Erythrocyte membrane lipidomics as a molecular tool for precision nutrition on children with obesity

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"La ciencia es una forma de pensar, mucho más que un conjunto de conocimientos"

Carl Sagan

AGRADECIMIENTOS

En primer lugar, quisiera agradecer a Azti la oportunidad que me ha brindado para desarrollar mi tesis doctoral durante estos 4 años., en especial, al área de Alimentación y Salud, que siempre ha estado ahí para ofrecer consejo, apoyo y guía durante este periplo. A la universidad, EHU/UPV, por poner a mi alcance todos los medios necesarios para desarrollar mi tesis y al Gobierno Vasco por financiar económicamente este proceso.

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SUMMARY

Obesity is defined as a pathological condition characterized by an abnormal or excessive accumulation of fat, to the extent that it is considered a risk factor or marker for several chronic diseases.

The criterion established to define the obese population has been a BMI $\geq$30 kg/m^2 for adults, and a BMI at or above the 95th percentile for children, although recent research points to a greater need to establish other types of markers that describe more specifically the metabolism of obesity, and its relationship with the development of other diseases such as hypertension, type II diabetes, cardiovascular diseases, or the increased probability of suffering from some types of cancer.

In this sense, molecular analysis tools such as metabolomics, genomics or the study of the intestinal microbiome allow us to advance in our knowledge of human metabolic behavior, and favor the development of new diagnostic methods, both for obesity and for certain associated diseases.

Lipids play a decisive role in obesity due to both their structural and molecular signaling functions. Although the fatty acid (FA) composition of each tissue is specific, the mature red blood cell (RBC) is considered a good reporter of the metabolic state of different organs, tissues and cells, as it is a circulating cell and its membrane reflects an overall picture of the metabolism of each individual.

For this reason, defining the mature RBC membrane FA profile will allow us to know the metabolic and nutritional status of the individuals studied, which is very useful in the molecular characterization of obesity.

Taking these considerations into account, the aim of this thesis is to define the RBC membrane FA profile that characterizes obese children, and to establish its relationship with metabolism and dietary habits, in order to be able to design precise

nutritional strategies. To this end, a study was carried out in a population of normal-weight, overweight and obese children.

In turn, a study was carried out to establish the respective differences in RBC membrane FA profile between adults and children with obesity, in order to be able to establish specific and personalized nutritional recommendations according to the differences observed by age, and derived from the metabolism of obesity itself.

Statistical clustering techniques were also used to isolate a subgroup of obese children with a similar lipid profile to normal-weight children. Analyzing the RBC membrane FA profile as a biomarker of inflammation allows us to generate knowledge that contributes to the molecular characterization of the so-called metabolically healthy obese. At the same time, the absence of an inflammatory profile in this group requires different nutritional recommendations tailored to their metabolic needs for effective interventions.

To re-establish the optimal composition of the RBC membrane FA profile, an adequate nutritional strategy must be established, comprised not only of an optimal diet adapted to specific metabolic needs, but in many cases, it must also be accompanied by the use of ω-3 supplements, as these FAs have anti-inflammatory properties and counteract the effects of excess ω-6 FAs observed in obese children.

From a precision nutrition point of view, knowing the RBC membrane FA profile of obese children, together with the integration of other molecular parameters, dietary habits, preferences and eating behavior, is of great interest to understand their relationship with the obesity metabolism, and to propose future nutritional intervention strategies, which may be effective in the long term, and reverse the increase in prevalence of obesity.

RESUMEN

La obesidad se define como una condición patológica caracterizada por una acumulación anormal o excesiva de grasa, hasta el punto en el que es considerado un factor de riesgo o marcador de varias enfermedades crónicas.

El criterio establecido para la definición de la población obesa ha sido el IMC≥30 kg/m2 para adultos y el IMC igual o mayor del percentil 95 para población infantil, aunque existe cierta controversia con respecto al uso de tablas específicas por edad y sexo para definir la obesidad en los niños. Para homogeneizar la definición de obesidad infantil, se utilizan algunas tablas internacionales, como el International Obesity Task Force o la Organización Mundial de la Salud (OMS). Estas tablas permiten comparar los diferentes estudios científicos realizados en diferentes países y permiten monitorear el desarrollo de la enfermedad en todo el mundo.

