alimentos específicos y los niveles de PCR-hs por por sexo y grupo de edad en niños europeos.

ARTICULO III: *Prospective associations between dietary patterns and high sensitivity C-Reactive protein in European children: The IDEFICS study.*
Objetivo: Describir los patrones dietéticos derivados de los análisis de conglomerados medidos en dos puntos: al inicio del estudio IDEFICS (T0) y en el seguimiento (T1) y asociar, de manera transversal y prospectiva, la relación entre estos patrones dietéticos identificados y la concentración de PCR, como marcador de inflamación.

ARTICULO IV: *Ideal cardiovascular health and inflammation in European adolescents: the HELENA study.*
Objetivo: Evaluar la asociación entre el índice de salud cardiovascular ideal y los marcadores inflamatorios en adolescentes europeos y examinar el uso de un índice inflamatorio para valorar la inflamación en los adolescentes.

ARTICULO V: *Inflammation and insulin resistance according to body composition in European adolescents: the HELENA study.*

Objetivo: Valorar la relación ente los marcadores inflamatorios y la resistencia a la insulina según composición corporal en una muestra de adolescentes europeos.

ARTICULO VI: *Inflammation in metabolically healthy and metabolically abnormal European adolescents: the HELENA study.*

Objetivo: Evaluar la asociación entre un conjunto de marcadores inflamatorios y el estado de salud metabólica, también según índice de masa corporal, en una muestra de adolescentes europeos.

OBJECTIVES

The **general objective** of this doctoral thesis is to evaluate the relationship between inflammation, measured by a set of inflammatory biomarkers, the metabolism and diet in European children and adolescents.
The specific objectives of the six manuscripts of the thesis are the following:

MANUSCRIPT I: *Whole-blood fatty acids and inflammation in European Children: the IDEFICS study.*
Objective: To assess the relationship between whole-blood fatty acids and concentration of high sensitivity C-reactive protein in European children.

MANUSCRIPT II: *Food intake and inflammation in European children: the IDEFICS study.*
Objective: To assess the association between frequency intake of specific food items and the levels of high sensitivity C-reactive protein in European children.

MANUSCRIPT III: *Prospective associations between dietary patterns and high sensitivity C-Reactive protein in European children: The IDEFICS study.*
Objective: Describe cluster analysis-derived dietary patterns in children at two time points: baseline (T0) and follow-up (T1) of the IDEFICS study and to assess the cross-sectional and prospective relationships between the identified dietary patterns and high sensitivity CRP, as a marker of inflammation.

MANUSCRIPT IV: *Ideal cardiovascular health and inflammation in European adolescents: the HELENA study.*
Objective: To assess the association between ideal cardiovascular health and inflammatory markers in European adolescents and to examine the use of an inflammatory score to assess the inflammatory status in adolescents.

MANUSCRIPT V: *Inflammation and insulin resistance according to body composition in European adolescents: the HELENA study.*

Objective: To evaluate the relationship between inflammatory markers and insulin resistance by body composition in a sample of European adolescents.

MANUSCRIPT VI: *Inflammation in metabolically healthy and metabolically abnormal European adolescents: the HELENA study.*

Objective: To assess the relationship between a set of inflammatory markers and the metabolic health status, also according to BMI status, in a sample of European adolescents.

MATERIAL Y MÉTODOS

MATERIAL AND METHODS

Estudio IDEFICS (Identification and Prevention of Dietary- and Lifestyle- induced Health Effects in Children and Infants)

ARTÍCULOS I-III

Muestra y diseño del estudio

El estudio IDEFICS es un estudio prospectivo multi-céntrico en niños europeos reclutados en escuelas de ocho países europeos: Bélgica, Chipre, Estonia, Alemania, Hungría, Italia, España y Suecia. El estudio IDEFICS también tenía integrado un estudio de intervención; para este fin, en cada país, se establecieron dos regiones de características similares. Se contactó a los participantes a través de las escuelas y guarderías. El tamaño total de la muestra a estudiar, se estableció en 16.000 niños, aproximadamente 2.000 niños por país. Esta muestra debía estar distribuida equitativamente por género, región y curso escolar. Se realizaron dos mediciones: inicio (T0) y seguimiento (T1), dos años después (Figura 3). Los niños preescolares y de primer y segundo curso de primaria, fueron incluidos en la medición inicial. La primera medición se realizó entre Septiembre de 2007 y Mayo de 2008, incluyendo un total de 16.228 niños con edades comprendidas entre 2 y 9 años, mientras que la medición de seguimiento, realizada entre Septiembre de 2009 y Mayo de 2010, incluyó 11.038 niños, con edades comprendidas entre 4 y 11 años (se obtuvo una tasa de respuesta del 68%).

Figura 3. *Cronograma de IDEFICS. Datos incluidos en esta memoria de: T0, medida basal, y T1, medida de seguimiento (follow-up).*

Para que un sujeto fuera considerado válido, debía haber completado el cuestionario con información general sobre el niño, cumplimentado por los padres, y tener, además, las medidas antropométricas de peso y altura.

En resumen, usando una serie de métodos armonizados y estandarizados, el estudio IDEFICS ha generado, por primera vez, datos comparables en niños menores de 10 años de 8 países europeos, no solo en cuanto a indicadores básicos de obesidad (peso, altura y otras medidas antropométricas) sino también en cuanto a factores ambientales, sociales, conductuales, de hábitos de vida, genéticos y factores sociales. La cohorte IDEFICS aporta información válida para analizar las relaciones entre estos factores en el desarrollo de alteraciones relacionadas con la dieta y los estilos de vida en niños.
La descripción del estudio así como sus principales características han sido publicadas anteriormente (68).

A continuación se describen únicamente los métodos utilizados para obtener los datos incluidos en los artículos de esta Tesis Doctoral. Para el desarrollo de estos artículos se seleccionaron sub-muestras de la muestra total de IDEFICS en base a haberse realizado las medidas necesarias (Figura 4).

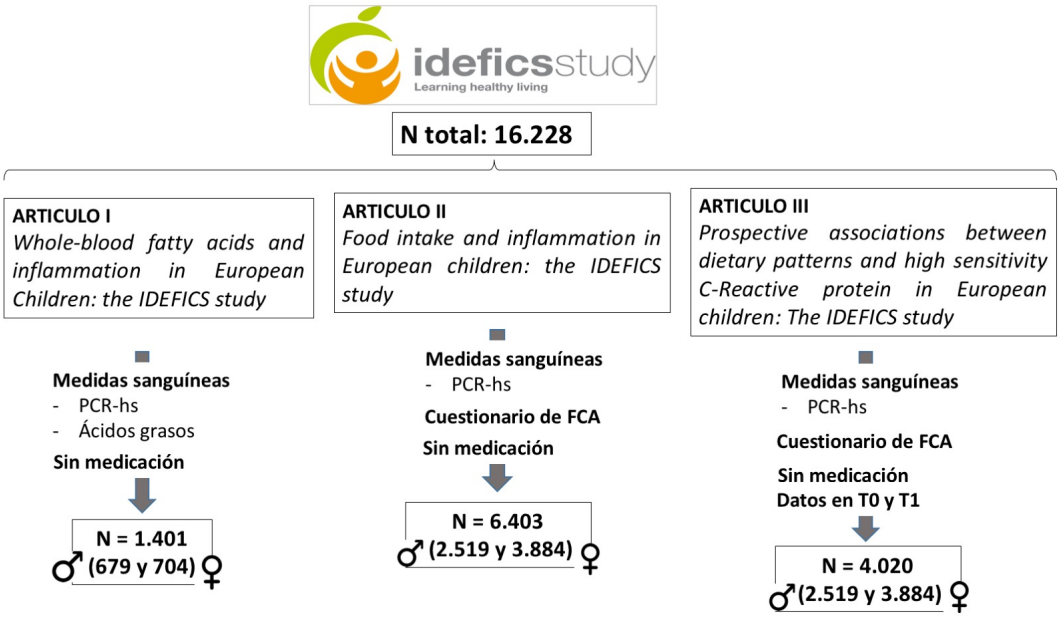

Figura 4. *Sub-muestras seleccionadas en cada uno de los artículos realizados en el estudio IDEFICS (Artículos I-III).*

COMITÉ DE ÉTICA

El estudio IDEFICS se realizó de acuerdo a la Declaración de Helsinki (1975), y las guías éticas de la revisión de Edimburgo de 2000. Se consiguió la aprobación de todos los comités éticos locales en los centros donde se desarrolló el estudio. Todos los niños fueron informados y dieron su consentimiento oral mientras que los padres leyeron una carta informativa y firmaron un consentimiento para participar en el estudio. En el caso de Aragón, el Comité de Ética (CEICA), aprobó la realización de todas las medidas.

MÉTODOS DE MEDIDA

Medidas antropométricas

El peso se midió en ropa interior con una balanza electrónica (TANITA BC 420 SMA, Tanita Europe GmbH, Sindelfingen, Alemania) con una precisión de 100 g, y la altura se midió con los pies descalzos usando un estadiómetro (SECA 225 m, Birmingham, UK) con una precisión de 0,1 cm. El IMC se calculó dividiendo el

peso por la altura en metros, al cuadrado. Los valores de z-score del IMC se calcularon siguiendo el criterio propuesto por Cole (69), teniendo en cuenta los valores específicos para cada edad y sexo. Todas las medidas antropométricas se realizaron por investigadores entrenados siguiendo un protocolo estandarizado (70).

Actividad física

Se valoró la actividad física, de manera objetiva, mediante acelerometría, y de manera subjetiva, mediante cuestionarios en los que los padres informaban de las horas de actividad física que realizaban sus hijos/as. Como no todos los sujetos disponían de la medida realizada con el acelerómetro, la variable de actividad física que se incluyó en los artículos descritos en la presente Tesis fue: 'horas de actividad física en un club deportivo'. Esta medida mostraba correlaciones significativas con la actividad física moderada/intensa obtenida mediante acelerometría (71).

Nivel educativo de los padres

El nivel de educación de ambos padres se categorizó de acuerdo a la Clasificación Estándar Internacional de Educación (ISCED en sus siglas en inglés) (72). Esta clasificación se utilizó para permitir la comparabilidad entre los distintos países participantes en el estudio.

Cuestionario de frecuencia de consumo de alimentos (CFCA) (artículo II y III)

El CFCA utilizado en este estudio, es un instrumento de medida validado, cuyo objetivo es valorar las frecuencias de consumo de un listado de alimentos (73, 74). Este cuestionario no fue diseñado para obtener una estimación de la ingesta energética total o de la cantidad total de ingesta de alimentos, sino para reflejar los hábitos dietéticos relacionados con la obesidad infantil. La fiabilidad del CFCA se ha evaluado mostrando un buen acuerdo en los estimadores de consumo grupal en los niños europeos (75, 76). Los padres/tutores de los niños completaban este cuestionario en el que informaban de las frecuencias de consumo para cada uno de los alimentos que consumían en casa en una semana considerada como normal, durante las cuatro semanas previas. El CFCA incluye

43 grupos de alimentos agrupados por sus características, en 14 grupos. Las posibles respuestas incluyen 7 categorías de frecuencia de consumo: 'nunca/menos de una vez a la semana', '1-3 veces por semana', '4-6 veces por semana', '1 vez al día', '2 veces al día', '3 veces al día' y '4 o más veces al día'; también 'no lo sé' era una posible respuesta. Estas categorías de frecuencia fueron transformadas a veces por semana. Por ejemplo, el valor 7 fue asignado a la categoría: '1 vez al día'. De esta manera conseguimos valores desde 0 a 30. Se realizó una imputación múltiple para estimar valores perdidos en función de género, edad, IMC y país.

Análisis bioquímico

A los niños se les pidió participar, de manera voluntaria, en una extracción sanguínea en ayunas. La descripción de la recogida de sangre y los procesos analíticos posteriores han sido detalladas anteriormente (77).

Las concentraciones de PCR-hs se determinaron en un laboratorio central, mediante nefelometría aumentada por látex (BN2-Nephelometer, Siemens, Deerfield, IL USA) con un límite de detección de 0,02 mg/dL.

Ácidos grasos en sangre total (artículo I)

En el estudio IDEFICS, se midieron 22 ácidos grasos en sangre total. La selección de estos ácidos grasos estuvo basada en su importancia como precursores de compuestos activos, como mayores componentes de la dieta o por su función estructural, en la membrana celular. En la presente Tesis, se seleccionaron 9 de estos ácidos grasos, por su relación con las vías de la activación inflamatoria: acido palmítico, oleico, ácido linoleico (LA), gamma linolenico, AA, alpha linolenico (ALA), eicosapentaenoico (EPA), docosapentaenoico y docosahexaenoico (DHA). Adicionalmente, también se evaluaron relaciones entre estos ácidos grasos así como los ácidos grasos altamente insaturados omega-3, omega-6 y la relación entre ácidos grasos altamente insaturados omega-3 y la suma de éstos con los ácidos grasos altamente insaturados omega-6.

El análisis de los ácidos grasos se realizó con una gota de sangre venosa u obtenida tras punción dactilar. La gota se absorbió en una tira de papel para cromatografía (Scheiler Schuel, Dassel, Alemania; Papel de cromatografía, preparative 165 gsm). La cantidad de sangre recogida fluctuaba entre 15 y 75

(insertar signo especifico) y las tiras de sangre se procesaban rápidamente y se almacenaban a 4 grados centígrados, en sobres individuales cerrados herméticamente (78) y se analizaron por cromatografía de gases (85.10; DANI Instruments S.p.A, Cologno Monzese, Italy).

Estudio HELENA (Healthy Lifestyle in Europe by Nutrition in Adolescence)

ARTÍCULOS IV-VI

Muestra y diseño del estudio

El estudio HELENA es un estudio transversal multi-céntrico realizado entre 2006 y 2007 en 10 ciudades europeas: Atenas, Heraklion, Dortmund, Gante, Lille, Pecs, Roma, Estocolmo, Viena y Zaragoza. Las edades de los adolescentes participantes estaba entre 12,5 y 17,5 años. La muestra total de sujetos es de 3.528 adolescentes europeos.

Para que un sujeto se considerara válido debía: 1) no estar participando simultáneamente en otro estudio clínico; 2) no haber estado enfermo durante la semana anterior a la toma de medidas, 3) tener medidas de peso y altura y 4) haber completado al menos el 75% del resto de pruebas.

Un subgrupo de aproximadamente 1000 adolescentes, de las 10 ciudades participantes, fueron elegidos aleatoriamente para participar en la extracción sanguínea. Como los parámetros sanguíneos tienen mucha menos variabilidad que el resto de variables, una muestra más pequeña es suficiente para ser representativa. El tamaño de los subgrupos (aproximadamente 100 adolescentes en cada ciudad) fue elegido teniendo en cuenta las medias de los parámetros

inmunológicos, ya que fueron las medidas sanguíneas que presentaban una mayor variabilidad entre todos los parámetros incluidos en el estudio (Figura 5).

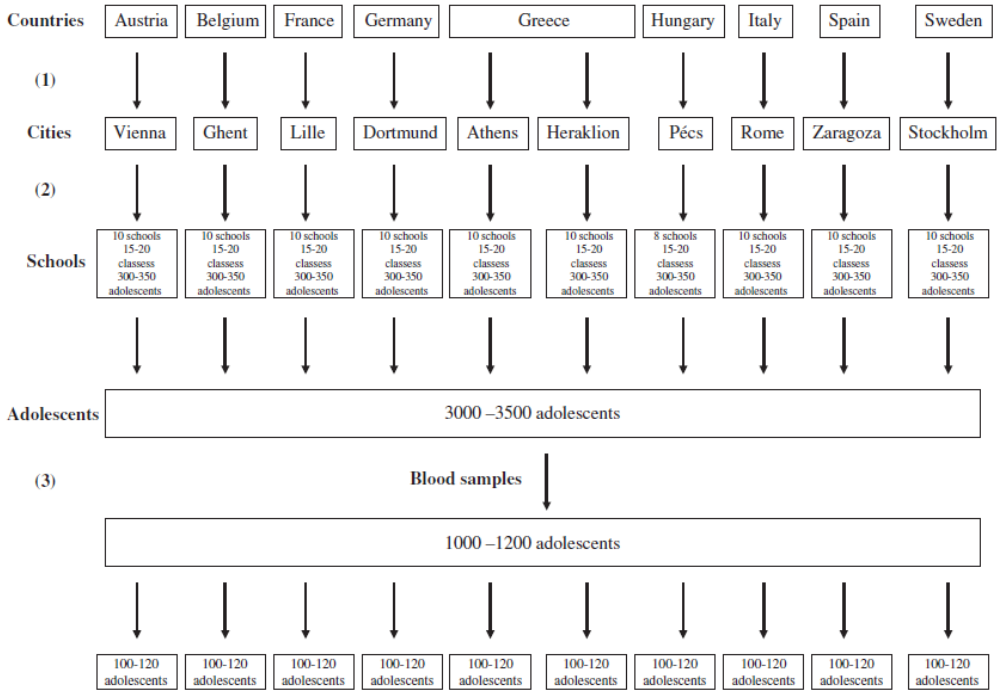

Figura 5. *Muestreo aleatorio por conglomerados en el estudio HELENA.*

Las sub-muestras seleccionadas de la muestra total de HELENA se describen a continuación (Figura 6).

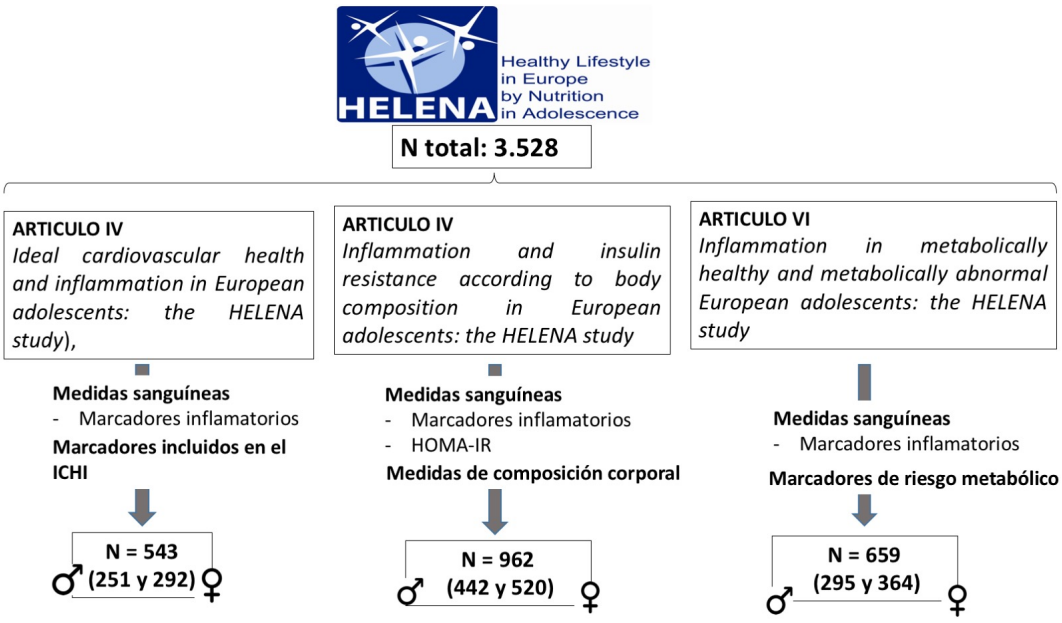

Figura 6. *Sub-muestras seleccionadas en cada uno de los artículos realizados en el estudio HELENA (Artículos IV-VI).*

La metodología general del estudio así como los procedimientos desarrollados para su realización han sido descritos previamente (79).

COMITÉ DE ÉTICA

El estudio HELENA se realizó de acuerdo a las guías éticas de la Declaración de Helsinki (1975), en su la revisión de Edimburgo de 2000 y también según la legislación de investigación epidemiológica de cada uno de los países participantes. Todos los comités éticos locales de los centros donde se desarrolló el estudio aprobaron el protocolo y el desarrollo del estudio. En Aragón, se obtuvo el permiso para la realización del estudio del CEICA Se recopilaron consentimiento informados de los adolescentes participantes y de sus padres/tutores.

MÉTODOS DE MEDIDA

Medidas antropométricas y exploración física

Para la medida del peso y la altura, los adolescentes se encontraban en ropa interior y con los pies descalzos. El peso se midió con una báscula electrónica (SECA 861, Seca Ltd, Birmingham, UK) con una precisión d 100 gramos, rango 0-150 kg, y la altura con un estadiómetro (SECA 225, Seca Ltd), precisión 0.1 cm, rango 70-200 cm. Además, el IMC se calculó como peso en kilogramos dividido por la altura en metros al cuadrado. El perímetro de la cintura se midió con una cinta no elástica. También se midieron los pliegues cutáneos, y de ellos se calculó el porcentaje de grasa, mediante la fórmula de Slaughter (80) y también se calculó el índice de masa grasa (kg/m2). El estadío puberal lo valoró un médico según los criterios de Tanner (5 categorías) (81).

Se midió también la tensión arterial, con un aparato de oscilometría automático (Omron M6)(82). Los adolescentes fueron sentados en una habitación tranquila con sus espaldas apoyadas y pies en el suelo. Se realizaban dos mediciones, con una diferencia de 5 minutos entre ellas. La media de los valores más bajos de las

mediciones era el que se usaba para el análisis de los datos. Todas las medidas fueron tomadas siguiendo un protocolo estandarizado.

Actividad física

Los niveles de actividad física fueron auto-referidos por los adolescentes, usando el cuestionario internacional de actividad física para adolescentes (IPAQ-A en sus siglas en inglés) (83). En este cuestionario se incluían: la actividad física en horario escolar (incluyendo las clases de actividad física y recreos), el transporte, los deberes y las actividades durante el tiempo de ocio. En este cuestionario se recogió el tiempo (y el número de días a la semana) dedicado cada día a estas actividades. Los adolescentes fueron clasificados según el tiempo dedicado a actividad física moderada e intensa (84) IPAQ. (http://www.ipaq.ki.se/ipaq.htm).

Nivel socioeconómico

Para evaluar el nivel socioeconómico de las familias de los adolescentes participantes, se utilizó una versión modificada de la escala de bienestar familiar: Family Afluence Scale (FAS en sus siglas en inglés). Los adolescentes completaban un cuestionario en el que se les preguntaba por el número de coches y de ordenadores que tenían en casa, si disponían de internet en el hogar y si los adolescentes tenían o no su propia habitación. El nivel socioeconómico oscilaba entre 0 (muy bajo nivel socioeconómico) y 8 (nivel socioeconómico muy alto).

Ingesta de alimentos (artículo IV)

Para valorar la ingesta dietética, se utilizó el recordatorio de 24 horas mediante un programa informático, HELENA-DIAT (85), basado en otro desarrollado previamente, llamado YANA-C (86, 87). En un periodo de dos semanas, los adolescentes, con el apoyo de los miembros del equipo investigador, rellenaron dos veces este cuestionario, en días no consecutivos. Los alimentos se agruparon de acuerdo a su composición nutricional en 31 grupos y se calculó el consumo habitual de cada uno de los grupos mediante el multiple source method (MSM), el cual tiene en cuenta la variación intra- e inter- individual (88). Por motivos logísticos, no se obtuvieron datos de Pécs y Heraklion. Se valoró la ingesta de los alimentos mediante las tablas de composición alemanas (German Food Code and

Nutrition Database) debido a que son las más completas a nivel Europeo en cuanto a nutrientes y alimentos. La ingesta de energía se expresa como kilocalorías (Kcal) por día.

Análisis bioquímico

La extracción sanguínea se realizaba a primera hora de la mañana, tras 10 horas de ayuno. Los niveles de PCR se cuantificaron por immunoturbidimetría (AU 2700, Olimpus, Rungis, France). Los adolescentes que presentaron valores superiores a 10 mg/dL de CRP fueron excluidos del estudio. Los factores C3 y C4 del complemento fueron analizados por nefelometría (Behring Diagnostics CA, USA). EL coeficiente de variación intra-ensayo fue de 1,9% para la PCR, 1,4% para el C3 y 1,2% para el C4. Los límites de detección (sensibilidad) fueron 0,007 mg/L para la PCR, 0,01 g /L para el C3 y 0,002 g/L para el C4. El recuento de leucocitos y sus correspondientes porcentajes se determinaron con contadores de células automatizados. La leptina sérica se midió usando el kit de ELISA RayoBio (Enzyme-Linked Immunosorbent Assay RayBiotech, Norcross, USA). La sensibilidad en la medición de leptina fue de 6 pg/mL con variaciones intra-ensayo e inter-ensayo de 10% y 12% respectivamente.

Las citoquinas séricas IL-6 y TNF-α se determinaron usando el kit de alta sensibilidad para citoquinas humanas MILLIPLEX™ MAP kit (Millipore Corp., Billerica, MA, USA) mediante la tecnología X-Map en un aparato Luminex-100 (v.2.3, Luminex Corporation, Austin, TX, USA). La precisión intra-e inter-ensayo fue de 3,5% y 4,5%, respectivamente, para la IL-6; y de 3,5% y 3,8%, respectivamente, para TNF-α. Los límites de detección (sensibilidad) para todos los análisis fueron: 0,1 pg/ml para la IL-6, y 0,05 pg/ml para el TNF-α. Los valores indetectables se establecieron en el límite de detección específico para cada analito. Los niños que presentaron valores de 0,12 pg/mL para el TNF-α y IL-6 fueron excluidos ya que se trataba de un valor asignado para las concentraciones inferiores al límite de detección. La molécula de adhesión sérica sL-selectina fue analizada utilizando un kit comercial ELISA (Diaclone, France), con una sensibilidad de 1 ng/mL.

Las moléculas L-Selectina, sE-Selectina, sVCAM-1 (*vascular cell adhesion molecule-1*, en sus siglas en inglés), sICAM-1 (*soluble intercellular adhesion molecule-1*, en sus

siglas en inglés) se cuantificaron de forma simultánea con un kit multiplex. mediante la tecnología X-Map en un aparato Luminex ®100 (Luminex Corporation, Austin, TX, USA). La sensibilidad de las determinaciones fue: 0,079 ng/mL para sE-Selectina, 0,016 ng/mL para sVCAM-1 y 0,009 ng/mL para sICAM-1. La variación intra-ensayo fue 11,2% para sE-Selectina, 4,5% para sVCAM-1 y 7,9% para sICAM-1.

Los triglicéridos séricos, la glucosa y la lipoproteína de alta densidad se midieron enzimáticamente en suero utilizando un sistema integrado de química clínica (Dade Behring, Schwalbach, Germany) de acuerdo a las instrucciones del fabricante. Los niveles de insulina se obtuvieron usando el Immulite 2000 analyser (DPC Bierman GmbH, Bad Nauheim, Alemania) y se calculó el índice HOMA (Homeostasis Model Assessment) (89) como insulina en ayunas (IU/mL) x glucosa en ayunas (mmol/l)/ 22.5.

ANÁLISIS ESTADÍSTICO

STATISTICAL ANALYSIS

El análisis estadístico de los artículos incluidos en la presente Tesis Doctoral ha seguido un esquema similar. De manera exploratoria se ha realizado inicialmente un test de Kolmogorov-Smirnov en todos los artículos, para valorar la normalidad de la distribución de las variables continuas y, en caso de no cumplimiento, se realizaba la pertinente transformación a la normalidad de la variable. Del mismo modo, en todos los artículos (excepto en el artículo III) se presenta una tabla descriptiva con las características principales de los participantes estratificados por sexo. En esta tabla se muestran los valores medios con su desviación estándar para aquellas variables continuas o porcentajes para aquellas cualitativas de todos los marcadores y variables que se han utilizado en el resto de los análisis. El test T de student fue utilizado para valorar las diferencias por sexo de las variables continuas mientras que el test chi cuadrado se usó para evaluar diferencias en aquellas variables cualitativas.

El análisis de la covarianza (ANCOVA) fue empleado para valorar las diferencias de concentraciones de marcadores por grupos, controlando el efecto de covariables seleccionadas. En el **artículo I**, el ANCOVA se realizó para compararlos valores medios de concentraciones de ácidos grasos en sangre total por grupos de PCR usando como covariables la edad, IMC y horas de actividad física en un club deportivo. Este análisis se realizó en chicos y en chicas por separado. En el **artículo II** se usó el ANCOVA para comparar las diferencias de frecuencia de consumo de alimentos por grupo de PCR, usando como covariable el valor estandarizado del IMC. En el **artículo IV** el ANCOVA se realizó para comparar las concentraciones medias del score inflamatorio por categoría del índice de salud cardiovascular ideal, ajustando por estadío puberal (Tanner)

como covariable y centro como factor aleatorio. En el **artículo V** se compararon las concentraciones medias de los marcadores inflamatorios por tertil de indicador de composición corporal (IMC, índice de masa grasa y circunferencia de cintura). Se utilizaron los residuos estandarizados de la regresión para cada uno de estos indicadores de composición corporal, teniendo en cuenta la edad. Finalmente en el **artículo VI** se realizó un ANCOVA para comparar las diferencias para cada biomarcador inflamatorio para las categorías de salud metabólica/status de IMC por sexo. Las covariables usadas en este análisis fueron: edad y actividad física de moderada a vigorosa valorada mediante cuestionarios.
La corrección de Bonferroni para contrastes múltiples *post-hoc* fue aplicada para para valorar las diferencias entre grupos 2 a 2.

Además del uso del ANCOVA, los artículos presentaban adicionalmente técnicas estadísticas más avanzadas, como modelos de regresión y modelos multivariantes, que pretendían responder a la hipótesis planteada en cada uno de ellos. Por ejemplo, en el **artículo I** se realiza una regresión logística ordinal (dependiente: PCR-hs, independiente: ácidos grasos) con varios modelos controlados por una serie de covariables en el modelo totalmente ajustado: edad, educación de la madre, país, IMC, lactancia materna y horas de actividad física en un club deportivo a la semana. Mientras que en el **artículo II** (dependiente: PCR-hs, independiente: grupos de alimentos) se realiza una regresión logística ordinal multinivel, siendo el país el nivel de agrupación. En este caso los modelos están controlados por: los valores z del IMC, educación de la madre, lactancia materna y horas de actividad física en un club deportivo a la semana. En el **artículo III** se realizó un análisis por conglomerados o *cluster analysis* para identificar agrupaciones de niños con comportamientos dietéticos similares. Posteriormente, una regresión logística multinivel, siendo el país el nivel de agrupación (dependiente: hs-CRP, independiente: patrones dietéticos de manera tranversal y longitudinal). Esta regresión se realizó de manera transversal con los patrones dietéticos con la muestra de inicio (T0) y la muestra de seguimiento (T1) y también se calcularon las Odds Ratio (OR) de los cambios, o persistencia, de los patrones dietéticos en el tiempo. En el **artículo IV** se realizó una regresión logística multinivel, siendo el país el nivel de agrupación (dependiente: índice ideal de salud cardiovascular, independiente: índice inflamatorio). Las

covariables usadas en el modelo totalmente ajustado, fueron: estadío puberal, nivel socioeconómico y fitness cardiorrespiratorio. En el **artículo V** (dependiente: resistencia a la insulina medida mediante HOMA, independiente: cada uno de los marcadores de inflamación medidos) se realizó la regresión lineal múltiple ajustando por centro. Se ajustaron por edad cada uno de los indicadores de composición corporal mediante el cálculo de los residuos estandarizados de la regresión. En el **artículo VI** se realizó una regresión logística multinivel para valorar la asociación entre las categorías de salud metabólica y de IMC (dependiente) y cada marcador de inflamación (independiente) ajustando por edad, sexo y actividad física de moderada a vigorosa.

Todos los análisis estadísticos se han realizado usando el paquete estadístico SPSS (versión 16.0, 19.0 y 21.0; SPSS,Inc) o STATA (versión 13.0). Las figuras y gráficos se han realizado con Sigma Plot (Systat Software, Inc), Prism (GraphPad Software) y Excel (Microsoft).

RESULTADOS

RESULTS

Los resultados de la presente Tesis Doctoral se muestran en forma de artículos científicos siendo el orden el siguiente:

ARTICULO I: *Whole-blood fatty acids and inflammation in European Children: the IDEFICS study.*

ARTICULO II: *Food intake and inflammation in European children: the IDEFICS study.*

ARTICULO III: *Prospective associations between dietary patterns and high sensitivity C-Reactive protein in European children: The IDEFICS study.*

ARTICULO IV: *Ideal cardiovascular health and inflammation in European adolescents: the HELENA study.*

ARTICULO V: *Inflammation and insulin resistance according to body composition in European adolescents: the HELENA study.*

ARTICULO VI: *Inflammation in metabolically healthy and metabolically abnormal European adolescents: the HELENA study.*

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ORIGINAL ARTICLE

Whole-blood fatty acids and inflammation in European children: the IDEFICS Study

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BACKGROUND/OBJECTIVES: Fatty acids are hypothesized to influence cardiovascular disease risk because of their effect on inflammation. The aim of this study is to assess the relationship between whole-blood fatty acids (WBFAs) and high-sensitivity C-reactive protein (hs-CRP) in European children.

SUBJECTS/METHODS: A total of 1401 subjects (697 boys and 704 girls) aged between 2 and 9 years from the IDEFICS (*I*dentification and prevention of *D*ietary- and lifestyle-induced health *EF*fects in *C*hildren and infant*S*) study were measured in this cross-sectional analysis. The sample was divided into three categories of hs-CRP. Associations between WBFA and hs-CRP were assessed by logistic regression models adjusting for body mass index (BMI), country, age, breastfeeding, mother's education and hours of physical activity.

RESULTS: Linoleic acid (LA) ($P = 0.013$, 95% confidence interval (CI): 0.822–0.977) and sum of n-6 WBFA ($P = 0.029$, 95% CI: 0.866–0.992) concentrations were associated with lower concentrations of hs-CRP in boys. In girls, a high ratio of eicosapentaenoic acid (EPA)/arachidonic acid (AA) was associated ($P = 0.018$, 95% CI: 0.892–0.989) with lower hs-CRP concentrations. In contrast, sum of blood n-6 highly unsaturated fatty acids ($P = 0.012$, 95% CI: 1.031–1.284), AA ($P = 0.007$, 95% CI: 1.053–1.395) and AA/LA ratio ($P = 0.005$, 95% CI: 1.102–1.703) were associated ($P < 0.05$) with higher concentrations of hs-CRP in girls.

CONCLUSIONS: The n-6 WBFAs (sum of n-6 FA and LA) were associated with lower hs-CRP in boys and with higher hs-CRP in girls (AA, sum of n-6 highly unsaturated and AA/LA ratio). More studies are needed to identify the optimal levels of WBFAs to avoid low-grade inflammation in children considering the differences by sex and BMI.

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INTRODUCTION

Low-grade chronic inflammation is related with obesity[1–3] and with the onset and development of atherosclerosis.[1,4] Atherosclerosis development is characterized by an interaction between vascular endothelial cells and circulating leukocytes,[5] especially in early stages of the process. High-sensitivity C-reactive protein (hs-CRP) is the most widely used biomarker of inflammation associated with adiposity[6–9] and atherosclerosis progression, as assessed by intima–media thickness, even in children.[7] In previous literature, food, nutrient intake and dietary patterns have also been shown to be associated with hs-CRP, as a marker of inflammation, and cardiovascular disease (CVD) risk factors.[10–12]

Among dietary factors associated with inflammation, fatty acids (FAs) seem to play a relevant role.[12] Inflammatory response can be modulated by some FAs by different mechanisms, such as transcriptional downregulation of proinflammatory cytokines and vascular surface expression of endothelial leukocyte adhesion molecules.[13] However, not all FAs play the same role in the inflammatory process. Consumption of polyunsaturated fatty acids (PUFAs), especially dietary n-3 PUFAs, has been suggested to reduce inflammation.[14–16] N-3 FA blood levels, especially long-chain FAs docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), are related with lower CVD risk.[17] Long-chain n-3 PUFAs decrease the production of inflammatory mediators, eicosanoids, cytokines and reactive oxygen species, and also the expression of adhesion molecules and are precursors of resolvins that are considered anti-inflammatory mediators.[18] In addition, plasma n-3 FAs have been independently associated with lower CRP concentrations.[19] In contrast, the most relevant long-chain n-6 PUFA, the arachidonic acid (AA), has been hypothesized to have proinflammatory properties by generating lipid mediators that cause vessel inflammation and endothelial and platelet dysfunction.[20]

An important issue in studies aimed to investigate associations between FA and inflammation/CVD is the assessment of the FA status on an individual basis. There is considerable individual variability in FA concentrations depending on dietary intake, absorption and metabolism, mainly because of genetic variations.[21] Therefore, the assessments of FA in blood (serum, plasma, erythrocytes, whole blood)

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provide an accurate measure of the FA status in contrast with the estimation of dietary intake.[17] Whole-blood measurement of FA is a recently developed method and is adequate for epidemiological studies as it is valid, noninvasive and time and cost saving.[22] This method, which reflects dietary intake only using fingertip puncture, presented no significant differences with samples obtained from venous blood.[22]

The aim of this exploratory study is to assess the relationship between whole-blood FA (WBFA) levels and hs-CRP to identify WBFAs associated with inflammation in European children.

MATERIALS AND METHODS

Subjects

IDEFICS (*I*dentification and prevention of *D*ietary- and lifestyle-induced health *EF*fects in *C*hildren and infant*S*) is a large European multicenter study on childhood obesity. A total of 16 224 children aged 2–9 years were recruited into a population-based baseline survey from 8 European countries (Sweden, Germany, Hungary, Italy, Cyprus, Spain, Belgium and Estonia). Parents reported sociodemographic, behavioral, medical, nutritional and other lifestyle information for their children and families. Examinations of children included anthropometry, blood pressure, physical fitness, physical activity, DNA from saliva and biochemical markers. Detailed information regarding design, characteristics and participation can be found elsewhere.[23]

This study was conducted according to the Declaration of Helsinki. Approvals for the ethics committee were obtained from the local authorities in each center. All children were informed and provided oral consent while the parents gave their written consent.

Out of the total of 16 228 children, 9601 provided blood samples. The hs-CRP was measured in 9038 of these children but 2647 were excluded as they had taken medication the week before blood drawing. On the other hand, 2600 of the total 16 228 children had the WBFA measured. Of these, 1413 children met the criteria of having hs-CRP and WBFA measured. Cyprus and Belgium were excluded from the study, as the number of subjects in these countries was very low, 3 and 9 respectively. For the current analysis, 1401 subjects were used (697 boys and 704 girls).

Biochemical analysis

Children were asked to participate, on a voluntary basis, in fasting blood draw. A detailed description of sample collection and analytical procedures can be found elsewhere.[24]

The hs-CRP concentrations were measured in a central laboratory with a high-sensitivity assay using latex-enhanced nephelometry (BN2-Nephelometer, Siemens, Deerfield, IL, USA) and the lower limit of detection of the assay was 0.02 mg/dl.

Whole-blood fatty acids

In the IDEFICS study, 22 WBFAs were measured. The selection of these WBFAs was based on their importance in different aspects: precursors of active compounds, as major components of the diet or for their structural function (cell membrane). For this study, 9 of these single WBFAs were selected because of their relation with the pathways of the inflammatory metabolism: palmitic acid, oleic acid, linoleic acid (LA), γ-linoleic acid, AA, α-linolenic acid, EPA, docosapentaenoic acid and DHA.[5,20,25–28] In addition, n-3 highly unsaturated fatty acids (n-3 HUFA), highly unsaturated n-6 (n-6 HUFA) and the ratio between n-3 HUFA and n-3 HUFA+n-6 HUFA (×100 n-3 HUFA) were measured. The ratios calculated are, in some cases, indexes of WBFA conversion (from the precursor to the product, for example, LA/AA) or a simple ratio (n-3/n-6) with possible implications in the synthesis of eicosanoids.

WBFA analysis

The drop of blood was obtained by punching the fingertip with a lancet from an automatic lancing device, and blood was absorbed on a strip of paper for chromatography (Schleicher Schuell, Dassel, Germany; Chromatography Paper, preparative, 165 gsm). If the children agreed to venous puncture, a drop of blood was taken from the sample to be applied to the WBFA strip. The amount of blood collected fluctuated between 15 and 75 μg (equivalent to the same values in μl). The strips with the drop of blood were either immediately processed or stored at 4 °C in individual cellophane envelopes with airtight closure for better storage. Further information can be found elsewhere.[29]

Covariates

The models were adjusted for potential confounders, selected on the basis of previously published associations with hs-CRP or associations with exposures or outcomes in the present analysis. Covariates used for the analysis were: age, body mass index (BMI), country, education of the mother, breastfeeding (BF) and self-reported hours of physical activity (PA) in a sport club per week. The International Standard Classification of Education (ISCED) was used to report mother's education.[30] BF was treated as a dichotomous variable, being yes if the mother stated months of BF, or no if the child was not breastfed. In order to maximize the sample, self-reported hours of PA in a sport club were taken. In the IDEFICS study, children's weekly hours in sports club activities was significantly correlated with data from accelerometers.[31] BMI was calculated using measured weight and height.[32]

Statistical analysis

Kolmogorov–Smirnov test was used to contrast normality. The analyses were performed separately in boys and girls, as sex was an effect modifier when assessing the associations.

Because ~50% of children had values less than the minimal detectable concentration, in this analysis hs-CRP is treated as a categorical rather than a continuous variable. The following three hs-CRP cutoffs were defined to categorize hs-CRP levels in our population: (1) hs-CRP under the detecti on limit (<0.02 mg/dl); (2) hs-CRP >0.02 mg/dl and $<$75th sex-specific percentile of those with hs-CRP values over the detection limit (<0.21 mg/dl in boys and 0.22 mg/dl in girls); and (3) hs-CRP $>$75th sex-specific percentile of those with hs-CRP values over the detection limit (>0.21 mg/dl in boys and >0.22 mg/dl in girls).[33] Analysis of covariance was performed, separately in boys and girls, to assess the differences in mean WBFA concentrations between categories of hs-CRP. The covariables entered in the model were the continuous ones: age, BMI and self-reported hours of PA per week. The *post hoc* comparisons between hs-CRP groups were conducted with a Bonferroni correction applied.

To assess the association between hs-CRP groups and concentration of each WBFA, we used ordinal logistic regression models. In order to explore mechanisms of the association, we used two hierarchical models in which we controlled for potential confounders (age, mother education, country, BMI, BF and self-reported hours of PA in sports club per week). Model 1, presented as Supplementary Material, included each WBFA, age, mother education and country. Model 2 was a fully adjusted model including BMI, BF and self-reported hours of PA in sports club per week in addition to the covariates assessed in model 1.

The odds ratio (OR) of the WBFA presented the percentage of being in the upper CRP group by increasing the WBFA concentration in 1 unit. In contrast, the ORs of the ratios, ratio DHA/AA, ratio EPA/AA, ratio AA/LA, ratio AA/dihommo-γ-linoleic acid and ratio n-6/n-3, showed the percentage of being in the upper CRP group by increasing the ratio in 0.1 units.

Data were managed and analyzed with the IBM SPSS Statistics v.19 (IBM Corp., New York, NY, USA, 2010).

RESULTS

Sample characteristics

Baseline characteristics by sex and distribution by country of participants are shown in Table 1. The mean serum concentration of hs-CRP was significantly higher in girls than in boys ($P<0.001$); in contrast, boys had significantly higher concentrations of some WBFAs such as docosapentaenoic acid ($P<0.001$), the sum of the n-6 highly unsaturated WBFA ($P<0.001$) and the ratio AA/LA ($P<0.001$).

In addition, analysis of covariance was performed using continuous covariables, BMI, age and self reported hours of PA in a sports club, to assess the differences between mean concentrations of each WBFA and the three categories of hs-CRP by gender. This analysis is presented in Supplementary Tables S1 and S2.