Por otro lado, las tablas de crecimiento también se utilizan a nivel nacional, ya que las tablas internacionales son menos representativas de la población local que los estándares aplicados. utilizando la metodología correcta en la población de referencia. Estas tablas internacionales se realizan mezclando poblaciones muy diversas, y tergiversan lo que es apropiado para las especificidades locales. Esto conduce a una clasificación errónea del estado de crecimiento de los niños y, por lo tanto, a una identificación errónea de los niños con sobrepeso u obesidad.

Según la OMS, la obesidad en todo el mundo casi se ha triplicado desde 1975, lo que indica que, en 2016, el 39% de los adultos mayores de 18 años tenía sobrepeso y el 13% padecía obesidad, lo que resultó en 1.900 millones de adultos con sobrepeso y 650 millones de adultos obesos en 2016. Además de afectar a la población adulta, la obesidad se está convirtiendo en un problema que afecta cada vez más a niños y adolescentes. Como afirma la OMS, más de 340 millones de niños y adolescentes,

alrededor de uno de cada tres de 5 a 19 años, tenían sobrepeso u obesidad en 2016, y 38 millones de niños menores de 5 años tenían sobrepeso u obesidad en 2019. Los estudios más recientes apuntan que, en España, la prevalencia de sobrepeso se sitúa alrededor del 34% con una tasa de prevalencia de obesidad del 10% para población infantil y adolescente.

Recientes investigaciones, apuntan a una mayor necesidad de establecer otro tipo de marcadores que describan de una forma más específica el metabolismo de la obesidad y su relación con el desarrollo de otras enfermedades como la hipertensión, la diabetes tipo II, las enfermedades cardiovasculares o el aumento de la probabilidad de sufrir algunos tipos de cáncer.

Esta relación con las enfermedades citadas, provoca que la obesidad sea la causa de 4.7 millones de muertes prematuras en el mundo en 2017, representando el 8% de las muertes totales mundiales. Además, se encontró que las personas obesas, tenían costos médicos que eran aproximadamente un 30% mayores que las personas con peso normal.

En este sentido, las herramientas moleculares de análisis como la metabolómica, la genómica o el estudio del microbioma intestinal, nos permiten avanzar en un mayor conocimiento del comportamiento metabólico humano y favorecen el planteamiento de nuevos métodos de diagnóstico, tanto de la obesidad, como de ciertas enfermedades asociadas.

Los lípidos tienen un papel determinante en la obesidad debido tanto a sus funciones estructurales, como de señalización molecular. Aunque la composición de ácidos grasos (AG) de cada tejido es específica, el eritrocito maduro es considerado un buen reportero del estado metabólico de diferentes órganos, tejidos y células, ya que es una célula circulante y su membrana refleja una imagen general del metabolismo de cada individuo. Al mismo tiempo, es una muestra de fácil obtención y presenta un ciclo de vida de aproximadamente 120 días, lo que permite recopilar

información sobre los cambios de composición de la membrana que se producen en todo el organismo, a partir de los 4 meses anteriores.

Por este motivo, definir el perfil lipídico de membrana de eritrocito maduro (PLME), permitirá conocer el estado metabólico y nutricional de los individuos estudiados, siendo de gran utilidad en la caracterización molecular de la obesidad.

Teniendo en cuenta estas consideraciones, el objetivo del presente trabajo de tesis consiste en definir el PLME que caracteriza a la población infantil obesa y establecer su relación con el metabolismo y los hábitos dietéticos, para poder diseñar estrategias nutricionales de precisión. Para ello, se realizó un estudio en población infantil entre 6 y 16 años con normopeso, sobrepeso y obesidad.

Se analizó la composición del PLM mediante cromatografía de gases con detector de ionización de llama (GC-FID). Los hábitos alimentarios se evaluaron mediante cuestionarios validados de frecuencia de consumo (FFQ) y el test para la medición de la adherencia a la dieta mediterránea para niños (KIDMED).

En comparación con los niños normopeso, los niños con obesidad mostraron un perfil inflamatorio en los AG de los eritrocitos maduros, evidenciado por niveles más altos de ácidos grasos poliinsaturados ω-6 (principalmente ácido araquidónico). Los niños con sobrepeso u obesidad presentaron niveles más bajos de AG monoinsaturados en comparación con los niños con normopeso, lo que resultó en un aumento de la proporción de ácidos grasos saturados/ monoinstaturados. A su vez, destacar que se observó una menor ingesta de frutos secos en los niños con obesidad.

A su vez, se llevó a cabo un estudio para establecer las diferencias respectivas al PLME entre adultos y niños con obesidad con el fin de poder establecer recomendaciones nutricionales específicas y personalizadas según las diferencias observadas por edad y derivadas del propio metabolismo de la obesidad.

Los niños con obesidad presentaron niveles más altos de ácidos grasos poliinsaturados ω-6 (principalmente ácido linoleico) y valores más bajos de ácidos grasos ω-3 (principalmente DHA) en comparación con los adultos. En cuanto a los parámetros bioquímicos, los niños con obesidad presentaron niveles más bajos de glucosa, colesterol LDL y alanina aminotransferasa en comparación con los adultos con obesidad. Estas diferencias deberán considerarse para proporcionar recomendaciones nutricionales específicas para diferentes grupos de edad, basadas en una ingesta adecuada de grasas.

También se utilizaron técnicas estadísticas de clusterización para aislar un subgrupo de población infantil obesa que presenta un PLME similar a los normopeso. El grupo obeso metabólicamente sano (MHO) mostró niveles de AG similares a los niños normopeso, caracterizados por valores más bajos de ácido araquidónico, ω-6 total, la ratio ω6/ω3 y valores más altos para EPA, DHA y ω-3 total en comparación con el resto de los niños con obesidad. El análisis del PLME como biomarcador de inflamación, permite generar conocimiento que contribuye a la caracterización molecular de los llamados obesos metabólicamente sanos. Al mismo tiempo, la ausencia de un perfil inflamatorio en este grupo requiere de unas recomendaciones nutricionales diferentes y adaptadas a sus necesidades metabólicas para llevar a cabo intervenciones efectivas.

Para reestablecer la composición óptima del PLME, es necesario establecer una adecuada estrategia nutricional compuesta no solo por una dieta óptima y adaptada a las necesidades metabólicas específicas, sino que, en muchos casos, debe acompañarse del uso de suplementos de ω-3, ya que estos AG presentan propiedades antiinflamatorias y contrarrestan los efectos derivados de un exceso de AG ω-6 observados en los niños con obesidad.

Sin embargo, en la situación actual, en la que las nuevas tendencias conducen a recomendaciones nutricionales y de salud individualizadas, el uso de tecnologías

ómicas para una suplementación más precisa y personalizada, en cuanto a dosis o diferentes suplementos de ácidos grasos, parece ser crucial para lograr los beneficios de salud. En este sentido, la aplicación de la lipidómica de la membrana celular brinda una opción válida para comprender los cambios estructurales y funcionales en la composición de ácidos grasos tanto en estados normales como patológicos. También proporciona una medida de las necesidades específicas de AG de cada individuo en función de sus niveles metabólicos basales, así como de los diferentes cambios metabólicos que se producen con la suplementación.

Además de determinar el perfil lipidómico de un individuo, también es necesario generar nuevos conocimientos a nivel molecular, integrando diferentes herramientas ómicas, con el fin de diseñar estrategias nutricionales precisas, monitorear el efecto de la intervención nutricional, o como una nueva forma de definir nuevos biomarcadores tempranos de la enfermedad estudiada.

En el campo cada vez más importante de la nutrición de precisión, el objetivo final es el de diseñar recomendaciones nutricionales personalizadas para tratar o prevenir los trastornos metabólicos. Más específicamente, la nutrición de precisión busca desarrollar pautas nutricionales únicas para cada individuo, combinando factores genéticos, ambientales y de estilo de vida para desarrollar enfoques efectivos. Para ello, los enfoques de nutrición de precisión incluyen diferentes ciencias ómicas como la genomica, la metabolómica o la microbiómica junto con otros factores como los hábitos alimentarios, el comportamiento alimentario y la actividad física.

Desde un punto de vista de la nutrición de precisión, conocer el PLME de la población infantil obesa, junto con la integración de otros parámetros moleculares, hábitos dietéticos, preferencias y conducta alimentaria, es de gran interés para comprender su relación con el metabolismo de la obesidad y plantear futuras estrategias de intervención nutricional, que puedan ser efectivas a largo plazo, y revertir el aumento de la prevalencia de la obesidad.

Por tanto, se espera que las investigaciones futuras basadas en la lipidómica tengan un impacto a nivel socioeconómico, ya que se pueden desarrollar nuevos productos (kits) con fines de diagnóstico, pronóstico e intervención, así como nuevos alimentos y suplementos para su uso en niños con obesidad.