Table 1. Descriptive characteristics of the study participants

	Boys, n = *697*		*Girls,* n = *704*		P-*value*
Country	*n* (%)		*n* (%)		
Estonia	38 (5.5%)		47 (6.7%)		—
Germany	190 (27.3%)		201 (28.6%)		—
Hungary	76 (10.9%)		89 (12.6%)		—
Italy	265 (38%)		260 (36.9%)		—
Spain	78 (11.2%)		55 (7.8%)		—
Sweden	50 (7.2%)		52 (7.4%)		—
Mother's educational level	*n* (%)		*n* (%)		
ISCED level 1	46 (7.1%)		52(8%)		
ISCED level 2	173 (26.8%)		153 (23.5%)		
ISCED level 3	245 (37.9%)		255 (39.2%)		
ISCED level 4	61 (9.4%)		661(10.2%)		
ISCED level 5	121 (18.7%)		124 (19.1%)		
Breast feeding (yes), *n* (%)	376 (53.9%)		383 (54.4%)		
Hs-CRP groups					
CRP I	260 (37.3%)		179 (25.4%)		
CRP II	329 (47.2%)		397 (56.5%)		
CRP III	108 (15.5%)		128 (18.2%)		
	Mean	s.d.	Mean	s.d.	
Age (years)	6.41	1.72	6.46	1.71	0.442
Physical activity[a] (h)	1.13	1.58	1.10	1.53	0.703
BMI (kg/m^2)	18.22	3.56	18.09	3.34	0.780
hs-CRP (mg/dl)	0.14	0.32	0.16	0.36	< **0.001**
Palmitic acid (% of total FA)	25.72	1.42	25.76	1.42	0.610
Oleic acid (% of total FA)	18.47	2.04	18.71	2.15	**0.035**
Linoleic acid (% of total FA)	18.1	2.0	18.3	2.04	0.065
GLA (% of total FA)	0.23	0.08	0.22	0.08	**0.006**
Arachidonic acid (% of total FA)	7.61	1.34	7.41	1.38	**0.006**
ALA (% of total FA)	0.19	0.07	0.2	0.08	0.976
EPA (% of total FA)	0.26	0.09	0.26	0.09	0.173
DPA (% of total FA)	0.54	0.16	0.51	0.15	< **0.001**
DHA (% of total FA)	1.19	0.41	1.15	0.41	**0.030**
Sum n-3 HUFA (% of total FA)	2.01	0.59	1.92	0.59	**0.007**
Sum n-6 HUFA (% of total FA)	10.27	1.79	9.91	1.85	< **0.001**
×100 n-3 HUFA (% of total FA)	16.31	3.57	16.18	3.68	0.390
Ratio DHA/AA	0.15	0.04	0.15	0.04	0.286
Ratio EPA/AA	0.03	0.01	0.03	0.01	0.903
Ratio AA/LA	0.42	0.08	0.4	0.01	< **0.001**
Ratio AA/DHGLA	6.1	1.25	6.3	1.31	**0.004**
Ratio n-6/n-3	13.81	3.97	14.23	4.16	0.055
Sum n-6 (% of total FA)	28.63	2.63	28.5	2.67	0.378
Sum n-3 (% of total FA)	2.21	0.61	2.13	0.61	**0.007**
SFA (% of total FA)	44.36	1.86	44.28	1.86	0.437
MUFA (% of total FA)	24.65	2.37	24.94	2.48	**0.026**
PUFA (% of total FA)	30.83	2.78	30.59	2.87	0.129

Abbreviations: AA, arachidonic acid; ALA, α-linolenic acid; BMI, body mass index; DHA, docosahexaenoic acid; DHGLA, dihommo-γ-linoleic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acid; GLA, γ-linolenic acid; hs-CRP, high-sensitivity C-reactive protein; ISCED, International Standard Classification of Education; LA, linoleic acid; MUFA, sum of monounsaturated fatty acids; n-3 HUFA, highly unsaturated n-3; n-6 HUFA, highly unsaturated n-6; ×100 n-3 HUFA, ratio between n-3 HUFA and n-3 HUFA+n-6 HUFA; (), PUFA, sum of all polyunsaturated fatty acids; SFA, sum of saturated fatty acids. Shown are percentages, mean values and s.d. values. [a]Self-reported hours of physical activity in a sport club per week. Bold entries indicate significant $P < 0.005$.

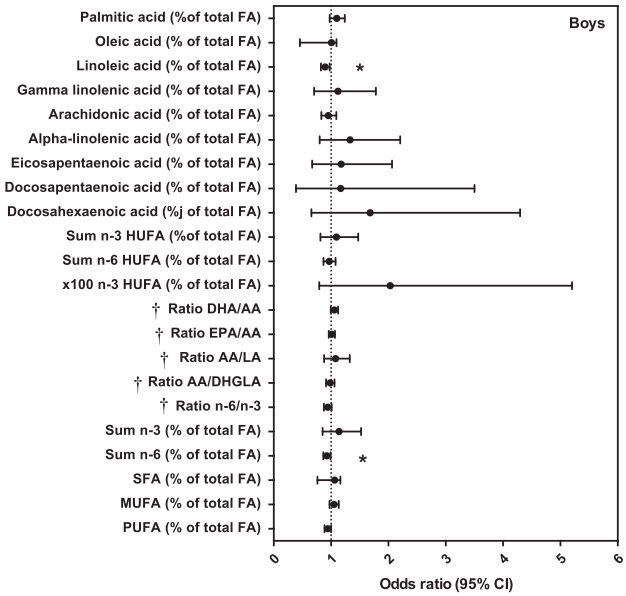

Figure 1. OR (95% CI) assessing the association between hs-CRP categories and blood fatty acid concentrations in boys. Logistic regression model adjusted by: age, education of the mother, country, BMI, BF and self-reported hours of PA in a sports club. DHA, n-3 HUFA, n-6 HUFA, ratio between n-3 HUFA and n-3 HUFA+ n-6 HUFA (×100 n-3 HUFA), AA, LA, dihommo-γ- linoleic acid (DHGLA), sum of saturated fatty acids (SFA), sum of monounsaturated fatty acids (MUFA) and sum of all polyunsaturated fatty acids.[42] [†]OR of the ratios, ratio DHA/AA, ratio EPA/AA, ratio AA/LA, ratio AA/DHGLA and ratio n-6/n-3, showed the percentage of being in the upper CRP group by increasing the ratio in 0.1 units. *$P < 0.05$.

Multivariate analysis

In the multivariate analysis, model 1 (Supplementary Tables S3 and S4) and model 2 (Figures 1 and 2), hs-CRP group is considered as dependent variable and each WBFA as independent variable.

In boys, blood concentrations of some WBFAs were significantly associated with higher concentration of hs-CRP. The highest OR observed in the model 1 (Supplementary Table S3) was the concentration of the n-6 γ-linolenic acid (OR = 1.82, 95% confidence interval (CI): 1.177–2.824, $P = 0.007$), meaning that for each unit increased of this acid, the probability of reaching upper level of hs-CRP group increased by 82% after controlling for age, mother education and country. When BMI, self-reported hours of PA per week in a sport club and BF (Figure 1) were added to model 1, some blood WBFAs decreased the probability of reaching the upper level of hs-CRP, such as in the case of LA (OR = 0.89, 95% CI: 0.822–0.977, $P = 0.013$) and sum of n-6 (OR = 0.92, 95% CI: 0.866–0.992, $P = 0.029$).

In girls (Supplementary Table S3), blood concentration of some WBFAs also significantly increased the probability of reaching the upper level of hs-CRP when increasing the concentration of the WBFA in 1 unit: γ-linolenic acid by 92% (OR = 1.92, 95% CI: 1.240–2.989, $P = 0.004$), AA by 24% (OR = 1.24, 95% CI: 1.089–1.415, $P < 0.001$) and for sum of n-6 HUFAs by 20% (OR = 1.20, 95% CI: 1.084–1.331, $P < 0.001$). In addition, when increasing the ratio AA/LA in 0.1 units, the probability of reaching the upper level of hs-CRP increased by 40% (OR = 1.404, 95% CI: 1.149–1.553, $P = 0.001$). In contrast, increases of 0.1 units in blood concentration of DHA/AA ratio decreased the probability of having a higher concentration of hs-CRP by 6% (OR = 0.939, 95% CI: 0.884–0.998, $P = 0.044$). Some of these relationships were also observed when adding the following covariables: BMI, self-reported hours of PA and BF (Figure 2) to model 1. This was the case for AA (OR = 1.21, 95% CI: 1.053–1.395, $P = 0.007$) the sum of n-6 HUFAs (OR = 1.15, 95% CI: 1.031–1.284, $P = 0.012$) and AA/ LA ratio (OR = 1.37, 95% CI: 1.102–1.703, $P = 0.005$) by increasing the concentration in 0.1 units. In contrast, blood EPA/AA ratio (OR = 0.939, 95% CI: 0.892–0.989, $P = 0.018$) significantly decreased the probability of having a higher concentration of hs-CRP.

Out of all covariables, in the ordinal logistic regression, BMI, as continuous variable, showed association with some of the WBFAs, specifically n-3 HUFAs ($P < 0.001$) and sum of n-3 ($P < 0.001$).

DISCUSSION

In a subsample of European children participating in the baseline IDEFICS cross-sectional study, associations were observed, stratifying by sex, between WBFA and hs-CRP concentrations after controlling by a set of potential confounders.

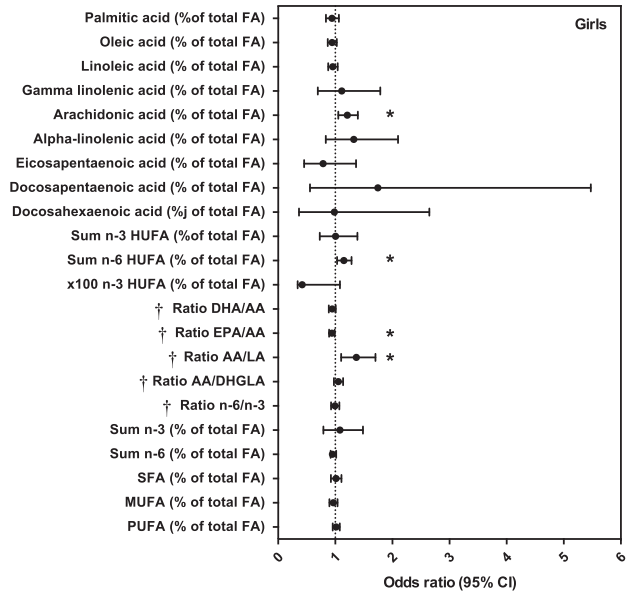

Figure 2. OR (95% CI) assessing the association between hs-CRP categories and blood fatty acid concentrations in girls. Logistic regression model adjusted by: age, education of the mother, country, BMI, BF and self-reported hours of PA in a sports club. DHA, n-3 HUFA, n-6 HUFA, ratio between n-3 HUFA and n-3 HUFA+ n-6 HUFA (×100 n-3 HUFA), AA, LA, dihommo-γ- linoleic acid (DHGLA), sum of saturated fatty acids (SFA), sum of monounsaturated fatty acids (MUFA) and sum of all polyunsaturated fatty acids.[42] †OR of the ratios, ratio DHA/AA, ratio EPA/AA, ratio AA/LA, ratio AA/DHGLA and ratio n-6/n-3, showed the percentage of being in the upper CRP group by increasing the ratio in 0.1 units. *$P < 0.05$.

Among the main findings of our study, LA concentration and sum of n-6 WBFA concentrations were inversely associated with hs-CRP concentrations in boys, whereas EPA/AA ratio was inversely associated with hs-CRP in girls. In addition, DHA/AA ratio was inversely associated with hs-CRP when adjusting by age, education and country in the logistic regression for both sexes.

These results found in boys are in line with previous studies: total PUFA concentrations have anti-inflammatory properties, as the presence of double bonds, regardless of the n-3 or n-6 bond position, seems to be crucial to the modulation of endothelial–leukocyte interaction.[5]

In girls, the DHA/AA and EPA/AA ratios were inversely associated with hs-CRP concentrations. In previous studies, the blood n-3 to n-6 PUFA ratio and DHA/AA ratio were negatively associated with change in plaque volume in patients with coronary artery disease,[34] whereas EPA/AA ratio was inversely related with major coronary events in a general Japanese population.[35] EPA has been described as a clinically relevant measurement[17] and, along with DHA, has the opposite influence with AA.[27,36] AA is the main n-6 long-chain FA and is a source of prostaglandins and leukotrienes, mediators that cause vessel inflammation and endothelial and platelets dysfunction, and has been related with ischemic heart disease.[20] EPA and DHA prevent the conversion of AA to pro-inflammatory eicosanoids and the formation of anti-inflammatory compounds.[37] This would shift the production of inflammatory eicosanoids synthesized from n-6 PUFA to n-3 PUFA, less inflammatory than their AA-derived eicosanoid compounds.[36]

Furthermore, in girls, the WBFAs associated with hs-CRP concentrations were: sum of n-6 HUFAs, AA and ratio AA/LA. As mentioned before, AA has a role in inflammation and immune function as it is precursor of prostaglandins, leukotrienes and related compounds.[38] In our study, high AA/LA ratios, index of the conversion rate of LA to AA, was positively correlated with enhanced proinflammatory conditions, in support of the concept that enhanced production of the proinflammatory AA may play a role.

Differences found by gender are in line with previous data suggesting more efficient PUFA metabolism in women than in men. Apparently, estrogens stimulates whereas testosterone inhibits the conversion of short chained FAs to their longer chain derivates, although the effect seems to be moderate.[39] These gender differences are higher regarding the synthesis of long-chain n-3 FA, especially DHA, in adults and adolescents.[40,41]

Among all the covariables, BMI showed the strongest association with hs-CRP concentrations in children, confirming previous literature[8] that suggest that BMI should be taking into account when assessing hs-CRP and WBFA.

The strengths of the study are the use of standardized data from children living in six European countries and the population, as there is no literature assessing the relationship between WBFA and inflammation in healthy young European children. In addition, the use of WBFA concentrations is a strength as, in addition to dietary intake, there are physiological and genetic mechanisms explaining the large variability on body FA status. Additionally, blood FA concentrations are an important clinical measurement to assess CVD risk.[17] The first limitation of the study is the use of hs-CRP alone, as the measurement of additional inflammatory biomarkers would have been useful to explain the mechanisms for these associations. Another limitation is that country is not considered separately for sample size reasons; however, country as covariable is considered in both models of the logistic ordinal regression. Finally, the cross-sectional study design does not allow examining causality.

In conclusion, the results of this exploratory study suggest that n-6 WBFAs presented more associations with hs-CRP in children than n-3 in both sexes. The n-6 WBFAs, specifically sum of n-6 FA and LA, were associated with lower hs-CRP in boys, and AA, sum of n-6 highly unsaturated and AA/LA ratio with higher hs-CRP in girls. In addition, higher EPA/AA and DHA/AA concentrations ratios were associated with low serum hs-CRP concentrations across the different analyses in girls. Furthermore, these results suggest that sex and BMI should be taken into account when assessing blood concentrations of WBFA. More studies in children are needed to identify the optimal levels of WBFA to avoid systemic low-grade inflammation that could lead to cardiovascular diseases in later life.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies this paper on European Journal of Clinical Nutrition website (http://www.nature.com/ejcn)

MATERIAL SUPLEMENTARIO

SUPPLEMENTARY MATERIAL

Table S1. Mean whole blood fatty acids concentrations (95% CI) by group of high sensitive C-reactive protein (hs-CRP) in boys, controlling by age, body mass index and self-reported hours of physical activity in a sport club per week.

Fatty acid (% of total FA)	I (n=254)	II (n=318)	III (n=107)	p for trend
Palmitic acid (% of total FA)	25.75(25.57-25.94)	25.63(25.48-25.79)	25.98(25.69-26.28)	0.103
Oleic acid (% of total FA)	18.52(18.26-18.78)	18.42(18.21-18.64)	18.51(18.1-18.92)	0.826
Linoleic acid (% of total FA)	18.38(18.12-18.64)	18.03(17.81-18.25)	17.7(17.29-18.11)*	0.022
Gamma linolenic acid (% of total	0.23(0.22-0.24)	0.24(0.23-0.25)	0.22(0.21-0.24)	0.082
Arachidonic acid (% of total FA)	7.51(7.34-7.68)	7.71(7.56-7.85)	7.55(7.28-7.83)	0.197
Alpha-linolenic acid (% of total FA)	0.19(0.18-0.21)	0.19(0.19-0.21)	0.19(0.18-0.21)	0.891
Eicosapentaenoic acid (% of total	0.26(0.25-0.27)	0.27(0.26-0.28)	0.25(0.23-0.27) †	0.009
Docosapentaenoic acid (% of total	0.53(0.51-0.55)	0.55(0.54-0.57)	0.53(0.5-0.57)	0.232
Docosahexaenoic acid (% of total	1.13(1.07-1.18)	1.24(1.2-1.29)*	1.18(1.09-1.26)	0.003
Sum n-3 HUFA (% of total FA)	1.93(1.86-2.01)	2.08(2.02-2.15)*	1.96(1.84-2.08)	0.005
Sum n-6 HUFA (% of total FA)	10.11(9.88-10.33)	10.41(10.22-10.61)	10.21(9.85-10.57)	0.118
x100 n-3 HUFA (% of total FA)	16(15.54-16.43)	16.67(16.29-17.05)*	16.03(15.32-16.74)	0.030
Ratio DHA/AA	0.15(0.14-0.15)	0.16(0.15-0.16)*	0.15(0.14-0.16)	0.005
Ratio EPA/AA	0.03(0.03-0.04)	0.03(0.03-0.04)	0.03(0.03-0.03)	0.197
Ratio AA/LA	0.41(0.4-0.42)	0.43(0.42-0.44)	0.43(0.41-0.45)	0.068
Ratio AA/DHGLA	6.08(5.92-6.24)	6.07(5.93-6.02)	6.15(5.89-6.4)	0.991
Ratio n-6/n-3	14.39(13.88-14.89)	13.35(12.92-13.77)*	13.93(13.13-14.72)	0.015
Sum n-3 (% of total FA)	2.13(2.06-2.21)	2.29(2.22-2.36)*	2.16(2.04-2.29)	0.006
Sum n-6 (% of total FA)	28.72(28.38-29.05)	28.72(28.44-29.01)	28.17(27.64-28.7)	0.178
SFA (% of total FA)	44.37(44.04-44.62)	44.26(44.05-44.46)	44.68(44.3-45.07)	0.143
MUFA (% of total FA)	24.58(24.28-24.89)	24.63(24.38-24.89)	24.83(24.35-25.32)	0.713
PUFA (% of total FA)	30.88(30.53-31.24)	30.95(30.65-31.25)	30.34(29.77-30.91)	0.168

*(I) CRP below the detection limit (<0.02mg/dL); (II) hs-CRP ≥ 0.02 to 0.21 (III) hs-CRP higher than 0.21. Highly unsaturated n-3 (n-3 HUFA), highly unsaturated n-6 (n-6 HUFA), ratio between n-3 HUFA and n-3 HUFA+n-6 HUFA (x100 n-3 HUFA), docosahexaenoic acid (DHA), arachidonic acid (AA), eicosapentaenoic acid (EPA), linoleic acid (LA), dihommo gamma linolenic acid (DHGLA), sum of saturated fatty acids (SFA), sum of monounsaturated fatty acids (MUFA) sum of all polyunsaturated fatty acids (PUFA). Post-hoc comparisons between hs-CRP groups with Bonferroni correction applied. *p<0.05 ref CRP I, † p<0.05 ref CRP II*

Blood fatty acids and CRP

In boys (Table S1) there were some statistically significant associations ($p<0.05$) between the group of hs-CRP and the concentration of some whole blood fatty acids (WBFA). The WBFA that showed significantly positive association with hs-CRP were: EPA ($p=0.009$), DHA ($p=0.003$), ratio DHA/AA ($p=0.005$), ratio between the two major long chain PUFAs in the n-3 and the n-6 series, respectively, sum of n-3 high unsaturated fatty acids (HUFAs) ($p=0.005$), ratio between n-3 HUFAs and n-3 HUFAs + n-6 HUFAs ($p=0.030$), sum of n-3 ($p=0.006$), ratio n-6/n-3 ($p=0.015$). LA showed inverse association with hs-CRP ($p=0.022$) with the CRP groups.

Table S2. Mean whole blood fatty acids concentration (95% CI) by group of high sensitive C-reactive protein (hs-CRP) in girls, controlling by age, body mass index and self-reported hours of physical activity in a sport club per week.

Fatty acid	I (n=172)	II (n=384)	III (n=121)	p for trend
Palmitic acid (% of total FA)	25.94(25.72-26.17)	25.74(25.59-25.88)	25.7(25.43-25.97)	0.275
Oleic acid (% of total FA)	18.89(18.56-19.22)	18.79(18.58-19.01)	18.32(17.92-18.72)	0.083
Linoleic acid (% of total FA)	18.42(18.11-18.72)	18.31(18.12-18.51)	18.07(17.7-18.45)	0.399
Gamma linolenic acid (% of total	0.22(0.21-0.24)	0.22(0.22-0.23)	0.23(0.21-0.24)	0.946
Arachidonic acid (% of total FA)	7.16(6.95-7.37)	7.37(7.23-7.5)	7.83(7.58-8.01)* †	0.001
Alpha-linolenic acid (% of total FA)	0.2(0.18-0.21)	0.21(0.19-0.21)	0.18(0.17-0.2)	0.384
Eicosapentaenoic acid (% of total	0.26(0.24-0.27)	0.26(0.25-0.27)	0.25(0.23-0.27)	0.544
Docosapentaenoic acid (% of total	0.51(0.48-0.53)	0.51(0.49-0.52)	0.52(0.49-0.55)	0.760
Docosahexaenoic acid (% of total	1.13(1.06-1.19)	1.14(1.1-1.18)	1.19(1.11-1.26)	0.546
Sum n-3 HUFA (% of total FA)	1.91(1.82-2)	1.91(1.85-1.97)	1.96(1.85-2.07)	0.741
Sum n-6 HUFA (% of total FA)	9.61(9.32-9.89)	9.87(9.69-10.05)	10.43(10.09-10.77)* †	0.002
x100 n-3 HUFA (% of total FA)	16.33(15.76-16.89)	16.27(15.91-16.63)	15.58(14.9-16.26)	0.232
Ratio DHA/AA	0.15(0.14-0.16)	0.15(0.15-0.16)	0.15(0.14-0.16)	0.529
Ratio EPA/AA	0.03(0.03-0.04)	0.03(0.03-0.04)	0.03(0.03-0.03)* †	0.015
Ratio AA/LA	0.39(0.38-0.4)	0.4(0.39-0.41)	0.43(0.42-0.45)* †	<0.001
Ratio AA/DHGLA	6.18(5.98-6.39)	6.23(6.1-6.36)	6.57(6.33-6.82)* †	0.036
Ratio n-6/n-3	14.5(13.86-15.14)	14.11(13.7-14.52)	14.21(13.44-14.98)	0.778
Sum n-3 (% of total FA)	2.09(2.01-2.19)	2.12(2.06-2.18)	2.15(2.04-2.26)	0.760
Sum n-6 (% of total FA)	28.31(27.9-28.72)	28.51(28.24-28.77)	28.69(28.19-29.18)	0.532
SFA (% of total FA)	44.33(44.04-44.62)	44.24(44.05-44.42)	44.4(44.05-44.75)	0.672
MUFA (% of total FA)	25.09(24.7-25.47)	25.02(24.78-25.27)	24.57(24.11-25.03)	0.197
PUFA (% of total FA)	30.41(29.96-30.85)	30.58(30.29-30.86)	30.81(30.27-31.34)	0.562

*(I) CRP under the detection limit (<0.02mg/dL); (II) hs-CRP ≥ 0.02 to 0.22 (III) hs-CRP higher than 0.22. Highly unsaturated n-3 (n-3 HUFA), highly unsaturated n-6 (n-6 HUFA), ratio between n-3 HUFA and n-3 HUFA+ n-6 HUFA (x100 n-3 HUFA), docosahexaenoic acid (DHA), arachidonic acid (AA), eicosapentaenoic acid (EPA), linoleic acid (LA), dihommo gamma linolenic acid (DHGLA), sum of saturated fatty acids (SFA), sum of monounsaturated fatty acids (MUFA) sum of all polyunsaturated fatty acids (PUFA). Post-hoc comparisons between hs-CRP groups with Bonferroni correction applied. *p<0.05 ref CRP I, † p<0.05 ref CRP II*

Blood fatty acids and CRP

In girls (Table S2), significant associations with hs-CRP were found with the following WBFA: AA (p=0.001), the sum of n-6 HUFAs (p=0.002), the ratio AA / LA (p<0.001) and ratio AA /dihomo gamma linolenic acid (DHGLA) (p=0.036). Also an inversely association was found between hs-CRP and the blood EPA/AA ratio (p=0.015).

Table S3. Odds ratio (OR) (95% confidence interval) assessing the association between hs-CRP categories and whole blood fatty acids concentrations, in boys. Logistic regression model adjusted by: age, education of the mother and country.

	Logistic regression		
Fatty acids (% of total FA)	OR	95% CI	p-value
Palmitic acid (% of total FA)	1.020	0.911-1.141	0.730
Oleic acid (% of total FA)	0.971	1.052-1.614	0.479
Linoleic acid (% of total FA)	0.949	0.876-1.029	0.211
Gamma linolenic acid (% of total FA)	1.824	1.177-2.824	0.007
Arachidonic acid (% of total FA)	1.048	0.921-1.192	0.477
Alpha-linolenic acid (% of total FA)	1.459	0.900-2.366	0.125
Eicosapentaenoic acid (% of total FA)	1.402	0.826-2.380	0.211
Docosapentaenoic acid (% of total	1.752	0.625-4.904	0.286
Docosahexaenoic acid (% of total FA)	2.096	0.864-5.078	0.102
Sum n-3 HUFA (% of total FA)	1.217	0.920-1.606	0.168
Sum n-6 HUFA (% of total FA)	1.063	0.960-1.176	0.243
x100 n-3 HUFA (% of total FA)	1.721	0.712-4.158	0.227
Ratio DHA/AA*	1.051	0.992-1.115	0.089
Ratio EPA/AA*	1.011	0.960-1.063	0.684
Ratio AA/LA*	1.122	0.922-1.365	0.249
Ratio AA/DHGLA*	0.950	0.884-1.020	0.163
Ratio n-6/n-3*	0.944	0.883-1.008	0.090
Sum n-3 (% of total FA)	1.239	0.944-1.626	0.123
Sum n-6 (% of total FA)	0.997	0.936-1.062	0.926
SFA (% of total FA)	0.946	0.871-1.027	0.185
MUFA (% of total FA)	1.033	0.961-1.110	0.379
PUFA (% of total FA)	1.008	0.613-1.068	0.772

*Docosahexaenoic acid (DHA), arachidonic acid (AA), highly unsaturated omega 3 (Omega 3 HUFA), highly unsaturated omega 6 (Omega 6 HUFA), ratio between omega 3 HUFA and omega 3 HUFA+ omega 6 HUFA (x100 omega 3 HUFA), eicosapentaenoic acid (EPA), linoleic acid (LA), dihommo gamma linolenic acid (DHGLA), sum of saturated fatty acids (SFA), sum of monounsaturated fatty acids (MUFA) sum of all polyunsaturated fatty acids (PUFA). *Values represent OR changes for 0.1 units.*

Table S4. Odds ratio (OR) (95% confidence interval) assessing the association between hs-CRP categories and whole blood fatty acids concentrations, in girls. Logistic regression model adjusted by: age, education of the mother and country.

	Logistic regression		
Fatty acids (% of total FA)	OR	95% CI	p-value
Palmitic acid (% of total FA)	0.976	0.871-1.093	0.672
Oleic acid (% of total FA)	0.944	0.872-1.022	0.156
Linoleic acid (% of total FA)	0.956	0.882-1.037	0.277
Gamma linolenic acid (% of total	1.925	1.240-2.989	0.004
Arachidonic acid (% of total FA)	1.241	1.089-1.415	0.001
Alpha-linolenic acid (% of total FA)	1.456	0.944-2.248	0.089
Eicosapentaenoic acid (% of total	0.962	0.577-1.603	0.880
Docosapentaenoic acid (% of total	2.002	0.678-5.900	0.209
Docosahexaenoic acid (% of total	0.947	0.376-2.385	0.908
Sum n-3 HUFA (% of total FA)	1.006	0.746-1.357	0.968
Sum n-6 HUFA (% of total FA)	1.202	1.084-1.331	<0.001
x100 n-3 HUFA (% of total FA)	0.422	0.173-1.031	0.058
Ratio DHA/AA*	0.939	0.884-0.998	0.044
Ratio EPA/AA	0.957	0.911-1.004	0.077
Ratio AA/LA*	1.404	1.149-1.553	0.001
Ratio AA/DHGLA*	0.998	0.928-1,073	0.964
Ratio n-6/n-3*	0.989	0.924-1.058	0.753
Sum n-3 (% of total FA)	1.074	0.803-1.435	0.634
Sum n-6 (% of total FA)	1.040	0.975-1.108	0.236
SFA (% of total FA)	0.950	0.871-1.036	0.245
MUFA (% of total FA)	0.975	0.909-1.046	0.484
PUFA (% of total FA)	1.029	0.972-1.091	0.321

*Docosahexaenoic acid (DHA), arachidonic acid (AA), highly unsaturated n-3 (n-3 HUFA), highly unsaturated n-6 (n-6 HUFA), ratio between n-3 HUFA and n-3 HUFA+ n-6 HUFA (x100 n-3 HUFA), eicosapentaenoic acid (EPA), linoleic acid (LA), dihommo gamma linolenic acid (DHGLA), sum of saturated fatty acids (SFA), sum of monounsaturated fatty acids (MUFA) sum of all polyunsaturated fatty acids (PUFA). *Values represent OR changes for 0.1 unit.*

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ORIGINAL CONTRIBUTION

Food intake and inflammation in European children: the IDEFICS study

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Abstract
Purpose This cross-sectional study assesses the relationship between consumption frequencies of food items and high-sensitivity C-reactive protein (hs-CRP) in European children.
Methods Out of the baseline sample ($N = 16.228$) of the IDEFICS study, 6.403 children (1.315 boys aged 2 to <6, 1.908 boys aged 6 to <10, 1.204 girls aged 2 to <6 and 1.976 girls aged 6 to <10 years) had hs-CRP measured and the Children's Eating Habits Questionnaire filled, including a food frequency questionnaire. Logistic regression adjusted for body mass index z-score, education of the mother, breast-feeding and self-reported hours of physical activity in a sport club per week was conducted.
Results Mean frequency intake of raw vegetable was lower in boys ($p = 0.022$ in young and $p = 0.020$ in old) and older girls ($p = 0.026$) with high hs-CRP concentration, while in younger girls ($p = 0.008$) the same occurred with the cooked vegetables. The probability of having higher hs-CRP concentration was significantly associated with having low consumption frequency of vegetables ($p = 0.004$ in older boys, raw vegetables; and $p = 0.0032$ in younger girls, cooked vegetables). Also, honey/jam intake decreased the probability of having higher concentration of

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hs-CRP, whereas soft drinks with sugar, mayonnaise and cereals milled increased this probability.

Conclusions Out of all food items associated with hs-CRP, frequency intake of vegetables presented more associations across all the analysis. Findings suggest that a high-frequency intake of vegetables is inversely related to an inflammatory status in children. More studies are needed to assess the association between diet and inflammation.

Keywords Food intake · Inflammation · European · Children · IDEFICS

Introduction

Chronic low-grade inflammation is associated with cardiovascular diseases (CVD) [1], due to its role in the development of atherosclerosis [2]. Adipose tissue contributes to the release of a large variety of inflammatory mediators into the bloodstream, including cytokines, acute phase reactants, growth factors and proteins involved in glucose homeostasis [3, 4]. High-sensitivity C-reactive protein (hs-CRP) represents the most commonly measured inflammatory biomarker in clinical and epidemiologic studies and is associated with adiposity and cardiovascular risk factors [5], even in children [6, 7].

Dietary habits, especially the consumption of specific foods and dietary components, seem to play a role in inflammation [8, 9]. Foods such as whole-grain foods are rich in bioactive compounds with anti-inflammatory properties [10]. Also, vegetables and fruits are low-energy dense foods with high contents of water, fiber and rich in antioxidant compounds and other anti-inflammatory phytochemicals [8]. Previous studies have shown inverse correlation between fruit and vegetable consumption and serum levels of inflammatory markers in adults [11] and adolescents [12]. In contrast, consumption of red meat [13] and high glycemic index (GI) foods may contribute to oxidative stress and chronic low-grade inflammation even in lean subjects [14, 15].

Regarding dietary components, the relationship between dietary n-3 fatty acids, alpha linoleic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) and inflammation, is widely accepted [16, 17]. Epidemiological studies have shown an inverse association between dietary fish or fish oil (EPA and DHA) consumption and biomarkers of inflammation [18, 19]. It appears that the overall quantity of fat intake, the sources and type of dietary fat, with special emphasis on some FA and the ratio of n-6/n-3, play a crucial role in modulating inflammation.

Most of the dietary studies have focused on intakes of nutrients or food components and not food intake. As these components are not consumed individually, but within food items, it is also important to study the effect that food items may have on the inflammatory status in different populations.

This study aims to assess the relationship between frequency intake of food items and hs-CRP in European children by sex and age group, independently of their body mass index z-score (zBMI) and of other potential confounders.

Materials and methods

Subjects

Data were obtained from the baseline survey of the IDEFICS study. Identification and prevention of dietary- and lifestyle-induced health effects in children and infants (IDEFICS) is a large European multicenter study addressing childhood obesity. A cohort of 16.228 children aged 2–9 years was recruited into a population-based baseline survey from eight European countries, ranging from the north to the south and from the east to the west (Sweden, Germany, Hungary, Italy, Cyprus, Spain, Belgium and Estonia). Parents reported socio-demographic, behavioral, medical, nutritional and other lifestyle information for their children and families. Examinations of children included anthropometry, blood pressure, physical activity (recorded with questionnaires and accelerometry), physical fitness, DNA from saliva and biochemical markers in blood and urine. Detailed information regarding the designs, characteristics and participation rates can be found elsewhere [20].

This study was conducted according to the guidelines laid down in the Declaration of Helsinki. Approvals by the Ethics committee were obtained from the local authorities in each participant center. All children were informed and provided oral consent, while the parents gave their written consent.

Out of the total of 16.228, 9.601 children provided blood samples. hs-CRP was measured in 9.038 out of them, but 2.635 were excluded as they had taken medications the week before blood drawing. Finally, 6.403 children (1.315 boys aged 2 to <6 years, 1.908 boys aged 6 to <10 years, 1.204 girls aged 2 to <6 years and 1.976 girls aged 6 to <10 years) met the inclusion criteria of the present study, i.e., having a valid hs-CRP measurement and providing the FFQ part of the Children's Eating Habits Questionnaire (CEHQ).

Food consumption

The CEHQ is a screening instrument that aims to assess usual consumption frequencies of listed food items and

of dietary behaviors associated with overweight, obesity and general health in children. The primary caregivers completed the food frequency section of the CEHQ in which they reported the consumption frequencies for each food item at home during a typical week over the previous four weeks. The CEHQ-FFQ included 43 food groups, which were further clustered in 14 food groups [21]. Responses included eight frequency categories of consumption: 'never/less than once a week,' '1–3 times per week,' '4–6 times per week,' '1 time per day,' '2 times per day,' '3 times per day' and '4 or more times per day'; also 'I have no idea' was a possible answer. Frequencies categories were converted into times per week, i.e., the value 7 was assigned to the category: '1 time per day.' In our sample, 'milk with sugar' and 'milk without sugar' were included in milk total, and 'pizza' and 'hamburgers/hot dogs' were included in processed meals; therefore, 41 food items were considered in the analysis. The CEHQ-FFQ was previously validated against objective biomarkers [21].

Biochemical analysis

Children were asked to participate, on a voluntary basis, in fasting blood drawing. A detailed description of sample collection and analytical procedures in the IDEFICS survey has been published [22]. To avoid pricking the finger of children who agreed to the venous puncture, one drop of venous blood was used for a point-of-care analysis. A second drop of blood was applied to a test strip for the analysis of the fatty acid profile.

hs-CRP concentrations were measured in a central laboratory with a high-sensitivity assay using latex-enhanced nephelometry (BN2-Nephelometer, Siemens, Deerfield, IL USA). The lower limit of detection of the assay was 0.02 mg/dL.

Anthropometric measurements

Weight was measured in light underwear with an electronic scale (TANITA BC 420 SMA, Tanita Europe GmbH, Sindelfingen, Germany), and height was measured without shoes using a stadiometer (SECA 225 m, Birmingham, UK). Body mass index (BMI) was calculated as weight divided by squared height. BMI z-score (zBMI) according to Cole et al. [23] was calculated to account for differences in BMI by age and gender.

Covariates

Covariates used for the analysis were: zBMI, self-reported education of the mother, breast-feeding (BF) and hours of physical activity (PA) in a sport club per week. The International Standard Classification of Education (ISCED) was used for mother's education in order to maintain equivalence between countries [24]. Maternal education has been identified as the most consistent predictor of children's diet [25]. As accelerometry data were available in a subsample, self-reported hours of physical activity in a sport club were used as a proxy of physical activity. In the IDEFICS study, children's weekly hours in sports club activities were significantly correlated with children's daily time spent in moderate-to-vigorous PA as measured by accelerometers [26].

Statistical analysis

Analyses were performed stratifying by sex and age group. hs-CRP was treated as a categorical variable. The following three hs-CRP cutoffs were used to categorize hs-CRP levels in our population: CRP I: hs-CRP detection limit (0.02 mg/dL); CRP II: hs-CRP > 0.02 mg/dL and <50th sex-specific percentile (0.06 mg/dL in boys and 0.07 mg/dL in girls) of those with hs-CRP values above the detection limit; and CRP III: hs-CRP ≥ 50th sex-specific percentile (0.06 mg/dL in boys and 0.07 mg/dL in girls) of those with hs-CRP values above the detection limit [27]. Analysis of covariance (ANCOVA) was performed to assess the differences in mean weekly consumption frequencies of food items at home between categories of hs-CRP with zBMI included. Post hoc comparisons between hs-CRP groups were conducted applying a Bonferroni correction.

To assess the association between hs-CRP groups and consumption frequencies per week of each food item, multilevel ordinal logistic regression models (level: country) were used to estimate odds ratios (OR) and 95 % confidence intervals. Since children were clustered by study design, a random effect coefficient for country was added. In order to explore associations between food items and hs-CRP, we performed a model for each of the 14 food groups, e.g., *raw vegetables* were included in the model together with *cooked vegetables* and *fries potatoes*. The resulting food groups were used in the logistic regression models to reduce the multiple testing problems and hence to receive more meaningful results. In addition, all models were controlled for potential confounders: zBMI, education of the mother, BF and self-reported hours of PA in sports club per week.

All food items that were significant ($p < 0.05$) in the previous analysis were included in a final model, adjusted for: zBMI, mother education, BF and self-reported hours of PA in sports club per week.

Data were managed and analyzed with STATA v.12.

Results

Sample characteristics

Baseline characteristics by sex and age group and distribution of participants are shown in Table 1. Mean serum hs-CRP concentration was significantly higher in girls than boys ($p < 0.05$), but only in younger children. Older boys spend more time doing physical activity in a sport club than girls of the same age group ($p < 0.001$).

Food intake and CRP

Tables S1 (S1.1 and S1.2) and S2 (S2.1 and S2.2) presented as supplementary material show the mean consumption frequencies of each food item according to the three categories of hs-CRP in boys and girls.

In younger boys (Table S1.1), mean frequency intakes of *raw vegetables* and *butter or margarine* decreased significantly across the ordinal groups of hs-CRP: $p = 0.022$ and $p = 0.040$, respectively. Differences that were also found in mean consumption frequencies of food items by group of hs-CRP were found with *processed meals* (*pizza*, *hot dog*, *hamburgers*…) ($p = 0.002$), *nuts, seeds and dried fruit* ($p = 0.042$) and *ice cream* ($p = 0.002$). In older boys (Table S1.2), mean frequency intakes of *raw vegetables* ($p = 0.020$) and *fresh fruits without sugar* ($p = 0.032$) decreased significantly across the ordinal groups of hs-CRP. Mean consumption frequency of *white bread* ($p = 0.049$) was also significantly related to hs-CRP.

In younger girls (Table S2.1), mean consumption frequencies of *cooked vegetables* ($p = 0.008$), *egg boiled* ($p = 0.026$) and *butter or margarine* ($p = 0.26$) decreased significantly across the ordinal groups of hs-CRP. In contrast, mean consumption frequencies of *chocolate-nut-based spread* ($p = 0.047$) and *cereals milled* ($p = 0.036$) increased across the ordinal groups of hs-CRP. Also mean frequency intakes of *meat replacement products* ($p = 0.018$) and *mayonnaise* ($p = 0.042$) were significantly related to hs-CRP concentrations. In older girls (Table S2.2), mean consumption frequencies of *raw vegetables* ($p = 0.026$) decreased significantly across the ordinal groups of hs-CRP. Finally, consumption frequency of *cheese* was related to hs-CRP ($p = 0.010$).

Multilevel ordinal logistic regression

In the multivariate analysis (Figs. 1, 2, 3, 4), hs-CRP level (low/medium/high) was considered as dependent variable and the weekly consumption frequencies of the different food groups as independent variables. The odds ratio (OR) of each food represented the probability of being in the upper CRP group by increasing the weekly consumption frequency by 1 unit.

Table 1 Characteristis of the study participants

Age groups	Aged 2 to <6 ($n = 2519$)			Aged 6 to <10 ($n = 3884$)		
	Boys ($n = 1315$)	Girls ($n = 1204$)	p	Boys ($n = 1908$)	Girls ($n = 1976$)	p
Age (Mean ± SD)	4.37 ± 0.903	4.39 ± 0.915	–	7.501 ± 0.8	7.51 ± 0.78	
Mother's education						
ISCED 1, *n* (%)	51 (4.1 %)	40 (3.5 %)	–	71 (4 %)	72 (3.9 %)	–
ISCED 2, *n* (%)	127 (10.2 %)	121 (10.5 %)	–	238 (13.3 %)	225 (12.2 %)	–
ISCED 3, *n* (%)	488 (39.2 %)	473 (41.2 %)	–	771 (43 %)	799 (43.4 %)	–
ISCED 4, *n* (%)	163 (13.1 %)	146 (12.7 %)	–	208 (11.6 %)	223 (12.1 %)	–
ISCED 5, *n* (%)	416 (33.4 %)	369 (32.1 %)	–	507 (28.2 %)	524 (28.4 %)	–
hs-CRP (mg/dL)	0.13 ± 0.32	0.18 ± 0.51	0.012	0.11 ± 0.3	0.12 ± 0.29	0.062
CRP I *n* (%)	549 (41.7 %)	374 (31.1 %)		947 (49.5 %)	710 (35.9 %)	
CRP II *n* (%)	373 (28.4 %)	436 (36.2 %)		494 (25.9 %)	650 (32.9 %)	
CRP III *n* (%)	393 (29.9 %)	394 (32.7 %)		467 (24.5 %)	616 (31.2 %)	
BMI	15.89 ± 1.7 (1315)	15.83 ± 1.69 (1204)	0.367	16.8 ± 2.84 (1908)	16.83 ± 2.83 (1976)	0.702
Hours of PA in a sports club	1.62 ± 1.074	1.64 ± 1.39	0.826	2.74 ± 1.67	2.40 ± 1.74	<0.001
BF (yes)	661 (58.1 %)	593 (55.7 %)	–	886 (46.4 %)	923 (55.5 %)	–

ISCED, International Standard Classification for Education; hs-CRP, high-sensitivity C-reactive protein; CRP I, hs-CRP detection limit (0.02 mg/dL); CRP II, hs-CRP > 0.02 mg/dL and <50th sex-specific percentile (0.06 mg/dL in boys and 0.07 in girls); of those with hs-CRP values over the detection limit CRP III, hs-CRP ≥ 50th sex-specific percentile (0.06 mg/dL in boys and 0.07 in girls) of those with hs-CRP values above the detection limit

BMI body mass index, *PA* physical activity, *BF* breast-feeding

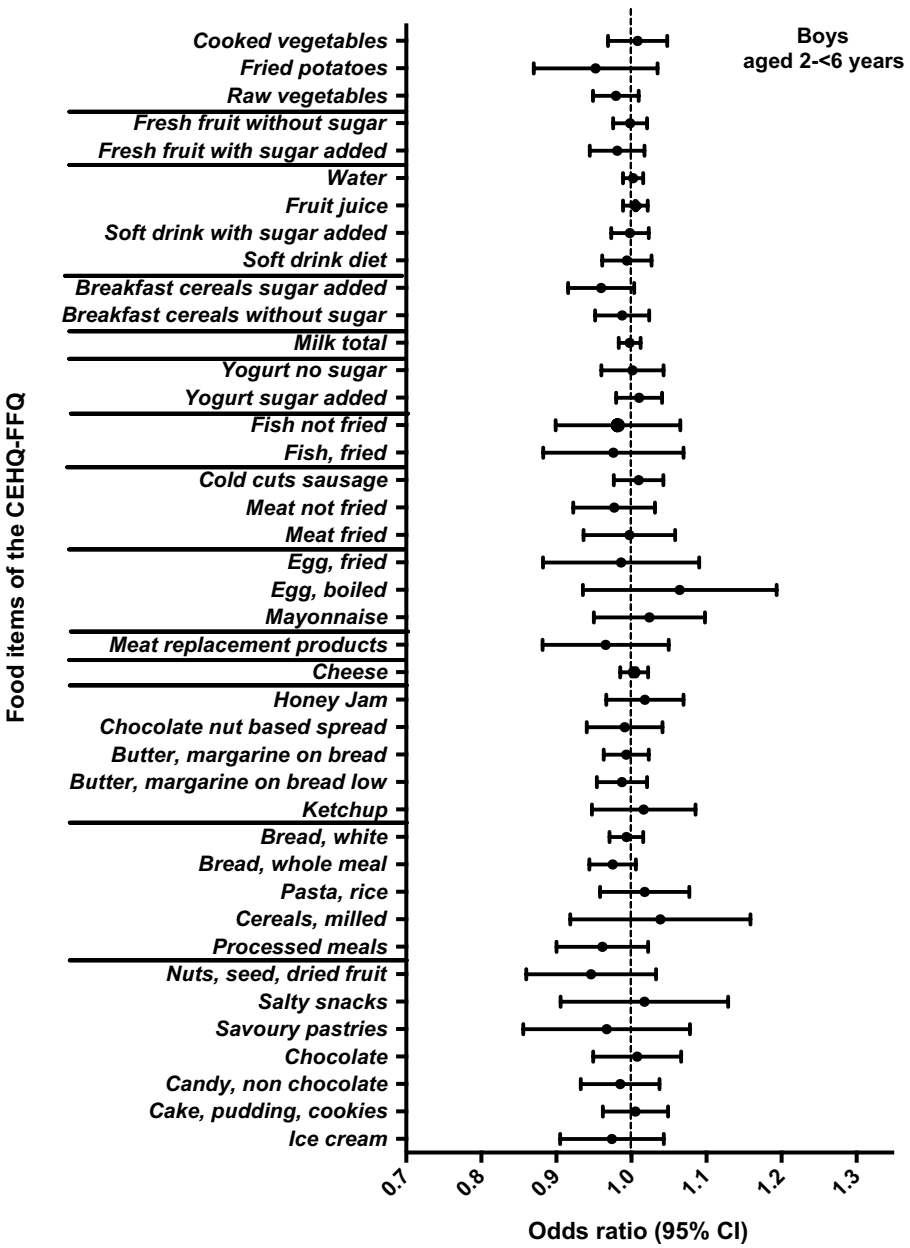

Fig. 1 Association between food consumption frequency and hs-CRP categories. Multilevel ordinal logistic regression. Odds ratio and confidence intervals for each food item of the CEHQ-FFQ in boys aged 2 to <6 years. Covariates: zBMI [23], mother education, BF and self-reported hours of physical activity in sports club per week
*$p < 0.05$

In young boys (Fig. 1), we found no significant associations. In contrast, in older boys (Fig. 2), a higher weekly consumption frequency of *raw vegetables* decreased the probability of being in a higher hs-CRP group by 4.1 % (OR 0.959; 95 % CI 0.932–0.986; $p = 0.004$).