Abbreviations

Δ5D	Delta-5-desaturase
Δ6D	Delta-6-desaturase
Δ9D	Delta-9 desaturase
AA	Arachidonic Acid
AACE	American Association of Clinical Endocrinologists
ALA	alpha- linolenic acid
AMA	American Medical Association
ANCOVA	Analysis of Covariance
BMI	Body Mass Index
C14:0	Myristic Acid
C16:0	Palmitic Acid
C16:1; 9c	Palmitoleic Acid
C18:0	Stearic Acid
C18:1; 11c	cis-vaccenic Acid
CDC	Centers for Disease Control and Prevention
CE	Elongase enzyme
COX	Cyclooxygenase
CVD	Cardiovascular diseases
DGLA	Dihomo-gamma-linolenic acid
DHA	Docosahexaenoic acid
DNL	De novo lipogenesis
DPA	Docosapentaenoic acid
EDTA	Ethylenediaminetetraacetic acid
EFA	Essential fatty acid
EFSA	European Food Safety Authority
ELO	Elongase
EPA	Eicosapentaenoic acid
FA	Fatty Acid
FAME	Fatty Acid Methyl Ester
FAS	Fatty Acid Synthase
FFQ	Food Frequency Questionnaire
FID	Flame Ionization Detector
GC	Gas Chromatography
IOTF	International Obesity Task Force
KMO	Kaiser-Meyer-Olkin
KOH	Potassium Hydroxide
LA	Linoleic Acid
MeOH	Methyl Alcohol
MHO	Metabolically healthy obesity
MS	Mass spectrometry
MONW	Metabolically obese normal weight
MUFA	Monousaturated Fatty acid
NAFLD	Non-alcoholic fatty liver disease
OA	Oleic Acid
PCA	Principal Component Analysis

PI	Peroxidation Index
PUFA	Polyunsaturated Fatty Acid
RBC	Red Blood Cell
SCD1	stearoyl-CoA desaturase-1
SD	Standard Deviation
SE	Standard Error
SEEDO	Spanish Society of Obesity
SFA	Saturated Fatty Acid
SI	Saturation Index
SNP	Single nucleotide polymorphisms
TFA	*Trans* Fatty Acid
Trans 18:1	Elaidic Acid
Trans 20:4	*Trans* Arachidonic Acid
UI	Unsaturation Index
WHO	World Health Organization
ω-3	Omega-3
ω-6	Omega-6

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1.Introduction

1.1. Obesity

Obesity is a condition in which fat accumulates in the body to a point where it is a risk factor or marker for a number of chronic diseases, including diabetes, cardiovascular diseases (CVDs) or cancer. Furthermore, it has adverse effects on overall health (2, 3). The pathophysiology of obesity is well understood. It occurs when the energy intake is greater than the energy expenditure and requirements. A more clinically-orientated definition describes obesity as an inflammation of body fat mass affecting health. As such, obesity can be interpreted as a failure of the body systems to use external and/or internal input in order to regulate energy reserves (4).

Obesity has, for a long time, been characterized only as a major and modifiable risk factor for premature mortality, morbidity, and disability (5-8). However, in recent times, several organizations, such as the American Association of Clinical Endocrinologists (AACE) or the American Medical Association (AMA), have officially declared that obesity is a disease *per se* and requires treatment (9).

Obesity is currently considered a global epidemic, and its implications in childhood and adulthood are increasingly important as it has been found to decrease health-related quality of life and overall life expectancy.

Body mass index (BMI) is a statistical index using a person's weight and height to provide an estimate of body fat in males and females. It is calculated as weight in kilograms (kg) divided by height in meters squared to define a person as underweight, normal weight, overweight, or obese. Overweight is defined as BMI of 25 to <30 kg/m² and obesity as BMI of >30 kg/m² (10).

In the pediatric population, BMI percentile charts are taken as reference to define the different categories, using an age- and sex-specific pediatric z-score table, normal weight being defined when the standard deviation (SD) of BMI is -1 <SD ≤ +1, overweight when it is +1<SD ≤ +2 and obesity when it is > +2. This means that a BMI at or above the 95th percentile for children is classified as obesity. There is some

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controversy regarding the use of age- and sex-specific tables for defining obesity in children (11). In order to homogenize the definition of childhood obesity, some international tables, such as the International Obesity Task Force (IOTF) (12), Centers for Disease Control and Prevention (CDC) (13) and the World Health Organization (WHO) are used (14, 15). These tables enable the comparison of the different scientific studies carried out in different countries and permit monitoring the development of the disease worldwide.

On the other hand, growth charts are also used at national level (e.g., Spain, France, Germany, Italy) (16, 17), as the international tables (IOTF and WHO) are less representative of the local population than the standards applied using the correct methodology in the reference population. These international tables are carried out by mixing very diverse populations (18), and they misrepresent what is appropriate for local specificities. This leads to a misclassification of children's growth status and thus, a misidentification of children with overweight or obesity (11).

Moreover, national growth charts (19, 20) have been used more than international tables (21) to study the evidence of the screening ability and relationship to morbidity, to classify childhood obesity. This fact supports the use of national tables for national studies.

For all these reasons, in this work, age- and sex-specific tables of the Spanish population will be used, since they are more representative to define obesity and overweight in the population studied (22, 23).

1.2. Obesity in numbers

According to the World Health Organization (WHO), worldwide obesity has nearly tripled since 1975, indicating that, in 2016, 39% of adults aged 18 years and over were overweight, and 13% suffered from obesity, resulting in 1.9 billion overweight adults and 650 million obese adults in 2016 (Figure 1) (24).

Share of adults that are overweight or obese, 2016

Being overweight is defined as having a body-mass index (BMI) greater than or equal to 25. Obesity is defined by a BMI greater than or equal to 30. BMI is a person's weight in kilograms divided by his or her height in metres squared.

No data 0%   10%   20%   30%   40%   50%   60%   70%   >80%

Source: WHO, Global Health Observatory

OurWorldInData.org/obesity • CC BY
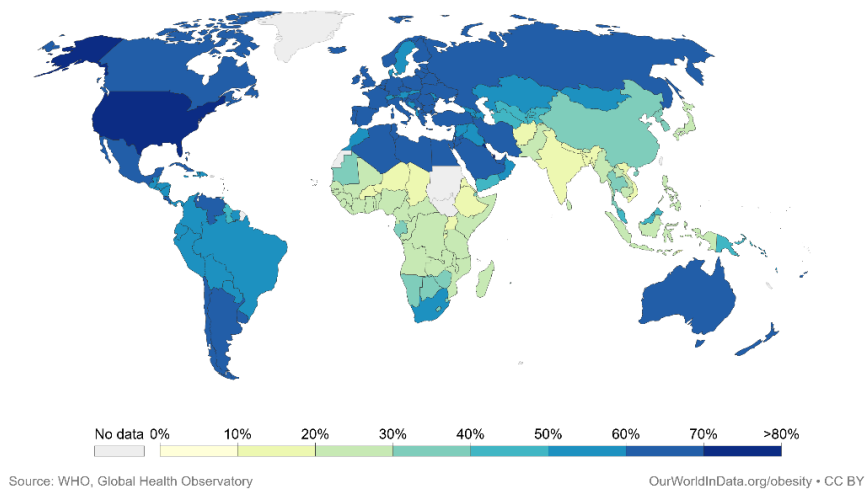
Figure 1: Prevalence of overweight and obesity among adults. Overweight defined as BMI ≥25 and obesity as BMI ≥30. (From Hannah Ritchie (2017)-"Obesity". Published online at OurWorldInData.org. Retrieved from: 'https://ourworldindata.org/obesity' [Online Resource])

In addition to affecting the adult population, obesity is becoming a problem that increasingly affects children and adolescents, too. As the WHO states, more than 340 million children and adolescents, around one in three from 5 to 19 years, were overweight or obese in 2016, and 38 million children under the age of 5 were overweight or obese in 2019 (24).
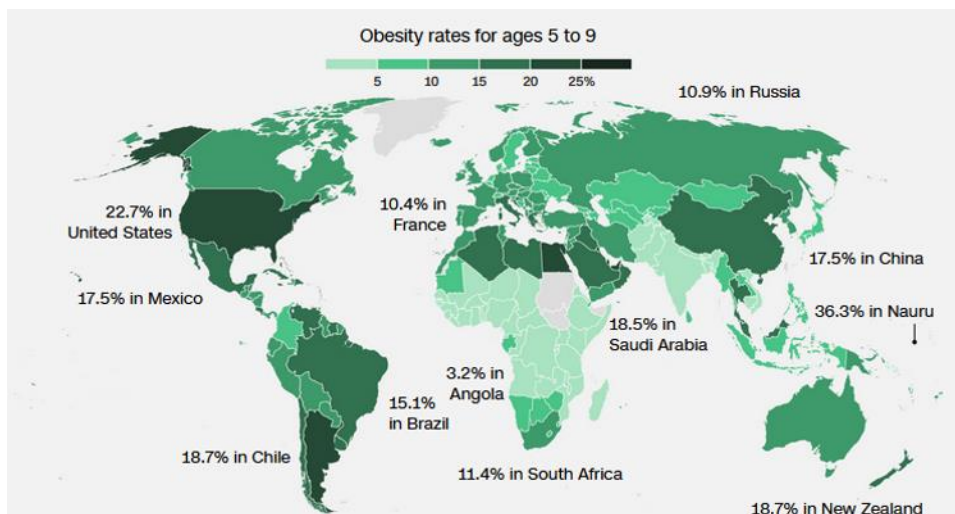
Figure 2: Prevalence of obesity among children between 5 and 9 years old, calculated as BMI > +2 standard deviations above the median. Data from 2016. Source: WHO.