In young girls (Fig. 3), consumption frequencies of *cooked vegetables* (OR 0.946; 95 % CI 0.9006–0.995; $p = 0.032$) and *honey–jam* (OR 0.942; 95 % CI 0.889–0.999; $p = 0.047$) decreased the probability of being in a higher group of hs-CRP by a 5.4 and 5.8 %, respectively, and for each increase in frequency of consumption of *soft drinks with sugar added* (OR 1.033; 95 % CI 1.006–1.062; $p = 0.017$), *mayonnaise* (OR 1.165, 95 % CI 1.063–1.276; $p = 0.001$), *white bread* (OR 1.028; 95 % CI 1.003–1.053; $p = 0.024$) and *cereals milled* (OR 1.17; 95 % CI 1.041–1.315; $p = 0.008$), the probability of being in a higher category of hs-CRP. In older girls (Fig. 4), no significant associations were found.

Finally, a fully adjusted model was carried out (Table 2). In older boys, intake of *raw vegetables* decreased the probability of being in a higher category of hs-CRP by 3 % (OR 0.970; 95 % CI 0.945–0.995, $p = 0.021$). In young girls, *cooked vegetables* (OR 0.941; 95 % CI 0.895–0.988, $p = 0.016$) and *honey–jam* (OR 0.938; 95 % CI 0.886–0.993, $p = 0.030$) decreased the probability of being in the upper group of hs-CRP, while *soft drinks with sugar added* (OR 1.034; 95 % CI 1.006–1.062, $p = 0.014$), *mayonnaise* (OR 1.128: 95 % CI 1.008–1.263, $p = 0.036$)

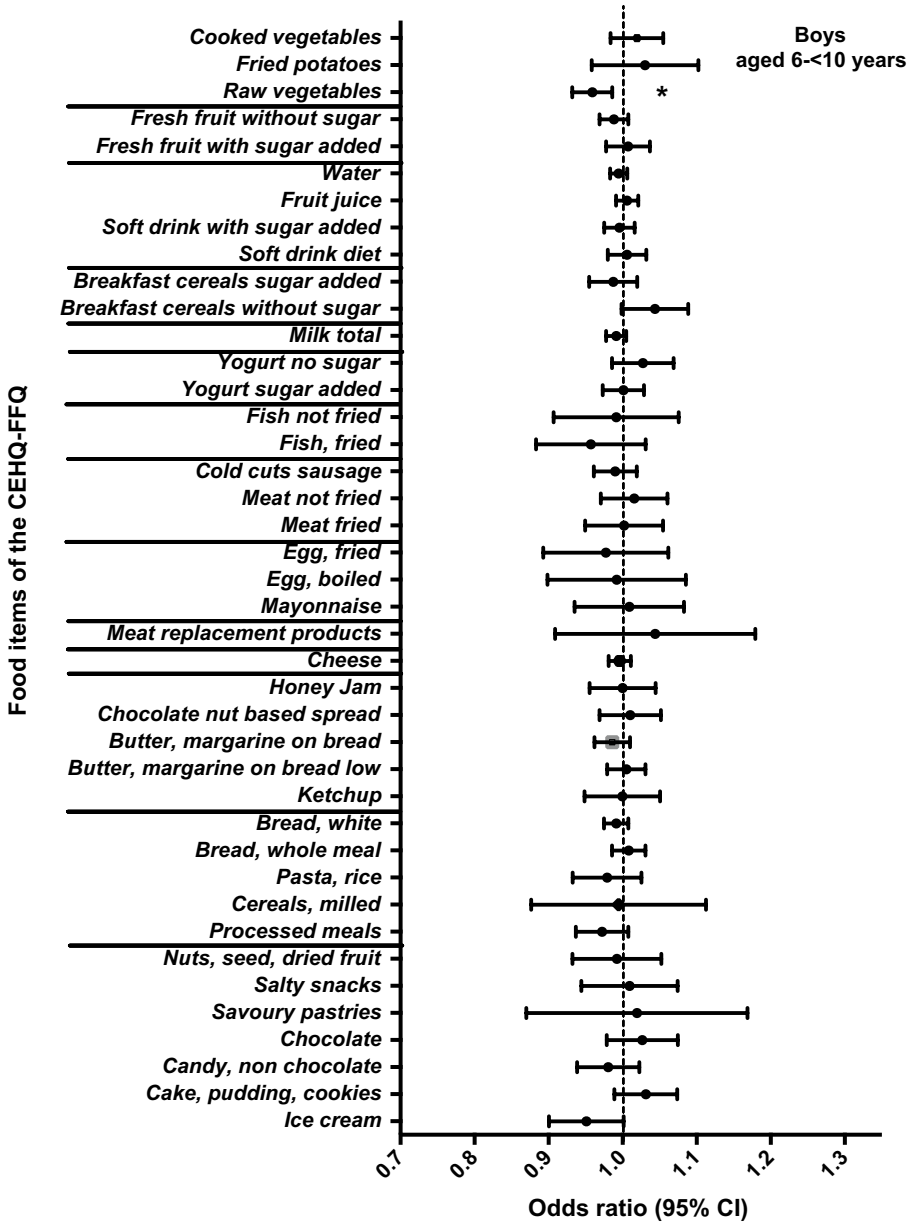

Fig. 2 Association between food consumption frequency and hs-CRP categories. Multilevel ordinal logistic regression. Odds ratio and confidence intervals for each food item of the CEHQ-FFQ in boys aged 6 to <10 years. Covariates: zBMI [23], mother education, BF and self-reported hours of physical activity in sports club per week
*$p < 0.05$

and *cereals milled* (OR 1.186; 95 % CI 1.050–1.340, $p = 0.006$) increased the probability of being in the upper group of hs-CRP.

Discussion

In our study, the consumption frequency of some food items was associated with the C-reactive protein in a large cohort of European children. Differences by age group and sex were found.

Among the food items, vegetables were the food group showing more associations with hs-CRP across the analyses performed. Mean consumption frequency of raw vegetables was higher in boys and older girls with low hs-CRP concentrations, while in younger girls the same occurred with the cooked vegetables in the ANCOVA analysis. The probability of having lower hs-CRP concentration was also significantly associated with a high consumption frequency of raw vegetables in older boys and of cooked vegetables in younger girls.

Our findings are consistent with previous literature using, however, different dietary assessment methods; for instance, in children participating in the NHANES, intake of vegetables and grains was negatively associated with hs-CRP, independently of body composition [28]. Similar associations between intake of vegetables and CRP were observed in adult men [29] and women [30]. Vegetables are very rich in antioxidants and other anti-inflammatory phytochemicals,

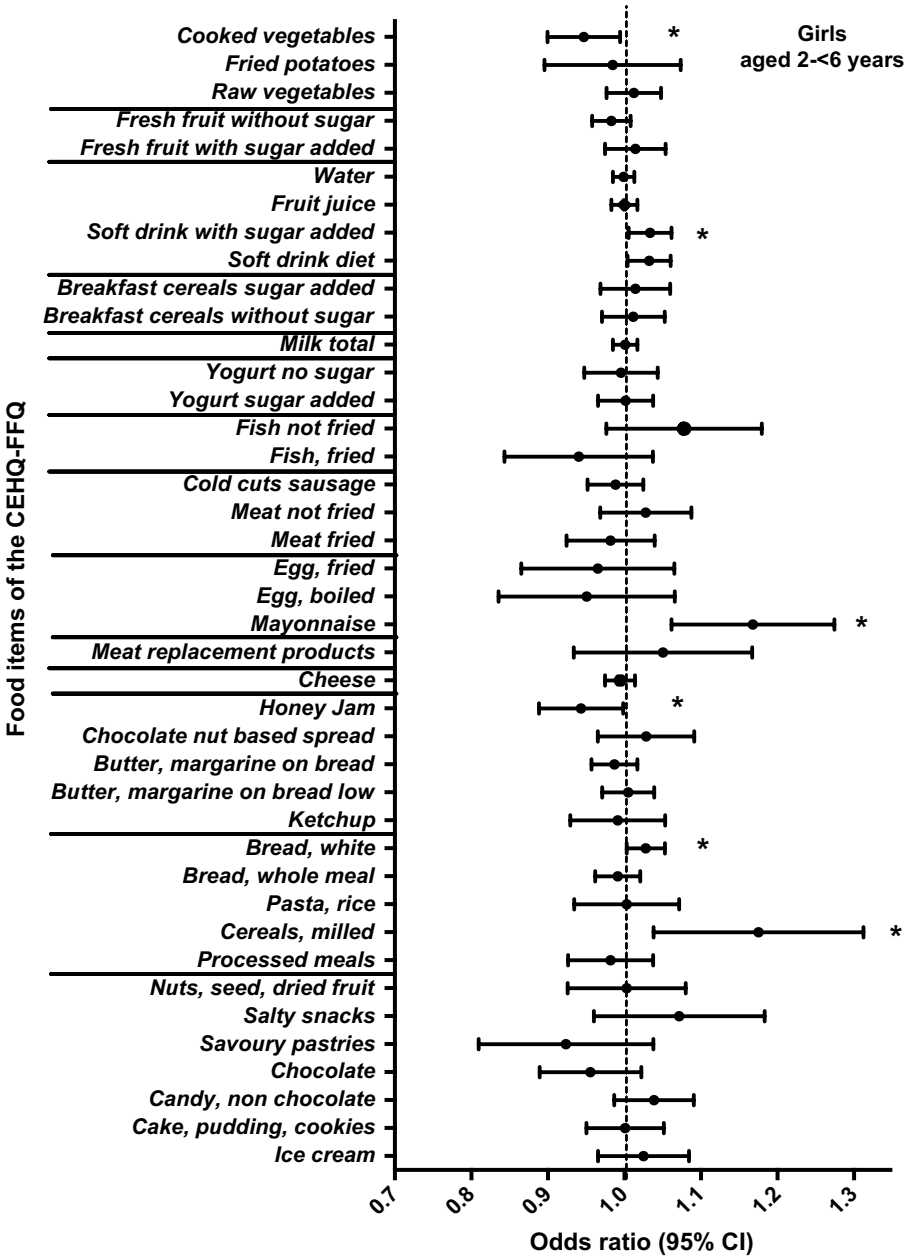

Fig. 3 Association between food consumption frequency and hs-CRP categories. Multilevel ordinal logistic regression. Odds ratio and confidence intervals for each food item of the CEHQ-FFQ in girls aged 2 to <6 years. Covariates: zBMI [23], mother education, BF and self-reported hours of physical activity in sports club per week
*$p < 0.05$

containing low amount of fat and high contents on water and fiber. These characteristics may explain the above results. Besides vegetables, other food items reduced the probability of having higher concentrations of hs-CRP, such as honey and jam in younger girls. Honey and jam are food items typically consumed at breakfast with medium GI [31]. In addition, breakfast foods consumption has been associated with lower CVD risk factors in adults [32]. Also, the main ingredient of jam is fruit, which has anti-inflammatory properties. In a previous study conducted in the IDEFICS population, girls with higher consumption of honey and jam were at lower risk of having clustered CVD compared with those with the lowest consumption [33].

Only in younger girls, consumption of soft drinks with sugar added, mayonnaise and cereals milled was positively associated with the increase in hs-CRP plasma concentrations. Soft drinks and cereals milled are associated with glycemic spikes that may contribute to oxidative stress and both acute and chronic inflammation even in lean subjects [14, 15]. Increased postprandial blood glucose concentrations are considered to yield nitric oxide generation, which in turn combines with superoxide to produce peroxynitrite, a potent long-lived pro-oxidant molecule [34]. Finally, mayonnaise is a high-fat product and its consumption could be related to non-healthy dietary patterns.

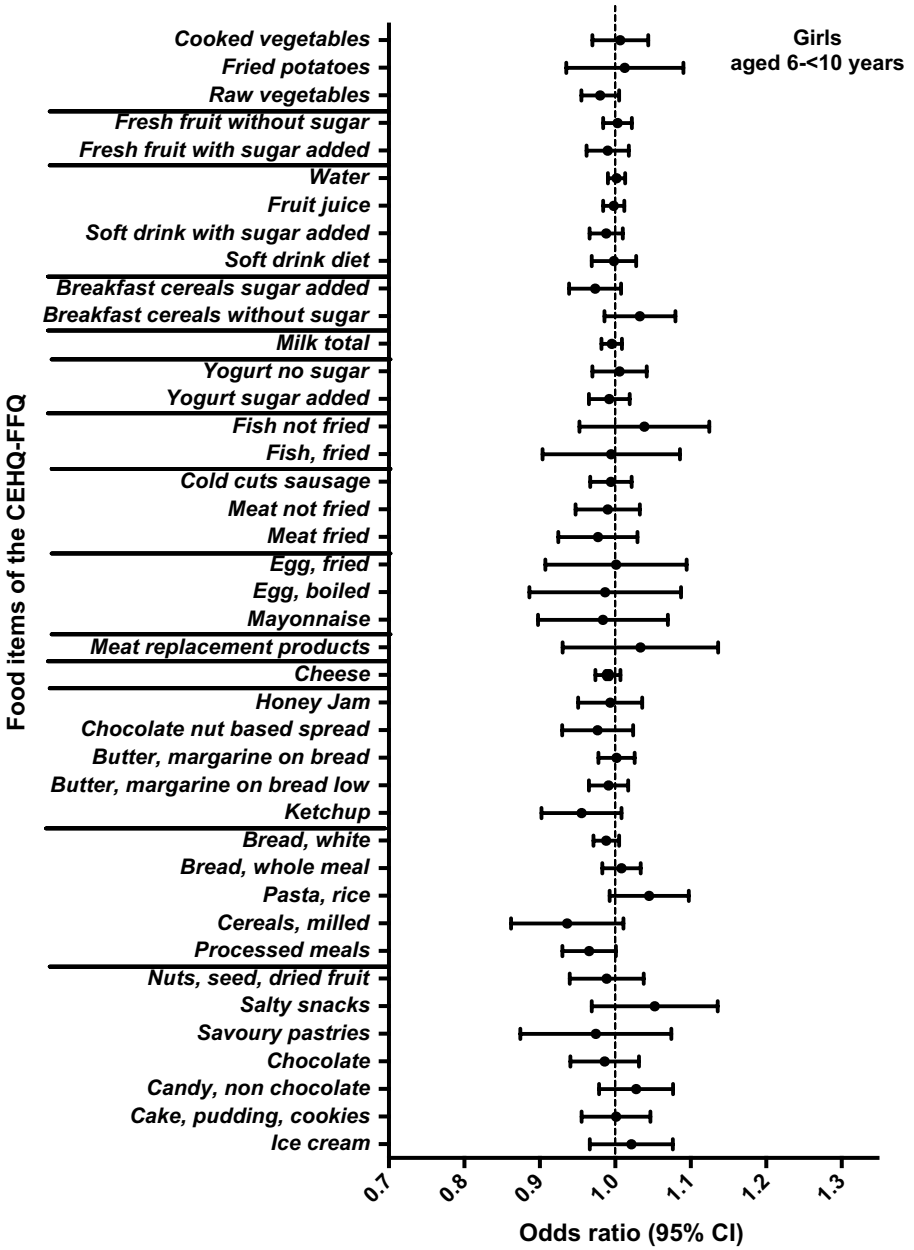

Fig. 4 Association between food consumption frequency and hs-CRP categories. Multilevel ordinal logistic regression. Odds ratio and confidence intervals for each food item of the CEHQ-FFQ in girls aged 6 to <10 years. Covariates: zBMI [23], mother education, BF and self-reported hours of physical activity in sports club per week
*$p < 0.05$

The present study has several limitations. Firstly, the cross-sectional design does not allow us to establish causal relationships between the consumption frequency of foods and hs-CRP although recent studies with genetic data suggest that genetically raised concentrations of CRP are not a cause of coronary heart disease [35]. Secondly, the CEHQ-FFQ is a proxy reported food frequency questionnaire that only takes into account the food consumption at home. However, the CEHQ-FFQ has previously been shown to give reproducible estimates of the frequency of food group consumption in European children [21, 36].

On the other hand, there are also strengths of the study. Firstly, the multilevel design took into account differences by country and the analysis were performed adjusting for a set of potential confounders. Also, the FFQ takes into account infrequently eaten foods that could be missed in the 24-h recalls and is a practical tool for epidemiological studies compared with other methods such as weighted dietary records. Moreover, literature in this age group is scarce and most of the dietary studies carried out in adults and children have focused on intakes of nutrients or food compounds, not on food consumption. Evidence is still scarce on the independent association between overall food intake and inflammation.

In conclusion, this exploratory study shows that consumption frequencies of specific food items presented association with hs-CRP. It seems that a high consumption frequency of vegetables is inversely related to an

Table 2 Multilevel ordinal logistic regression, odds ratios (OR) and the 95 % confidence intervals

	OR	*p*	CI
Aged 2 to <6			
Female			
Cooked vegetables	0.941	0.016	0.895–0.988
Soft drinks with sugar added	1.034	0.014	1.006–1.062
Mayonnaise	1.128	0.036	1.008–1.263
Honey, Jam	0.938	0.030	0.886–0.993
Cereals milled	1.186	0.006	1.050–1.340
Aged 6 to <10			
Male			
Raw vegetables	0.970	0.021	0.945–0.995

Those food items significantly associated ($p < 0.05$) with hs-CRP in the previous models were analyzed together in a final multilevel ordinal logistic regression model including all potential confounders: zBMI [23], mother education, BF and self-reported hours of physical activity in sports club per week

inflammatory status in children, independently of the body mass index. However, more studies are needed to assess the associations between food items and low-grade inflammation in order to explore the underlying mechanisms and to prevent an early onset of the low-grade inflammation.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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MATERIAL SUPLEMENTARIO

SUPPLEMENTARY MATERIAL

Table S1.1. Supplementary material. Mean consumption frequencies and 95% confidence interval (CI), by high sensitive C-reactive protein (hs-CRP) categorie in younger boys, controlling by body mass index z-score [23].

	Aged 2-<6 years			
	CRP I	**CRPII**	**CRPIII**	**p-value**
Food items	**Mean (95% CI)**	**Mean (95% CI)**	**Mean (95% CI)**	
Cooked vegetables	4.469 (4.176-4.762)	4.525 (4.173-4.876)	4.318 (3.973-4.663)	0.688
Fried potatoes	1.099 (0.965-1.232)	0.973 (0.811-1.135)	1.078 (0.92-1.235)	0.479
Raw vegetables	4.638 (4.24-5.036)	4.286 (3.809-4.762)	3.768 (3.297-4.239)*	0.022
Fresh fruit	7.69 (7.190-8.190)	8.016 (7.411-8.621)	7.483 (6.889-8.079)	0.460
Fresh fruit witth sugar added	1.932 (1.609-2.254)	1.511 (1.122-1.900)	1.886 (1.507-2.266)	0.229
Water	20.253 (19.336-21.170)	20.780 (19.670-21.889)	21.411 (20.324-22.497)	0.281
Fruit juice	6.988 (6.282-7.694)	6.308 (5.462-7.154)	7.379 (6.542-8.216)	0.201
Soft drink with sugar added	2.554 (2.065-3.044)	1.965 (1.371-2.559)	2.800 (2.218-3.382)	0.127
Soft drink, diet	1.020 (0.689-1.350)	0.847(0.447-1.248)	1.004 (0.608-1.400)	0.790
Breakfast cereals, sugar added	2.339 (2.084-2.594)	1.880 (1.573-2.188)	2.169 (1.867-2.471)	0.081
Breakfast cereals, no sugar	2.218(1.899-2.536)	2.360(1.981-2.738)	1.746 (1.366-2.125)	0.059
Milk total	11.502 (10.780-12.223)	11.272 (10.400-12.143)	11.359 (10.509-12.209)	0.920
Yoghurt, no sugar	1.522 (1.252-1.792)	1.667 (1.346-1.989)	1.448 (1.129-1.767)	0.624
Yoghurt, sugar added	4.107 (3.713-4.5)	4.292 (3.819-4.765)	4.458 (3.991-4.924)	0.526
Fish not fried	1.087 (0.950-1.223)	1.086 (0.923-1.250)	1.219 (1.059-1.378)	0.396
Fish, fried	1.14 (0.998-1.282)	1.080 (0.910-1.251)	1.248 (1.081-1.416)	0.371
Cold cuts, sausage	4.165 (3.819-4.510)	4.008 (3.59-4.426)	4.043 (3.634-4.451)	0.830
Meat,not fried (cooked)	2.215(2.008-2.422)	1.974(1.724-2.225)	2.108(1.865-2.352)	0.351

Table S1.2. Supplementary material. Mean consumption frequencies and 95% confidence interval (CI), by high sensitive C-reactive protein (hs-CRP) categorie in older boys, controlling by body mass index z-score [23].

	Aged 6-<10 years			
	CRP I	**CRP II**	**CRP III**	**p-value**
Food item	**Mean (95% CI)**	**Mean (95% CI)**	**Mean (95% CI)**	
Cooked vegetables	4.264 (4.047-4.481)	4.024(3.727-4.320)	4.052(3.739-4.365)	0.359
Fried potatoes	0.983 (0.883-1.082)	1.110 (0.974-1.246)	0.987(0.843-1.132)	0.295
Raw vegetables	4.574 (4.287-4.862)	3.940(3.546-4.333)*	4.041(3.624-4.457)	0.020
Fresh fruit	7.975(7.582-8.368)	7.243 (6.704-7.782)	7.184 (6.618-7.749)	0.032
Fresh fruit with sugar added	1.651(1.380-1.922)	2.191 (1.822-2.561)	1.806 (1.418-2.195)	0.069
Water	20.398 (19.668-21.127)	19.665 (18.627-20.683)	20.173 (19.118-21.228)	0.514
Fruit juice	6.707 (6.180-7.233)	7.517 (6.798-8.236)	7.223 (6.462-7.983)	0.187
Soft drinks with sugar added	2.865 (2.479-3.251)	3.356 (2.826-3.886)	2.755 (2.197-3.314)	0.230
Soft drink, diet	1.241 (0.958-1.524)	1.295 (0.909-1.681)	1.209 (0.800-1.618)	0.953
Breakfast cereals, sugar added	1.790 (2.565-3.015)	3.105 (2.800-3.411)	2.761 (2.439-3.083)	0.196
Breakfast cereals, no sugar	1.386 (1.198-1.575)	1.310(1.050-1.570)	1.591 (1.320-1.861)	0.311
Milk total	10.688 (10.172-11.204)	10.514 (9.810-11.219)	10.282 (9.538-11.027)	0.689
Yoghurt, no sugar	1.217 (1.028-1.405)	1.469(1.213-1.724)	1.564 (1.294-1.834)	0.088
Yoghurt, sugar added	3.650(3.389-3.911)	3.976(3.617-4.335)	3.366 (2.987-3.744)	0.068
Fish not fried	0.976 (0.880-1.073)	0.992(0.861-1.123)	1.013 (0.875-1.152)	0.916
Fish, fried	1.103 (1.004-1.203)	0.996(0.860-1.132)	1.044 (0.902-1.186)	0.450
Cold cuts, sausage	4.628(4.359-4.898)	4.232 (3.864-4.600)	4.372 (3.982-4.761)	0.213
Meat,not fried (not cooked)	2.306 (2.144-2.467)	2.478(2.258-2.697)	2.538(2.305-2.771)	0.226
Meat, fried	2.644(2.494-2.794)	2.357(2.152-2.562)	2.561(2.342-2.779)	0.086

Egg, fried	0.964(0.868-1.060)	0.920(0.788-1.052)	0.963(0.822-1.103)	0.858
Egg, boiled	0.776 (0.686-0.866)	0.779(0.656-0.903)	0.679 (0.549-0.808)	0.436
Mayonnaise	0.665(0.562-0.767)	0.777(0.636-0.918)	0.657(0.509-0.804)	0.383
Meat replacement products	0.090(0.041-0.139)	0.090(0.023-0.157)	0.153(0.082-0.224)	0.325
Cheese	8.599(8.136-9.063)	8.494(7.857-9.131)	8.042(7.377-8.707)	0.409
Honey Jam	1.784(1.604-1.965)	1.745(1.497-1.993)	1.775(1.513-2.036)	0.969
Chocolate_nut_based_spread	2.111(1.899-2.323)	2.270(1.980-2.560)	2.041(1.735-2.347)	0.532
Butter, margarine on bread	4.246 (3.869-4.624)	3.716(3.198-4.234)	4.089(3.549-4.630)	0.269
Butter, margarine on bread low fat	2.898 (2.563-3.233)	2.723(2.260-3.186)	2.841(2.357-3.325)	0.836
Ketchup	1.777(1.625-1.929)	1.772(1.564-1.979)	1.814(1.595-2.032)	0.955
Bread, white	7.506 (7.051-7.962)	8.077(7.455-8.699)	6.955(6.300-7.610)	0.049
Bread, wholemeal	3.648(3.314-3.981)	3.665(3.208-4.122)	3.706 (3.226-4.187)	0.981
Pasta,rice	2.931(2.777-3.084)	2.740(2.530-2.949)	2.875(2.653-3.097)	0.355
Cereals, milled,	0.351(0.280-0.422)	0.405(0.307-0.503)	0.458(0.356-0.561)	0.247
Processed meals: pizza, main dished, hot dogs, hamburguers..	2.120(1.894-2.346)	2.454(2.144-2.763)	2.002(1.678-2.326)	0.104
Nuts,seed, dried fruit	0.847 (0.737-0.958)	0.865(0.714-1.016)	0.737(0.579-0.896)	0.452
Salty snacks	1.019(0.913-1.125)	1.239(1.094-1.383)	1.120(0.968-1.273)	0.056
Savoury pastries	0.643(0.556-0.730)	0.762 (0.643-0.882)	0.788(0.663-0.912)	0.118
Chocolate	1.931(1.760-2.103)	2.208(1.974-2.442)	2.070(1.823-2.317)	0.172
Candy, non chocolate	2.030 (1.835-2.225)	1.876(1.608-2.143)	1.874 (1.592-2.156)	0.557
Cake, pudding, cookies	2.433(2.224-2.643)	2.461(2.175-2.747)	2.850(2.549-3.151)	0.074
Ice cream	1.656(1.503-1.809)	1.599(1.389-1.809)	1.544(1.322-1.765)	0.717

*CRP I: hs-CRP detection limit (0.02mg/dL); CRP II: hs-CRP > 0.02 mg/dL and < 50th sex-specific percentile (0.06 mg/dL in boys and 0.07 in girls); of those with hs-CRP values over the detection limit CRP III: hs-CRP ≥ 50th sex-specific percentile (0.06 mg/dL in boys and 0.07 in girls) of those with hs-CRP values above the detection limit. Post-hoc comparisons between hs-CRP groups with Bonferroni correction applied. *p<0.05 ref CRP I, † p<0.05 ref CRP II.*

Table S2.1. Supplementary material. Mean consumption frequencies and 95% confidence interval (CI), by high sensitive C-reactive protein (hs-CRP) categorie in younger girls, controlling by body mass index z-score [23].

	Aged 2-<6 years			
	CRP I	**CRP II**	**CRP III**	**p-value**
Food items	**Mean (95% CI)**	**Mean (95%CI)**	**Mean (95%CI)**	
Cooked vegetables	4.427(4.108-4.747)	4.385(4.095-4.674)	3.801(3.490-4.112)* †	0.008
Fried potatoes	0.887(0.737-1.037)	0.930(0.791-1.068)	0.905(0.758-1.052)	0.918
Raw vegetables	4.852 (4.382-5.323)	4.395(3.967-4.824)	4.068(3.608-4.527)	0.068
Fresh fruit	8.229 (7.649-8.808)	7.523(7.001-8.045)	7.542(6.981-8.103)	0.149
Fresh fruit with sugar added	1.588 (1.201-1.975)	1.583(1.232-1.933)	1.634(1.259-2.009)	0.978
Water	20.439 (19.311-21.567)	20.176 (19.155-21.198)	20.860(19.760-21.960)	0.668
Fruit juice	6.900(6.013-7.787)	7.370(6.571-8.169)	6.893(6.035-7.751)	0.635
Soft drink, sugar added	1.806(1.240-2.373)	2.534(2.017-3.051)	2.551(1.997-3.104)	0.111
Soft drink, diet	0.579(0.198-0.960)	0.989 (0.639-1.338)	0.948(0.577-1.319)	0.251
Breakfast cereals, sugar added	2.017(1.677-2.357)	2.252(1.944-2.560)	2.139(1.809-2.468)	0.606
Breakfast cereals, no sugar	1.882 (1.532-2.233)	1.773(1.453-2.093)	1.609(1.268-1.949)	0.547
Milk total	10.957(10.098-11.815)	11.403(10.616-12.190)	10.836(9.987-11.685)	0.590
Yoghurt, no sugar	1.404(1.105-1.703)	1.527(1.254-1.801)	1.489(1.197-1.781)	0.835
Yoghurt, sugar added	3.618(3.217-4.019)	3.740(3.378-4.102)	3.649(3.260-4.037)	0.896
Fish not fried	1.009(0.856-1.163)	1.161(1.023-1.299)	1.180(1.032-1.328)	0.234
Fish, fried	1.033 (0.888-1.179)	1.147(1.014-1.279)	0.981(0.838-1.124)	0.226
Cold cuts, sausage	4.153(3.763-4.543)	4.124(3.767-4.481)	3.953(3.571-4.335)	0.736
Meat,not fried (cooked)	2.295(2.029-2.560)	2.237(1.993-2.482)	2.337(2.077-2.598)	0.857
Meat, fried	2.448(2.178-2.718)	2.667(2.423-2.912)	2.552(2.291-2.812)	0.496
Egg, fried	1.017(0.868-1.166)	0.955(0.820-1.091)	0.938(0.794-1.083)	0.740
Egg, boiled	1.001(0.866-1.136)	0.888(0.765-1.011)	0.740(0.608-0.871)*	0.026

Mayonnaise	0.571(0.406-0.736)	0.547(0.396-0.698)*	0.810(0.648-0.972)	0.042
Meat replacement products	0.044(-0.052-0.140)	0.233(0.145-0.321)	0.144(0.051-0.237)	0.018
Cheese	8.699 (8.016-9.381)	7.689(7.073-8.306)	8.176(7.512-8.839)	0.100
Honey Jam	1.872(1.609-2.134)	1.701(1.461-1.941)	1.497(1.240-1.755)	0.141
Chocolate_nut_based_spread	1.518(1.246-1.791)	1.818(1.570-2.065)	1.999(1.733-2.264)	0.047
Butter, margarine on bread	4.060(3.537-4.583)	3.864(3.382-4.346)	3.101(2.584-3.619)	0.026
Butter, margarine on bread low fat	2.387(1.947-2.827)	2.438(2.036-2.840)	2.317(1.883-2.750)	0.922
Ketchup	1.491(1.271-1.711)	1.593(1.393-1.793)	1.427(1.211-1.643)	0.530
Bread, white	5.784(5.161-6.406)	6.286(5.720-6.853)	6.777(6.167-7.386)	0.087
Bread, wholemeal	4.147(3.637-4.657)	4.008(3.539-4.478)	3.495(2.994-3.997)	0.169
Pasta,rice	2.878(2.641-3.115)	2.871(2.655-3.087)	2.938(2.705-3.171)	0.905
Cereals, milled,	0.399(0.236-0.561)	0.507(0.358-0.655)	0.694(0.537-0.851)*	0.036
Processed meals: pizza, main dished, hot dogs, hamburguers..	1.870(1.546-2.194)	1.808(1.513-2.102)	1.697(1.378-2.015)	0.752
Nuts,seed, dried fruit	0.989(0.756-1.222)	0.948(0.738-1.158)	1.128(0.902-1.354)	0.497
Salty snacks	0.909(0.756-1.062)	0.968(0.828-1.107)	0.984(0.834-1.134)	0.773
Savoury pastries	0.676(0.539-0.812)	0.675(0.550-0.800)	0.525(0.391-0.659)	0.193
Chocolate	1.823(1.560-2.087)	1.983(1.741-2.224)	1.842(1.582-2.102)	0.622
Candy, non chocolate	2.091(1.770-2.413)	2.260(1.968-2.553)	2.261(1.944-2.578)	0.697
Cake, pudding, cookies	2.431(2.109-2.753)	2.513(2.219-2.807)	2.721(2.403-3.038)	0.433
Ice cream	1.853(1.591-2.114)	1.662(1.422-1.902)	1.688(1.430-1.947)	0.539

*CRP I: hs-CRP detection limit (0.02mg/dL); CRP II: hs-CRP > 0.02 mg/dL and < 50th sex-specific percentile (0.06 mg/dL in boys and 0.07 in girls); of those with hs-CRP values over the detection limit CRP III: hs-CRP ≥ 50th sex-specific percentile (0.06 mg/dL in boys and 0.07 in girls) of those with hs-CRP values above the detection limit. Post-hoc comparisons between hs-CRP groups with Bonferroni correction applied. *p<0.05 ref CRP I, † p<0.05 ref CRP II.*

Table S2.2. Supplementary material. Mean consumption frequencies and 95% confidence interval (CI), by high sensitive C-reactive protein (hs-CRP) categorie in older girls, controlling by body mass index z-score [23].

	Aged 6-<10 years			
	CRP I	**CRPII**	**CRPIII**	**p-value**
Food items	**Mean (95% CI)**	**Mean (95% CI)**	**Mean (95% CI)**	
Cooked vegetables	4.334(4.079-4.590)	4.329(4.071-4.5889	4.065(3.789-4.342)	0.305
Fried potatoes	0..959(0.840-1.079)	1.031(0.910-1.152)	1.080(0.951-1.209)	0.424
Raw vegetables	4.985(4.627-5.344)	4.668(4.306-5.030)	4.230(3.843-4.617)*	0.026
Fresh fruit	7.672(7.205-8.138)	7.527(7.058-7.996)	7.481(6.980-7.982)	0.854
Fresh fruit witth sugar added	1.783(1.453-2.114)	1.833(1.501-2.165)	1.939(1.583-2.295)	0.827
Water	20.537(19.704-21.370)	20.919(20.065-21.773)	21.652(20.740-22.565)	0.226
Fruit juice	6.749(6.137-7.361)	6.544(5.922-7.165)	6.658(5.996-7.320)	0.898
Soft drink with sugar added	2.776(2.343-3.209)	2.745(2.308-3.183)	2.586(2.116-3.057)	0.836
Soft drink, diet	1.227(0.912-1.542)	1.086(0.765-1.407)	1.062(0.720-1.405)	0.757
Breakfast cereals, sugar added	2.661(2.407-2.916)	2.596(2.338-2.854)	2.509(2.233-2.785)	0.745
Breakfast cereals, no sugar	1.384(1.169-1.600)	1.411(1.192-1.630)	1.501(1.268-1.734)	0.770
Milk total	10.393(9.740-11.047)	9.788(9.126-10.450)	10.003(9.296-10.710)	0.436
Yoghurt, no sugar	1.325(1.100-1.550)	1.257(1.029-1.484)	1.378(1.136-1.619)	0.770
Yoghurt, sugar added	3.553(3.268-3.837)	3.454(3.164-3.744)	3.236(2.927-3.544)	0.348
Fish not fried	1.045(0.917-1.172)	0.987(0.858-1.117)	1.118(0.981-1.255)	0.397
Fish, fried	0.989(0.889-1.089)	1.000(0.899-1.101)	1.060(0.951-1.168)	0.629
Cold cuts, sausage	4.538(4.215-4.860)	4.032(3.706-4.359)	4.198(3.851-4.545)	0.091
Meat,not fried (cooked)	2.397(2.198-2.596)	2.193(1.992-2.393)	2.482(2.266-2.697)	0.130
Meat, fried	2.569(2.386-2.752)	2.503(2.317-2.690)	2.453(2.255-2.652)	0.717
Egg, fried	0.985(0.883-1.087)	0.960(0.857-1.063)	1.010(0.900-1.121)	0.806

Egg, boiled	0.859(0.755-0.962)	0.828(0.723-0.933)	0.791(0.678-0.904)	0.705
Mayonnaise	0.788(0.646-0.930)	0.660(0.515-0.804)	0.650(0.496-0.804)	0.352
Meat replacement products	0.108(0.039-0.176)	0.159(0.090-0.229)	0.102(0.028-0.176)	0.447
Cheese	9.380(8.855-9.906)	8.261(7.728-8.794)*	8.513(7.946-9.079)	0.010
Honey Jam	1.723(1.526-1.921)	1.554(1.353-1.756)	1.462(1.248-1.676)	0.219
Chocolate_nut_based_spread	2.189(1.965-2.413)	1.922(1.694-2.150)	2.053(1.806-2.299)	0.259
Butter, margarine on bread	4.342(3.925-4.759)	3.685(3.259-4.110)	3.831(3.376-4.285)	0.081
Butter, margarine on bread low fat	2.709(2.352-3.066)	2.286(1.925-2.646)	2.698(2.309-3.087)	0.177
Ketchup	1.789(1.607-1.970)	1.494(1.309-1.679)	1.578(1.378-1.777)	0.076
Bread, white	7.707(7.196-8.218)	7.670(7.152-8.189)	7.199(6.645-7.753)	0.368
Bread, wholemeal	3.710(3.340-4.080)	3.385(3.012-3.759)	3.129(2.729-3.530)	0.127
Pasta,rice	3.029(2.830-3.228)	2.976(2.773-3.179)	3.005(2.788-3.222)	0.935
Cereals, milled,	0.582(0.468-0.696)	0.463(0.345-0.580)	0.369(0.243-0.494)	0.057
Processed meals: pizza, hamburguer, hot dog, falafel	2.202 (1.936-2.467)	1.993(1.722-2.263)	2.025(1.738-2.312)	0.520
Nuts,seed, dried fruit	0.982(0.828-1.135)	0.907(0.750-1.063)	0.732(0.566-0.898)	0.103
Salty snacks	1.036(0.905-1.166)	1.051(0.918-1.184)	1.011(0.869-1.152)	0.922
Savoury pastries	0.679(0.564-0.793)	0.605(0.487-0.722)	0.652(0.527-0.777)	0.667
Chocolate	2.028(1.820-2.235)	1.941(1.730-2.152)	2.197(1.972-2.422)	0.263
Candy, non chocolate	2.000(1.776-2.224)	1.889(1.661-2.117)	2.212(1.968-2.456)	0.163
Cake, pudding, cookies	2.358(2.125-2.591)	2.438(2.202-2.674)	2.583(2.330-2.836)	0.459
Ice cream	1.514(1.350-1.678)	1.637(1.471-1.804)	1.775(1.597-1.953)	0.125

*CRP I: hs-CRP detection limit (0.02mg/dL); CRP II: hs-CRP > 0.02 mg/dL and < 50th sex-specific percentile (0.06 mg/dL in boys and 0.07 in girls); of those with hs-CRP values over the detection limit CRP III: hs-CRP ≥ 50th sex-specific percentile (0.06 mg/dL in boys and 0.07 in girls) of those with hs-CRP values above the detection limit. Post-hoc comparisons between hs-CRP groups with Bonferroni correction applied. *p<0.05 ref CRP I, † p<0.05 ref CRP II.*

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ORIGINAL CONTRIBUTION

Prospective associations between dietary patterns and high sensitivity C-reactive protein in European children: the IDEFICS study

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Abstract
Purpose This prospective study explores high sensitivity C-reactive protein (hs-CRP) levels in relation to dietary patterns at two time points in European children.
Methods Out of the baseline sample of the IDEFICS study ($n = 16{,}228$), 4020 children, aged 2–9 years at baseline, with available hs-CRP levels and valid data from a food frequency questionnaire (FFQ) at baseline (T0) and 2 years later (T1) were included. K-means clustering algorithm based on the similarities between relative food consumption frequencies of the FFQ was applied. hs-CRP was dichotomized according to sex-specific cutoff points. Multilevel logistic regression was performed to assess the relationship between dietary patterns and hs-CRP adjusting for covariates.
Results Three consistent dietary patterns were found at T0 and T1: 'animal protein and refined carbohydrate', 'sweet and processed' and 'healthy'. Children allocated to the 'protein' and 'sweet and processed' clusters at both time points had significantly higher odds of being in the highest category of hs-CRP (OR 1.47; 95% CI 1.03–2.09 for 'animal protein and refined carbohydrate' and OR 1.44; 95% CI 1.08–1.92 for 'sweet and processed') compared to the 'healthy' cluster. The odds remained significantly higher for the 'sweet and processed' pattern (OR 1.39; 95% CI 1.05–1.84) when covariates were included.

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Conclusions A dietary pattern characterized by frequent consumption of sugar and processed products and infrequent consumption of vegetables and fruits over time was independently related with inflammation in European children. Efforts to improve the quality of the diet in childhood may prevent future diseases related with chronic inflammation.

Keywords Dietary patterns · Inflammation · C-reactive protein · European · Children · IDEFICS

Introduction

Chronic low-grade inflammation is related with metabolic disorders [1] and cardiovascular diseases (CVD) due to its role in the development of atherosclerosis [2]. In obese individuals, the endocrine function of the adipose tissue is impaired and it contributes to the production and release of pro-inflammatory cytokines; this condition is already observed in children [3, 4]. Among the available inflammatory biomarkers, high sensitivity C-reactive protein (hs-CRP) is the most commonly measured biomarker in clinical and epidemiologic studies and it is associated with adiposity and cardiovascular risk factors [5], even in children [6, 7].

Dietary intake, and its relation with low-grade inflammation, has been previously investigated in adults, taking into account nutrients, specific food items or dietary patterns [8–10]. In the baseline sample of the IDEFICS study, cross-sectional associations with hs-CRP were found between fatty acids intake, assessed via whole blood [11], and consumption frequencies of specific foods measured using a food frequency questionnaire (FFQ) [12] and hs-CRP. Dietary pattern analysis seems a good way to assess the diet as a whole as it considers also the possible interactions between the foods consumed and not only specific food items or isolated components. Additionally, dietary patterns could give an accurate insight into dietary behaviors in a population [13] and are useful to link specific dietary habits with chronic diseases [14].

Recent literature suggests that unhealthy patterns, i.e., those characterized by a westernized diet with high intake of animal proteins, free sugars and/or processed foods and low intake of vegetables/fruits, are positively related with inflammation while patterns with high intake of fruits and/or vegetables, i.e., plant-based patterns, are inversely associated with the inflammatory state [15–18].

Out of different approaches to derive dietary patterns, cluster analysis identifies diet patterns by grouping individuals into non-overlapping groups that reflect relatively homogeneous dietary behaviors within groups and relatively different dietary behavior between groups. Principal component analysis (PCA) is the most commonly used method to assess dietary patterns. However, PCA provides linear combinations of food, instead of referring to identifiable groups of subjects. A previous study [19] compared PCA and cluster analysis assessment methods, finding similar patterns and comparable long-term associations with coronary heart disease and stroke. Cluster analysis has been used to describe homogeneous groups of subjects with similar dietary patterns [20], it seems that this approach could be useful to give a good insight of dietary patterns. Previous longitudinal studies in adults have linked cluster analysis-derived dietary patterns and chronic diseases [21]. However, similar studies following young populations are scarce. Identifying young individuals with persistent healthy or unhealthy patterns over time may help to understand the cumulative impact of dietary habits on hs-CRP that could lead to future chronic diseases.

Thus, the first aim of this study was to describe cluster analysis-derived dietary patterns in children at two time points [baseline (T0) and follow-up (T1)] of the identification and prevention of dietary- and lifestyle-induced health effects in children and infants (IDEFICS) study. The second aim of this study was to assess the cross-sectional and prospective relationships between the identified dietary patterns and hs-CRP, as a marker of inflammation.

Materials and methods

Study design

The IDEFICS study is a multicentre population-based study of European children between 2 and 9 years old at time of recruitment in schools of eight countries: Belgium, Cyprus, Estonia, Germany, Hungary, Italy, Spain and Sweden. The general design and main procedures of the IDEFICS study have been described in detail elsewhere [22]. Two main surveys were performed in the present study: baseline (T0) and follow-up (T1) 2 years later. Children of pre-school and first or second grade of primary education were included at baseline. The baseline survey was performed between September 2007 and May 2008 and included 16,228 children from 2 to 9 years, while the follow-up survey performed between September 2009 and May 2010 included 11,038 children aged 4–11 years (overall response rate of 68%).

Authorization was obtained from the ethics committees of all participating countries. Parents provided written informed consent and children provided oral consent. The

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study was performed according to the ethical guidelines of the Edinburgh revision of the 1964 Declaration of Helsinki (2000).

Study sample

8754 children from the baseline sample of the IDEFICS study had data on hs-CRP and less than 50% of missing values in the food frequency questionnaire (FFQ). On the other hand, 6688 children from the follow-up sample of the IDEFICS study had the already mentioned data. Out of the total baseline and follow-up sample, 4174 children had less than 50% of missing values in the food frequency questionnaire (FFQ) and hs-CRP measured, at T0 and T1.

Then, children with hs-CRP concentrations higher than 10 mg/dL and those who took any medication the previous 24 h to blood collection that could potentially affect the hs-CRP values, i.e., anti-inflammatory drugs, steroids and/or corticoids, were excluded from the present analysis. Finally, 4020 children were included in the present analysis.

Measurements

The FFQ for obtaining the dietary data was the Children's Eating Habits Questionnaire FFQ (CEHQ-FFQ) [23, 24], a validated screening tool where the parents recorded their children's frequency of consumption of specific food items during the previous 4 weeks. The CEHQ-FFQ which comprised 43 food items within 14 food groups and was not designed to provide an estimate of total energy intake or total amount of food but to reflect dietary habits. Responses included seven frequency categories of consumption: 'never/less than once a week,' '1–3 times per week,' '4–6 times per week,' '1 time per day,' '2 times per day,' '3 times per day' and '4 or more times per day'. Also 'I have no idea' was a possible answer. Frequency categories were converted into times per week, represented by a number ranging from 0 to 30. Multiple imputation was applied to estimate missing values using gender, age, BMI and country as predictors for the rest of missing values and the pooled data from the imputed databases was retrieved.

Children were asked to participate in fasting blood collection, on a voluntary basis. A description of blood sampling and analytical procedures in the IDEFICS survey has been published elsewhere [25]. The hs-CRP concentrations were measured in a central laboratory with a high-sensitivity assay using latex-enhanced nephelometry (BN2-Nephelometer, Siemens, Deerfield, IL, USA) and the lower limit of detection of the assay was 0.02 mg/dL.

Parental education level (the highest level of both parents) was categorized according to the International Standard Classification of EDucation (ISCED) [26]. As the previous 24 h medication intake was recorded for the day of the blood collection, the type of medication, other than the medication mentioned as exclusion criteria, was used as confounder in the analysis as a categorical variable.

Finally, trained staff performed the anthropometric measurements, at T0 and T1, following standardized procedures. Body height was measured with bare feet in a portable stadiometer (SECA 225). Weight was measured in a child-adapted Tanita BC 420 SMA with the children in fasting status. BMI was calculated as the ratio between weight (kg) and squared height (m^2).

Statistical analyses

K-means cluster analysis was performed to identify clusters of children with similar dietary patterns [27]. The same procedures as in a previous IDEFICS study were followed [28]. Out of the 43 food items included in the FFQ, 'meat replacement products' were excluded from the analysis as more than 95% of the subjects reported to consume: 'never/less than once per week'. Correlations between the single items were calculated to assess multi-collinearity, showing no redundant variables. For the 42 food items, relative frequencies of consumption were calculated: the frequency of consumption of each one was divided by the sum of the consumption of all food items for each subject. *Z* scores of the relative frequencies of each food item were calculated to standardize values and to avoid large differences between food items [29]. The k-means algorithm was applied with a pre-defined maximum of 100 iterations, generated until no changes in the centroids were shown, to create cluster solutions for two to six clusters. Several solutions were obtained with different starting seeds to find stable cluster patterns. Randomly splitting the database in two halves to repeat the same procedure in baseline and follow-up datasets was used to examine the stability of the final solution both in baseline and follow-up datasets. Cohen's kappa values for the selected solution were 0.892 and 0.963 for baseline and follow-up, respectively.

The criteria to choose the clusters were based on stability of the cluster solution and interpretability. The clusters were labeled based on the corresponding *z* score values of the types of foods they included. Three clusters over time were found: 'healthy', 'animal protein and refined carbohydrate' and 'sweet and processed'. In addition, radar plots showing the maximum and minimum *z* score values in comparison with the other clusters were created to identify and to describe visually each cluster at each time point.

Distribution of children in different clusters was calculated, stratified by gender, age, BMI status and country at baseline and follow-up. Cluster memberships at baseline and follow-up were cross tabulated, to assess the percentage of children characterized by persistent dietary patterns and of those who changed dietary pattern from T0 to T1.

The distribution of hs-CRP was skewed as approximately a third of the sample had the value 'under detection limit': 0.02 mg/dL, in T0 and T1. Thus, subjects were allocated into two groups or categories, i.e., the first and second sex-specific hs-CRP tertiles vs. the third sex-specific tertile.

For the prospective analysis, each possible combination of dietary patterns over time was treated as a separate category. For example, being allocated in the 'sweet and processed' in T0 and 'healthy' in T1 was considered one category. Those children who stayed in the 'Healthy pattern' over time, at T0 and T1, were considered as the reference category. In addition, being persistently allocated to the same cluster at baseline and follow-up was considered as additional categories.

Finally, multilevel logistic regression (levels: country and school) was performed using the hs-CRP at both time points as dependent variable to assess the odds ratio (OR) for having a higher inflammatory status when presenting a specific dietary pattern at baseline and follow-up separately. Additionally, ORs for having a high inflammatory state when being persistently allocated to the same cluster at baseline and follow-up (i.e., 'animal protein and refined carbohydrate', 'sweet and processed' or 'healthy') or when changing from one of the three clusters to another were calculated. The 'healthy' cluster was always considered as the reference. Two models with different covariates were applied. Model 1 was adjusted by levels: country and school, while model 2 was additionally adjusted for age, gender, study region (intervention vs. control), parental education level, BMI and medication. These covariates were assessed at both time points, T0 and T1. The analysis with the combination of T0 and T1 patterns included: hs-CRP of T1 as dependent and was adjusted for age at T1, gender, study region (intervention vs. control), parental education level at T1, BMI at T1, hs-CRP at T0 and medication.

The analyses were performed using Statistical Package for the Social Sciences (version 21.0; SPSS, Inc.) and Stata (version 13.0) for the multilevel logistic regression. The radar plots were performed with Excel (Microsoft).

Results

Based on the food items and their z score values, a three-cluster solution was considered the most interpretable and stable. The following names were assigned to the clusters: 'healthy' ($n=1245$ at T0 and $n=1335$ at T1), 'sweet and processed' ($n=1472$ at T0 and $n=1306$ at T1) and 'animal protein and refined carbohydrate' ($n=1303$ at T0 and $n=1379$ at T1).

Tables 1 and 2 present the z scores of the 42 food items and standard deviations for each cluster. The cluster solutions obtained were similar in terms of interpretability at both time points. The mean relative frequency of the majority of the food items differed significantly between the three clusters (Tables 1, 2).

At both time points, the 'animal protein and refined carbohydrate' cluster presented higher relative frequencies of consumption of water, sweetened fruit, white bread, pasta, rice and also foods like sweetened milk, sweet yogurt, fish (fresh or fried), meat and fried eggs. Food items such as whole bread, spreads, cold cuts, fried meat, plain milk, hamburgers or sweetened and diet drinks scored lowest. In contrast, 'the sweet and processed' cluster had consistently higher relative consumption frequencies for sugar-rich products such as fruit juices, sweetened drinks, diet drinks, sweetened breakfast cereals, chocolate/nut-spread, ketchup, chocolate-candy bars, candies, biscuits/pastries and ice-cream. Also, at both time points, this cluster had higher relative frequencies for fried potatoes, cold cuts, fried meat, mayonnaise and hamburgers/hot dogs/kebabs, whereas food items such as cooked vegetables, fresh fruit, water, muesli, plain yogurt, fresh fish, cheese or pasta scored the lowest. Finally, the 'healthy' cluster presented at both time points higher relative consumption frequencies for cooked vegetables, raw vegetables, fresh fruits, muesli, plain milk, plain yogurt, boiled eggs, reduced-fat products on bread, whole-meal bread, dish of milled cereals and nuts/seeds. Food items such as fried potatoes, fruits with added sugar, sweetened breakfast cereals, sweetened milk, sweet yogurt, fried eggs, mayonnaise, chocolate/nut spreads, white bread, pizza as main dish, crisps, savoury pastries, chocolate/candy bars or biscuits scored the lowest.

Table 3 shows the main characteristics of the participants in the three clusters. The percentage of girls in the 'healthy' cluster was slightly higher than in the other clusters, while a higher percentage of boys were observed in the 'sweet and processed' and 'animal protein and refined carbohydrate' cluster. Also, age differences by cluster are presented. A higher percentage of older children in T0 and T1 were allocated to the 'sweet and processed' cluster compared to the other clusters. Regarding BMI differences, the 'animal protein and refined carbohydrate' cluster included a higher percentage of overweight and obese children compared with the other two clusters. In contrast, the 'healthy' cluster had lower percentages of obese children compared with the 'animal protein and refined carbohydrate' or 'sweet and processed' cluster over time. There were also differences between the distributions by country per cluster, i.e., certain countries allocated subjects up to 51.7% on one cluster. The 'animal protein and refined carbohydrate' cluster was mainly represented by Spain and Italy; the 'sweet and processed' cluster by Hungary, Belgium, Estonia and Germany while the 'healthy' cluster predominated in Sweden, Estonia, Hungary and Germany.

Table 1 *Z* scores of relative consumption frequencies in the three clusters at baseline [mean values and standard deviation (SD)]

Food items	Animal protein and refined carbohydrate ($n=1303$)		Sweet and processed ($n=1472$)		Healthy ($n=1245$)	
	Mean	SD	Mean	SD	Mean	SD
Cooked vegetables, potatoes, beans	-0.07^{b}	0.96	$-0.14^{b,*}$	0.92	$0.24^{a,\dagger}$	1.07
Fried potatoes, potato croquettes	0.02^{c}	0.93	$0.27^{a}\dagger$	1.17	$-0.35^{b,*}$	0.67
Raw vegetables	$-0.40^{b,*}$	0.70	-0.25^{c}	0.74	$0.72^{a,\dagger}$	1.12
Fresh fruits without added sugar	0.11^{c}	1.07	$-0.42^{b}*$	0.72	$0.38^{a,\dagger}$	1.01
Fresh fruits with added sugar	$0.11^{a,\dagger}$	1.17	0.06^{a}	1.01	$-0.18^{b}*$	0.73
Water	$0.76^{a,\dagger}$	0.76	$-0.52^{b,*}$	0.89	-0.17^{c}	0.83
Fruit juices	-0.17^{b}	0.76	$0.30^{a,\dagger}$	1.21	$-0.17^{b}*$	0.83
Sweetened drinks	$-0.30^{b,*}$	0.39	$0.46^{a,\dagger}$	1.43	-0.22^{b}	0.04
Diet drinks	-0.15^{b}	0.37	$0.26^{a,\dagger}$	1.54	$-0.15^{b,*}$	0.36
Breakfast cereals, muesli, sweetened	-0.08^{c}	1.01	$0.29^{a,\dagger}$	1.10	$-0.26^{b,*}$	0.74
Porridge, oat meal, gruel, cereals, muesli, unsweetened	-0.21^{c}	0.91	$-0.30^{b}*$	0.54	$0.58^{a,\dagger}$	1.23
Plain unsweetened milk	$-0.41^{b,*}$	0.83	-0.09^{c}	0.87	$0.54^{a,\dagger}$	1.05
Sweetened milk	$0.63^{a,\dagger}$	1.22	-0.13^{c}	0.78	$-0.51^{b,*}$	0.48
Plain unsweetened yogurt or kefir	-0.16^{b}	0.84	$-0.18^{b}*$	0.68	$0.38^{a,\dagger}$	1.31
Sweet yogurt, fermented milk beverages	$0.27^{a,\dagger}$	1.23	-0.10^{b}	0.81	$-0.16^{b,*}$	0.85
Fresh or frozen fish, not fried	$0.53^{a,\dagger}$	1.15	$-0.50^{b,*}$	0.63	0.02^{c}	0.87
Fried fish, fish fingers	$0.23^{a,\dagger}$	1.16	$-0.16^{b,*}$	0.88	-0.05^{c}	0.89
Cold cuts, preserved, ready to cook meat products	-0.12^{c}	0.86	$0.36^{a,\dagger}$	1.07	$-0.29^{b,*}$	0.90
Fresh meat, not fried	$0.31^{a,\dagger}$	1.11	$-0.13^{b,*}$	0.92	-0.16^{c}	0.87
Fried meat	$-0.22^{b,*}$	0.92	$0.14^{a,\dagger}$	1.05	0.05^{a}	0.96
Fried or scramble eggs	$0.30^{a,\dagger}$	1.09	-0.06^{c}	0.95	$-0.24^{b,*}$	0.85
Boiled or poached eggs	$-0.19^{b,*}$	0.86	-0.12^{b}	0.91	$0.35^{a,\dagger}$	1.13
Mayonnaise, mayonnaise-based products	-0.16^{b}	0.74	$0.34^{a,\dagger}$	1.32	$-0.23^{b,*}$	0.58
Cheese	-0.07^{a}	0.95	$-0.05^{b,*}$	0.96	$0.06^{a,\dagger}$	1.08
Jam-honey	$-0.29^{b,*}$	0.74	$0.16^{a}\dagger$	1.02	0.11^{a}	1.12
Chocolate or nut based spreads	0.06^{c}	0.87	$0.29^{a}\dagger$	1.20	$-0.42^{b,*}$	0.65
Butter, margarine on bread	$-0.56^{b,*}$	0.34	0.25^{c}	1.04	$0.29^{a,\dagger}$	1.14
Reduced-fat products on bread	$-0.46^{b}*$	0.31	0.07^{c}	0.97	$0.39^{a,\dagger}$	1.27
Ketchup	$-0.31^{b,*}$	0.83	$0.22^{a}\dagger$	1.09	0.06^{c}	0.96
White bread, white roll, white crispbread	$0.34^{a,\dagger}$	1.09	0.13^{c}	0.97	$-0.51^{b,*}$	0.65
Wholemeal bread, dark roll, dark crispbread	$-0.50^{b,*}$	0.61	-0.02^{c}	0.94	$0.56^{a,\dagger}$	1.09
Pasta, noodles, rice	$0.26^{a,\dagger}$	1.23	$-0.30^{b,*}$	0.70	0.07^{c}	0.92
Dish of milled cereals	$-0.27^{b,*}$	0.41	-0.01^{c}	0.88	$0.29^{a,\dagger}$	1.40
Pizza as main dish	$0.35^{a,\dagger}$	1.34	-0.04^{c}	0.85	$-0.31^{b,*}$	0.47
Hamburguers, hot dogs, kebabs, wraps, falafel	$-0.35^{b,*}$	0.52	$0.35^{a,\dagger}$	1.28	-0.05^{c}	0.84
Nuts, seeds, dried fruits	$-0.06^{b,*}$	0.91	-0.06^{b}	0.90	$0.15^{a,\dagger}$	1.16
Crisps, maize (corn) crisps, popcorn	$0.16^{a,\dagger}$	1.13	0.07^{a}	1.01	$-0.25^{b,*}$	0.74
Savoury pastries, fritters	0.01^{c}	1.06	$0.17^{a,\dagger}$	1.12	$-0.23^{b,*}$	0.66
Chocolate, candy bars	-0.12^{c}	0.84	$0.38^{a,\dagger}$	1.24	$-0.32^{b,*}$	0.59
Candies, loose candies, marshmallows	-0.13^{b}	0.83	$0.26^{a,\dagger}$	1.29	$-0.17^{b}*$	0.62
Biscuits, packaged cakes, pastries, puddings	0.02^{c}	0.99	$0.19^{a,\dagger}$	1.20	$-0.26^{b,*}$	0.58
Ice cream, milk- or fruit-based bars	$-0.30^{b,*}$	0.76	$0.29^{a,\dagger}$	1.24	-0.02^{c}	0.77

*The lowest mean value within a row

†The highest mean value within a row

^{a,b,c}Mean values within a row with unlike superscript letters were significantly different ($p<0.05$)

Table 2 *Z* scores of relative consumption frequencies in the three clusters at follow-up [mean values and standard deviation (SD)]

Food items	Animal protein and refined carbohydrate ($n=1303$)		Sweet and processed ($n=1306$)		Healthy ($n=1335$)	
	Mean	SD	Mean	SD	Mean	SD
Cooked vegetables, potatoes, beans	0.02^{c}	1.01	$-0.15^{b,*}$	0.93	$0.12^{a,\dagger}$	1.04
Fried potatoes, potato croquettes	-0.07^{c}	0.94	$0.42^{a,\dagger}$	1.12	-0.33^{b*}	0.74
Raw vegetables	-0.25^{c}	0.78	-0.41^{b*}	0.61	$0.66^{a,\dagger}$	1.15
Fresh fruits without added sugar	0.20^{a}	1.07	-0.46^{b*}	0.73	$0.24^{a}\dagger$	0.98
Fresh fruits with added sugar	$0.13^{a,\dagger}$	1.26	-0.01^{c}	0.85	-0.13^{b*}	0.78
Water	$0.69^{a,\dagger}$	0.81	$-0.43^{b,*}$	0.94	-0.29^{c}	0.82
Fruit juices	$-0.08^{b,*}$	0.88	$0.15^{a,\dagger}$	1.16	-0.06^{b}	0.91
Sweetened drinks	$-0.26^{b,*}$	0.41	$0.49^{a,\dagger}$	1.52	-0.21^{b}	0.44
Diet drinks	$-0.16^{b,*}$	0.34	$0.29^{a,\dagger}$	1.58	-0.11^{b}	0.53
Breakfast cereals, muesli, sweetened	0.08^{b}	1.09	$0.13^{a,\dagger}$	1.03	$-0.21^{a,*}$	0.81
Porridge, oat meal, gruel, cereals, muesli, unsweetened	-0.26^{b}	0.74	$-0.28^{b,*}$	0.61	$0.55^{a,\dagger}$	1.26
Plain unsweetened milk	$-0.41^{b,*}$	0.76	-0.18^{c}	0.81	$0.60^{a,\dagger}$	1.08
Sweetened milk	$0.55^{a,\dagger}$	1.17	-0.08^{c}	0.90	$-0.49^{b,}{}_{*}$	0.47
Plain unsweetened yogurt or kefir	-0.11^{b}	0.91	$-0.17^{b,}{}_{*}$	0.72	$0.29^{a,\dagger}$	1.22
Sweet yogurt, fermented milk beverages	$0.19^{a,\dagger}$	1.14	-0.04^{c}	0.92	$-0.15^{b,*}$	0.86
Fresh or frozen fish, not fried	$0.57^{a,\dagger}$	1.08	$-0.47^{b,*}$	0.71	-0.12^{c}	0.84
Fried fish, fish fingers	$0.08^{a,\dagger}$	1.10	0.02^{a}	1.01	$-0.10^{b,*}$	0.84
Cold cuts, preserved, ready to cook meat products	$-0.21^{b,*}$	0.84	$0.28^{a,\dagger}$	1.08	-0.05^{c}	0.99
Fresh meat, not fried	$0.42^{a,\dagger}$	1.01	-0.16^{c}	0.98	$-0.27^{b,*}$	0.85
Fried meat	$-0.23^{b,*}$	0.86	$0.20^{a,\dagger}$	1.05	0.04^{c}	1.02
Fried or scramble eggs	$0.20^{a,\dagger}$	1.03	0.05^{c}	1.05	$-0.25^{b,*}$	0.84
Boiled or poached eggs	$-0.22^{b,*}$	0.83	-0.02^{c}	1.01	$0.25^{a,\dagger}$	1.09
Mayonnaise, mayonnaise-based products	-0.18^{b}	0.74	$0.46^{a,\dagger}$	1.33	$-0.25^{b,*}$	0.61
Cheese	$0.07^{a,\dagger}$	1.06	$-0.08^{b,*}$	0.97	0.01^{b}	0.94
Jam-honey	$-0.25^{b,*}$	0.82	0.07^{c}	0.96	$0.19^{a,\dagger}$	1.13
Chocolate or nut based spreads	-0.02^{c}	0.81	$0.44^{a,\dagger}$	1.26	$-0.40^{b,}{}_{*}$	0.62
Butter, margarine on bread	$-0.49^{b,*}$	0.47	$0.11^{a,\dagger}$	0.97	0.40^{c}	1.18
Reduced-fat products on bread	$-0.39^{b,*}$	0.41	0.03^{c}	0.91	$0.37^{a,\dagger}$	1.31
Ketchup	$-0.30^{b,}{}_{*}$	0.75	$0.37^{a,\dagger}$	1.27	-0.05^{c}	0.78
White bread, white roll, white crispbread	$0.34^{a,\dagger}$	1.13	0.06^{c}	0.94	$-0.41^{b,}{}_{*}$	0.71
Wholemeal bread, dark roll, dark crispbread	$-0.49^{b,}{}_{*}$	0.58	-0.05^{c}	0.91	$0.57^{a,\dagger}$	1.12
Pasta, noodles, rice	$0.19^{a,\dagger}$	1.23	$-0.18^{b,*}$	0.81	-0.02^{c}	0.84
Dish of milled cereals	$-0.28^{b,}{}_{*}$	0.37	-0.03^{c}	0.91	$0.32^{a,\dagger}$	1.36
Pizza as main dish	0.13^{a}	1.06	$0.18^{a,\dagger}$	1.21	-0.32^{b*}	0.49
Hamburguers, hot dogs, kebabs, wraps, falafel	$-0.32^{b,}{}_{*}$	0.54	$0.31^{a,\dagger}$	1.28	0.02^{c}	0.94
Nuts, seeds, dried fruits	-0.07^{b}	0.91	$-0.08^{b,}{}_{*}$	0.87	$0.15^{a,\dagger}$	1.16
Crisps, maize (corn) crisps, popcorn	-0.13^{c}	0.89	$0.38^{a,\dagger}$	1.19	$-0.24^{b,}{}_{*}$	0.75
Savoury pastries, fritters	-0.06^{c}	0.91	$0.31^{a,\dagger}$	1.24	$-0.24^{b,}{}_{*}$	0.68
Chocolate, candy bars	-0.22^{b}	0.77	$0.50^{a,\dagger}$	1.24	-0.25^{b*}	0.72
Candies, loose candies, marshmallows	$-0.26^{b,}{}_{*}$	0.72	$0.39^{a,\dagger}$	1.34	-0.11^{c}	0.68
Biscuits, packaged cakes, pastries, puddings	-0.12^{c}	0.87	$0.35^{a,\dagger}$	1.28	$-0.21^{b,}{}_{*}$	0.64
Ice cream, milk- or fruit-based bars	$-0.25^{b,}{}_{*}$	0.85	$0.28^{a,\dagger}$	1.21	-0.01^{c}	0.83

*The lowest mean value within a row

†The highest mean value within a row

^{a,b,c}Mean values within a row with unlike superscript letters were significantly different ($p<0.05$)

Table 3 Description of the included study population by cluster membership at baseline (T0) and follow-up (T1)

	Animal protein and refined carbohydrate				Sweet and processed				Healthy				Total T0	
	T0		T1		T0		T1		T0		T1			
	n	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
Total	1303		1379		1472		1306		1245		1335		4020	
Gender														
Boys	666	51.1	708	51.3	766	52.0	683	52.3	605	48.6	646	48.4	2037	50.7
Girls	637	48.9	671	48.7	706	48.0	623	47.7	640	51.4	689	51.6	1983	49.3
Age														
<6 years	522	40.1	220	16.0	566	38.5	148	11.3	526	42.2	202	15.1	1614	40.1
≥6 years	781	59.9	1159	84.0	906	61.5	1158	88.7	719	57.8	1133	84.9	2406	59.9
BMI status														
Underweight	97	7.4	90	6.5	212	14.4	179	13.7	160	12.9	157	11.7	469	11.8
Normal weight	833	63.9	859	62.3	1074	73	879	67.3	933	74.9	964	72.2	2840	70.6
Overweight	234	18.0	295	21.4	117	7.9	179	13.7	113	9.1	161	12.1	464	11.5
Obese	139	10.7	135	9.8	69	4.7	69	5.3	39	3.1	53	4.0	247	6.1
Country														
Italy	497	38.1	458	33.2	91	6.2	133	10.2	30	2.4	27	2.0	618	15.4
Estonia	10	0.8	15	1.1	280	19	208	15.9	218	17.5	285	21.4	508	12.6
Cyprus	4	0.3	7	0.5	30	2.0	26	2.0	15	1.2	16	1.2	49	1.2
Belgium	54	4.1	58	4.2	339	23.1	324	24.8	100	8.1	111	8.3	493	12.3
Sweden	5	0.4	17	1.2	30	2.0	45	3.4	537	43.1	510	38.2	572	14.2
Germany	11	0.8	13	1.0	176	12	140	10.7	99	8.0	133	10.0	286	7.1
Hungary	49	3.8	133	9.6	472	32.2	364	27.9	196	15.7	222	16.6	719	17.9
Spain	673	51.7	678	49.2	52	3.5	66	5.1	50	4.0	31	2.3	775	19.3

Table 4 Cross tabulation between the cluster memberships of children at baseline (T0) and follow-up (T1). (Number of participants and percentages)

Cluster membership at T1	Cluster membership at T0						
	Animal protein and refined carbohydrate		Sweet and processed		Healthy		Total, *n*
	n	%	*n*	%	*n*	%	
Animal protein and refined carbohydrate	1056	76.6	187	13.6	136	9.9	1379
Sweet and processed	183	14.0	964	73.8	159	12.2	1306
Healthy	64	4.8	321	24	950	71.2	1335
Total	1303	32.4	1472	36.6	1245	31.0	4020

Table 4 summarizes the percentages of children allocated to the same, or different, clusters at baseline and follow-up. The cluster presenting the highest stability was the 'animal protein and refined carbohydrate' pattern with 76% of the children being allocated there both in T0 and T1. 73.8% of the children remained in the 'sweet and processed' cluster over time while 71.2% remained in the 'healthy' cluster from T0 to T1. Table 5 summarizes the percentages of children allocated in the different categories of hs-CRP over time. Most of the children, 79.9%, remained in the lowest category of the hs-CRP at both time points, i.e., were in the first or second tertile of the hs-CRP.

Table 5 Cross tabulation between the high sensitivity C-reactive protein (hs-CRP) categories at baseline (T0) and follow-up (T1). (Number of participants and percentages)

hs-CRP categories at T1	hs-CRP categories at T0				
	Category I		Category II		Total, *n*
	n	%	*n*	%	
Category I	2618	79.9	658	20.1	3276
Category II	540	72.6	204	27.8	744
Total	3158	78.6	862	21.4	4020

Category I being in the first or second tertile of hs-CRP by gender

Category II being in the highest tertile of hs-CRP by gender

In contrast, 27.8% remained in the highest tertile of hs-CRP over time.

Finally, Table 6 shows the OR and 95% CI for the associations between the hs-CRP categories and the identified dietary patterns.

In the cross-sectional analyses, there were no associations of diet with CRP at T0. When diet assessed at T1 was compared to hs-CRP at T1, children allocated to the 'sweet and processed' cluster had a 28% higher probability of being in the upper category of hs-CRP compared with those allocated in the 'healthy' cluster (OR = 1.28; 95% CI 1.03, 1.61) in the full-adjusted model. In the analysis of the cluster combinations, children allocated to the 'animal protein and refined carbohydrate' or to the 'sweet and processed' cluster at both times presented, respectively, a 47% (OR = 1.47; 95% CI 1.03, 2.09) and a 44% (OR = 1.44; 95% CI 1.08, 1.92) higher probability of being in the upper hs-CRP category compared with those allocated to the 'healthy' cluster both times in the unadjusted model. When all the co-variables were included in the analyses, those allocated in the 'sweet and processed' cluster still presented significantly higher odds of being in the highest hs-CRP category (OR = 1.39; 95% CI 1.05, 1.84) compared to those in the 'healthy cluster'.

The *z* scores of the relative frequency of the food items that defined the clusters, i.e., the highest or lowest z value from Tables 1 and 2 in comparison with the other patterns over time, are presented as radar plots in the supplementary material (Supplementary Figs. 1–12).

Discussion

This study in European children identified three dietary patterns at two time points (T0 and T1) using cluster analysis. The so-labeled 'animal protein and refined carbohydrate' pattern was characterized for having a relatively

Table 6 Associations between high sensitivity C-reactive protein (hs-CRP) in each time point and cluster membership in each time point (T0 and T1)* and the combinations of clusters over time

	hs-CRP		hs-CRP	
	Model 1		Model 2	
	OR**	95% CI	OR**	95% CI
T0				
Healthy cluster	1		1	
Animal protein and refined carbohydrate	1.21	0.96, 1.52	1.19	0.95, 1.50
Sweet and processed	1.11	0.90, 1.35	1.10	0.90, 1.35
Animal protein and refined carbohydrate or sweet and processed	1.14	0.95, 1.38	1.14	0.94, 1.37
T1				
Healthy cluster	1		1	
Animal protein and refined carbohydrate	1.25	0.93, 1.67	1.22	0.95, 1.56
Sweet and processed	**1.28**	**1.02, 1.61**	**1.28**	**1.03, 1.61**
Animal protein and refined carbohydrate or sweet and processed	**1.27**	**1.02, 1.59**	**1.26**	**1.02, 1.54**
Cluster combinations over time[†]				
Healthy at two time points ($n = 950$)	1		1	
Animal protein and refined carbohydrate cluster at two time points ($n = 1056$)	**1.47**	**1.03, 2.09**	1.13	0.97, 1.76
Sweet and processed cluster at two time points ($n = 964$)	**1.44**	**1.08, 1.92**	**1.39**	**1.05, 1.84**
Animal protein and refined carbohydrate or sweet/processed to healthy cluster, ($n = 385$)	1.25	0.88, 1.79	1.12	0.78, 1.60
Healthy cluster to sweet/processed and animal protein and refined carbohydrate ($n = 295$)	1.19	0.82, 1.73	1.12	0.76-1.63
Animal protein and refined carbohydrate or sweet/processed to animal protein and refined carbohydrate or sweet/processed, ($n = 370$)	1.42	0.98, 2.04	1.28	0.90, 1.81

All models of the multilevel logistic regression include random effects (country, school) to account for the study design

Model 1: unadjusted multilevel logistic regression

Model 2: multilevel logistic regression adjusted for age, gender, study region (intervention vs. control), parental education level, BMI and medication

Bold value indicates $p < 0.005$

*T0: baseline, T1: follow

**Odds for being allocated to the highest hs-CRP tertile

[†]Model 1 and Model 2 for the cluster combination included hs-CRP of T1 as dependent variable. Model 1 was unadjusted, while Model 2 was adjusted for age at T1, sex, study region (intervention vs. control), parental education level at T1, BMI at T1, hs-CRP at T0 and medication

high frequency of protein foods, water and some carbohydrate foods; the 'sweet and processed' pattern showed a high relative frequency of both sweet products and sweet drinks and a low relative frequency of fruit and vegetables, whereas the named 'healthy' pattern showed high relative frequency of fruits and vegetables, whole grain foods and low consumption of sweet products. These patterns were consistently similar at both time points, which allowed us to explore the associations of persistency/changes of dietary patterns in children and hs-CRP.

Although dietary patterns are dependent on the specific study group sample and not comparable between studies, it should be mentioned that a previous dietary patterns analysis in the IDEFICS cohort was performed; similar patterns were found using PCA [30]. In the previous study, similar 'animal protein and refined carbohydrate', 'healthy' and 'sweet and processed' patterns were found but were allocated to different names. In addition, they identified a fourth pattern named 'snacking' which was not identified in our analysis and this could be due to the different statistical approach or the different sub-sample. Nevertheless, other studies have obtained similar dietary patterns using different assessment methods in the same sample of adults [31, 32], and even in children [33]. Also, another study performed with IDEFICS data [28] found a similar 'healthy' dietary pattern using cluster analysis. This study also obtained a 'processed' cluster and a 'sweet' cluster, with similar characteristics as the 'sweet and processed' pattern found in this study, whereas no pattern related with protein intake was found. This could be due to the differences in sample size and characteristics: 9301 children were included in that analysis compared with 4020 children included in the present study. Importantly, these studies found persistent patterns in both time points.

In the current study, lower percentages of obese children were included in the 'healthy' pattern in comparison with the proportion of children in the other two patterns. In contrast, higher percentages of overweight and obese children were observed in the 'animal protein and refined carbohydrate' pattern when compared to both 'sweet and processed' and 'healthy' patterns.

The present study also found positive associations between the 'sweet and processed' pattern and inflammation at T1 as compared to the 'healthy' pattern. In the literature, a review identified the western-type diet, characterized by a high consumption of meat, as the dietary pattern more related with inflammation, while the 'healthy' pattern with high consumption of fruits and vegetables was inversely related with inflammation [10]. However, this review included only cross-sectional observational studies. It seems that westernized dietary patterns characterized by higher intakes of red and processed meats, sweets, desserts, fried foods, and refined grains are positively related to an increase of inflammation molecules, endothelial adhesion molecules and atherogenic promoters [34, 35]. Also another review found similar results regarding the western dietary pattern comparing studies using different ways to obtain the dietary patterns [36]. Another study [37] found that the 'eggs and sweets' pattern was associated with high levels of CRP, as well as the 'pasta and meat' pattern, while the 'olive oil and vegetables' pattern was negatively associated with CRP. Therefore, results from literature suggest that the relationship between unhealthy dietary patterns and inflammation is not as consistent as for the 'healthy' pattern. This could be explained because the statistical approach is *a posteriori* method, meaning that different clusters could appear in different samples. In addition, the definition of an unhealthy pattern is wider than for the 'healthy' pattern, often characterized for a high consumption of vegetables and fruits. The beneficial combination of antioxidant vitamins or compounds, fiber and other anti-inflammatory phytochemicals, which are contained in vegetal foods, may underlie the inverse association with CRP, or inflammation [38].

Results from the present study regarding the 'sweet and processed' pattern, in comparison with other studies, could be explained by the population sample, as children are more likely to eat sweet products than adults in a regular basis. Also, soft drinks or sugar-rich foods are associated with glycemic spikes that may contribute to oxidative stress and both to acute and chronic inflammation even in lean subjects [39]. Also the 'animal protein and refined carbohydrate' cluster included foods with high glycemic index, which also have been related with inflammation. However, the 'sweet and processed' pattern was also characterized by a low relative frequency of vegetables and fruits. Therefore, the combination of these frequencies of consumption of these specific foods could explain the relationship between this pattern and inflammation, measured by the hs-CRP.

The present study is subject to a number of limitations. The CEHQ-FFQ was not designed to capture total food intake but to record information on parent-supervised meals. However, the CEHQ-FFQ has previously been shown to give reproducible estimates of the frequency of food group consumption in European children [23, 24]. Also, the number of meals under parental control varied between countries, which could partially explain the differences observed in dietary patterns between countries. In addition, hs-CRP was the only inflammatory marker measured in the IDEFICS study; more markers could have provided a better insight of their effect in the inflammatory process. Moreover, recent Mendelian randomization studies with genetic CRP marker data do not support a causal role for CRP in the etiology coronary heart disease [40]. Also, in cluster analysis, clusters are not exactly the same at different time points, although in the present study we

found high similarities at both time points for each cluster. Finally, the FFQ covered the 4 previous weeks; therefore, potential differences due to seasonality could not be considered in our analysis. However, the measurements of the subjects were performed in the same period over time. On the other hand, this study also presents some strengths: Firstly, the use of standardized and harmonized information from eight European countries and the use of a validated dietary instrument, providing reproducible estimates of food frequency consumptions. Secondly, the multilevel design, which takes into account differences by country and schools adjusting for a set of relevant confounders such as BMI. Finally, the prospective design of the analysis is a strength as it gives a better insight of a long-term behavior such as diet consumption and its relation with inflammation.

To our knowledge, this is the first prospective study to assess the association between dietary patterns and inflammation, measured by hs-CRP, in a sample of European children. In conclusion, this study shows that a 'sweet and processed' pattern was associated with hs-CRP cross-sectionally and over time. It seems that a long-term pattern characterized by a high relative consumption frequency of sugar and processed products and a low relative consumption frequency of vegetables and fruits is independently related with inflammation already in childhood. Efforts to reduce the frequency of sugar and processed products consumption and to increase the frequency of fruits and vegetables consumption should be undertaken in children, to avoid potential future diseases related with chronic inflammation. These results provide further insight to better understand the association between dietary patterns and inflammation.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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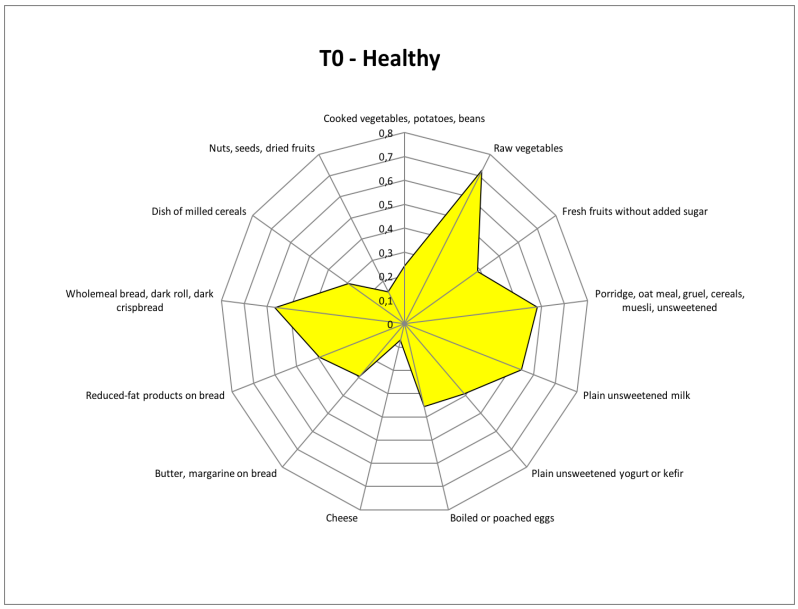
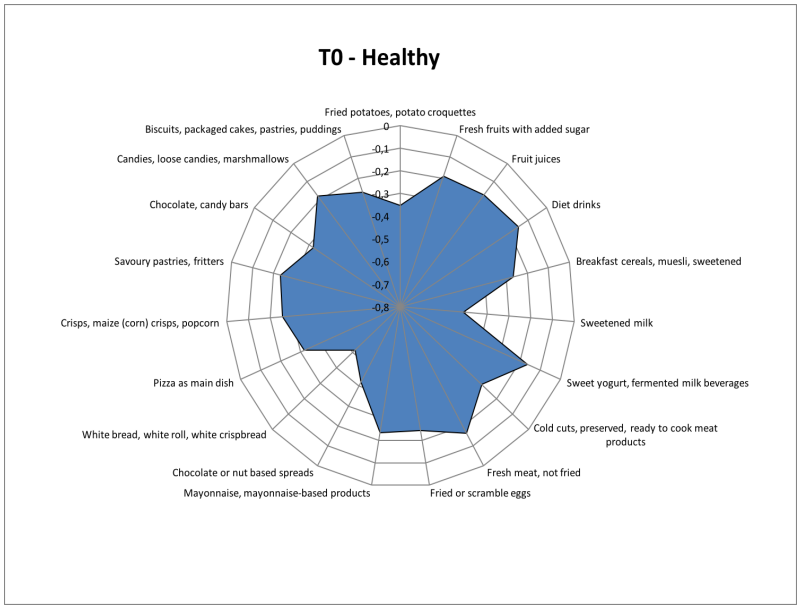
MATERIAL SUPLEMENTARIO

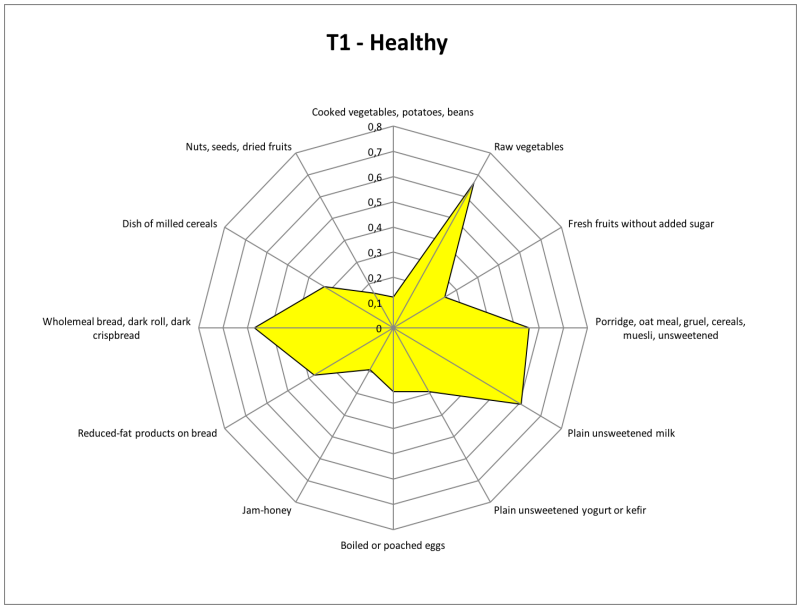
SUPPLEMENTARY MATERIAL



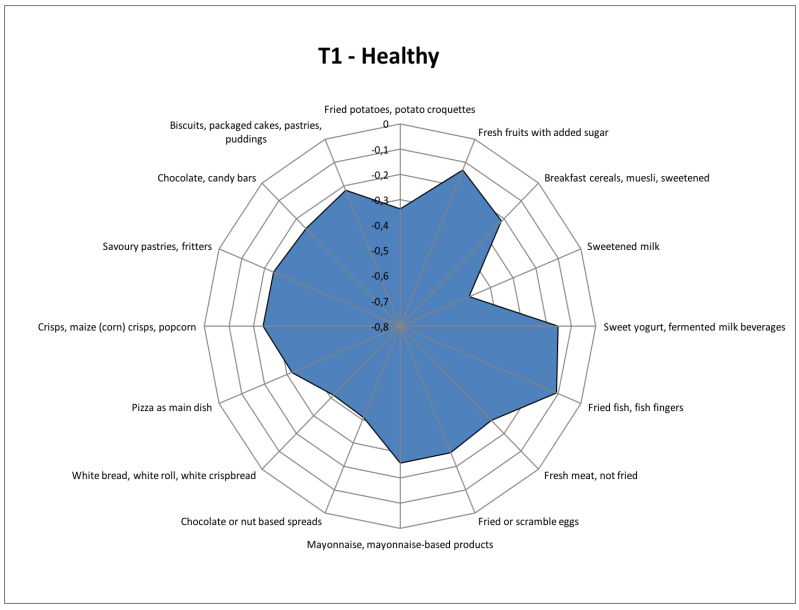
Supplementary figure 1. Z-Scores of relative consumption frequencies in the 'healthy' pattern in baseline (T0). Highest mean values per food item in comparison with the other two patterns.



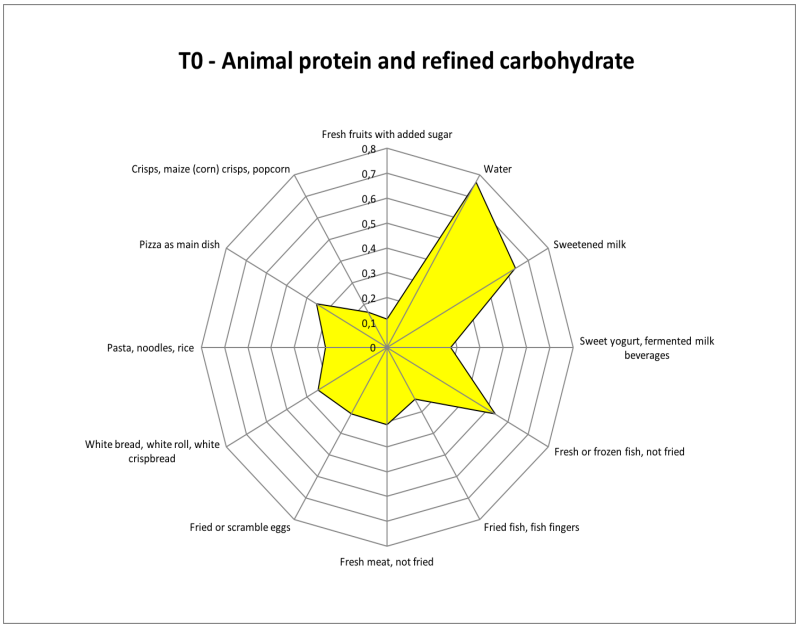
Supplementary figure 2. Z-Scores of relative consumption frequencies in the 'healthy' pattern in baseline (T0). Lowest mean values per food item in comparison with the other two patterns.



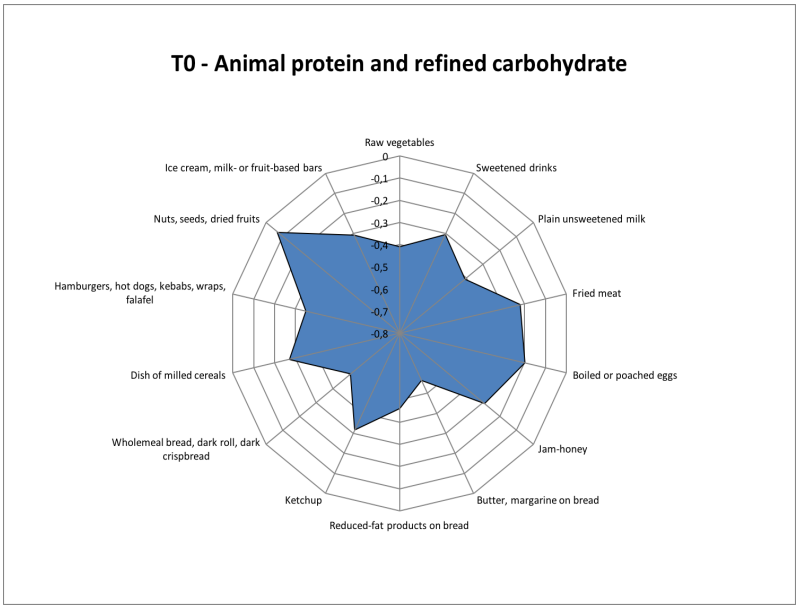
Supplementary figure 3. Z-Scores of relative consumption frequencies in the 'healthy' pattern in follow-up (T1). Highest mean values per food item in comparison with the other two patterns.



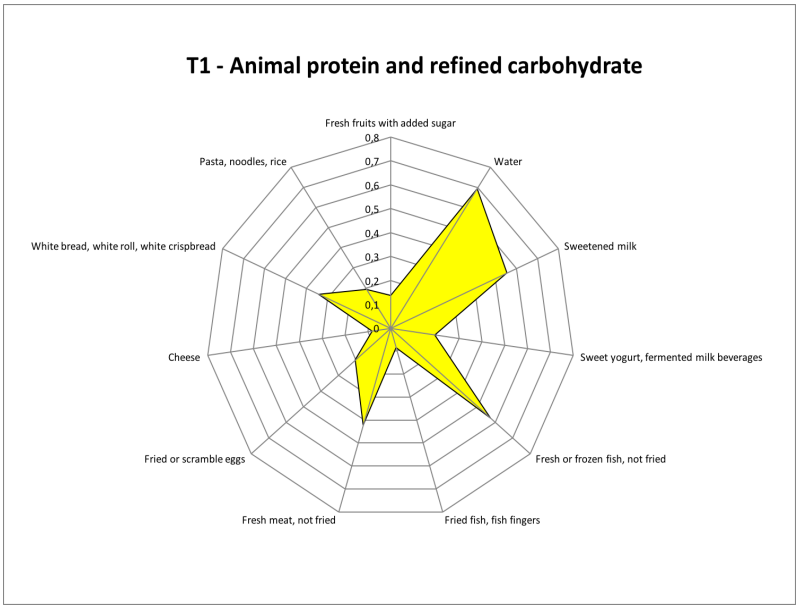
Supplementary figure 4. Z-Scores of relative consumption frequencies in the 'healthy' pattern in follow-up (T1). Lowest mean values per food item in comparison with the other two patterns.



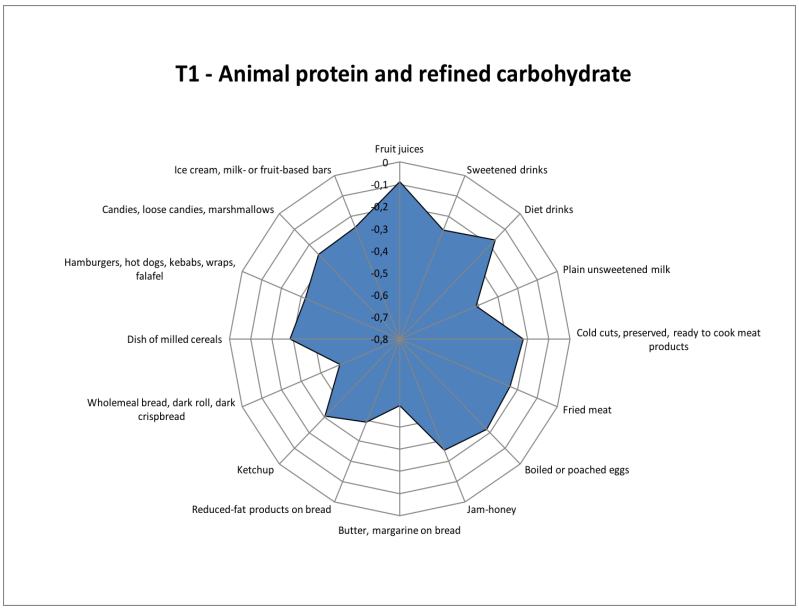
Supplementary figure 5. Z-Scores of relative consumption frequencies in the 'animal protein and refined carbohydrate' pattern in baseline (T0). Highest mean values per food item in comparison with the other two patterns.



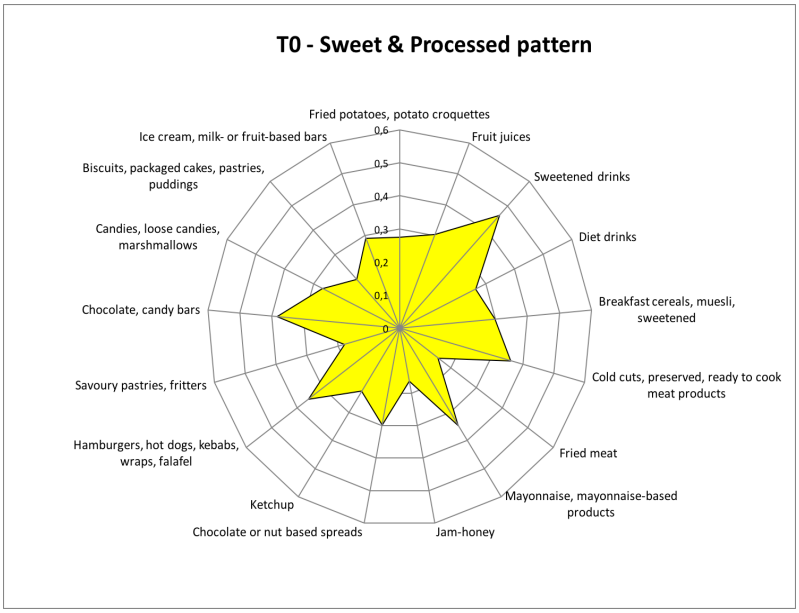
Supplementary figure 6. Z-Scores of relative consumption frequencies in the 'animal protein and refined carbohydrate' pattern in baseline (T0). Lowest mean values per food item in comparison with the other two patterns.



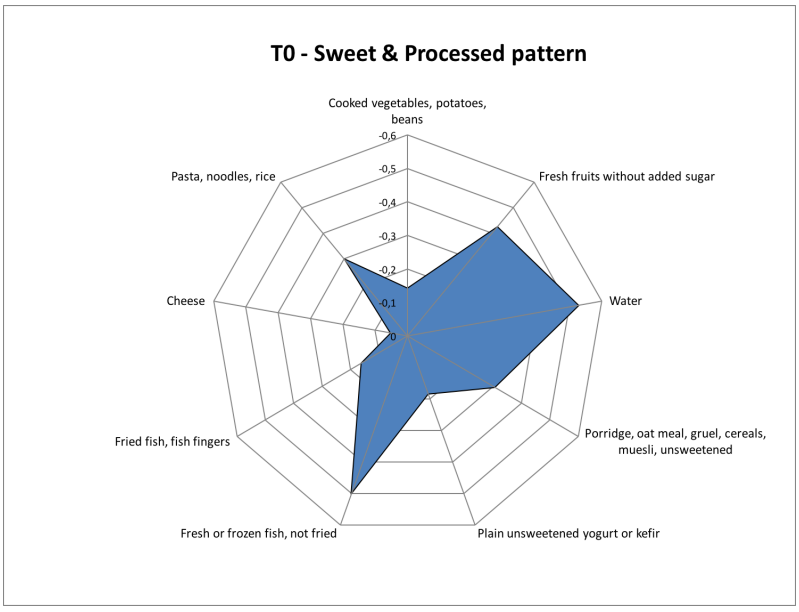
Supplementary figure 7. Z-Scores of relative consumption frequencies in the 'animal protein and refined carbohydrate' pattern in follow-up (T1). Highest mean values per food item in comparison with the other two patterns.



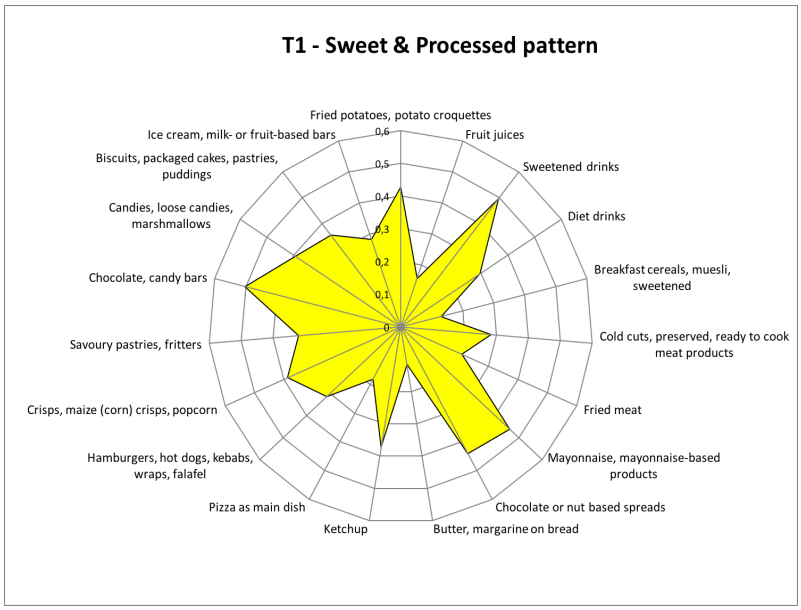
Supplementary figure 8. Z-Scores of relative consumption frequencies in the 'animal protein and refined carbohydrate' pattern in follow-up (T1). Lowest mean values per food item in comparison with the other two patterns.



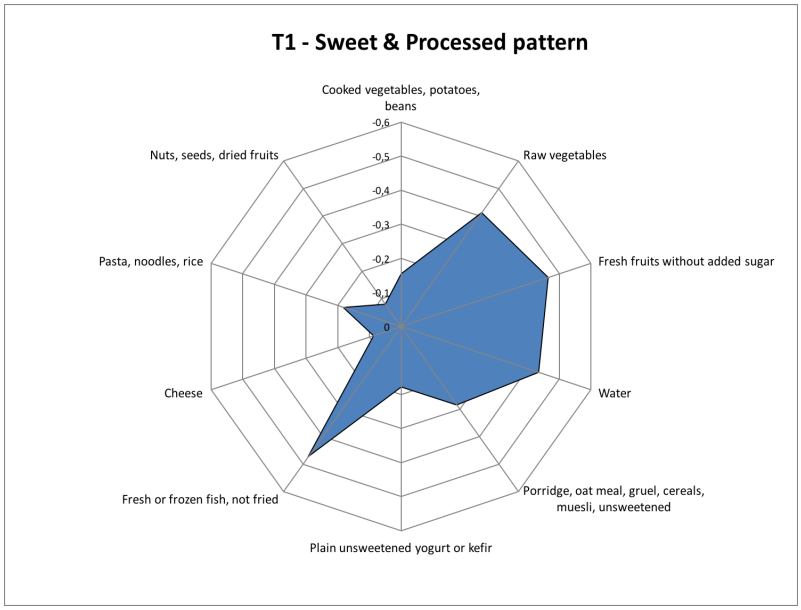
Supplementary figure 9. Z-Scores of relative consumption frequencies in the 'sweet and processed' pattern in baseline (T0). Highest mean values per food item in comparison with the other two patterns.



Supplementary figure 10. Z-Scores of relative consumption frequencies in the 'sweet and processed' pattern in baseline (T0). Lowest mean values per food item in comparison with the other two patterns.



Supplementary figure 11. Z-Scores of relative consumption frequencies in the 'sweet and processed' pattern in follow-up (T1). Highest mean values per food item in comparison with the other two patterns.



Supplementary figure 12. Z-Scores of relative consumption frequencies in the 'sweet and processed' pattern in follow-up (T1). Lowest mean values per food item in comparison with the other two patterns.

IDEAL CARDIOVASCULAR HEALTH AND INFLAMMATION IN EUROPEAN ADOLESCENTS: THE HELENA STUDY.

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ABSTRACT

Background and aims: Inflammation plays a key role in atherosclerosis and this process seems to appear in childhood. The ideal cardiovascular health index (ICHI) has been inversely related to atherosclerotic plaque in adults. However, evidence regarding inflammation and ICHI in adolescents is scarce. The aim is to assess the association between the ICHI and inflammation in European adolescents.

Methods and results: 543 adolescents (251 boys and 292 girls) from the Healthy Lifestyle in Europe by Nutrition in Adolescence (HELENA) study, a cross-sectional multi-center study including 9 European countries, were measured. C-reactive protein (CRP), complement factors C3 and C4, leptin and white blood cell counts were used to compute an inflammatory score. Multilevel linear models and multilevel logistic regression were used to assess the association between ICHI and inflammation controlling by covariates. Higher ICHI was associated with a lower inflammatory score, as well as with several individual components, both in boys and girls ($p<0.01$). In addition, adolescents with at least 4 ideal components of the ICHI had significantly lower inflammatory score and lower levels of the study biomarkers, except CRP. Finally, the multilevel logistic regression showed that for every unit increase in the ICHI, the probability of having an inflammatory profile decreased by 28.1% in girls.

Conclusion: Results from this study suggest that a better ICHI is associated with a lower inflammatory profile already in adolescence. Improving these health behaviors, and health factors included in the ICHI, could play an important role in CVD prevention.

Keywords: Cardiovascular health; inflammation; European adolescents.

INTRODUCTION

Cardiovascular diseases (CVD), such as coronary artery disease, are the result of atherosclerosis progression (1). Evidence suggest that inflammation has a key role in the origin and development of atherosclerosis (2) as it triggers the formation of the fatty streak and its development into complex plaque (3). Atherosclerosis has its origins in childhood and is associated with early risk factors (4), yet symptoms may appear later in life (5). The relationship between inflammation and cardiovascular diseases is present already in childhood (6).
High concentrations of C-reactive protein (CRP) seem to track from childhood to adulthood (7). However, there are other biomarkers contributing to the characterization of the inflammatory process such as cytokines,(8) e.g. tumor necrosis factor alpha (TNF-alpha), or interleukins, e.g. interleukin 6 (IL-6). Nevertheless, other biomarkers have also been considered (9).
In addition, CRP is not always associated with atherosclerosis diagnosed by image techniques,(10) therefore, the use of a score that combines several inflammatory biomarkers could provide an overall estimation of the inflammatory status. A previous study (11) developed an inflammatory score, which included CRP, complement factors C3 and C4, leptin and white blood cells (WBC) being selected due to their high correlation with fatness and traditional cardio-metabolic risk factors.

In 2010, the American Heart Association (AHA) released the ideal cardiovascular health index (ICHI), (12) including four health behaviors and three health factors. The behavior-related criteria were: non-smoking, being physically active, having normal body mass index (BMI), and eating a healthy diet, while the health factors included were: normal blood pressure, plasma total cholesterol and glucose. The ICHI has been inversely related to the presence of atherosclerotic plaque in adults (13); therefore, it could represent a useful epidemiological tool to assess the cardiovascular profile.
Although there are some studies assessing the relationship between cardiovascular profile and metabolic risk factors in adolescents or young adults,(14, 15) there is not sufficient evidence on the association between inflammation and cardiovascular health in young populations.

The aims of the present study were to to assess the association between ICHI and inflammatory markers in European adolescents and to examine the use of an inflammatory score to assess the inflammatory status in adolescents (14).

METHODS

Study design

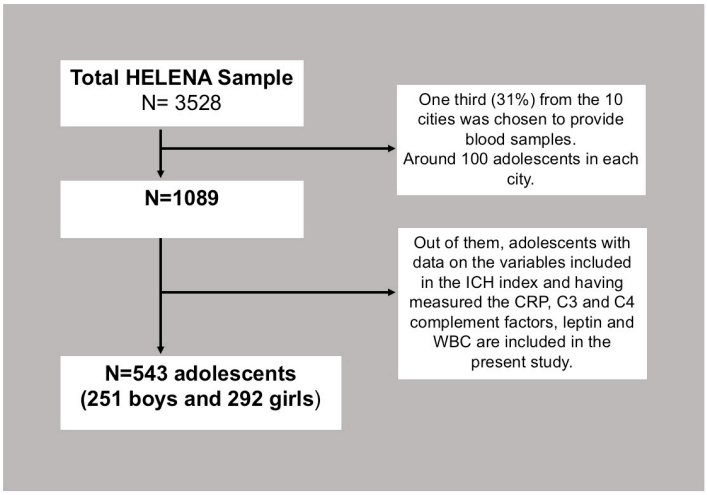
The HELENA study is a cross-sectional multi-center study (n=3528) conducted in 10 European cities: Athens and Heraklion in Greece, Dortmund in Germany, Ghent in Belgium, Lille in France, Pecs in Hungary, Rome in Italy, Stockholm in Sweden, Vienna in Austria and Zaragoza in Spain. HELENA study has been previously described(16).

The study was performed according to the ethical guidelines of the Edinburgh revision of the 1964 Declaration of Helsinki (2000). The local Ethics Committees of each center approved the protocol and written informed consent was obtained.

Study population

Out of the total HELENA sample, one third from the 10 cities was chosen to provide blood samples (n=1089, 31%). Therefore, around 100 adolescents in each city were selected by means of the immunological parameters which were those with the highest variability within the blood measurements that were included in the study (16). Overall, 543 participants (251 boys and 292 girls) met the inclusion criteria for the present analysis: having data on the variables included in the ICH index and having measured the CRP, C3 and C4 complement factors, leptin and WBC. (Supplementary Figure 1)

Supplementary Figure 1. Flow diagram of the study population.



Physical examinations

Weight and height were measured in underwear and barefoot with a SECA 861 (Seca Ltd) and with a stadiometer SECA 225 (Seca Ltd). In addition, body mass index (BMI) was calculated as body weight in kilograms divided by the square of height in meters. Pubertal maduration was examined by a clinician and was assessed according Tanner (5-point-scale). Systolic and diastolic blood pressure was measured with an automatic oscillometric device (Omron M6). Participants were seated in a quiet room for ten minutes with their backs supported and feet on the ground. The lowest value of the two measurements, taken with a difference of 5 minutes, was recorded and the mean was used in data analysis. All anthropometric measures were taken following a standardized protocol.

Socioeconomic status

A modified version of the family affluence scale (FAS) was used as a proxy of socioeconomic status (SES). The adolescents completed a questionnaire asking about the numbers of cars and computers at home, having internet and whether the adolescent had his or her own room. In the HELENA study, the FAS was modified by replacing 'frequency of family holidays' by 'Internet availability at home'. Adolescents were scored from 0 (very low SES) to 8 (very high SES).

Blood analysis

Blood withdrawal was performed in fasting status. WBC counts and percentages were determined with automated blood cell counters. C-reactive protein (CRP) levels were quantified by immunoturbidimetry (AU 2700, Olimpus, Rungis, France). Serum C3 and C4 complement factors were analyzed by nephelometry (Behring Diagnostics, CA, USA). The coefficient of variation (inter-assay precision) was 1.9% for CRP, 1.4% for C3, and 1.2% for C4. Detection limits (sensitivity) were 0.007 mg/L for CRP, 0.01 g/L for C3, and 0.002 g/L for C4. Serum leptin (ng/mL) was measured using the RayBio Human Leptin ELISA (Enzyme-Linked Immunosorbent Assay; RayBiotech, Norcross, GA, USA) kit. The sensitivity of the leptin assay was <6 pg/mL, with intra-assay and interassay coefficients of variation of <10% and <12%.

Ideal cardiovascular health index

The AHA released the ICHI in 2010 (12) with the cut off values for adolescents.

Health behaviors

Four health behaviors were considered for the ICH index: smoking behavior, physical activity, BMI and diet.

Smoking status was categorized considering those who had never smoked as having an ideal smoking behavior. Adolescents who performed more than 60 min of moderate to vigorous self-reported exercise every day were classified as having an ideal physical activity level. BMI z-score and BMI categories were derived using the British 1990 Growth Reference Data from the Child Growth.(17, 18)

To assess dietary intake the HELENA-Dietary Assessment Tool (HELENA-DIAT)(19), a self-report dietary recall based on six meal occasions, was used. The dietary indicators used to assess ideality of the diet were: consumption of fruit and vegetables (more than 400 g per day), fish and fish products (at least 28 g per day), fiber (at least 1.1 grams per 10 g of carbohydrates per day), sodium (less than 1500 mg per day), and soft drinks (less than 145 mL per day). Having at least 4 of these indicators classified as 'ideal' was considered as ideal healthy diet.

Health factors

The cut-off for the biomarkers assessed to consider them ideal was <170mg/dL for plasma total cholesterol and <100mg/dL for glucose.

The lower value of the diastolic blood pressure and systolic blood pressure was used in the analysis to classify blood pressure status as ideal when lower than the 90th centile for the blood pressure (12).

Inflammatory score

A continuous score was computed from some inflammatory biomarkers: CRP, C3, C4, WBC and leptin. The selection of these biomarkers was based on a preliminary analysis with fatness and traditional cardio-metabolic risk factors as previously assessed within the HELENA study (11) (Supplementary material table 1).

Standardized values of the biomarkers were calculated for boys and girls and by 1-year age groups with the following formula: standardize value= (value – mean) / standard deviation (SD), as has been done elsewhere.(11) Z-scores from biomarkers were summed up to create a score of inflammation.

Statistical analysis

Analyses were stratified by sex. Normality assumption was checked and transformation was performed if required. Partial correlations, adjusted for age, sex, pubertal stage and center, between traditional and nontraditional cardio metabolic biomarkers were performed for the selection of the inflammatory biomarkers for the inflammatory score.

Student t test and chi-squared test were performed for the differences between the study participants by sex. Additionally, ANCOVA was performed to assess mean value of the inflammatory score by the ideal category and non-ideal category of each component of the ICHI, adjusting by tanner as covariate and center as random factor.

Multilevel linear models (level: center) were used to assess the associations between the inflammatory score (dependent variable) and the ICHI. Two

different models were carried out. In the first model, the covariates used were Tanner and SES while in the second model the cardiorespiratory fitness was included. Frequencies between number of components of the ICHI and inflammatory score were assessed and the p for trend was calculated.

Finally, a multilevel logistic regression (level: center) was performed. The inflammatory index was transformed into a categorical variable using the median value in order to split the sample into two groups (I: > -0.737; II: ≤ -0.737 for boys and I: > -0.268; II: ≤-0.268 for girls) and the ideal cardiovascular health index was considered as independent variable. Two different models were performed. In the first model, the covariates used were Tanner and SES while in the second model the cardiorespiratory fitness was included. Interactions between covariates and dependent variable were assessed before calculating the multivariate regression model using Wald test in both multilevel models: linear and logistic, and no statistical significance was observed in any. Also, multicollinearity was assessed by means of variance inflation factor values calculation for covariates in each multilevel linear model, and all were < 10.

Data were managed and analyzed with SPSS Statistics v.19 and R software with lme4 package for multilevel regression models and AED package to test for multicollinearity.

RESULTS

Baseline characteristics are shown in Table 1. There were significant differences by sex in some of the ICHI components and some biomarkers. None of the boys and only 9% of the girls followed a healthy diet, almost 47% of the girls had high total cholesterol levels and 40% of the girls did not comply with PA guidelines. Results for the selection of the inflammatory biomarkers are found in Supplementary table 1. Differences in mean concentration of the inflammatory score by the categories of each ICHI component are presented in Supplementary table 2. Significant sex differences were found in BMI, physical activity and blood pressure. Plasma glucose showed significant differences by category of ICHI component in boys.

Table 1. Characteristics of the study participants.

Mean ± SD	*Boys (n=251)*	*Girls (n=292)*	*p*
Age (years)	14.80±1.28	14.81±1.17	0.911
Tanner I % (n)	0.8 (2)	0 (0)	0.126
Tanner II % (n)	12.4 (31)	6.9 (20)	**0.028**
Tanner III % (n)	17.6 (44)	24.5 (71)	0.054
Tanner IV % (n)	46.0 (115)	45 (132)	0.887
Tanner V % (n)	23.2 (58)	23.1 (67)	0.964
Moderate-vigorous PA (min/day)	121.73±91.47	90.89±72.21	**<0.001**
BMI (kg/m^2)	21.04±3.96	21.14±3.38	0.763
Systolic blood pressure (mm Hg)	120.10±14.04	112.85±11.19	**<0.001**
Diastolic blood pressure (mm Hg)	64.02±8.61	65.04±8.72	0.174
Glucose (mg/dL)	92.21±6.93	88.43±5.99	**<0.001**
Total cholesterol (mg/dL)	152.60±26.06	167.67±27.49	**<0.001**
Inflammatory score	-0.02±3.23	0.14±3.06	0.517
CRP (mg/L)	0.82±1.18	0.85±1.27	0.781
C3 (g/L)	1.11±0.16	1.13±0.16	0.089
C4 (g/L)	0.20±0.06	0.21±0.06	0.271
Leptin (ng/mL)	9.17±14.93	29.1±25.06	**<0.001**
WBC (10x3/ μL)	6.06±1.34	6.45±1.55	**0.002**
Ideal health behaviors			
Smoking % (n)	61.8 (155)	59.2 (173)	0.552
Body mass index % (n)	78.5 (197)	82.9 (242)	0.195
Physical activity % (n)	70.5 (177)	59.9 (175)	**0.010**
Diet % (n)	0 (0)	9 (3.1)	**-**
Ideal health factors			
Total cholesterol % (n)	78.5 (197)	53.4 (156)	**<0.001**
Blood pressure % (n)	88.8 (223)	90.1 (263)	0.643
Plasma glucose % (n)	84.5 (212)	96.9 (283)	**<0.001**

Physical activity. BMI: Body mass index. CRP: C-reactive protein. C: Complement factor. WBC: Whole blood cells count. ICHI: Ideal cardiovascular health index. Those in bold had a significance level lower than 0.005.

Results for the multilevel linear models of the ICHI are presented in Table 2, for boys, and Table 3, for girls. In model 1, the ICHI was significantly and inversely related to the inflammatory score and its components: inflammatory score ($p<0.001$ for boys and girls), C3 ($p=0.001$ for boys and $p<0.001$ for girls), C4 ($p=0.002$ for boys and $p=0.001$ for girls), WBC ($p=0.017$ for girls) and log-leptin ($p<0.001$ for boys and girls). In model 2, the biomarkers significantly and inversely associated with the ICH index were: inflammatory score ($p=0.005$ for boys and $p=0.005$ for girls), C3 ($p=0.001$ in girls), C4 ($p=0.004$ in boys and $p=0.039$ in girls) and log-leptin ($p<0.001$ in boys and $p=0.006$ in girls). Also,

lower levels of inflammation were associated with a higher number of components of the ICH index in boys ($p<0.001$) and girls ($p<0.001$) (Figure 1).

Table 2. Multilevel linear models of the ideal cardiovascular health index and inflammation in boys.

	Model 1			**Model 2**		
BOYS	**Inflammatory score**			**Inflammatory score**		
Ideal cardiovascular health index	Beta	95% CI	**P**	Beta	95% CI	**P**
	-0.794	-1.146, -0.442	<0.001	-0.597	-1.014, -0.181	0.005
	CRP*			**CRP***		
	Beta	95% CI	**P**	Beta	95% CI	**P**
	-0.040	-0.183, -0.096	0.540	0.029	-0.140, 0.199	0.732
	C3			**C3**		
	Beta	95% CI	**P**	Beta	95% CI	**P**
	-0.297	-0.047, -0.011	0.001	-0.017	-0.037, 0.003	0.110
	C4			**C4**		
	Beta	95% CI	**P**	Beta	95% CI	**P**
	-0.011	-0.018, -0.004	0.002	-0.012	-0.021, -0.004	0.004
	WBC			**WBC**		
	Beta	95% CI	**P**	Beta	95% CI	**P**
	-0.077	-0.229, 0.074	0.315	-0.045	-0.226, 0.136	0.625
	Leptin*			**Leptin***		
	Beta	95% CI	**P**	Beta	95% CI	**P**
	-0.393	-0.509, -0.227	<0.001	-0.298	-0.432, -0.164	<0.001

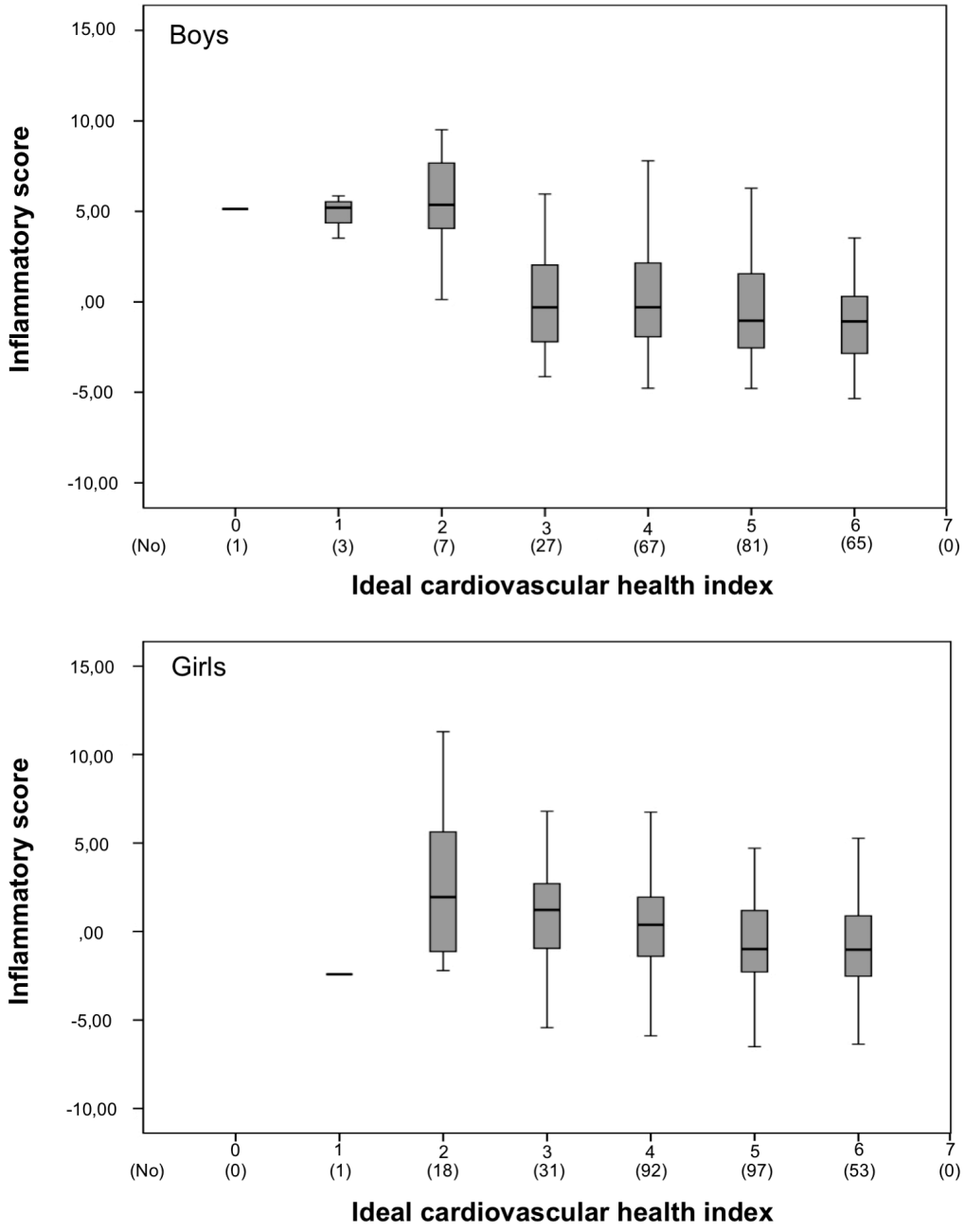
95% CI: Confidence Interval. CRP: C-reactive protein. C: Complement factor. WBC: Whole blood cells count.
**CRP and Leptin are log-transformed.*
Model 1: Adjusted by tanner and socioeconomic status (SES)
Model 2: Adjusted by tanner, SES, and cardiorespiratory fitness.

Table 3. Multilevel linear models of the ideal cardiovascular health index and inflammation in girls.

	Model 1			Model 2		
GIRLS	**Inflammatory score**			**Inflammatory score**		
Ideal cardiovascular health index	Beta	95% CI	**P**	Beta	95% CI	**P**
	-0.646	-0.973, -0.319	<0.001	-0.52	-0.885, -0.155	0.005
	CRP*			**CRP***		
	Beta	95% CI	**P**	Beta	95% CI	**P**
	-0.102	-0.240, 0.035	0.145	-0.017	-0.180, 0.145	0.831
	C3			**C3**		
	Beta	95% CI	**P**	Beta	95% CI	**P**
	-0.036	-0.054, -0.019	<0.001	-0.034	-0.053, -0.015	0.001
	C4			**C4**		
	Beta	95% CI	**P**	Beta	95% CI	**P**
	-0.012	-0.019, -0.005	0.001	-0.008	-0.016, -0.0004	0.039
	WBC			**WBC**		
	Beta	95% CI	**P**	Beta	95% CI	**P**
	-0.202	-0.366, -0.037	0.017	-0.157	-0.358, 0.043	0.123
	Leptin*			**Leptin***		
	Beta	95% CI	**P**	Beta	95% CI	**P**
	-0.170	-0.257, -0.082	<0.001	-0.143	-5.469, -1.963	0.006

95% CI: Confidence Interval. CRP: C-reactive protein. C: Complement factor. WBC: Whole blood cells count.
**CRP and Leptin are log-transformed.*
Model 1: Adjusted by tanner and socioeconomic status (SES)
Model 2: Adjusted by tanner, SES, and cardiorespiratory fitness.

Figure 1. Association between inflammatory score and Ideal Cardiovascular Health index.



Finally, the multilevel logistic regression (Table 4) showed the probability of having a higher or lower inflammatory state when increasing one unit of the ICHI. For boys, when increasing the ICHI with one unit, the probability of having a higher inflammatory status decreased 30.7% (OR=0.693, 95%CI: 0.544-0.883, p=0.003) in the model 1, while this probability decreased 26.5% (OR=0.735, 95%CI: 0.533-1.014 p=0.061) in model 2. In girls, when increasing the ICHI with one unit the probability of having a higher inflammatory status decreased 22.3% (OR=0.677, 95%CI: 0.539-0.850, p<0.001) in the first model and 28.1% (OR=0.719, 95%CI: 0.534-0.969, p=0.031) for model 2.

Table 4. Multilevel logistic regression.

	Model 1			**Model 2**		
BOYS	***OR***	***95% CI***	***p***	***OR***	***95% CI***	***p***
ICHI	0.693	0.544-0.883	0.003	0.735	0.533-1.014	0.061
GIRLS	***OR***	***95% CI***	***p***	***OR***	***95% CI***	***p***
ICHI	0.677	0.539-0.850	<0.001	0.719	0.534-0.969	0.031

OR: Odds ratio
Model 1: Adjusted by tanner and socioeconomic status (SES)
Model 2: Adjusted by tanner, SES, and cardiorespiratory fitness.
CI: Confidence Interval.

DISCUSSION

Findings from this study suggest that the ICHI proposed by the AHA is negatively associated with inflammation, measured by biomarkers and an inflammatory score, in a sample of European adolescents.

Less than optimal cardiovascular health during adolescence seems to be critical in the development of future CVD.(20) A very low prevalence of the ICHI has been shown in a U.S sample of adolescents, especially regarding both behavioral components, physical activity and diet.(21) Furthermore, in another study in adolescents, the ICHI was inversely associated with aortic intima-

media and directly associated with aortic elasticity, already in adolescence, supporting the relevance of this tool as part of a primary prevention of future cardiovascular events.(22)

However, none of the European adolescents included in our study sample met the 7 components of the ICHI. This result is in line with previous studies reporting the same outcome in adolescents (14, 20, 22). Maybe these results are due to the low scores of the ideal diet score component; this component includes at least four ideal diet criteria out of five, and was also the component least often met in our sample, 1.7%. In studies performed in adults, the ideal diet score was also the less frequent component; prevalence being <1%(23) and 0.4%(24). In our sample, among the diet components, the optimum level of sodium intake was achieved only by 8.7% of the adolescents, being the most difficult criteria to meet, but also one of the most challenging criteria to measure accurately. In contrast, having <100mg/dL for glucose was the most commonly achieved component of the ICHI since 91.2% of our sample met this criteria.

In our sample, we observed a negative association between ICHI and the inflammatory score, suggesting that the higher the ICHI the lower the inflammatory score. To our knowledge, there are no previous studies assessing the relation between ICHI and inflammation in adolescence. However, a previous study observed that ICHI in adolescence was a good predictor of cardio-metabolic health in adulthood (20). As, individually, the components of the ICHI, such as cardiovascular risk factors, have been already related to biomarkers of inflammation(25). It seems that cardiovascular risk could be mediated through inflammation.

In the current study, the observed associations were found using the ICHI as a 7-component variable and the inflammatory score, independently of sex. However, the ICHI was associated with all the individual biomarkers of the inflammatory score except CRP. This protein is the most widely clinical biomarker of inflammation because it is easily and reliably measured and it has been related to adiposity and cardiovascular risk factors in healthy children.(26) Moreover, CRP has been related to the prediction of coronary heart disease (27) and atherosclerosis in adults (28). However, based on our findings, it would be recommended to investigate other biomarkers related to traditional metabolic risk factors, in addition to CRP, to evaluate the inflammatory status.

Cardiorespiratory fitness can be considered as a marker of cardiovascular health in children and adolescents (29) and has been related to an increased prevalence of CVD risk factors in adolescents and adults (30). A previous study with HELENA data showed that higher levels of cardiorespiratory fitness were positively associated with the ICHI in adolescents.(14) Our results show that the ICHI was associated with inflammation independently of cardiorespiratory fitness in girls.

There were several limitations to our findings. First, the cross-sectional nature of the study is a limitation. The inflammatory score is sample specific and each biomarker weighted equally for the prediction of cardio-metabolic risk. Blood samples only reflect inflammation at this specific time point. However, this study has many strengths including the use of an inflammatory score that sums up several inflammatory biomarkers, related to cardio-metabolic risk to assess an overall cardio metabolic status as well as the use of standardized and harmonized information from 9 European countries.

In conclusion, results from the current study show that there is an association between the ideal cardiovascular health in adolescence and inflammatory status. Despite not being significant for CRP, results were strongly associated with a composite index of inflammation including CRP, WBC, C3, C4 and leptin, in both gender, and, in girls, independently of the cardiorespiratory. Since the most difficult ICHI criteria to achieve was ideal diet, we should concentrate efforts to improve consumption of those food items included in the index, especially emphasizing the reduction of salt intake. These results provide further insight to better understand the association between lifestyle and cardiovascular risk. Longitudinal studies in adolescent populations measuring the association between inflammation and cardiovascular risk are needed to confirm these results and to prevent future related diseases.

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CONFLICT OF INTEREST: None declared

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Table S1. Partial correlations (r, adjusted for age, sex, pubertal stage (Tanner) and center) between traditional and nontraditional cardiometabolic biomarkers

	BMI[a]	*Waist circumf.*[a]	*Sum of 4 skinfolds*[a]	*Systolic BP*	*Diastolic BP*	*TG*[a]	*TC/HDL*[a]	*HOMA-IR*[a]
CRP[a] (mg/L)	0.231***	0.197***	0.263***	0.057	0.087*	0.114**	0.116**	0.111*
C3[b] (g/L)	0.34***	0.339***	0.398***	0.176***	0.195***	0.366***	0.269***	0.235***
C4[b](g/L)	0.222***	0.209***	0.222***	0.045	0.085*	0.255***	0.178***	0.146***
Leptin[a](ng/mL)	0.609***	0.566***	0.671***	0.150**	0.127*	0.311***	0.138**	0.309***
WBC[a](10x3/ μL)	0.150**	0.146**	0.153**	0.085*	0.122**	0.183***	0.083*	0.140**
IL-6[a] (pg/mL)	0.020	0.038	0.048	0.017	0.022	0.016	-0.044	0.048
TNF-α1 [a](ng/mL)	0.039	0.027	0.057	-0.010	0.087*	-0.033	-0.014	0.069
TGF- β [b](ng/mL)	0.011	0.039	-0.002	0.038	0.043	-0.020	0.022	0.005
L-selectin[b] (ng/mL)	0.072	0.045	0.057	-0.032	-0.066	0.078	0.005	-0.027
sE-selectin[a] (ng/mL)	0.085*	0.083*	0.065	0.013	0.075	-0.028	0.013	-0.015
sVCAM-1[b] (ng/mL)	-0.93*	-0.057	-0.045	0.038	0.013	-0.106*	-0.086*	-0.017
sICAM-1[a] (ng/mL)	0.091*	0.069	0.100*	0.002	0.059	-0.072	0.035	-0.084*

*CRP: C-reactive protein. C: Complement factor. WBC: White blood cell count. IL: interleukin. TNF: Tumor Necrosis Factor. TGF: Transforming growth factor. VCAM: Vascular cell adhesion protein. ICAM: Intercellular Adhesion Molecule. *p<0.005 **p<0.01 and ***p<0.000. P values are one-tailed.*

aValues were natural log transformed before analysis . bValues were square root transformed before analysis

Table S2. Inflammatory score mean and SD by ideal cardiovascular health components. From analysis of covariance with tanner as covariate and centre as random factor.

	Boys					***Girls***				
	Ideal		**Non-ideal**			**Ideal**		**Non-ideal**		
	N	**Mean ± SD**	**N**	**Mean ± SD**	**P**	**N**	**Mean ± SD**	**N**	**Mean ± SD**	**P**
Health behaviours										
Smoking	155	-0.03±3.18	96	-0.01±3.32	0.862	173	0.028±3.01	119	0.32±3.14	0.544
Body mass index	197	-0.65±2.76	54	2.24±3.78	**<0.001**	242	-0.37±2.72	50	2.65±3.38	**<0.001**
Physical activity	177	-0.40±3.03	74	0.87±3.53	**0.004**	175	-0.16±3.05	117	0.61±3.01	**0.046**
Diet	-	-	251	-0.02±3.23	-	9	0.41±3.38	283	0.14±3.05	0.807
Health factors										
Total cholesterol	197	-0.21±3.11	54	0.62±3.6	0.105	156	0.07±3.1	136	0.22±3.01	0.696
Blood pressure	223	-0.25±3.17	28	1.8±3.19	**0.001**	263	-0.01±2.96	29	1.66±3.49	**0.005**
Plasma glucose	212	-0.21±2.99	39	1±4.21	**0.031**	283	0.13±3.08	9	0.56±2.33	0.631
	≥ 4 ideal components		**<4 components**			**≥ 4 ideal components**		**<4 components**		
TOTAL	146	-0.65±2.84	105	0.84±3.54	**<0.001**	150	-0.56±2.79	142	0.89±3.15	**<0.001**

*Those in **bold** had a significance level lower than 0.005*

INFLAMMATION AND INSULIN RESISTANCE ACCORDING TO BODY COMPOSITION IN EUROPEAN ADOLESCENTS: THE HELENA STUDY.

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ABSTRACT

Introduction: Inflammation is related to insulin resistance in adults, especially on those individuals with high levels of body composition.

Objectives: The aim of this study is to assess the relationship between a set of inflammatory biomarkers and insulin resistance by levels of body composition in a sample of European adolescents.

Material and methods: 962 adolescents (442 boys and 520 girls) from 9 European countries met the inclusion criteria of having measurements for the homeostasis model assessment (HOMA) and a set of inflammation-related biomarkers: C-reactive protein (CRP), tumor necrosis factor alpha (TNF-α), interleukin (IL-6), complement factors C3 and C4 and selected cell adhesion molecules. Body mass index (BMI), fat mass index (FMI) and waist circumference (WC) were categorized using tertiles. To assess the associations stratifying by body composition indexes, ANOVA and linear regression models were performed.

Results: Mean biomarkers' concentrations differed across BMI, FMI and WC tertiles ($p<0.05$), by sex. In both sexes, insulin, HOMA, CRP, C3 and C4 were significantly different between categories ($p<0.001$), always showing the highest mean concentration in the upper category of BMI, FMI and WC. The most consistent finding was an association between insulin resistance and C3 concentrations ($p<0.05$), in the adolescents in the highest tertile of BMI, FMI and WC, except in the case of FMI in girls.

Conclusion: Inflammatory and glucose metabolism markers differed by tertiles of body composition, being usually higher in the highest tertile. C3 complement factor was associated with insulin resistance in adolescents, especially those with high total and abdominal adiposity.

Keywords: Inflammation, insulin resistance, body composition, European adolescents.

INTRODUCTION

Obesity is a condition in which the adipose tissue mass is increased due to a high adipocytes number and size (1). In obese individuals, the endocrine function of the adipose tissue is impaired and the adipocytes, as well as the pre-adipocytes, macrophages, and adipose stem cells, contribute to the production of pro-inflammatory cytokines (2). Both the hypertrophied adipocytes and other adipose tissue immune cells could lead to chronic inflammation through innate and adaptive immune responses (3). Additionally, the adipose tissue dysfunction is related with the development of co-morbidities such as insulin resistance, type 2 diabetes and cardiovascular diseases (4). The inflammatory state triggered by the impaired function of the adipose tissue, seems also to be related with these comorbidities (5, 6).

Among a large number of inflammatory-related biomarkers, C-reactive protein (CRP) has been the one most widely used. Hepatic synthesis of CRP, complement factors C3 and C4, depends on pro-inflammatory cytokines released by the adipose tissue such as tumor necrosis factor alpha (TNF-α) or interleukin 6 (IL-6) (7). Moreover, cell adhesion molecules are elevated during inflammatory conditions and have been suggested as markers for atherosclerosis (8).

Inflammation seems to be an important step in the pathogenesis of insulin resistance (IR) (9). In obese subjects, the inflammatory response can lead to altered insulin mediated signaling pathway by directly inhibiting insulin receptors (10). The relation between traditional inflammatory cells, cytokines, and chemokines and insulin resistance, has been studied in adult populations (10). In children, obesity-related adipose tissue dysfunction develops early in childhood and is related to IR (11). In a previous study in adolescents, an association between IR and some inflammatory biomarkers was observed, and these relationships were stronger in the obese subjects (12). However, another study performed in adolescents and young adults suggested that low grade inflammation did not appear to play a role in the development of IR (13). Due to these controversial findings, there is a need of further research in early stages of life, as it is a critical period for the development of future co-morbidities. Body composition, especially the body fat, could determine the associations between inflammatory markers and insulin resistance. Thus, the aim of this study is to

assess the relationship between inflammatory markers and insulin resistance by body composition in a sample of European adolescents.

MATERIAL AND METHODS

2.1 Study design

The HELENA study is a cross-sectional multi-center study (n=3528) conducted between 2006 and 2007 in 10 European cities: Athens and Heraklion in Greece, Dortmund in Germany, Ghent in Belgium, Lille in France, Pecs in Hungary, Rome in Italy, Stockholm in Sweden, Vienna in Austria and Zaragoza in Spain. General procedures and methodology of the HELENA study have been previously described (14).

The study was performed according to the ethical guidelines of the Edinburgh revision of the 1964 Declaration of Helsinki (2000), the International Conferences on Harmonization for Good Clinical Practice and the legislation on clinical research from each of the participating countries. The local Ethics Committees of each center approved the protocol. Written informed consent was obtained from the adolescents and their parents.

2.2 Study sample

Blood collection was randomly performed in approximately a third of the total sample (n=1089, 31%). 962 participants (442 boys and 520 girls) met the inclusion criteria of having measured the homeostasis model assessment (HOMA) and the set of biomarkers related with inflammation: TNF-α, IL-6, CRP, complement factors C3 and C4 and cell adhesion molecules: vascular cell adhesion molecule-1 (sVCAM-1), intercellular adhesion molecule-1 (sICAM-1), sE-selectin and L-selectin.

2.3 Physical measurements

Weight and height were measured in underwear and barefoot with an electronic scale (SECA 861, Seca Ltd, Birmingham, UK) and a stadiometer (SECA 225, Seca Ltd). In addition, body mass index (BMI) was calculated as body weight in kilograms divided by the square of height in meters. From Skinfolds, percentage of fat was calculated using the Slaughter formula and then the fat mass index

was also calculated (kg/m^2) (15). Waist circumference was measured with an unelastic tape. All anthropometric measures were taken following a standardized protocol. Inter-observer reliability for skinfold thicknesses and circumferences measurements was always greater than 90% (16).

2.4 Blood analysis

Blood withdrawal was performed after 12 hours overnight fast. C-reactive protein (CRP) levels were quantified by immunoturbidimetry (AU 2700, Olimpus, Rungis, France). Adolescents with higher CRP concentrations than 10 mg/dL were excluded. Serum C3 and C4 complement factors were analyzed by nephelometry (Behring Diagnostics, CA, USA). The coefficient of variation (inter-assay precision) was 1.9% for CRP, 1.4% for C3, and 1.2% for C4. Serum cytokines IL-6 and TNF-α were determined using the High Sensitivity Human Cytokine MILLIPLEX™ MAP kit (Millipore Corp., Billerica, MA, USA) and collected by flow cytometry (Luminex-100 v.2.3, Luminex Corporation, Austin, TX, USA). The intra- and inter-assay precision CVs were: 3.5% and 4.5%, respectively, for IL-6; and 3.5% and 3.8%, respectively, for TNF-α. Detection limits (sensitivity) for all the analyses were 0.007 mg/l for CRP, 0.01 g/l for C3, 0.002 g/l for C4, 0.1 pg/ml for IL-6, and 0.05 pg/ml for TNF-α. Undetectable values were recorded as the specific detection limit. Children with values of 0.12 pg/mL for TNF-α and IL-6 were excluded as it was an assigned value for children with concentration values under the detection curve. The serum adhesion molecule sL-selectin was analysed through commercial ELISA kits (Diaclone, France), the sensitivities of this kit was less than 1 ng/mL for L-selectin. The analysed by Universal Microplate Spectophotometer (Power WaveTM XS, Biotek® Instruments, INC USA).

The multiplex assay kit, were used to detect for the simultaneous quantification of the following molecules sE-Selectin, sVCAM-1, sICAM-1, in serum. The samples were analysed by citometry (Luminex ®100). The sensitivities of these assay were: Min DC 0.079 ng/mL for sE-Selectin, 0.016 ng/mL for sVCAM-1 and 0.009 ng/mL for sICAM-1. The intra-assay CV were 11.2% for sE-Selectin, 4.5% for sVCAM-1 and 7.9% for sICAM-1.

2.5 Statistical analysis

Analyses were performed separately for boys and girls. Normality of distributions was assessed with the Kolmogorov–Smirnov test. Glucose, Insulin, HOMA, CRP, IL6, TNF-α, L-selectin, sE-selectin and sICAM were normalized by natural logarithm transformation. T tests were used for comparisons of continuous variables by sex.

For BMI, FMI and WC, the standardized regression residuals by age were calculated and then categorized in tertiles.

Analysis of variance (ANOVA) with Bonferroni post-hoc correction was applied to compare mean differences of each biomarker between the categories of each indicator of body composition.

Finally, multiple linear regressions were performed to assess the association between HOMA-IR (dependent) and each marker of inflammation (independent) adjusted by center within body composition tertiles (BMI, FMI or WC). The effect of the city of residence was controlled in all regressions by using dummy variables. The dependent variable, HOMA-IR, was log-transformed. For markers of inflammation that were also log-transformed, results were expressed as percentage of change of the geometrical mean of the HOMA-IR for 10% increases of the corresponding biomarker. If the independent variable was normally distributed results were expressed as percentage of change of the geometrical mean of HOMA per unit increase of the corresponding biomarker.

Data were managed and analyzed with the IBM SPSS Statistics v.21 (IBM Corp., New York, NY, USA, 2012).

Results

Descriptive characteristics are presented in Table 1. Boys were significantly taller and heavier than girls and had significantly higher values of waist circumference whereas girls had higher levels of FMI. Regarding the biomarkers measured, boys had significantly higher concentrations of glucose, HOMA, IL-6, TNF-α, L-selectin, sE-selectin, sVCAM-1 and sICAM-1 than girls while girls had significantly higher levels of insulin and C3 than boys.

Table 1. Characteristics of the study participants

	Boys (N=442)		**Girls (N=520)**		
Center (country)	n	%	n	%	-
Athens (Greece)	29	6.6	39	7.5	-
Dortmund (Germany)	60	13.6	47	9.0	-
Gent (Belgium)	54	12.2	52	10.0	-
Heraklion (Greece)	44	10.0	42	8.1	-
Lille (France)	34	7.7	50	9.6	-
Pecs (Hungary)	44	10.0	79	15.2	-
Roma (Italy)	46	10.4	48	9.2	-
Stockholm (Sweden)	46	10.4	49	9.4	-
Vienna (Austria)	45	10.2	58	11.2	-
Zaragoza (Spain)	40	9.0	56	10.8	-
	Mean	**SD**	**Mean**	**SD**	**p**
Age (years)	14.77	1.21	14.73	1.16	0.616
Height (cm)	169.94	9.89	161.84	6.96	<0.001
Weight (kg)	61.83	13.66	55.86	10.12	<0.001
BMI	21.27	3.74	21.27	3.30	0.980
Fat mass index (Kg/m^2)	13.33	10.01	15.09	6.37	0.001
Waist circumference (cm)	74.43	8.95	70.58	7.86	<0.001
Glucose (mg/dL)	92.80	7.30	89.48	6.58	<0.001
Insulin (μlU/mL)	10.16	9.19	10.38	6.77	0.666
HOMA	2.36	2.34	2.32	1.66	0.725
CRP (mg/L)	0.86	1.25	0.83	1.25	0.766
IL-6 (pg/mL)	27.19	37.07	19.89	25.53	0.001
TNF-α (pg/mL)	7.35	5.99	5.65	3.47	<0.001
C3 (g/L)	1.12	0.16	1.14	0.17	0.134
C4 (g/L)	0.20	0.06	0.21	0.06	0.167
L-selectin (ng/mL)	3836.56	1603.85	3700.91	1510.49	0.180
sE-selectin (ng/mL)	41.76	21.07	35.89	18.79	<0.001
sVCAM-1 (ng/mL)	1419.78	406.28	1221.47	378.49	<0.001
sICAM-1 (ng/mL)	181.56	158.93	151.51	102.97	0.001

SD: standard deviation; BMI: Body mass index; HOMA: Homeostasis model assessment; CRP: C-reactive protein; IL-6: interleukin 6; TNF-α: tumor necrosis factor alpha; C3 and C4: complement factors; sVCAM-1: soluble vascular cell adhesion protein 1; sICAM-1: soluble Intercellular Adhesion Molecule.

Results for the ANOVA are presented in Table 2, 3 and 4. Significant differences were found in mean values of the measured biomarkers across categories of each marker of body composition, by sex. When BMI increased, the mean concentrations of insulin and HOMA in boys, CRP in girls and C3 and C4, in both sexes, significantly increased ($p<0.001$). Also, CRP and sE-selectin in boys, and insulin, HOMA and sVCAM-1 in girls presented also significantly different mean values across categories. Also, when FMI increased, the mean levels of glucose in boys, CRP in girls and insulin, HOMA, C3 and C4, in both sexes, significantly

increased (p<0.001). Additionally, CRP in boys and sVCAM-1 presented also significant differences (p<0.001) across categories. Finally, when WC increased, the mean level of insulin in boys and CRP in girls increased (p<0.001); HOMA, CRP, C3 and C4 significantly increased (p<0.001), in both sexes. Mean values of sVCAM-1 presented also significant differences (p=0.024) between waist circumference categories but only in girls.

Table 2. Mean differences and standard deviations of biomarkers in boys and girls by body mass index categories

	BMI I		BMI II		BMI III		
Boys	N=146		N=147		N=149		
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**p**
Glucose (mg/dL)†	91.69	6.70	92.94	8.09	93.75	6.94	0.056
Insulin (μlU/mL)†	7.34	4.33	10.21	8.24	12.89*+	12.31	**<0.001**
HOMA†	1.67	1.01	2.35	2.02	3.05*+	3.22	**<0.001**
CRP (mg/L)†	0.70	1.21	0.67	0.81	1.20*+	1.55	**<0.001**
IL-6 (pg/mL)†	31.12	42.63	25.17	31.08	25.44	37.20	0.642
TNF-α (pg/mL)†	7.11	3.86	7.13	6.73	7.85	6.84	0.277
C3 (g/L)	1.07	0.14	1.09	0.14	1.20*+	0.17	**<0.001**
C4 (g/L)	0.19	0.06	0.19	0.05	0.23*+	0.07	**<0.001**
Lselectin (ng/mL)†	3958.25	1679.11	3659.34	1434.20	3894.13	1682.69	0.206
sE-selectin (ng/mL)†	42.43	19.08	37.58	18.48	45.40+	24.57	**0.005**
sVCAM-1 (ng/mL)	1458.04	411.79	1392.11	383.19	1409.95	423.39	0.363
sICAM-1 (ng/mL)†	173.56	96.41	173.28	96.27	198.07	240.01	0.733
Girls	N=169		N=176		N=175		
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**p**
Glucose (mg/dL)†	89.84	7.16	89.28	6.24	89.34	6.33	0.733
Insulin (μlU/mL)†	9.63	8.03	9.18	4.16	12.33*+	7.14	**<0.001**
HOMA†	2.18	2.08	2.03	0.96	2.74*+	1.69	**<0.001**
CRP (mg/L)†	0.60	0.95	0.81	1.24	1.08*+	1.46	**<0.001**
IL-6 (pg/mL)†	17.65	23.73	19.99	27.13	22.08*	25.55	**0.045**
TNF-α (pg/mL)†	5.61	2.79	5.76	4.03	5.56	3.50	0.997
C3 (g/L)	1.08	0.17	1.13	0.16	1.19*+	0.16	**<0.001**
C4 (g/L)	0.19	0.06	0.20	0.06	0.23*+	0.06	**<0.001**
L-selectin (ng/mL)†	3656.23	1355.57	3566.20	1600.20	3878.23	1552.44	0.190
sE-selectin (ng/mL)†	37.12	18.83	35.94	22.18	34.65	14.68	0.459
sVCAM-1 (ng/mL)	1300.20	404.84	1184.45*	352.42	1181.63+	367.01	**0.005**
sICAM-1 (ng/mL)†	164.63	141.47	150.31	91.68	139.95	58.82	0.247

*BMI: body mass index; SD: Standard deviation; HOMA: Homeostasis model assessment; CRP: C-reactive protein; IL-6: interleukin 6; TNF-α: tumor necrosis factor alpha; C3 and C4: complement factors; sVCAM-1: soluble vascular cell adhesion protein 1; sICAM-1: soluble Intercellular Adhesion Molecule 1. †Biomarkers log-transformed. Post-hoc test for multiple comparisons: Bonferroni. *p<0.05 ref CRP I, + p<0.05 ref CRP II.*

Table 3. Mean differences and standard deviations of biomarkers in boys and girls by fat mass index categories.

	FMI I		FMI II		FMI III		
Boys	N=132		N=137		N=137		
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**p**
Glucose (mg/dL)†	91.78	7.51	92.06	7.49	94.22*+	6.92	**0.010**
Insulin (μlU/mL)†	7.95	5.40	9.27	7.67	13.61*+	12.78	**<0.001**
HOMA†	1.79	1.15	2.13	1.93	3.24*+	3.35	**<0.001**
CRP (mg/L)†	0.72	1.22	0.68	0.89	1.13*+	1.43	**<0.001**
IL-6 (pg/mL)†	30.19	36.14	21.24	27.75	25.15	37.20	0.184
TNF-α (pg/mL)†	7.26	3.91	6.67	3.44	7.58	6.88	0.260
C3 (g/L)	1.05	0.13	1.10	0.14	1.20*+	0.17	**<0.001**
C4 (g/L)	0.18	0.05	0.19	0.05	0.22*	0.07	**<0.001**
L-selectin (ng/mL)†	3910.89	1604.22	3622.45	1524.49	3833.36	1561.94	0.163
sE-selectin (ng/mL)†	42.80	19.19	38.50	18.89	44.18	25.08	0.099
sVCAM-1 (ng/mL)	1452.09	395.95	1409.38	410.73	1369.82	410.35	0.263
sICAM-1 (ng/mL)†	169.16	62.44	179.27	121.36	193.51	249.90	0.977
Girls	N=166		N=176		N=172		
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**p**
Glucose (mg/dL)†	89.95	6.95	89.56	6.57	89.05	6.24	0.480
Insulin (μlU/mL)†	8.94	5.56	10.01	7.41	12.17*+	6.87	**<0.001**
HOMA†	2.01	1.34	2.24	1.93	2.70*+	1.61	**<0.001**
CRP (mg/L)†	0.62	0.96	0.78	1.23	1.10*+	1.47	**<0.001**
IL-6 (pg/mL)†	18.66	23.20	20.17	28.13	21.28	25.18	0.157
TNF-α (pg/mL)†	5.38	2.70	6.11	4.70	5.43	2.47	0.625
C3 (g/L)	1.08	0.18	1.13*	0.16	1.19*+	0.17	**<0.001**
C4 (g/L)	0.19	0.05	0.20	0.05	0.23*+	0.05	**<0.001**
L-selectin (ng/mL)†	3650.69	1513.99	3580.29	1487.21	3877.74	1524.27	0.256
sE-selectin (ng/mL)†	37.29	18.54	34.51	19.27	36.06	18.70	0.181
SVCAM-1 (ng/mL)	1315.54	390.64	1166.80*	380.15	1186.32*	352.50	**<0.001**
SICAM-1 (ng/mL)†	170.01	144.46	138.62*	83.33	146.71	64.18	**0.011**

*FMI: fat mass index; SD: Standard deviation; HOMA: Homeostasis model assessment; CRP: C-reactive protein; IL-6: interleukin 6; TNF-α: tumor necrosis factor alpha; C3 and C4: complement factors; sVCAM-1: soluble vascular cell adhesion protein 1; sICAM-1: soluble Intercellular Adhesion Molecule 1. †Biomarkers log-transformed. Post-hoc test for multiple comparisons: Bonferroni. *p<0.05 ref CRP I, + p<0.05 ref CRP II.*

Table 4. Mean differences and standard deviations of biomarkers in boys and girls by waist circumference categories.

	FMI I		FMI II		FMI III		
Boys	N=132		N=137		N=137		
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**p**
Glucose (mg/dL)†	91.78	7.51	92.06	7.49	94.22*†	6.92	**0.010**
Insulin (μlU/mL)†	7.95	5.40	9.27	7.67	13.61*†	12.78	**<0.001**
HOMA†	1.79	1.15	2.13	1.93	3.24*†	3.35	**<0.001**
CRP (mg/L)†	0.72	1.22	0.68	0.89	1.13*†	1.43	**<0.001**
IL-6 (pg/mL)†	30.19	36.14	21.24	27.75	25.15	37.20	0.184
TNF-α (pg/mL)†	7.26	3.91	6.67	3.44	7.58	6.88	0.260
C3 (g/L)	1.05	0.13	1.10	0.14	1.20*†	0.17	**<0.001**
C4 (g/L)	0.18	0.05	0.19	0.05	0.22*	0.07	**<0.001**
L-selectin (ng/mL)†	3910.89	1604.22	3622.45	1524.49	3833.36	1561.94	0.163
sE-selectin (ng/mL)†	42.80	19.19	38.50	18.89	44.18	25.08	0.099
sVCAM-1 (ng/mL)	1452.09	395.95	1409.38	410.73	1369.82	410.35	0.263
sICAM-1 (ng/mL)†	169.16	62.44	179.27	121.36	193.51	249.90	0.977
Girls	N=166		N=176		N=172		
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**p**
Glucose (mg/dL)†	89.95	6.95	89.56	6.57	89.05	6.24	0.480
Insulin (μlU/mL)†	8.94	5.56	10.01	7.41	12.17*†	6.87	**<0.001**
HOMA†	2.01	1.34	2.24	1.93	2.70*†	1.61	**<0.001**
CRP (mg/L)†	0.62	0.96	0.78	1.23	1.10*†	1.47	**<0.001**
IL-6 (pg/mL)†	18.66	23.20	20.17	28.13	21.28	25.18	0.157
TNF-α (pg/mL)†	5.38	2.70	6.11	4.70	5.43	2.47	0.625
C3 (g/L)	1.08	0.18	1.13*	0.16	1.19*†	0.17	**<0.001**
C4 (g/L)	0.19	0.05	0.20	0.05	0.23*†	0.05	**<0.001**
L-selectin (ng/mL)†	3650.69	1513.99	3580.29	1487.21	3877.74	1524.27	0.256
sE-selectin (ng/mL)†	37.29	18.54	34.51	19.27	36.06	18.70	0.181
SVCAM-1 (ng/mL)	1315.54	390.64	1166.80*	380.15	1186.32*	352.50	**<0.001**
SICAM-1 (ng/mL)†	170.01	144.46	138.62*	83.33	146.71	64.18	**0.011**

*WC: waist circumference; SD: Standard deviation; HOMA: Homeostasis model assessment; CRP: C-reactive protein; IL-6: interleukin 6; TNF-α: tumor necrosis factor alpha; C3 and C4: complement factors; sVCAM-1: soluble vascular cell adhesion protein 1; sICAM-1: soluble Intercellular Adhesion Molecule 1. †Biomarkers log-transformed. Post-hoc test for multiple comparisons: Bonferroni. *p<0.05 ref CRP I, † p<0.05 ref CRP II.*

Tables 5, 6 and 7 show results of the linear regression between HOMA-IR and the markers of inflammation for each body composition index. Table 5 show the results of the linear regression by tertiles of BMI. In the lowest tertile, in girls, HOMA-IR increased in a 31.1% and 22.7% per each additional g/L of C3 and C4, respectively, and in 2.0% and in 2.4% when TNF-alpha and IL-6 increases by 10%,

respectively. Also, in the second tertile of BMI, we expect about 23.7% increase in HOMA-IR per each additional g/L of C3 and about 14.8% decrease in HOMA-IR per each additional ng/ml of sICAM-1. Moreover, in the highest tertile of BMI, in girls, HOMA-IR increased by 22.3% and 17.7% per each additional ng/ml of sICAM-1 and 1.5% when sE-selectin increases by 10%. Significant associations between HOMA-IR and C3 in the highest tertile of BMI in both sexes were found: HOMA-IR increased in a 17.7% per each additional g/L of C3 in girls and 27.7% per each additional g/L of C3 in boys.

Table 5. Linear regression with HOMA-IR as dependent variable and the markers of inflammation as independent variables for each category of body mass index (adjust: center).

Boys		**BMI I**		**BMI II**		**BMI III**	
		N=146		N=147		N=149	
		ß	**p**	**ß**	**p**	**ß**	**p**
	CRP (mg/L)†	-0.115	0.185	-0.062	0.482	-0.002	0.983
	IL-6 (pg/mL)†	0.106	0.270	0.027	0.783	0.047	0.595
	TNF-α (pg/mL)†	0.017	0.863	-0.028	0.761	0.027	0.767
	C3 (g/L)	-0.058	0.518	0.004	0.968	0.245	**0.004**
HOMA-IR†		-0.067	0.445	0.082	0.361	0.032	0.704
	C4 (g/L)						
	Lselectin (ng/mL)†	-0.171	0.064	-0.048	0.583	-0.031	0.724
	sE-selectin (ng/mL)†	-0.056	0.531	-0.137	0.122	0.083	0.327
	SVCAM-1 (ng/mL)	-0.067	0.443	-0.069	0.424	-0.067	0.443
	SICAM-1 (ng/mL)†	-0.097	0.269	-0.107	0.238	-0.055	0.506
Girls		N=169		N=176		N=175	
		ß	**p**	**ß**	**p**	**ß**	**p**
	CRP (mg/L)†	0.100	0.198	0.003	0.966	-0.046	0.550
	IL-6 (pg/mL)†	0.247	**0.007**	-0.048	0.579	-0.048	0.557
	TNF-α (pg/mL)†	0.208	**0.014**	0.112	0.191	-0.077	0.369
	C3 (g/L)	0.271	**0.001**	0.213	**0.008**	0.163	**0.029**
HOMA-IR†		0.205	**0.009**	0.048	0.551	0.026	0.739
	C4 (g/L)						
	Lselectin (ng/mL)†	0.012	0.883	-0.053	0.519	0.029	0.702
	sE-selectin (ng/mL)†	-0.159	0.062	-0.072	0.373	0.160	**0.044**
	sVCAM-1 (ng/mL)	0.100	0.200	-0.003	0.972	0.100	0.200
	sICAM-1 (ng/mL)†	-0.074	0.368	-0.161	**0.048**	0.201	**0.015**

BMI: body mass index; HOMA: Homeostasis model assessment; CRP: C-reactive protein; IL-6: interleukin 6; TNF-α: tumor necrosis factor alpha; C3 and C4: complement factors; sVCAM-1: soluble vascular cell adhesion protein 1; sICAM-1: soluble Intercellular Adhesion Molecule 1. †Biomarkers log-transformed.

Table 6 present the results of the linear regression between HOMA-IR and the markers of inflammation by tertiles of FMI. In the lowest tertile, HOMA-IR increased in a 1.8% when IL-6 increased 10% and in a 31.5% per each additional g/L of C3, in girls. Furthermore, in the second tertile of FMI, we expect about

1.9% increase in HOMA-IR when TNF-α increases by 10%, in girls. Moreover, in the highest tertile of BMI, HOMA-IR increased by 20.5% per each additional g/L of C3, in boys.

Table 6. Linear regression with HOMA-IR as dependent variable and the markers of inflammation as independent variables for each category of fat mass index (adjust: center).

Boys		FMI I		FMI II		FMI III	
		N=132		N=137		N=137	
		ß	p	ß	p	ß	p
HOMA-IR†	CRP (mg/L)†	-0.095	0.309	0.048	0.594	-0.074	0.419
	IL-6 (pg/mL)†	0.121	0.243	0.085	0.424	-0.024	0.795
	TNF-α (pg/mL)†	0.051	0.623	-0.059	0.557	0.108	0.231
	C3 (g/L)	-0.065	0.466	0.002	0.983	0.187	**0.040**
	C4 (g/L)	-0.057	0.527	-0.052	0.581	0.056	0.527
	Lselectin (ng/mL)†	-0.108	0.259	0.001	0.990	-0.108	0.259
	sE-selectin (ng/mL)†	-0.107	0.234	-0.038	0.685	0.076	0.395
	sVCAM-1 (ng/mL)	-0.055	0.549	-0.050	0.607	-0.053	0.552
	sICAM-1 (ng/mL)†	-0.127	0.170	-0.019	0.835	-0.079	0.375
Girls		N=166		N=176		N=172	
		ß	p	ß	p	ß	p
HOMA-IR†	CRP (mg/L)†	0.058	0.455	-0.027	0.723	0.045	0.555
	IL-6 (pg/mL)†	0.190	**0.035**	0.013	0.885	0.014	0.861
	TNF-α (pg/mL)†	0.153	0.076	0.196	**0.021**	-0.056	0.503
	C3 (g/L)	0.274	**0.001**	0.147	0.073	0.130	0.090
	C4 (g/L)	0.146	0.062	0.039	0.629	0.054	0.499
	Lselectin (ng/mL)†	-0.061	0.438	0.018	0.822	-0.061	0.438
	sE-selectin (ng/mL)†	-0.102	0.246	-0.056	0.499	0.028	0.724
	sVCAM-1 (ng/mL)	-0.042	0.604	-0.019	0.816	0.079	0.323
	sICAM-1 (ng/mL)†	-0.058	0.485	-0.037	0.658	-0.016	0.853

FMI: fat mass index; HOMA: Homeostasis model assessment; CRP: C-reactive protein; IL-6: interleukin 6; TNF-α: tumor necrosis factor alpha; C3 and C4: complement factors; sVCAM-1: soluble vascular cell adhesion protein 1; sICAM-1: soluble Intercellular Adhesion Molecule 1. †Biomarkers log-transformed.

Finally, Table 7 show the results of the linear regression by tertiles category of WC. In the lowest tertile of WC, HOMA-IR decreased 1.9% when sE-selectin increased 10% for boys; while in girls, HOMA-IR increased 2.2% when IL-6 increased 10% and 25.3% and 25.9% per each additional g/L of C3 and C4, respectively. In the second tertile of waist circumference, HOMA-IR increases 1.9% when TNF-α increases 10%, in girls. In the highest tertile of waist circumference, significant associations between C3 were observed in both sexes: HOMA-IR increased 24.9% and 32.8% per each additional g/L of C3 in boys and girls, respectively.

Table 7. Linear regression with HOMA-IR as dependent variable and the markers of inflammation as independent variables for each category of waist circumference (adjust: country).

Boys		WC I		WC II		WC III	
		N=145		N=149		N=148	
		ß	p	ß	p	ß	p
HOMA-IR†	CRP (mg/L)†	-0.083	0.348	0.003	0.976	-0.066	0.458
	IL-6 (pg/mL)†	0.140	0.149	-0.033	0.724	-0.001	0.993
	TNF-α (pg/mL)†	-0.071	0.454	0.013	0.889	0.073	0.418
	C3 (g/L)	-0.076	0.404	0.064	0.431	0.233	**0.007**
	C4 (g/L)	-0.084	0.345	0.107	0.207	0.007	0.936
	L-selectin (ng/mL)†	-0.086	0.326	-0.109	0.195	-0.075	0.404
	sE-selectin (ng/mL)†	-0.204	**0.020**	0.013	0.874	0.089	0.299
	sVCAM-1 (ng/mL)	-0.153	0.092	0.038	0.655	-0.056	0.520
	sICAM-1 (ng/mL)†	-0.145	0.103	-0.041	0.649	-0.067	0.426
Girls		N=173		N=167		N=174	
		ß	p	ß	p	ß	p
HOMA-IR†	CRP (mg/L)†	0.043	0.564	-0.015	0.855	0.046	0.546
	IL-6 (pg/mL)†	0.227	**0.007**	-0.008	0.930	0.018	0.820
	TNF-α (pg/mL)†	0.142	0.087	0.203	**0.023**	-0.058	0.484
	C3 (g/L)	0.226	**0.004**	0.100	0.236	0.284	**<0.001**
	C4 (g/L)	0.231	**0.002**	-0.062	0.459	0.091	0.243
	Lselectin (ng/mL)†	-0.036	0.633	0.030	0.725	0.027	0.740
	sE-selectin (ng/mL)†	-0.117	0.146	-0.036	0.675	0.035	0.662
	sVCAM-1 (ng/mL)	-0.099	0.196	0.056	0.494	-0.003	0.968
	sICAM-1 (ng/mL)†	-0.083	0.295	-0.072	0.369	0.020	0.822

WC: waist circumference; HOMA: Homeostasis model assessment; CRP: C-reactive protein; IL-6: interleukin 6; TNF-α: tumor necrosis factor alpha; C3 and C4: complement factors; sVCAM-1: soluble vascular cell adhesion protein 1; sICAM1: soluble Intercellular Adhesion Molecule 1. †Biomarkers log-transformed.

DISCUSSION

The main finding of this study is the consistent significant association between C3 complement factor and insulin resistance, irrespective of total and abdominal fat deposition. To our knowledge, this is the first study assessing the relationship between different inflammatory markers and insulin resistance in a relatively large sample of European adolescents, from different cities.

In our sample, mean concentrations of glucose, insulin, HOMA and some inflammatory markers such as CRP or C3 and C4 were significantly higher in the highest tertile of each marker of body composition. Even in children, obesity has been related with low-grade inflammation (17). Adipocyte hypertrophy has been

associated with HOMA- insulin resistance and inflammation in obese children (11). Results from our study also support the hypothesis that, even in adolescence, there is a link between adiposity, glucose metabolism and inflammation as some of these biomarkers were increased in the highest levels of total and abdominal adiposity.

Furthermore, in the present study there were linear associations between some inflammatory markers and HOMA as dependent variable, by categories of body composition indices. Previous studies suggest that inflammatory markers can interfere with insulin action by directly inhibiting insulin receptors (18). However, there are some discrepancies between studies regarding the relationship between inflammation and insulin resistance in adolescents (12, 13). A recent study in obese adolescents failed to show a significant relation between obesity and IR mediated by low-grade inflammation using traditional inflammatory markers (19).

Although previous studies have associated some traditional inflammatory biomarkers with the development of diabetes or insulin resistance (20-22), we did not find any relationship between the traditional inflammatory markers and the HOMA for adolescents with the highest levels of BMI, FMI and WC. Out of all the inflammatory markers measured in the present study, only C3 was consistently related with insulin resistance, measured by HOMA, especially in the highest tertiles of total and abdominal adiposity, except FMI in girls. Our results are in line with some previous studies. Serum C3 was the strongest inflammatory marker related with insulin resistance in a study in an elderly population (23). This complement factor is an emerging cardio metabolic risk factor related with some comorbities such as type 2 diabetes (24). In a sample of Spanish adolescents serum C3 levels were related with body fat, especially with abdominal obesity (25) and were higher in adult subjects with insulin resistance (26). A previous study performed in adults showed that low-grade inflammation and insulin resistance might represent two independent pathways by which body fat leads to elevated C3 (27). However, it seems that changes in C3 levels over a 7-year follow-up period were associated with changes in several measures of insulin resistance and that baseline C3 was associated with the 7-year incidence of type 2 diabetes (28). Although main production of the C3 is in the liver, C3 is also synthesized by activated macrophages (29) and adipocytes (30) as an inflammatory cytokine or an adipokine. Its hepatic production is induced

by cytokines, such as interleukin-6 and TNF-α (31) which may interfere with insulin receptor functioning and cause insulin resistance (32). In addition, the complement system is a regulator of both the innate and adaptive system and, as a part of the inflammatory response, could also contribute to insulin resistance. We also found associations between insulin resistance and C4 complement factor in girls. Production of C4 depends, as production of C3, on proinflammatory cytokines released by the adipose tissue such as tumor necrosis factor alpha (TNF-α) or interleukin 6 (IL-6) (7). However, literature on the relationship between C4 and insulin resistance is scarce.

In our study, associations between TNF-α and insulin resistance were found in girls, in the lowest tertiles of body fat composition but not in the highest tertiles. TNF-α interferes negatively with the insulin signaling pathway, but also induces insulin resistance indirectly by altering adipocyte differentiation and adipocyte lipid metabolism (10).

This study has strengths as well as some limitations. First, the cross-sectional design which does not allow drawing conclusions on causality; however, in adults it was observed that C3 was associated with the development of insulin resistance in a longitudinal study (28). Furthermore, the study is limited by the fact that blood samples only reflect inflammation, glucose and insulin concentrations at a given specific time point. On the other hand, the strengths of the study are: the use of traditional and non-traditional inflammatory markers that could be also involved in the pathogenesis of insulin resistance, the use of standardized and harmonized information on body composition of adolescents from 9 European countries.

4.1 Conclusions

In conclusion, results from the current study show that there is an association between C3 and HOMA in a multicenter sample of adolescents, especially in those with high levels of total and abdominal adiposity. To avoid chronic insulin resistance, efforts should be made to reduce deposition of total and abdominal fat in obese children and adolescents. This may impact on the reduction of serum C3 concentrations and prevent future insulin-related diseases such as diabetes. Longitudinal studies assessing this relationship between C3 and insulin resistance are needed to confirm these results.

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Disclosure statement

The authors declare no conflict of interest

Author contributions

FG, MGG, AK, KW, JD, YS, DM, AM and LAM designed and directed the study, SGM, LED and AM conducted and directed the laboratory analysis, EMGG performed the statistical analysis and wrote the manuscript. EMGG, LGM, JS, DM, FA, FG, AA, AK, KW, YM, AS, MGG, SGM, LED, CL, JD, AM and LAM critically reviewed the manuscript.

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INFLAMMATION IN METABOLICALLY HEALTHY AND METABOLICALLY ABNORMAL EUROPEAN ADOLESCENTS: THE HELENA STUDY.

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ABSTRACT

Background: Inflammation may influence the cardio-metabolic profile which relates with the risk of chronic diseases.

Objectives: To assess the inflammatory status by metabolic health/body mass index (BMI) category and to assess how inflammation can predict the cardio-metabolic profile in European adolescents, considering BMI.

Methods: 659 adolescents (295 boys) from a cross-sectional European study were included. Adolescents were classified by metabolic health based on age- and sex-specific cut-off points for glucose, blood pressure, triglycerides, high density cholesterol and BMI. C-reactive protein (CRP), tumor necrosis factor alpha (TNF-α), interleukin (IL-6), complement factors (C3, C4) and cell adhesion molecules were assessed.

Results: Metabolically abnormal (MA) adolescents had higher values of C3 ($p<0.001$) and C4 ($p=0.032$) compared to those metabolically healthy (MH). C3 concentrations significantly increased with the deterioration of the metabolic health and BMI ($p<0.001$). Adolescents with higher values of CRP had higher probability of being in the overweight/obese-MH group than those allocated in other categories. Finally, high C3 and C4 concentrations increased the probability of having an unfavorable metabolic/BMI status.

Conclusions: Metabolic/BMI status and inflammatory biomarkers are associated, being the CRP, C3 and C4 the most related inflammatory markers with this condition. C3 and C4 were associated with the cardio-metabolic health consistently.

INTRODUCTION

Obesity is characterized by an increase of the adipose tissue and this condition is often related with cardio-metabolic risk factors and inflammation (1). Body mass index (BMI) is the most used anthropometric index to assess obesity and is a strong predictor of CVD mortality (2, 3). However, literature suggests that some subjects with obesity could present a normal or healthy metabolic profile: the metabolically healthy obesity (MHO), in contrast to the so-called metabolically abnormal obesity (MAO) (4, 5). Currently, there is a lack of consensus on the definition of the MHO; usually, it is based on the definition for the metabolic syndrome (MS). In the adult literature, the prevalence of MHO ranges from 10 to 40% (6). In youth, there is a lack of information on prevalence due to the different definitions used. Ortega et al. (5) recently proposed a definition for youth and adults, aiming to harmonize the MHO definition, the latter based on the one proposed by Jolliffe and Janssen (7).

A review on the characterization of the MHO individuals concludes that low concentrations of inflammatory markers, low visceral adipose tissue deposition and preserved insulin sensitivity contribute to the MHO phenotype (8). Furthermore, the potential addition of some of these markers to the definition of the MHO has also been proposed (9). Evidence suggest that inflammation has a key role in the origin and development of metabolic disorders (1) and atherosclerosis (10). C-reactive protein (CRP) has been the biomarker most widely used in epidemiological studies, although other biomarkers are also related with inflammation. For instance, complement factors C3 and C4, depends on pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) or interleukin 6 (IL-6) (11). Also, cell adhesion molecules have been suggested as markers for atherosclerosis and their concentrations are elevated during inflammatory processes (12).

A previous study showed that MHO subjects with low CRP levels had a trend towards lower coronary heart disease risk than those MHO with high CRP levels and a similar risk to that of healthy non-obese subjects (13). This study suggests that CRP could help to identify those MHO individuals who are at low coronary heart disease risk. However, one study showed similarly adverse cardio metabolic profile in MHO and MAO adults (14).

Adolescents usually have lower prevalence of obesity in comparison with adults. Specifically, the HELENA sample presented a 3.8 % of obesity in boys and 7.3% in girls (15). Therefore, the study of the metabolically health status, or the metabolic health (MH) in overweight/obese adolescents, instead of the MHO seems to be a more feasible approach in this population.

To our knowledge there are no studies measuring inflammation in metabolically healthy and/or metabolically abnormal overweight/obese adolescents. The aims of this study are: to assess the inflammatory status by metabolic health/body mass index (BMI) category and, on the other hand, to assess how the increases of the inflammatory concentrations can predict the cardio-metabolic health profile in European adolescents, considering also the weight status.

METHODS

Study design

A cross-sectional multi-center projetct (n=3528), the HELENA (Healthy Lifestyle in Europe by Nutrition in Adolescence) study, was conducted between 2006 and 2007 in 10 European cities: Athens and Heraklion in Greece, Dortmund in Germany, Ghent in Belgium, Lille in France, Pecs in Hungary, Rome in Italy, Stockholm in Sweden, Vienna in Austria and Zaragoza in Spain. The description of the methodology and general procedure of this study have been previously described elsewhere (16).

This study was performed according to the ethical guidelines of the Edinburgh revision of Declaration of Helsinki (2000), the International Conferences on Harmonization for Good Clinical Practice and the legislation on clinical research from each of the participating countries. In addition, the local Ethics Committees of each center approved the protocol. Written informed consents were obtained from the adolescents and their caregivers.

Study sample

Out of the total HELENA sample, blood collection was randomly performed in approximately a third of the total sample (n=1089, 31%). 659 participants (295 boys and 364 girls) met the inclusion criteria of having measured the metabolic

biomarkers for the definition of the healthy/abnormal metabolic status, the confounders and the set of biomarkers related with inflammation.

Physical measurements

Weight was measured to the nearest 50 g in underwear and in fasting condition with an electronic scale (SECA 861, Seca Ltd) and height were measured to the nearest 0.1 cm in underwear, barefoot and in the Frankfort plane with a stadiometer (SECA 225, Seca Ltd), by trained researchers (17). In addition, body mass index (BMI) was calculated as body weight in kilograms divided by the square of height in meters. Systolic and diastolic blood pressure was measured with an automatic oscillometric device (Omron M6). The lowest value of the two measurements, taken with a difference of 5 minutes, was recorded.

Physical activity

Levels of physical activity were self-reported using the International Physical Activity Questionnaire for Adolescents (IPAQ-A) (18). School-related physical activity (including physical education classes and breaks), transportation, housework, and activities during leisure time were included in this questionnaire. The time periods per day (and the numbers of days per week) involved in these activities were recorded. Data were cleaned and truncated and then classified into moderate and vigorous activity according to the guidelines of IPAQ (http://www.ipaq.ki.se/ipaq.htm).

Blood analysis

Detailed description of blood sampling procedures has been published (19). Blood withdrawal was performed after 10 hours overnight fast. All parameters were centrally analysed. Serum triglycerides, glucose and high density lipoprotein were measured enzymatically in fresh seru, on the Dimension RxL clinical chemistry syste (Dade Behring, Schwalbach, Germany) using the manufacturer's reagents and instructions. The assessment methods for the CRP, C3 and C4 have been previously described elsewhere (20). Serum cytokines IL-6 and TNF-α were determined using the High Sensitivity Human Cytokine MILLIPLEX™ MAP kit (Millipore Corp., Billerica, MA, USA) and collected by flow cytometry (Luminex-100 v.2.3, Luminex Corporation, Austin, TX, USA). The intra- and inter-assay precision coefficients of variability (CVs) were: 3.5%

and 4.5%, respectively, for IL-6; and 3.5% and 3.8%, respectively, for TNF-α. Detection limits (sensitivity) for all the analyses were 0.007 mg/l for CRP, 0.01 g/l for C3, 0.002 g/l for C4, 0.1 pg/ml for IL-6, and 0.05 pg/ml for TNF-α. Undetectable values were recorded as the specific detection limit. Children with values of 0.12 pg/mL for TNF-α and IL-6 were excluded as it was an assigned value for children with concentration values under the detection curve. The serum adhesion molecule sL-selectin was analysed through commercial ELISA kit (Diaclone, France), the sensitivities of this kit was less than 1 ng/mL for L-selectin, analyzed by Universal Microplate Spectophotometer (Power WaveTM XS, Biotek® Instruments, INC USA).

The multiplex assay kit was used to detect for the simultaneous quantification of the following molecules sE-Selectin, sVCAM-1, sICAM-1, in serum. The samples were analysed by cytometry (Luminex ®100). The sensitivities of these assays were: Min DC 0.079 ng/mL for sE-Selectin, 0.016 ng/mL for sVCAM-1 and 0.009 ng/mL for sICAM-1. The intra-assay CVs were 11.2% for sE-Selectin, 4.5% for sVCAM-1 and 7.9% for sICAM-1.

Definition of MHO and MAO

There is no consensus for the definition of MHO as it depends on the definition for the metabolic syndrome that is not establish either. Ortega et al. (5), recently suggested an harmonization for the definition in youth, based on the one by Jolliffe and Janssen for metabolic syndrome (7). In this study, age- and gender-specific cut-off points for each marker of metabolic syndrome were developed except for the glucose criteria, considered a marker when the value was higher than 5.6 mmol/l or 100mg/dl. MHO is defined as 1) being obese/overweight according to the BMI cut-off points for youth by Cole et al. (21) and 2) no criterion of the following for the metabolic syndrome: high serum triglycerides (≥150mg/dL), fasting glucose(≥100mg/dL), systolic or diastolic blood pressure (Systolic ≥ 130 and diastolic ≥85 mm Hg) and low high density lipoprotein cholesterol (<40mg/dL in men and <50mg/dL in women). MAO was defined when 1 or more of the previous criteria were met. Waist circumference was excluded as criterion since high waist circumference is expected in overweight/obese individuals.

In contrast, metabolically healthy (MH) are those who accomplish having no criterion of the metabolic syndrome while metabolically abnormal (MA) are those with 1 or more than 1 criterion, both independently of their BMI status.

Statistical analysis

Normality of distributions was assessed with the Kolmogorov–Smirnov test. CRP, IL-6, TNF-α, L-selectin, sE-selectin and sICAM were normalized by natural logarithm transformation. T-tests were used for comparisons of continuous variables by metabolic health. Chi-square test was performed to test the differences between categories. Student t-test was performed to assess the mean differences between the adolescents allocated in the MH group and the MA group.

Statistical analysis was performed in both directions to get a better insight of the relationship between the inflammatory markers and the metabolic health according to BMI. Firstly, assessing the mean concentrations of the inflammatory biomarkers by category of metabolic health/BMI and, secondly, evaluating the probability of being in a worse category of metabolic health/BMI by the increase of the inflammatory biomarkers.

Two-way analysis of covariance (ANCOVA) with Bonferroni post-hoc correction was applied to compare mean differences of each biomarker between these categories of metabolic health/BMI status by sex. The confounders included in this analysis were age and the self-reported moderate-to-vigorous physical activity.

Finally, four categories combining BMI status and metabolic health were created: normal weight MH, normal weight MA, overweight/obese-MH and overweight/obese-MA, and then multinomial logistic regression was performed to assess the association between these categories of metabolic health (dependent) and each marker of inflammation (independent) adjusting by age, sex and moderate-to-vigorous physical activity. Results for the C3 and C4 were expressed in change for 0.1 g/L.

Data were managed and analyzed with the IBM SPSS Statistics v.21 (IBM Corp., New York, NY, USA, 2012).

RESULTS

Descriptive characteristics are presented in **Table 1**. Boys were more frequently allocated in the MA group (50.7%) than girls (49.3%). Also, those adolescents in the MA group had higher mean value of BMI (p<0.001). Differences between percentages of adolescents by BMI status were also found. Adolescents with a MA profile had higher values of all the metabolic markers included in the definition for metabolic health: blood pressure (systolic and diastolic), glucose, triglycerides and lower concentrations of high density lipoprotein cholesterol in comparison with those in the MH group (all p<0.001). Regarding the inflammatory biomarkers measured, MA adolescents presented higher concentrations of CRP (p=0.002), C3 (p<0.001) and C4 (p=0.032) than those classified as MH.

Table 1. Characteristics of the study sample by cardio-metabolic health status.

	MH (n=383)	**MA (n=276)**	
	Mean±SD	**Mean±SD**	**p-value**
Age (years)	14.59±1.12	14.96±1.20	**<0.001**
Sex	**N(%)**	**N(%)**	
Boys	155 (40.5)	140 (50.7)	**0.009**
Girls	228 (59.5)	136 (49.3)	
BMI (kg/m²)	20.17±2.85	22.08±3.63	**<0.001**
BMI group by Cole et al.	**N(%)**	**N(%)**	
Normal weight	338 (88.3)	194 (70.3)	**<0.001**
Overweight/Obese	45 (11.7)	82 (29.7)	
MVPA	779.03±570.08	784.95±607.99	0.898
Blood pressure			
Systolic (mm Hg)	110.84±8.68	124.09±13.83	**<0.001**
Diastolic (mm Hg)	61.88±7.28	68.13±8.97	**<0.001**
Glucose (mg/dL)	89.20±5.84	92.50±8.18	**<0.001**
Triglycerides (mg/dL)	62.15±22.31	82.90±47.76	**<0.001**
HDL-cholesterol (mg/dL)	59.09±9.00	51.59±11.22	**<0.001**
CRP* (mg/L)	0.66±1.04	0.91±1.28	**0.002**
IL6* (pg/mL)	24.40±32.99	19.59±24.68	0.085
TNF-α * (pg/mL)	6.65±5.25	6.16±4.77	0.141
C3 (g/L)	1.09±0.15	1.15±0.17	**<0.001**
C4 (g/L)	0.19±0.06	0.21±0.06	**0.032**
L-Selectin* (ng/mL)	3624.85±1583.08	3505.61±1355.87	0.985
sE-Selectin* (ng/mL)	37.19±18.89	36.97±20.95	0.335
sVCAM-1 (ng/mL)	1293.41±388.97	1270.42±413.48	0.472
sICAM-1* (ng/mL)	155.43±75.20	173.22±185.95	0.197

MH: metabolically healthy (no criterion of the following: high serum triglycerides (≥150mg/dL), fasting glucose(≥100mg/dL), systolic or diastolic blood pressure (Systolic ≥ 130 and diastolic ≥85 mm Hg) and

*low high density lipoprotein cholesterol (<40mg/dL in men and <50mg/dL in women); MA: metabolically abnormal (1 or more criteria of the following: high serum triglycerides (≥150mg/dL), fasting glucose(≥100mg/dL), systolic or diastolic blood pressure (Systolic ≥ 130 and diastolic ≥85 mm Hg) and low high density lipoprotein cholesterol (<40mg/dL in men and <50mg/dL in women); SD: standard deviation; BMI: Body mass index; MVPA: moderate to vigorous physical activity; HDL-cholesterol: High density lipoprotein; CRP: C-reactive protein; IL-6: interleukin 6; TNF-α: tumor necrosis factor alpha; C3 and C4: complement factors; sVCAM-1: soluble vascular cell adhesion protein 1; sICAM-1: soluble Intercellular Adhesion Molecule. *Log-trangsformed.*

In the ANCOVA (**Table 2**), mean biomarkers concentrations were different according to the categories of BMI status/metabolic health. In boys (Table 2), significant mean differences were found in CRP (p=00.001), C3 (p<0.001) and C4 (p<0.001). C3 concentrations increased with the deterioration of the metabolic health and the BMI status, being higher in the last group, i.e: overweight/obese-MA adolescents. Overweight/obese-MH adolescents had the highest value of CRP and C4 in boys. In girls, significant mean differences were found for C3 (p<0.001) being the highest mean concentration value found in the group of overweight/obese-MA adolescents.

Table 2. Mean differences of the inflammatory biomarkers by group of metabolic/BMI status in boys and girls. Age and moderate-to-vigorous activity were used as confounders.

	Normal weight		**Overweight/Obese**		
Boys	**MH (n=134)**	**MA (n=98)**	**MH (n=21)**	**MA (n=42)**	**p**
	Mean ±SD	Mean ±SD	Mean ±SD	Mean ±SD	
CRP (mg/L)*	0.58±0.77	0.79±1.16	1.23±1.98[c]	1.01±1.16	**0.001**
IL6 (pg/mL)*	27.62±35.61	22.65±26.91	34.34±58.25	24.53±31.31	0.176
TNF-α (pg/mL)*	7.99±7.19	7.03±6.85	7.17±5.22	6.74±.3.44	0.381
C3 (g/L)	1.06±0.13	1.10±0.16	1.17±0.14[c]	1.20±0.15[c]	**<0.001**
C4 (g/L)	0.18±0.06	0.19±0.05	0.23±0.07[c]	0.22±0.07[c]	**<0.001**
L-Selectin (ng/mL)*	3785.64±1618.29	3607.07±1344.40	3782.98±2120.99	3369.90±1280.04	0.908
sE-selectin (ng/mL)*	39.39±18.51	38.39±19.12	43.45±23.52	47.45±31.24	0.649
sVCAM-1(ng/mL)	1432.63±370.18	1370.99±404.49	1227.95±309.48	1408.76±398.84	0.388
sICAM-1(ng/mL)*	169.70±69.40	174.10±97.17	167.71±75.09	239.21±416.15	0.886
Girls	**MH (n=204)**	**MA (n=96)**	**MH (n=24)**	**MA (n=40)**	**p-value**
	Mean ±SD	Mean ±SD	Mean ±SD	Mean ±SD	
CRP (mg/L)*	0.63±1.04	0.87±1.29	0.97±1.06	1.16±1.16	0.070
IL6 (pg/mL)*	21.32±28.24	16.13±21.65	23.89±22.88	15.20±14.89	0.179
TNF-α (pg/mL)*	5.78±3.52	5.64±3.00	5.65±2.44	4.66±2.06	0.897
C3 (g/L)	1.09±0.16	1.16±0.18[c]	1.17±0.13[c]	1.21±0.18[c]	**0.001**
C4 (g/L)	0.19±0.06	0.21±0.06	0.24±0.08	0.22±0.05	0.089
L-Selectin (ng/mL)*	3454.23±1448.89	3401.14±1337.38	4039.05±1851.72	3647.63±1514.56	0.802
sE-selectin (ng/mL)*	34.80±18.88	31.93±16.32	39.66±13.99	34.38±17.57	0.508
sVCAM-	1225.61±292.78	1155.70±287.10	1149.72±210.45	1150.92±292.92	0.168

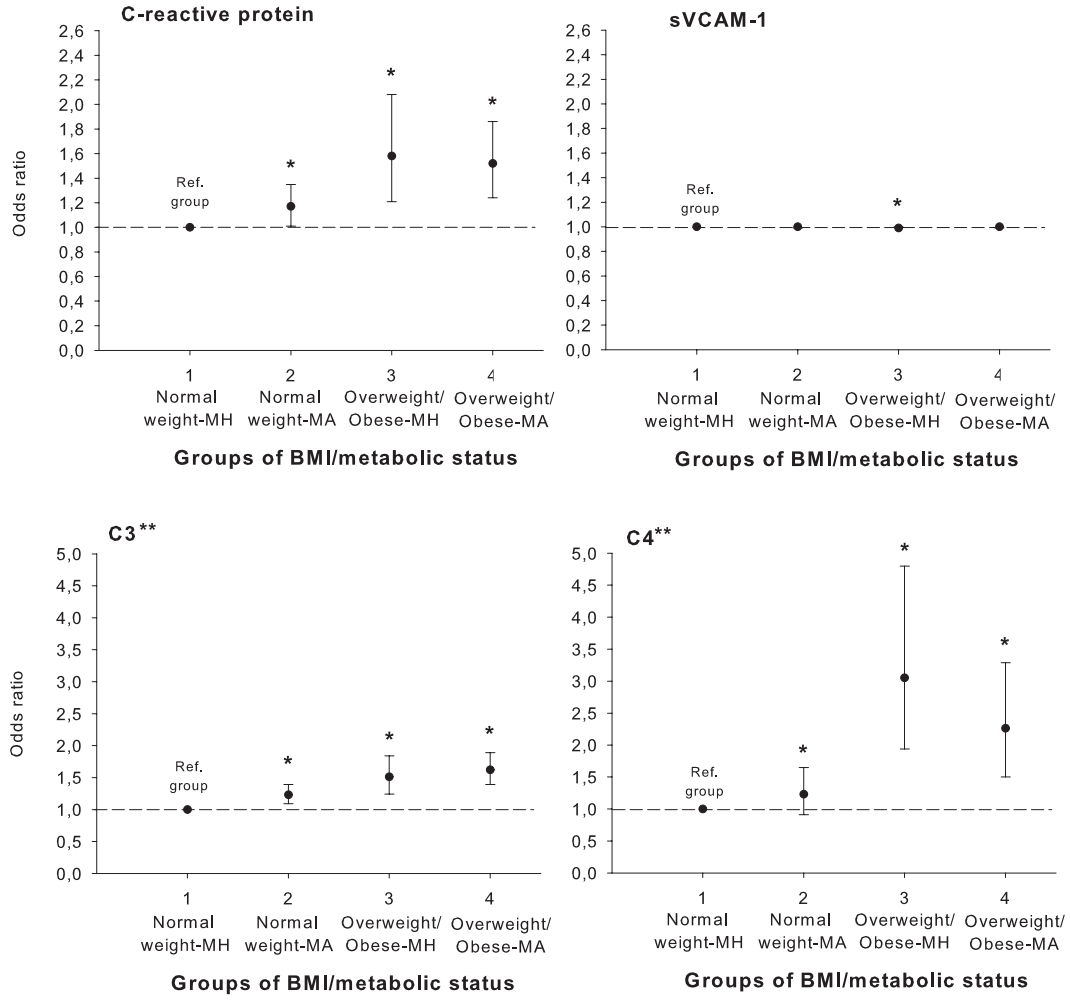
*MH: metabolically healthy (no criterion of the following: high serum triglycerides (≥150mg/dL), fasting glucose(≥100mg/dL), systolic or diastolic blood pressure (Systolic ≥ 130 and diastolic ≥85 mm Hg) and low high density lipoprotein cholesterol (<40mg/dL in men and <50mg/dL in women); MA: metabolically abnormal (1 or more criteria of the following: high serum triglycerides (≥150mg/dL), fasting glucose(≥100mg/dL), systolic or diastolic blood pressure (Systolic ≥ 130 and diastolic ≥85 mm Hg) and low high density lipoprotein cholesterol (<40mg/dL in men and <50mg/dL in women); MA: metabolically abnormal; SD: standard deviation; CRP: C-reactive protein; IL-6: interleukin 6; TNF-α: tumor necrosis factor alpha; C3 and C4: complement factors; sVCAM-1: soluble vascular cell adhesion protein 1; sICAM-1: soluble Intercellular Adhesion Molecule. *Log-transformed. Bonferroni: p<0.05 ref. normal weight-MH.*

Finally, the **supplementary table 1** shows the results for the multinomial logistic regression. This table presents the probability of being in a more unfavorable group of BMI/metabolic health by the increase of the concentration of the inflammatory biomarkers. The probability of being in the normal weight-MA or overweight/obese-MH increased in a 17% and in a 58%, respectively, and in a 52% for the overweight/obese MA per each increase of mg/L of the CRP. Per each additional 0.1 g/L in C3, the probability of being in the normal weight-MA category was 23%, in the overweight/obese a 51% and in the overweight/obese-MA a 62%.

In relation to C4, per each 0.1 g/L increases, the probability of being in the normal weight-MA increased in a 23%; and in a 205% and 126% of being overweight/obese-MH and overweight/obese-MA, respectively. Additionally, per each mg/L increase in sVCAM-1, the probability of being overweight/obese-MH decreased in a 1%. Finally, **Figure 1** present the significant results found in the multinomial logistic regression.

Figure 1: Significant results from the multinomial logistic regression to assess the association between inflammatory biomarkers and BMI/metabolic status adjusted by sex, tanner and moderate-to-vigorous physical activity. (**) C3 and C4 present changes per 0.1 g/L. *p<0.05

Normal weight-MH was set as reference group (i.e. value 1).



DISCUSSION

Findings from this study suggest that differences in inflammatory biomarkers' concentrations were found between MH and MA adolescents. Also, the probability of being in an unfavorable metabolic/BMI status increased with increasing C3 concentrations.

The prevalence of adults with obesity categorized as MHO, based on 10 population-based cohort studies in 7 European countries has been estimated as 12.1% (5, 22) . In youth, information on MHO is scarce, due to the lack of agreed definition in this population group. In our sample, 0.8% of the adolescents were classified as MHO while 6.8% were classified as MH/overweight-obese. This low prevalence of MHO could be due to the low sample size of this study and the different definitions used for MHO. In previous studies, prevalence of MH overweight/obese and obese adolescents ranged from 6 to 68% (23, 24). In our study, 2.7% of the adolescents were allocated in the MAO group. This highlights the importance of a common definition already in adolescence to identify those subjects with a favorable metabolic profile.

A previous study on adult population measured cardio-metabolic risk factors and inflammation in MHO and MAO individuals and found similar adverse inflammatory profile in both groups (19). However, when we assessed some inflammatory marker concentrations by groups, combining cardio-metabolic health and BMI status by sex, differences in mean concentrations were found. In both sexes, MHO had significantly higher CRP concentrations than their counterparts. This result contrast with previous literature as MHO seems to present a favorable inflammatory profile(4, 13, 25). Moreover, in our sample, adolescents with high values of CRP, C3, C4 and sVCAM-1 had higher probability of being in the overweight/obese-MH group in comparison with the normal weight-MH. Similar gene expression of visceral adipose tissue and liver has been found in a previous study, from MHO and MAO patients, with no differences in CRP levels (14). Also, a comparative study stated that the associations between inflammatory markers and MHO depend on the definition used (26).

However, the most consistent results across all the analysis were found for the C3. Overweight/obese-MA subjects had the highest concentrations of the C3 complement factor. In addition, high C3 concentrations increased the probability of being in an unfavorable metabolic/BMI status. C3 is recognized as a cardiovascular risk factor as it has been related with an increased likelihood of future cardiovascular heart disease (CHD) (27). It has also been associated with metabolic disorders, like adiposity, dyslipidemia, insulin resistance, liver dysfunction and diabetes (28, 29). Results from the present study suggest that this relationship between C3 and metabolic disorders is found already in

adolescence, especially on those individuals with the worse metabolic/BMI status. A previous study in adult population found that C3 concentrations were consistently lower in the MH individuals (25). Therefore, C3 could be an important marker for the characterization of the metabolic health. Also C4 was associated with the probability of being in an unfavorable metabolic/BMI status. C4, along with C3, has been related with metabolic syndrome (30) and this relationship could be due to their involvement in the development of visceral adiposity in children and adolescence.

This study has strengths as well as some limitations. First, the cross-sectional design does not allow establishing causality. Also, blood samples only reflect inflammatory status at one-time point. The low sample size could be also a limitation. In contrast, this study has some strengths: the use of traditional and non-traditional biomarkers to reflect the inflammatory status and the use of a definition for MH based on age- and sex-specific cut-off points as the basis to establish health risk in adolescents, which is more appropriate from a clinical perspective. Finally, the use of standardized and harmonized information of adolescents from 9 European countries is another strength to be acknowledged.

In conclusion, these results show that there is an association between some inflammatory biomarkers and metabolic/BMI status. C3 and C4 seem to be emerging biomarker related to the cardio-metabolic health, already in adolescence. Likewise, the increase of some inflammatory markers increased the probability of being in an overweight/obese-MH status. A unique definition for metabolic health is necessary to corroborate these results. Further longitudinal studies are needed to understand how these inflammatory markers influence the development of future cardiovascular diseases.

Conflict of interest

No conflicts of interest were declared

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Supplementary table 1. Multinomial logistic regression to assess the association between inflammatory biomarkers and BMI/metabolic status adjusted by sex, age and moderate-to-vigorous physical activity.

	Normal weight-MH (n=338)	**Normal weight-MA (n=194)**	**p-value**	**Overweight/Obese-MH (n=45)**	**p-value**	**Overweight/Obese-MA (n=82)**	**p-value**
		OR (95% CI)		**OR (95% CI)**		**OR(95% CI)**	
CRP (mg/L)*	Ref	1.17(1.01-1.35)	**0.028**	1.58(1.21-2.08)	**0.001**	1.52(1.24-1.86)	**<0.001**
IL6(pg/mL)*	Ref	0.93(0.81-1.07)	0.323	1.17(0.91-1.49)	0.201	0.93(0.78-1.12)	0.493
TNF-α (pg/mL)*	Ref	0.82(0.59-1.14)	0.825	1.04(0.59-1.85)	0.876	0.65(0.43-1.00)	0.060
C3 (per 0.1 g/L)	Ref	1.23 (1.09-1.39)	**0.001**	1.51(1.24-1.84)	**<0.001**	1.62(1.39-1.89)	**<0.001**
C4 (per 0.1 g/L)	Ref	1.23 (0.91-1.65)	**0.165**	3.05 (1.94-4.80)	**<0.001**	2.26 (1.56-3.29)	**<0.001**
L-Selectin(ng/mL)*	Ref	0.99 (0.69-1.41)	0.964	1.12(0.60-2.07)	0.709	0.98(0.62-1.57)	0.964
sE-Selectin(ng/mL)*	Ref	0.82(0.55-1.22)	0.338	1.50(0.77-2.92)	0.232	1.25(0.74-2.12)	0.391
sVCAM-1(ng/mL)	Ref	1.00(0.99-1.00)	0.237	0.99(0.99-1.00)	**0.013**	1.00(0.99-1.00)	0.357
sICAM-1(ng/mL)*	Ref	1.18(0.79-1.76)	0.407	1.08(0.57-2.07)	0.795	1.27(0.75-2.14)	0.358

*MH: metabolically healthy (no criterion of the following: high serum triglycerides (≥150mg/dL), fasting glucose(≥100mg/dL), systolic or diastolic blood pressure (Systolic ≥ 130 and diastolic ≥85 mm Hg) and low high density lipoprotein cholesterol (<40mg/dL in men and <50mg/dL in women); MA: metabolically abnormal (1 or more criteria of the following: high serum triglycerides (≥150mg/dL), fasting glucose(≥100mg/dL), systolic or diastolic blood pressure (Systolic ≥ 130 and diastolic ≥85 mm Hg) and low high density lipoprotein cholesterol (<40mg/dL in men and <50mg/dL in women); OR: Odds ratio; CI: confidence interval; CRP: C-reactive protein; IL-6: interleukin 6; TNF-α: tumor necrosis factor alpha; C3 and C4: complement factors; sVCAM-1: soluble vascular cell adhesion protein 1; sICAM-1: soluble Intercellular Adhesion Molecule. *Log-trangsformed. Signification level at p<0.05*

DISCUSIÓN

DISCUSSION

Los cambios en los estilos de vida que se han producido en las últimas décadas han propiciado el aumento de las enfermedades que están asociadas con un inflamación crónica. Este proceso inflamatorio se observa cada vez a edades más tempranas y su desarrollo puede ser debido a estilos de vida poco saludables en relación con la alimentación, la falta de actividad física y los comportamientos sedentarios, entre otros.

Dieta e inflamación

La dieta parece jugar un papel relevante en el estado inflamatorio. Esta relación ha sido investigada previamente en adultos, teniendo en cuenta nutrientes, alimentos específicos y patrones dietéticos (17, 18, 35).

1. Ácidos grasos e inflamación (artículo I)

Entre estos factores dietéticos asociados con la inflamación, los ácidos grasos parecen tener un papel relevante (19). Especialmente la relación de los ácidos grasos omega 3: ALA, EPA y DHA, con el estado inflamatorio (18, 21).

En el estudio IDEFICS se han observado asociaciones entre algunos ácidos grasos en sangre total y PCR-hs, tras controlar por potenciales confusores, en una muestra de niños europeos. En chicos, de todos los ácidos grasos estudiados, las concentraciones de ácido linoleico y la suma de ácidos grasos omega-6 en sangre total, se asociaron inversamente con las concentraciones de PCR-hs. Estos resultados parecen estar en la línea de estudios previos ya que la presencia de dobles enlaces, independientemente de la posición del enlace omega-3 o omega-6, parece ser crucial en la modulación de la interacción endotelial leucocitaria (90). Por otro lado, en chicas, la relación EPA/AA se asoció inversamente con la PCR-hs, aunque esta asociación no alcanzó la significación estadística al ajustar por algunas variables de confusión. Finalmente, en la regresión logística ajustada por edad, educación de la madre y país, se observó una asociación inversa entre

la relación DHA/AA y la PCR-hs, en ambos sexos. En la literatura científica se ha observado que la relación entre omega-3 y omega-6 y la relación DHA/AA se asocia negativamente con el volumen de la placa de ateroma en pacientes con enfermedad coronaria y/o de las arterias (91). En línea con los resultados hallados en la presente tesis doctoral, la relación EPA/AA también se asocia inversamente con la incidencia de enfermedades cardiacas graves (92). Esto es debido a que el EPA y el DHA limitan la formación de AA, precursor de mediadores inflamatorios, evitando así el estado inflamatorio. Finalmente, como en estudios previos, se observaron diferencias por sexo, sugiriendo que desde el punto de vista de la activación de los mecanismos de inflamación, el metabolismo de los ácidos grasos poliinsaturados es más eficiente en mujeres que en hombres (93).

2. Alimentos e inflamación (artículo II)

La ingesta de nutrientes y de componentes no nutricionales de los alimentos, no se realiza de forma aislada ya que estos se encuentran englobados dentro de los alimentos que consumimos. Por ello, en la presente tesis de doctoral, se valoraron 41 grupos de alimentos consumidos por los niños participantes en el estudio IDEFICS. En nuestro estudio, la frecuencia de consumo de algunos alimentos estaba relacionada con la PCR-hs, encontrando diferencias por grupo de edad y sexo.

Los vegetales fueron el grupo de alimentos que presentó una mayor asociación, siendo significativa la probabilidad de tener concentraciones más bajas de PCR-hs cuanto mayor era la frecuencia de consumo de verduras crudas en niños de 6 a 10 y de verduras cocidas, en niñas de 2 a 6 años. Estos resultados concuerdan con los descritos previamente en la literatura científica, tanto en niños (42) como en adultos (27, 94). Esta asociación inversa entre consumo de verduras e inflamación, puede deberse a la riqueza de estos alimentos en nutrientes y fitoquímicos con capacidad antioxidante y, a su vez, a la baja cantidad de grasa y alto contenido en agua y fibra.

Además de los vegetales, otros alimentos aumentaban la probabilidad de presentar concentraciones más bajas de PCR-hs, como la miel y la mermelada en

niñas jóvenes. Este resultado puede ser debido a que son alimentos de desayuno, que pueden estar relacionados con un estilo de vida saludable. En un estudio previo, en otra muestra de IDEFICS, las niñas con un alto consumo de miel y mermelada tenían menor riesgo cardiovascular (95). Finalmente, y sólo en niñas de 2 a 4 años, el consumo de bebidas refrescantes azucaradas, mayonesa y cereales refinados se asociaron positivamente con el aumento de los valores de PCR-hs. Este tipo de bebidas y los cereales refinados se han asociado con los picos de glucemia que pueden contribuir al estrés oxidativo y tanto a la inflamación aguda como a la inflamación crónica (30, 31, 96). Finalmente, la mayonesa es un producto con elevado contenido en grasas y cuyo consumo podría relacionarse con unos patrones dietéticos no saludables. Estos resultados sugieren que la alimentación juega un papel importante en la inflamación desde la infancia.

3. Patrones dietéticos e inflamación (artículo III)

Los patrones dietéticos aportan información global sobre los hábitos alimentarios, al tener en cuenta las combinaciones en el consumo de los distintos tipos de alimentos que se presentaban en los mismos grupos de individuos. En el tercer artículo de esta Tesis, en niños del estudio IDEFICS, se identificaron tres patrones, con características similares a lo largo del seguimiento: el "patrón saludable", el patrón de "proteína animal y carbohidratos refinados" y el patrón "dulces y alimentos procesados". Estos resultados coinciden con los obtenidos previamente en otra muestra del estudio IDEFICS, habiéndose descrito patrones similares, tales como "proteico", "saludable" y "dulce y procesado", aunque con nombres distintos a los descritos previamente (97). Adicionalmente, se observó un cuarto patrón, denominado "snacking", que no fue identificado en este análisis. Esto puede ser debido a que la técnica estadística empleada fue distinta: análisis por conglomerados (*cluster analysis*) *versus* análisis de componentes principales (PCA) en el estudio previo (97), y a que la sub-muestra utilizada es distinta.

Otro estudio prospectivo realizado con datos de IDEFICS (98) encontró un patrón saludable similar, usando el análisis de *clusters*. Este estudio también observó un patrón "procesados" y otro "dulces", con características similares al patrón "dulces y procesados" observado en este análisis. Sin embargo, no se

observó ningún patrón proteico similar al presentado en esta Tesis. Estas diferencias pueden deberse a que, en el estudio previo, se incluyeron 9.301 niños, mientras que en el presente estudio, se incluyeron 4.020 niños. Ello es debido a que en esta última muestra, se incluyeron aquellos niños para los que estaba disponible la medida de la PCR-hs.
En cuanto a la distribución de los sujetos del estudio, se observó un menor porcentaje de niños con obesidad, en el patrón "saludable", en comparación con la proporción de niños con obesidad, en los otros dos patrones. En cambio, se encontraron altos porcentajes de niños con sobrepeso/obesidad en el patrón de "proteína animal y carbohidratos refinados", en comparación con los porcentajes encontrados en los patrones "saludable" y "dulces y procesados".

Se observaron asociaciones positivas entre el patrón "dulces y procesados" y la inflamación en el análisis transversal de la valoración de seguimiento y, a su vez, de manera prospectiva, en comparación con el patrón "saludable". En la literatura científica, una revisión basada en estudios transversales, identificaba la dieta occidental, caracterizada por un alto consumo de carne, como el patrón dietético más relacionado con la inflamación, mientras que el patrón "saludable", con alto consumo de frutas y verduras, estaba inversamente relacionado con la inflamación (35).
Parece que los patrones dietéticos occidentales, caracterizados por elevadas ingestas de carnes rojas y procesadas, dulces, postres, alimentos fritos y granos refinados, se relacionan positivamente con un aumento de las moléculas inflamatorias, moléculas de adhesión endotelial y promotores aterogénicos (36, 37). Otra revisión de la literatura científica encontró resultados similares en cuanto a las dietas occidentales, al comparar estudios con distintas técnicas estadísticas para la obtención de los patrones dietéticos (99). Además, las conclusiones de otro estudio (100) establecieron que un patrón caracterizado por alto consumo de huevos y dulces se asociaba con altos niveles de PCR así como el patrón denominado "pasta y carne", mientras que el patrón denominado "aceite de oliva y verduras" estaba asociado negativamente con la PCR.

Por lo tanto, resultados previos sugieren que la relación entre los patrones dietéticos considerados no saludables y la inflamación, no son tan consistentes como los encontrados para la relación con el patrón "saludable". Una posible

explicación puede ser que el análisis estadístico es un análisis *a posteriori*, lo que significa que diferentes patrones pueden aparecer en diferentes muestras. Por otro lado, la definición de un patrón no-saludable es más amplia que la de un patrón saludable, caracterizado habitualmente por un alto consumo de verduras y frutas. La combinación beneficiosa de las vitaminas antioxidantes o compuestos, fibra, y otros fitoquimicos antiinflamatorios, que se encuentran en los alimentos de origen vegetal, parecen subyacer en la asociación inversa con la inflamación (16).

Riesgo cardio-metabólico e inflamación

1. Salud cardiovascular e inflamación (artículo IV)

La AHA propone que se debería valorar la salud cardiovascular de todos los niños y adolescentes, usando el ISCI. Ninguno de los adolescentes europeos del estudio HELENA, incluidos en nuestro estudio sobre salud cardiovascular, cumplía los criterios saludables de los siete componentes del ISCI. Este resultado coincide con los encontrados previamente en otros estudios en población adolescente (52, 55, 101). Esto es debido a los valores bajos del componente del índice referido a la dieta; este componente incluye al menos cuatro criterios de dieta ideal sobre cinco, y fue también el componente con menor cumplimiento en nuestra muestra, con un 1,7%. En estudios realizados en adultos, el índice de dieta ideal fue también el componente observado con menor frecuencia, de los que componen el ISCI, siendo la prevalencia en algunos estudios menor de 1%(102) y 0,4% (103). En la muestra estudiada, de todos los componentes de la dieta ideal, sólo un 8,7% de los adolescentes alcanzó el nivel óptimo de consumo de sodio, siendo el criterio más difícil de cumplir, además de uno de los criterios más difíciles de medir de manera precisa. Por el contrario, tener menos de 100 mg/dL de glucosa en sangre fue el componente del ISCI con mayor porcentaje de cumplimiento, representando un 91,2% de los adolescentes.

En nuestra muestra, se observó una asociación negativa entre la salud cardiovascular ideal y el score inflamatorio, es decir, a mayores valores del índice cardiovascular ideal, menores valores del índice inflamatorio. No hay estudios previos que valoren esta asociación en la adolescencia. Además, un estudio

previo observó que los valores del índice de salud cardiovascular en adolescentes era un buen predictor de la salud cardio-metabólica en adultos (52). Estudios previos en adultos ya han relacionado los componentes de este índice, con los biomarcadores de inflamación (104).
Las asociaciones entre el ISCI y el índice inflamatorio se hallaron en ambos sexos. El índice de salud cardiovascular ideal se asoció con todos los marcadores individuales del índice inflamatorio, excepto con la PCR. Esta proteína es el marcador más comúnmente usado de inflamación, ya que se ha relacionado con adiposidad y factores de riesgo cardiovascular en niños (105) y con la predicción de enfermedad coronaria (8) y aterosclerosis (106) en adultos.

La condición física cardiorespiratoria se puede considerar un marcador de salud cardiovascular en niños y adolescentes (107) y, adicionalmente, se ha relacionado con un aumento de la prevalencia de factores de riesgo cardiovascular en la adolescencia y en adultos (108). Un estudio previo, con datos del estudio HELENA, mostró que mayores niveles de condición física cardiorespiratoria se asociaban con el índice de salud cardiovascular ideal en adolescentes (109). Nuestros resultados muestran que el índice de salud cardiovascular se asociaba con la inflamación, independientemente de la condición física cardiorespiratoria, en chicas.

2. Resistencia a la insulina e inflamación (artículo V)

En una elevada proporción de los niños y adolescentes con obesidad se observa resistencia a la insulina, que puede ser considerada un precursor en el desarrollo de la diabetes de tipo 2. En los adolescentes del estudio HELENA, encontramos una asociación significativa entre el factor C3 del complemento y la resistencia a la insulina, independientemente de la grasa total y de la grasa abdominal. Este es el primer estudio que valora la relación entre diferentes marcadores inflamatorios y la resistencia a la insulina, en una muestra de adolescentes europeos. Las concentraciones medias de glucosa, insulina, HOMA y algunos marcadores inflamatorios como la PCR o la C3 y C4, fueron significativamente mayores en el tertil superior de cada marcador de adiposidad. En estudios previos en niños, la obesidad se ha relacionado con la inflamación (45) y, a su vez, la hipeplasia adipocitaria derivada de esta condición, se ha asociado con el

índice HOMA, como marcador de resistencia a la insulina en niños con obesidad (60). Los resultados de este estudio, apoyan la hipótesis de que existe una relación entre adiposidad, metabolismo glucídico e inflamación, incluso en la adolescencia, ya que varios de estos biomarcadores se encontraron elevados en los niveles más altos de adiposidad total y abdominal.

Además, se encontraron asociaciones lineales entre algunos de los marcadores inflamatorios y el índice HOMA, como variable dependiente, teniendo en cuenta las distintas categorías de los índices de composición corporal. Estudios previos, sugieren que los marcadores inflamatorios, pueden interferir con la acción de la insulina directamente, inhibiendo los receptores insulínicos (110). Sin embargo, existen algunas discrepancias entre los estudios que valoran la asociación entre la inflamación y la resistencia a la insulina, en adolescentes (63, 65). Un estudio reciente, en adolescentes con obesidad, no observó una relación significativa entre la obesidad y la resistencia a la insulina mediada por la inflamación, usando marcadores inflamatorios tradicionales (64).

A pesar de que estudios previos han asociado algunos marcadores tradicionales de infalamación, con el desarrollo de diabetes o resistencia a la insulina (58, 59, 111), no se ha observado ninguna relación entre los marcadores tradicionales de inflamación y el índice HOMA, en los adolescentes con los mayores niveles de IMC, índice de masa grasa (fórmula de Slaughter) y circunferencia de cintura. De todos los marcadores inflamatorios medidos en el presente estudio, solo el C3 se relacionaba de manera consistente con la resistencia a la insulina, valorada mediante el índice HOMA, en los adolescentes, especialmente en aquellos con los mayores niveles de grasa total y abdominal, excepto con el índice de masa grasa, en chicas. Nuestros resultados están en línea con estudios previos. C3 fue el marcador inflamatorio relacionado más intensamente con la resistencia a la insulina, en una población anciana (112). Este factor del sistema complemento es un factor emergente de riesgo cardio-metabólio relacionado con algunas comorbilidades como la diabetes de tipo 2 (113). En una muestra de adolescentes españoles, los niveles de C3 estaban relacionados con la grasa corporal, especialmente con la grasa abdominal (114) y fueron mayores en sujetos adultos con resistencia a la insulina (115). Un estudio previo, en adultos, mostraba que la inflamación y la resistencia a la insulina pueden ser dos rutas metabólicas por las que la grasa corporal origina altas concentraciones de C3 (116). Sin embargo, parece que los cambios en los niveles de C3 en un periodo de seguimiento de 7

años, se asociaron con cambios en algunas medidas de resistencia a la insulina y que los valores de C3 se asociaron con la incidencia de diabetes de tipo 2, en dicho periodo (117). Aunque la mayoría de la producción de factor C3 del complemento se realiza en el hígado, esta proteína también se sintetiza por macrófagos activados y adipocitos, como una citoquina inflamatoria o una adipokina (118, 119). Su producción hepática se encuentra inducida por citoquinas, como interleukin-6 y TNF-α (10, 120) que pueden interferir con el receptor insulínico y causar resistencia a la insulina (121). Además, el sistema del complemento es un regulador del sistema adaptativo e innato y, como parte de la respuesta inflamatoria, puede también contribuir a la resistencia a la insulina. Finalmente, se encontraron algunas relaciones entre la inflamación y el factor C4 del complemento o el TNF-α pero, en el caso de esta último, en chicas y solo en las categorías más bajas de composición grasa corporal.

3. Salud metabólica e inflamación (artículo VI)

En los individuos con obesidad se ha propuesto recientemente clasificarlos según la afectación cardio-metabólica que presentan. De esta manera, se suele hablar de "obesidad metabólicamente sana" y "obesidad metabólicamente no sana". En este estudio, los resultados sugieren que existen diferencias en los marcadores inflamatorios entre los adolescentes metabólicamente sanos y los metabólicamente no sanos. Además, la probabilidad de encontrarse en los grupos sobrepeso/obesidad, metabólicamente no sanos, aumentaba cuando aumentaban las concentraciones de C3.

En la adolescencia, la información sobre la prevalencia de la ObMS es escasa, debido a la falta de consenso en la definición para este grupo poblacional. En nuestra muestra, 0,8% de los adolescentes fueron clasificados como ObMS mientras que 6,8% fueron clasificados como sobrepeso-obesidad metabólicamente sanos (SObsMS). Esta baja prevalencia de ObMS puede ser debida al pequeño tamaño de la muestra y, además, a las diferencias que se encuentran según la definición utilizada. En estudios previos, la prevalencia de adolescentes SObsMS varía entre el 6 y el 68% (122, 123). En nuestro estudio, 2,7% de los adolescentes se clasificaron en el grupo de obesidad metabólicamente no sana (ObMNS). Esto pone de manifiesto la importancia de una definición común ya en la adolescencia, para identificar a aquellos sujetos con un perfil metabólico

saludable. Un estudio previo en población adulta valoró los factores de riesgo cardio-metabólicos y la inflamación en ObMS y ObMNS y, al contrario que en nuestro estudio, encontraron un perfil inflamatorio adverso similar en ambos grupos (124).

En ambos sexos, los adolescentes ObMS tenían significativamente mayores concentraciones de PCR que el resto. Este resultado contrasta con la literatura previa ya que los ObMS parecen presentar un perfil inflamatorio favorable (125-127). Además, en nuestra muestra, los adolescentes con valores elevados de PCR, C3, C4 and sVCAM-1 tenían mayor probabilidad de estar clasificados como SObMS, en comparación con los normo-peso metabólicamente sanos (NMS). En otro estudio previo, se ha observado una expresión genética similar del tejido adiposo visceral y del hígado de pacientes ObMS y ObMNS, sin diferencias en los niveles de PCR (128). También un estudio comparativo establecía que las asociaciones entre los marcadores inflamatorios y la ObMS dependían de la definición utilizada (129). Sin embargo, el resultado más consistente encontrado en nuestros análisis, se observó para el factor C3. Los adolescentes clasificados como sobrepeso/obeso metabólicamente no sanos (SObMNS) presentaban las mayores concentraciones del factor C3. Además, las concentraciones elevadas de C3 aumentaban la probabilidad de estar en los grupos sobrepeso/obesidad, metabólicamente no sanos.

El factor C3 del complemento se ha reconocido como otro factor de riesgo cardiovascular y se ha relacionado con un aumento en la probabilidad de enfermedad cardiaca futura (130). También, se ha asociado con alteraciones metabólicas como adiposidad, dislipidemia, resistencia a la insulina, disfunción hepática y diabetes (131, 132).

Resultados del presente estudio sugieren que esta relación entre el factor C3 y los desórdenes metabólicos, se encuentra ya en la adolescencia, especialmente en aquellos individuos clasificados en el grupo sobrepeso/obesidad, metabólicamente no sanos. Un estudio previo, en población adulta, observó que las concentraciones del factor C3 eran consistentemente menores en los individuos metabólicamente sanos (127). Por lo tanto, el factor C3 se puede considerar un importante marcador para la caracterización de la salud metabólica. El factor C4 también se asoció con la probabilidad de estar clasificado en el grupo sobrepeso/obesidad, metabólicamente no sano. El factor C4, al igual que el C3, se asoció con el síndrome metabólico (133) y este hallazgo puede ser

debido a su relación con el desarrollo de la adiposidad visceral en niños y adolescentes.

CONSIDERACIONES METODOLÓGICAS

Los artículos incluidos en la presente Tesis Doctoral están basados en el estudio transversal HELENA y en la valoración inicial (T0) y en la de seguimiento (T1) de IDEFICS; de este último se analizaron también los cambios de manera prospectiva.

Estudio IDEFICS

El principal objetivo del diseño de este estudio era conseguir regiones comparables para el desarrollo de la intervención, incluyendo también un grupo control. Debido a limitaciones económicas y consideraciones prácticas, el estudio IDEFICS no pretendía obtener una muestra representativa de cada país. Además, al tratarse de un estudio en el que se contactaba con los padres/tutores a través de las escuelas, es posible que aquellos individuos sin problemas de salud o menos concienciados con la salud de sus hijos, hayan presentado una menor motivación a participar. También, la participación de grupos de bajos y altos niveles socioeconómicos suele ser baja en este tipo de estudios. Al no tener información sobre los no-participantes en el estudio, no se puede cuantificar este sesgo. Estas limitaciones deben ser tenidas en cuenta cuando se interpreten los datos de manera transversal, estratificando por nivel de educación, ingresos, estado migratorio y otras características sociodemográficas. En cuanto a la distribución socioeconómica en los distintos países participantes, se observó una baja representación de familias de bajo nivel socioeconómico en las muestras de algunos países. Finalmente, el diseño transversal de este estudio no nos permite valorar causalidad.

Limitaciones y fortalezas de los artículos de IDEFICS

La principal limitación de todos los artículos basados en el estudio IDEFICS e incluidos en la presente tesis doctoral, es el uso exclusivo de la CRP-hs como

marcador para el estudio de la inflamación. Hubiera sido útil el uso de otros marcadores inflamatorios adicionales, para valorar mejor el estado inflamatorio.

En el **artículo I** de esta tesis doctoral, se ha valorado la asociación entre distintos factores relacionados con la alimentación y el estado inflamatorio.
En primer lugar se ha valorado la asociación con nutrientes previamente relacionados con el estado inflamatorio, como son los ácidos grasos, especialmente los de cadena larga. La medida de los ácidos grasos mediante el método descrito en esta memoria ha demostrado ser válido, no invasivo y sencillo. Además, en análisis de los ácidos grasos en sangre total reflejan la ingesta grasa dietética, como se ha demostrado para carne y pescado (134).

En el **artículo II** y **artículo III** se ha utilizado un CFCA, para valorar la ingesta de alimentos. Este cuestionario era rellenado por los padres/tutores y sólo tenía en cuenta aquellos alimentos consumidos en casa. Además, el número de comidas bajo control parental varía entre países. En el **artículo III** se ha realizado una valoración de los patrones dietéticos mediante el análisis de conglomerados (*cluster analysis*). En este artículo, se realiza un análisis longitudinal de los resultados; sin embargo, los patrones creados no son exactos en los dos momentos de medida, aunque los patrones obtenidos en la medición inicial (T0) y en la seguimiento (T1) eran similares.

En cuanto a las principales fortalezas de los artículos incluidos en la primera parte de esta tesis doctoral, se encuentra: el uso de datos estandarizados de niños de seis países europeos y el grupo de población, ya que la investigación sobre factores relacionados con la inflamación, en edad pediátrica, es escasa. El uso de las concentraciones de ácidos grasos en sangre total **(artículo I)** en vez de la ingesta de ácidos grasos es otra fortaleza ya que existe una gran variabilidad en el estatus de los mismos, explicada por la alta variabilidad individual en los mecanismos fisiológicos y genéticos. El CFCA aporta estimaciones reproducibles de la frecuencia de consumo de los distintos grupos de alimentos en niños europeos (73, 74). Otra ventaja del CFCA es que tiene en cuenta alimentos que se toman de manera poco frecuente, que podrían ser obviados con los recordatorios de 24 horas. Además, por razones de practicidad, el CFCA se considera un buen reflejo de la ingesta dietética habitual en grandes estudios epidemiológicos. El

análisis multinivel o, el uso de país como nivel de agrupación tiene en cuenta las diferencias que pueden existir por país en el consumo de determinados alimentos ya que culturalmente existen grandes diferencias entre las distintas zonas europeas. Finalmente, el análisis prospectivo de los resultados **(artículo III)** aporta una mayor perspectiva sobre los comportamientos dietéticos a largo plazo y su relación con la inflamación, en población pediátrica.

Estudio HELENA

La muestra de adolescentes en el estudio HELENA es representativa de cada una de las 10 ciudades europeas seleccionadas. La selección de las mismas, no fue aleatorio, sino que se decidió de acuerdo a unos criterios establecidos (79), siendo el más importante de ellos, la existencia de un grupo de investigación con capacidad para realizar este estudio. Además, las ciudades debían tener una población mayor de 100.000 habitantes y ser equivalentes y comparables en relación con cada uno de los países y con una muestra lo suficientemente grande como para asegurar la diversidad de la población. Las escuelas fueron seleccionadas al azar tras estratificación por distrito escolar. Otra vez, este modo de contacto con los padres/tutores y los propios adolescentes puede dar lugar a un sesgo, al sólo consentir a la participación aquellos sujetos que tienen una preocupación real por la salud de sus hijos adolescentes (135).

Limitaciones y fortalezas de los artículos del estudio HELENA

El diseño transversal del estudio HELENA es la principal limitación de todos estos artículos. Sin embargo, la valoración del estado inmuno-inflamatorio incluia un número importante de biomarcadores (79). En el **artículo IV,** se utiliza un score inflamatorio, que utiliza biomarcadores seleccionados previamente por presentar asociaciones positivas con marcadores de riesgo cardio-metabólico (136). Para cada uno de ellos, se calculaba el z-score y posteriormente la suma de todos ellos. Este score presenta como principal limitación considerar que todos los biomarcadores tienen el mismo peso en el metabolismo inflamatorio. Finalmente, las muestras sanguíneas solo reflejan el estado inflamatorio en un momento determinado del tiempo.

En relación con las fortalezas de los artículos incluidos en esta segunda parte destaca el uso de un score inflamatorio que puede ser considerado como un mejor reflejo del estado inflamatorio frente al uso de un único marcador. Además, este score usa marcadores inflamatorios tradicionales y no tradicionales, que se asocian con el riesgo cardio-metabólico. En el **artículo VI**, se valora la salud cardio-metabólica basada en puntos de corte específicos por edad y sexo, lo cual es apropiado desde una perspectiva clínica. Finalmente, el uso de información armonizada y estandarizada de 9 países europeos es también una fortaleza importante que debe ser tenida en cuenta.

Implicaciones para la salud pública

Los resultados obtenidos en la presente Tesis Doctoral apoyan la teoría de que, por un lado, los factores de riesgo cardio-metabólico asociados a la inflamación se encuentran presentes ya en la adolescencia y, por otro lado, que la dieta juega un papel importante en el estado de inflamación crónica subclínica, desde etapas precoces de la vida.

Estos resultados justifican la implantación de estrategias preventivas sobre los estilos de vida, con especial énfasis en la alimentación, para prevenir la aparición de enfermedades cardiovasculares en la edad adulta.

Existen algunas guías para la valoración del riesgo cardiovascular en población infanto-juvenil. A nivel internacional, destaca la del Panel de expertos sobre Pautas Integradas para la Salud Cardiovascular y la reducción del riesgo en Niños y Adolescentes: Summary Report (137). A nivel de España, la estrategia para la nutrición, actividad física y prevención de salud: NAOS. Si bien es cierto que la estrategia NAOS tenía como fin la prevención de la obesidad, ésta se considera un factor importante de la salud cardiovascular, siendo por ello similares las indicaciones para reducir el riesgo de ambas patologías. Aunque estas guías tienen una sección específica sobre alimentación, las que ha realizado el panel de expertos a nivel internacional se focalizan más en el consumo de nutrientes específicos, vitaminas, minerales o fibra.

En el **artículo I** de la presente Tesis, se ha observado que la inflamación, como precursor de las enfermedades cardiovasculares, se asocia con los ácidos grasos de cadena larga, ya desde la infancia. Este resultado es importante ya que la ingesta de nutrientes desde edades precoces, influye en el estado inflamatorio que, mantenido en el tiempo, puede derivar en enfermedades cardiovasculares. En la práctica, el consumo de nutrientes se realiza de manera combinada en forma de alimentos. Por esta razón, la realización de estudios que valoren la asociación entre alimentos e inflamación, como el análisis realizado en el **artículo II** de esta Tesis, es de mayor relevancia clínica a la hora de diseñar las guías de práctica clínica, para prevenir las enfermedades cardiovasculares. Los resultados de este artículo apoyan las recomendaciones encontradas en la guía del Panel de Expertos y la estrategia NAOS, en las que se propone aumentar el consumo de frutas y verduras y reducir el consumo de bebidas azucaradas, de manera que aumente el aporte de minerales, vitaminas y fibra, a la vez que disminuye el

aporte energético. Adicionalmente, en el **artículo III** de esta Tesis, hemos analizado los patrones dietéticos y su relación con el estado inflamatorio. Los resultados de este artículo tienen gran implicación clínica al demostrar que existe una relación entre patrones dietéticos e inflamación, incluso a largo plazo. Así pues, no sólo los alimentos de manera individual, sino la dieta entendida en su conjunto, deben ser tenidos en cuenta a la hora de establecer unas guías preventivas del riesgo cardiovascular, intentando evitar el desarrollo de un estado inflamatorio crónico de bajo grado. A la hora de proponer estrategias de prevención, debe ser la dieta en su conjunto el objetivo principal.

Por lo que se refiere a la relación entre inflamación y los factores de riesgo cardio-metabólico, hay que considerar que la inflamación es precursora de las enfermedades cardiovasculares, las cuales, a su vez, también están relacionadas con las alteraciones cardio-metabólicas. El **artículo IV** ha mostrado que, ya desde la adolescencia, un bajo índice de salud cardiovascular ideal se asocia con la inflamación. Por otro lado, en el **artículo V** se observa una asociación entre la inflamación y la resistencia a la insulina y en el **artículo VI** se relaciona la inflamación con un perfil metabólico más desfavorable, unido al sobrepreso/obesidad. Además de la importancia de conocer la existencia de estas relaciones, desde la adolescencia, otra contribución importante radica en observar que, de todos los marcadores inflamatorios, el factor C3 del sistema complemento ha sido el que ha demostrado resultados más consistentes a lo largo de todos los análisis. Por esta razón, el estudio de nuevos marcadores inflamatorios puede dar una mejor visión global del estado inflamatorio y la oportunidad de identificar a aquellos sujetos con riesgo de desarrollar futuras enfermedades asociadas a la inflamación.

Se ha encontrado una carencia de artículos sobre la relación entre la alimentación y la inflamación, por lo que esta Tesis contribuye a aumentar la evidencia científica sobre la importancia de una alimentación saludable, desde etapas precoces de la vida. En el futuro, se debe promover la realización de estudios longitudinales que exploren estas asociaciones, para confirmar los resultados obtenidos y poder así fomentar estrategias de prevención efectivas, con el objetivo de reducir el riesgo cardiovascular en la etapa adulta.

APORTACIONES PRINCIPALES DE LA TESIS DOCTORAL

ARTICULO I: La relación entre los ácidos grasos y la PCR-hs, como marcador inflamatorio, se encuentra presente desde la infancia. Tras la selección de unos ácidos grasos específicos, basada en la relevancia biológica de éstos, se observó que esta relación varía según el tipo de ácido graso y el sexo, siendo los ácidos grasos omega-6 los que presentaron más asociaciones.

ARTICULO II: La frecuencia de consumo de alimentos específicos se asocia con la PCR-hs según el sexo y el grupo de edad, en una muestra de niños europeos. Estos resultados muestran que se encuentran diferencias entre alimentos y su relación con el nivel inflamatorio ya desde las primeras etapas de la vida.

ARTICULO III: Los adolescentes incluidos en un patrón dietético identificado como desfavorable tenían mayores probabilidades de presentar mayores concentraciones de PCR-hs en comparación con los asignados al patrón dietético saludable. Esta asociación se encontró en el análisis transversal y en el longitudinal, destacando la importancia de un patrón dietético saludable desde la edad pediátrica.

ARTICULO IV: En adolescentes, el índice de salud cardiovascular ideal puede ser útil como herramienta para identificar aquellos con un estado inflamatorio alterado. Además, el uso de nuevos marcadores inflamatorios, en combinación con los tradicionales, puede ser útil desde el punto de vista clínico.

ARTICULO V: La composición corporal juega un papel importante en la relación entre la resistencia a la insulina y la inflamación, medida con varios marcadores inflamatorios. Esta asociación entre la resistencia a la insulina y el factor C3 del

complemento fue especialmente relevante para aquellos adolescentes con mayores niveles de grasa corporal.

ARTICULO VI: Aquellos adolescentes con un perfil metabólico más desfavorable, unido al sobrepreso/obesidad, presentan un estado inflamatorio alterado. De todos los marcadores estudiados, los factores C3 y C4 del complemento, se asociaron consistentemente con la salud cardio-metabólica.

MAIN CONTRIBUTIONS OF THE THESIS

MANUSCRIPT I: The relationship between the fatty acids and the hs-CRP, as an inflammatory marker, is present from childhood. After the selection of specific fatty acids, based on their biological relevance, it was observed that this relation varies according to the type of fatty acid and sex, being the omega-6 those which presented more associations.

MANUSCRIPT II: The food frequency consumption of specific food items is associated with the hs-CRP depending on sex and age group, in a sample of European children. This results shows that there are differences between food items and their relationship with the inflammatory level from first stages of life.

MANUSCRIPT III: Adolescents included in an unfavourable dietary patterns showed higher probability of having higher concentrations of hs-CRP in comparison with those allocated to the healthy dietary pattern. This association was found in a cross-sectional and in a longitudinal way, highlighting the importance of a healthy dietary pattern since paediatric age.

MANUSCRIPT IV: In adolescents, the ideal cardiovascular health index could be useful as a tool in adolescents to assess those with an alterated inflammatory state. Furthermore, the use of new inflammatory markers, in combination with the traditional ones, could be useful from the clinical point of view.

MANUSCRIPT V: The body composition plays an important role in the relationship between insulin resistance and inflammation, measured with a set of inflammatory biomarkers. This association between insulin resistance and the

complement factor C3 was especially relevant for those adolescents with higher levels of body fat.

MANUSCRIPT VI: Those adolescents with an unfavourable metabolic profile, along with overweight/obesity, present an altered inflammatory state. Out of all inflammatory markers, complement factors C3 and C4 were associated with the metabolic health consistently.

CONCLUSIONES

ARTICULO I: En chicos, concentraciones elevadas de suma de omega 6 y LA en sangre total, se asociaron con menores concentraciones de PCR-hs; en chicas, concentraciones elevadas de AA, suma de ácidos grasos omega 6 altamente insaturados y la relación AA/LA se asociaron con mayores concentraciones de hs-PCR. Se necesitan más estudios para identificar los valores óptimos de ácidos grasos en sangre total que podrían evitar la inflamación en niños.

ARTICULO II: Aunque la frecuencia de consumo de diferentes alimentos se asocia con las concentraciones de PCR-hs, la frecuencia de consumo de vegetales mostró los resultados más consistentes. Es importante valorar los factores alimentarios que pueden tener más impacto en la aparición precoz de un estado inflamatorio alterado.

ARTICULO III: Un patrón dietético caracterizado por un consumo frecuente de azúcar y productos procesados, junto con un consumo infrecuente de verduras y frutas mantenido durante dos años de seguimiento, se asocia independientemente con la inflamación en niños europeos. La mejora de la calidad de la dieta en la infancia puede prevenir futuras enfermedades relacionadas con la inflamación crónica.

ARTICULO IV: El índice de salud cardiovascular ideal (ISCI) se asocia con un perfil inflamatorio bajo, ya desde la adolescencia. La mejora de todos los factores y comportamientos relacionados con la salud incluidos en el ISCI, puede jugar un papel importante en el control de la inflamación crónica.

ARTICULO V: En adolescentes, las concentraciones de marcadores del metabolismo glucídico e inflamatorio difieren por tertiles de composición

corporal y son superiores en el tercil superior de composición grasa corporal. El factor C3 del complemento se asoció con la resistencia a la insulina, especialmente en aquellos con elevados niveles de adiposidad total o abdominal. El control adecuado del exceso de grasa corporal y de las alteraciones metabólicas asociadas, entre las cuales es clave la resistencia a la insulina, podría contribuir a evitar la aparición de un estado inflamatorio alterado.

ARTICULO VI: Los adolescentes con un perfil metabólico más desfavorable, unido al sobrepreso/obesidad, presentan riesgo elevado de tener concentraciones superiores de PCR, C3 y C4. Los factores C3 y C4 del complemento se asociaron consistentemente con la salud cardio-metabólica. En la práctica clínica se debe, no solo identificar el exceso de grasa corporal, sino también los factores de riesgo cardio-metabólicos asociados.

CONCLUSIONS

MANUSCRIPT I: In boys, high concentrations of sum of omega-6 and LA, measured in whole blood, were associated with low hs-CRP concentrations; in girls, high concentrations of AA, sum of omega-6 highly unsaturated FA and the ratio AA/LA were associated with high hs-CRP concentration. More studies are needed to identify the optimal levels of WBFAs to avoid low-grade inflammation in children.

MANUSCRIPT II: Although the frequency of consumption of different food items showed associations with hs-CRP, frequency of vegetables consumption showed the most consistent results. It is important to assess the food factors that could have more impact in the early onset of an altered inflammatory state.

MANUSCRIPT III: A dietary pattern characterized by frequent consumption of sugar and processed food products, along with infrequent consumption of vegetables and fruits, maintained over two years of follow-up, was independently related with inflammation in European children. Efforts to improve the quality of the diet in childhood may prevent future diseases related with chronic inflammation.

MANUSCRIPT IV: The ICHI is associated with a lower inflammatory profile, already in adolescence. Improving these behaviours and factors related with health, included in the ICHI, could play an important role in CVD prevention.

MANUSCRIPT V: In adolescents, concentrations of the markers from glucose and inflammatory metabolisms differed by tertiles of body composition and they were higher in the highest tertile of body fat composition. C3 complement factor

was associated with insulin resistance, especially on those with high levels of total and abdominal adiposity. The adequate control of the excess of body fat and the metabolic related alterations, out of which the insulin resistance is key, could contribute to avoid the appearance of an altered metabolic state

MANUSCRIPT VI: Adolescents with an unfavourable metabolic profile, along with overweight/obesity, present a high risk of having high concentrations of CRP, C3 and C4. C3 and C4 were consistently associated with cardio-metabolic health. In clinical practice, it is necessary not only to identify the excess of body fat but the associated cardio-metabolic risk factors.

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Lista de abreviaturas

List of abbreviations

AA	Ácido Araquidónico
AHA	American Heart Association
ALA	Ácido alfa-linolénico
ANCOVA	Análisis de la covarianza
BMI	Body mass index
CEICA	Comité ético de investigaciones científicas de Aragón
CFCA	Cuestionario de frecuencia de consumo de alimentos
DHA	Ácido docosahexaenoico
ELISA	Enzyme-Linked ImmunoSorbent Assay
EPA	Ácido eicosapentaenoico
FAS	Family Affluence Scale
IDEFICS	Identification and prevention of dietary- and lifestyle- induced health effects in children and infants
IL	Interleucina
IMC	Índice de masa corporal
IPAQ-A	International Physical Activity Questionnaire- Adolescents
ISCED	International Standard Classification of Education (ISCED)
ISCI	Índice de salud cardiovascular ideal
HELENA	Healthy lifestyle in Europe by Nutrition in Adolescence-Cross-sectional Study
HOMA	Homeostasis Model Assesment

LA	Ácido linoleico (linoleic acid)
MSM	Multiple Source Method
NMS	Normo-peso metabólicamente sano
ObMNS	Obesidad metabólicamente no sana
ObMS	Obesidad metabólicamente sana
SObsMNS	Sobrepeso/Obeso metabólicamente no sano
SObsMS	Sobrepeso/Obeso metabólicamente sano
OR	Odds ratio
PCR-hs	Proteína C-reactiva de alta sensibilidad
sICAM-1	Soluble intercellular adhesion molecule-1
SPSS	Statistical Package for the Social Sciences
sVCAM-1	Soluble vascular cell adhesion molecule 1
T0	Medida basal de IDEFICS
T1	Medida de seguimiento de IDEFICS
TNF-α	Factor de necrosis tumoral-alfa

APÉNDICE

APPENDIX

Factor de impacto y cuartil de las revistas en "*ISI Web of Knowledge – Journal Citation Report (JCR)*" en su área correspondiente para el año correspondiente de publicación (en caso de publicación del JCR para el año en cuestión, en caso contrario, aparece el factor de impacto del año anterior).

(MODO TABLA de INDICE)

ARTICULO I: *Whole-blood fatty acids and inflammation in European Children: the IDEFICS study (2016).* **European Journal of Clinical Nutrition**. Impact Factor 2.935 (2015). Rank 31/80 (Nutrition & Dietetics)

ARTICULO II: *Food intake and inflammation in European children: the IDEFICS study (2016).* **European Journal of Nutrition.** Impact Factor 3.239 (2015). Rank 27/80 (Nutrition & Dietetics)

ARTICULO III: *Prospective associations between dietary patterns and high sensitivity C-Reactive protein in European children: The IDEFICS study (2017).* **European Journal of Nutrition.** Impact Factor 3.239 (2015). Rank 27/80 (Nutrition & Dietetics)

ARTICULO IV: *Ideal cardiovascular health and inflammation in European adolescents: the HELENA study (2016).* **Nutrition Metabolism and Cardiovascular Diseases**. Impact Factor 3.390 (2015). Rank 37/124 (Cardiac & Cardiovascular systems). Rank 22/80 (Nutrition & Dietetics). Rank 48/133 (Endocrinology & Metabolism)

ARTICULO V: *Inflammation and insulin resistance according to body composition in European adolescents: the HELENA study (2017).* **Nutrición Hospitalaria.** Impact Factor 1.497 (2015). Rank 60/80 (Nutrition & Dietetics).

ARTICULO VI: *Inflammation in metabolically healthy and metabolically abnormal European adolescents: the HELENA study (2017).* (Submitted to Pediatric Obesity).

Los artículos anteriormente mencionados se han realizado con datos de dos proyectos europeos:

- **Estudio IDEFICS (Identification and prevention of dietary- and lifestyle- induced health effects in children and infants).** Proyecto financiado por la Unión Europea: European Union Sixth RTD Framework Programme (contract FOOD-CT-2006-016181-2). Coordinador: Wolfgang Ahrens. www.idefics.eu

- **Estudio HELENA (Healthy lifestyle in Europe by Nutrition in Adolescence-Cross-sectional Study).** Proyecto financiado por la Unión Europea: European Union Sixth RTD Framework Programme (Contract FOOD-CT-2005-007034). Coordinador: Luis Alberto Moreno Aznar. www.helenastudy.com

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"Muchas manos y corazones y mentes generalmente contribuyen a los grandes logros de cualquier persona"

'Many hands and hearts and minds generally contribute to anyone's notable achievements'

Walt Disney

Ya no tengo más excusas para retrasar la escritura de este apartado, el cual simboliza el cierre de una etapa. Es imposible condensar, en unas pocas páginas, la gratitud que siento hacia todas aquellas personas que han compartido estos años conmigo. Sin la intención de caer en tópicos, creo firmemente que esta tesis no habría salido adelante sin la ayuda, el apoyo y la paciencia de quienes han vivido este proceso a mi lado. Todos vosotros habéis ayudado a escribir estas páginas.

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ANEXOS

ANNEX

Estudio IDEFICS (Identification and Prevention of Dietary- and Lifestyle- induced Health Effects in Children and Infants)

CUESTIONARIOS INCLUIDOS EN LOS ARTICULOS I-III

1. Preguntas relacionadas con la Actividad Física del Cuestionario de padres

48. ¿Es miembro su hijo/a de algún club deportivo?

❍ 1 Sí

❍ 2 No ➔➔➔ **Por favor, continúe con la pregunta 49**

¿Cuánto tiempo pasa a la semana haciendo ejercicio en el club deportivo?

|___|___| horas |___|___| minutos

¿Qué tipo de deporte practica su hijo/a en el club deportivo?
Por favor, marque la opción que corresponda.

❍ 1 fútbol

❍ 1 natación

❍ 1 tenis

❍ 1 gimnasia rítmica

❍ 1 Otra. Por favor, especificar:

_____

2. Pregunta relacionada con la clasificación internacional estándar de educación (ISCED, en sus siglas en inglés).

66. ¿Cuál es el nivel más alto de educación escolar que usted y su cónyuge/pareja tienen?
Por favor, marcar solamente uno por persona.

	Yo	*Cónyuge/pareja*
Primaria /EGB	○ 1	○ 1
Secundaria /ESO	○ 2	○ 2
Formación profesional	○ 3	○ 3
Ciclos formativos de grado superior	○ 4	○ 4
Bachillerato/ BUP/COU	○ 5	○ 5
Sin graduación (todavía)	○ 8	○ 8
Otros/desconocido	○ 9	○ 9

3. Preguntas relacionadas con la lactancia materna.

28. ¿Qué tipo de alimentación recibió su hijo/a antes de ser totalmente incluido en la dieta que se lleva normalmente en casa?
Por favor, marque todas las opciones que correspondan y anote la edad que tenía su hijo/a cuando recibió cada tipo de alimentación específico.

	Tipo de alimentación	*Edad de comienzo*	*Edad de finalización*
○ 1	Exclusivamente lactancia	____ mes	____ mes
○ 1	Lactancia combinada con otros tipos de alimentación	____ mes	____ mes
○ 1	Leche de fórmula	____ mes	____ mes
○ 1	Otros tipos de alimentación infantil – por favor, especifique: _____	____ mes	____ mes

4. Cuestionario de Frecuencia de consumo de alimentos

	Nunca/ menos de una vez por semana	1 - 3 veces por semana	4 – 6 veces por semana	1 vez al día	2 veces al día	3 veces al día	4 o más veces al día	No lo sé
Vegetales								
Verduras, patatas y legumbres cocinadas (también combinadas en el mismo plato)	○ 1	○ 2	○ 3	○ 4	○ 5	○ 6	○ 7	○ 8
Patatas fritas, croquetas de patata	○ 1	○ 2	○ 3	○ 4	○ 5	○ 6	○ 7	○ 8
Vegetales crudos (mezclados en la ensalada, zanahoria, pepino, lechuga, tomate, etc.)	○ 1	○ 2	○ 3	○ 4	○ 5	○ 6	○ 7	○ 8
Frutas								
Frutas frescas (también licuadas) ***sin*** azúcar añadido	○ 1	○ 2	○ 3	○ 4	○ 5	○ 6	○ 7	○ 8
Frutas frescas (también licuadas) ***con*** azúcar añadido	○ 1	○ 2	○ 3	○ 4	○ 5	○ 6	○ 7	○ 8
Bebidas								
Agua	○ 1	○ 2	○ 3	○ 4	○ 5	○ 6	○ 7	○ 8
Zumos de frutas (zumo de naranja, manzana, melocotón, piña,etc.)	○ 1	○ 2	○ 3	○ 4	○ 5	○ 6	○ 7	○ 8
Bebidas edulcoradas incluyendo bebidas deportivas, té en lata o embotellado, refrescos, etc.	○ 1	○ 2	○ 3	○ 4	○ 5	○ 6	○ 7	○ 8
Coca-cola light o bebidas refrescantes sin azúcar	○ 1	○ 2	○ 3	○ 4	○ 5	○ 6	○ 7	○ 8

	Nunca/ menos de una vez por semana	1 - 3 veces por semana	4 – 6 veces por semana	1 vez al día	2 veces al día	3 veces al día	4 o más veces al día	No lo sé
Cereales de desayuno								
Cereales de desayuno azucarados o que se les ha añadido azúcar y muesli azucarado (ej. Corn flakes, crispies, etc.)	❍ 1	❍ 2	❍ 3	❍ 4	❍ 5	❍ 6	❍ 7	❍ 8
Papillas, copos de avena, cereales no azucarados, muesli natural	❍ 1	❍ 2	❍ 3	❍ 4	❍ 5	❍ 6	❍ 7	❍ 8
Leche								
Leche no azucarada	❍ 1	❍ 2	❍ 3	❍ 4	❍ 5	❍ 6	❍ 7	❍ 8
Leche azucarada (ej. con azúcar, chocolate, cola-cao, miel, etc.)	❍ 1	❍ 2	❍ 3	❍ 4	❍ 5	❍ 6	❍ 7	❍ 8
Qué tipo de leche consume su hijo/a habitualmente:	❍ 1 Entera ❍ 2 Semi-desnatada /desnatada							
Yogur								
Yogur natural o kéfir sin azúcar	❍ 1	❍ 2	❍ 3	❍ 4	❍ 5	❍ 6	❍ 7	❍ 8
Yogur azucarado y bebidas lácteas fermentadas (ej. Actimel®, LC1®, etc.)	❍ 1	❍ 2	❍ 3	❍ 4	❍ 5	❍ 6	❍ 7	❍ 8
Qué tipo de yogur consume su hijo/a habitualmente:	❍ 1 Entera ❍ 2 Semi-desnatada /desnatada							

	Nunca/ menos de una vez por semana	1 - 3 veces por semana	4 – 6 veces por semana	1 vez al día	2 veces al día	3 veces al día	4 o más veces al día	No lo sé
Pescado								
Pescado fresco o congelado, sin freír	❍ 1	❍ 2	❍ 3	❍ 4	❍ 5	❍ 6	❍ 7	❍ 8
Pescado frito y varitas de pescado	❍ 1	❍ 2	❍ 3	❍ 4	❍ 5	❍ 6	❍ 7	❍ 8
Carne y productos cárnicos								
Productos en lonchas y conservados, o listos para cocinar (ej. fiambres, embutidos, jamón, hamburguesas etc.)	❍ 1	❍ 2	❍ 3	❍ 4	❍ 5	❍ 6	❍ 7	❍ 8
Carne fresca, sin freír (chuletas, bistec, bovino, cerdo, aves, etc.)	❍ 1	❍ 2	❍ 3	❍ 4	❍ 5	❍ 6	❍ 7	❍ 8
Carne frita (chuletas, bistec, bovino, cerdo, aves, etc.)	❍ 1	❍ 2	❍ 3	❍ 4	❍ 5	❍ 6	❍ 7	❍ 8
Huevos								
Huevos fritos o huevos revueltos	❍ 1	❍ 2	❍ 3	❍ 4	❍ 5	❍ 6	❍ 7	❍ 8
Huevos duros o escalfados	❍ 1	❍ 2	❍ 3	❍ 4	❍ 5	❍ 6	❍ 7	❍ 8
Mayonesa y productos derivados de la mayonesa (ej. Ligeresa, salsa rosa, etc.)	❍ 1	❍ 2	❍ 3	❍ 4	❍ 5	❍ 6	❍ 7	❍ 8
Productos sustitutivos de la carne y productos de soja								
Tofu, tempé, leche de soja, yogures de soja, etc.)	❍ 1	❍ 2	❍ 3	❍ 4	❍ 5	❍ 6	❍ 7	❍ 8

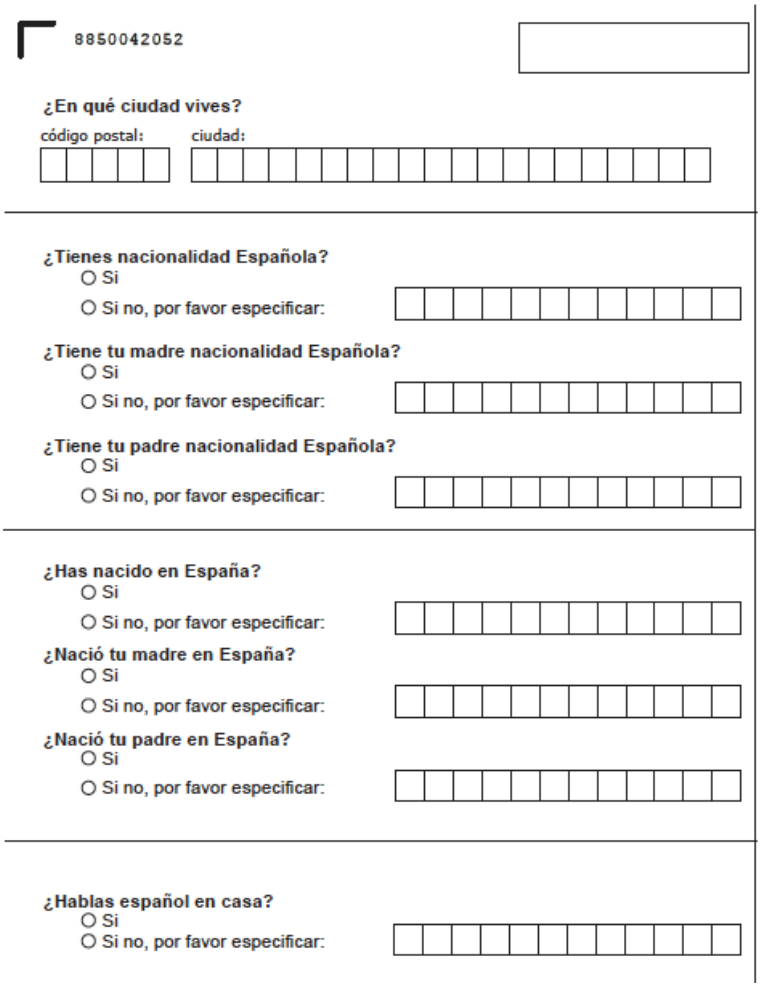
	Nunca/ menos de una vez por semana	1 - 3 veces por semana	4 – 6 veces por semana	1 vez al día	2 veces al día	3 veces al día	4 o más veces al día	No lo sé
Queso								
Queso (ej.curado, semicurado, tierno, fresco, tranchetes. etc.)	❍ 1	❍ 2	❍ 3	❍ 4	❍ 5	❍ 6	❍ 7	❍ 8
Queso para untar (ej. Philadelphia, etc)	❍ 1	❍ 2	❍ 3	❍ 4	❍ 5	❍ 6	❍ 7	❍ 8
Queso rallado	❍ 1	❍ 2	❍ 3	❍ 4	❍ 5	❍ 6	❍ 7	❍ 8
Productos para untar								
Mermelada, miel	❍ 1	❍ 2	❍ 3	❍ 4	❍ 5	❍ 6	❍ 7	❍ 8
Nocilla o crema de avellanas para untar	❍ 1	❍ 2	❍ 3	❍ 4	❍ 5	❍ 6	❍ 7	❍ 8
Mantequilla, margarina en pan	❍ 1	❍ 2	❍ 3	❍ 4	❍ 5	❍ 6	❍ 7	❍ 8
Productos bajos en grasa en pan (ej. Mermelada, etc.)	❍ 1	❍ 2	❍ 3	❍ 4	❍ 5	❍ 6	❍ 7	❍ 8
Ketchup	❍ 1	❍ 2	❍ 3	❍ 4	❍ 5	❍ 6	❍ 7	❍ 8
Productos hechos a base de cereales								
Pan blanco, panecillos blancos, biscotes blancos	❍ 1	❍ 2	❍ 3	❍ 4	❍ 5	❍ 6	❍ 7	❍ 8
Pan integral , panecillos integrales, biscotes integrales	❍ 1	❍ 2	❍ 3	❍ 4	❍ 5	❍ 6	❍ 7	❍ 8
Pasta, fideos, arroz	❍ 1	❍ 2	❍ 3	❍ 4	❍ 5	❍ 6	❍ 7	❍ 8
Cuscús, bulgur, etc.	❍ 1	❍ 2	❍ 3	❍ 4	❍ 5	❍ 6	❍ 7	❍ 8
Pizza como plato principal	❍ 1	❍ 2	❍ 3	❍ 4	❍ 5	❍ 6	❍ 7	❍ 8
Sandwiches (rellenos con queso, carne, vegetales, etc)	❍ 1	❍ 2	❍ 3	❍ 4	❍ 5	❍ 6	❍ 7	❍ 8

	Nunca/ menos de una vez por semana	1 - 3 veces por semana	4 – 6 veces por semana	1 vez al día	2 veces al día	3 veces al día	4 o más veces al día	No lo sé
Aperitivos								
Frutos secos y semillas y frutas secas (ej. Pipas, cacahuetes, pasas etc.)	❍ 1	❍ 2	❍ 3	❍ 4	❍ 5	❍ 6	❍ 7	❍ 8
Patatas fritas, aperitivos de maíz, palomitas de maíz, etc (ej. *Cheetos, Lay's, risketos*, etc.)	❍ 1	❍ 2	❍ 3	❍ 4	❍ 5	❍ 6	❍ 7	❍ 8
Tortas o bollos, pasteles (ej. Tarta de manzana, crepes, palmeras de hojaldre, etc.)	❍ 1	❍ 2	❍ 3	❍ 4	❍ 5	❍ 6	❍ 7	❍ 8
Chocolate, barritas de chocolate (Mars, Lions, Kit Kat, etc.)	❍ 1	❍ 2	❍ 3	❍ 4	❍ 5	❍ 6	❍ 7	❍ 8
Caramelos, chucherías, gominolas, etc.)	❍ 1	❍ 2	❍ 3	❍ 4	❍ 5	❍ 6	❍ 7	❍ 8
galletas, pasteles envasados, tartas (ej.Donuts, bollycao, cañas de chocolate, etc.)	❍ 1	❍ 2	❍ 3	❍ 4	❍ 5	❍ 6	❍ 7	❍ 8
Helados, polos, sorbetes de fruta(ej. Mágnum, calippo etc.)	❍ 1	❍ 2	❍ 3	❍ 4	❍ 5	❍ 6	❍ 7	❍ 8

Estudio HELENA (Healthy Lifestyle in Europe by Nutrition in Adolescence)

CUESTIONARIOS INCLUIDOS EN LOS ARTICULOS IV-VI

1. Cuestionario de nivel socioeconómico



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¿En qué ciudad vives?

código postal: ciudad:

¿Tienes nacionalidad Española?
O Si
O Si no, por favor especificar:

¿Tiene tu madre nacionalidad Española?
O Si
O Si no, por favor especificar:

¿Tiene tu padre nacionalidad Española?
O Si
O Si no, por favor especificar:

¿Has nacido en España?
O Si
O Si no, por favor especificar:

¿Nació tu madre en España?
O Si
O Si no, por favor especificar:

¿Nació tu padre en España?
O Si
O Si no, por favor especificar:

¿Hablas español en casa?
O Si
O Si no, por favor especificar:

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¿Has fumado tabaco alguna vez?

- ○ Sí
- ○ No

¿Con qué frecuencia fumas tabaco actualmente?

- ○ Cada día
- ○ Al menos una vez a la semana, pero no cada día
- ○ Menos de una vez a la semana
- ○ No fumo

¿Cuántos cigarros fumas por semana?

- ○ Ninguno
- ○ Menos de 5
- ○ Entre 5 y 10
- ○ Entre 11 y 20
- ○ Más de 20

¿Cuánto mides descalzo? 1 m ☐☐ cm

¿Cuánto pesas sin ropa? ☐☐☐,☐

Por favor, indicanos la afirmación más apropiada para tu madre:

- ○ tiene sobrepeso/obesidad
- ○ tiene peso normal
- ○ esta delgada/muy delgada
- ○ no lo sé

Por favor, indicanos la afirmación más apropiada para tu padre:

- ○ tiene sobrepeso/obesidad
- ○ tiene peso normal
- ○ esta delgado/muy delgado
- ○ no lo sé

El término familia se refiere a miembros viviendo juntos en la misma casa: padre, madre, hermanos
Para aquellos que vivan en dos familias, contesta acerca de la familia con la que vivas la mayor parte del tiempo.

¿Con quien vives la mayoría del tiempo?

- ○ con tus dos padres
- ○ con tu madre sóla
- ○ con tu madre y su nuevo compañero
- ○ con tu padre sólo
- ○ con tu padre y su nueva compañera
- ○ con tu madre la mitad del tiempo y tu padre la otra mitad
- ○ con tus abuelos
- ○ con padres adoptivos
- ○ en un centro de acogida
- ○ algún otro lugar

¿Cuantos de tus hermanas y/o hermanos incluyendo hermanastras y/o hermanastros viven en casa (excluyéndote a ti)?

○ 0 ○ 1 ○ 2 ○ 3 ○ 4 ○ más de 4

¿Dispones de tu propia habitación para ti sólo?

○ no ○ si

¿Cuantos coches posee tu familia? (por "familia" queremos decir miembros que viven juntos: padre, madre y hermanos)

○ 0 ○ 1 ○ 2 ○ más de 2

¿Tienes conexión a internet en casa?

○ no ○ si

¿Cómo le va a tu familia económicamente?

- ○ Fantásticamente bien
- ○ Muy bien
- ○ Normal
- ○ No muy bien
- ○ Mal

8965042059

Las siguientes preguntas son respecto a tu madre y a tu padre.
Si tienes madre y madrastra o padre y padrastro, contesta en relación a la persona más importante en tu educación.

Por favor indicar el nivel educativo más alto de tu madre y tu padre :

	PADRE	MADRE
educación elemental	○	○
terminó la ESO (BUP)	○	○
terminó Bachiller (COU)	○	○
termino educación superior (universitaria)	○	○

¿Cuál es la ocupación de tu padre ?

- ○ trabaja jornada completa
- ○ trabaja media jornada
- ○ amo de casa
- ○ retirado o enfermo
- ○ aprendiz/estudiante
- ○ en paro
- ○ temporalmente sin trabajar (ej: baja paternal)
- ○ nunca le veo
- ○ falleció
- ○ no lo sé

¿Cuál es la ocupación de tu madre ?

- ○ trabaja jornada completa
- ○ trabaja media jornada
- ○ ama de casa
- ○ retirada o enferma
- ○ aprendiz/estudiante
- ○ en paro
- ○ temporalmente sin trabajar (ej: baja maternal)
- ○ nunca la veo
- ○ falleció
- ○ no lo sé

Por favor, anota el tipo de trabajo que tienen tus padres. Si tienen más de un trabajo, indícalos

	PADRE	MADRE
1. Personal administrativo Presidente, Director de administración pública, Consejo de Administración (Jefe de Departamento o equivalente)	○	○
2. profesiones intelectuales y científicas Personal cualificado (matemático o especialista en ciencias y salud, especialistas técnicos :arquitectos, ingenieros, informáticos, biólogos, famrmacéuticos, médicos, abogados, profesor universitario, psicólogo, sociólogo, etc.)	○	○
3 Profesiones intermedias: Técnicos o peritos y otros trabajos intermedios (electricista, mecánico, enfermero/a, dietista, empleado de oficina, maestro, representante comercial y profesionales asociados)	○	○
4. Administración/Oficinas Banca, contabilidad, seguros, bibliotecarios, etc.	○	○
5. Empresas de negocios Ventas, marketing, publicidad, comunicaciones, etc.	○	○
6. Trabajadores cualificados de agricultura y pesca Granjeros, pescadores, guardabosques, etc.	○	○
7. Artesanos, manufactura y oficios relacionados Peluquero/a, mecánico, operario, artesano, mecánico, operario en industria textil, calzado, etc.	○	○
8. Operarios de maquinaria y montadores Trabajadores industriales y operarios de máquinas, conductores de grúas, etc.	○	○
9. Trabajos y ocupaciones elementales vendedores, empleados del hogar, albañiles, vigilantes de seguridad, limpiadores, etc.	○	○
10. fuerzas armadas	○	○
11. otro nombre (descríbelo detalladamente)	○	○
12. no trabaja	○	○

2. Pregunta de actividad física del cuestionario IPAQ-Q.

Parte 4: ACTIVIDAD FÍSICA DURANTE EL TIEMPO DE OCIO, DEPORTE Y TIEMPO LIBRE

Esta sección es sobre toda la actividad física que has hecho en los últimos 7 días, únicamente respecto al tiempo de ocio, de práctica deportiva, entrenamiento o placer. Por favor, no incluyas actividades que ya has mencionado.

Durante los últimos 7 días, cuántos hiciste una de las siguientes actividades por al menos 10 minutos sin parar, durante tu tiempo libre…

No incluyas actividades que duraran menos de 10 minutos ininterrumpidos.

… **CAMINAR**

❍ nada | ❍ 1 día | ❍ 2 días | ❍ 3 días | ❍ 4 días | ❍ 5 días | ❍ 6 días | ❍ 7 días

¿Cuánto tiempo empleas normalmente uno de esos días en **caminar** en tu tiempo libre?
___horas ___minutos por día

… **Actividad física VIGOROSA, que conlleva un gran esfuerzo físico y te hace respirar mucho más fuerte de lo normal,** como ejercicio aeróbico, correr, andar en bici o nadar rápido…

❍ nada | ❍ 1 día | ❍ 2 días | ❍ 3 días | ❍ 4 días | ❍ 5 días | ❍ 6 días | ❍ 7 días

¿Cuánto tiempo empleas normalmente uno de esos días en **actividad física vigorosa** en tu tiempo libre?
___horas ___minutos por día

… **Actividad física MODERADA, que conlleva un moderado esfuerzo físico y te hace respirar un poco más fuerte de lo normal,** como bailar, nadar o montar en bicicleta despacio …

❍ nada | ❍ 1 día | ❍ 2 días | ❍ 3 días | ❍ 4 días | ❍ 5 días | ❍ 6 días | ❍ 7 días

¿Cuánto tiempo empleas normalmente uno de esos días en **actividad física moderada** en tu tiempo libre?
___horas ___minutos por día