In a study on the prevalence of obesity among adults and children in the United States, carried out by the CDC, an upward trend in obesity can be observed for both population groups in the 21st century (Figure 3) (25).
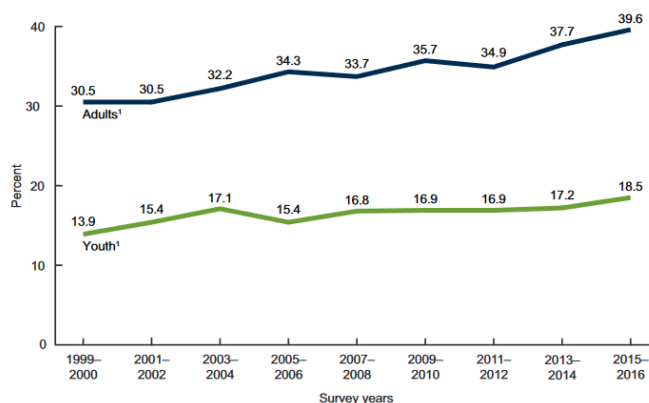
Figure 3: Trends in obesity prevalence among adults aged 20 and over (age adjusted) and youth aged 2–19 years: United States(25). SOURCE: NCHS, National Health and Nutrition Examination Survey, 1999–2016.

The Global Burden of Disease is a major global study on the causes and risk factors for death and disease published in the medical journal *"The Lancet"* (26). Obesity is a risk factor for several of the world's leading causes of death, including

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heart disease, stroke, type II diabetes (27, 28) and various types of cancer (29). Obesity is not the direct cause of any of these health impacts, but it can increase the likelihood of them occurring.

According to the Global Burden of Disease study, 4.7 million people died prematurely in 2017 because of obesity (Figure 4). To put this into context: this was close to four times the number that died in road accidents, and close to five times the number that died from HIV/AIDS in 2017.

Risk Factor	Deaths
High blood pressure	10.44 million
Smoking	7.1 million
High blood sugar	6.53 million
Air pollution (outdoor & indoor)	4.9 million
Obesity	4.72 million
Outdoor air pollution	3.41 million
Diet high in sodium	3.2 million
Diet low in whole grains	3.07 million
Alcohol use	2.84 million
Diet low in fruits	2.42 million
Diet low in nuts and seeds	2.06 million
Indoor air pollution	1.64 million
Diet low in vegetables	1.46 million
Diet low in seafood omega-3 fatty acids	1.44 million
Low physical activity	1.26 million
Unsafe water source	1.23 million
Secondhand smoke	1.22 million
Low birth weight	1.1 million
Child wasting	1.08 million
Unsafe sex	1.03 million
Diet low in fiber	873,408
Poor sanitation	774,241
No access to handwashing facility	707,248
Drug use	585,348
Diet low in legumes	534,767
Low bone mineral density	327,314
Vitamin-A deficiency	232,777
Child stunting	220,678
Diet low in calcium	184,760
Non-exclusive breastfeeding	160,983
Iron deficiency	59,882
Zinc deficiency	28,595
Diet high in red meat	24,833
Discontinued breastfeeding	10,012

Figure 4: Number of deaths by Risk Factor worldwide, across all age groups and both sexes. Source: IHME. Global Burden of Disease (GBD)

Globally, 8% of the deaths in 2017 were the result of obesity, representing an increase from 4.5% in 1990.

More specifically, in Europe, although the prevalence of obesity in general has increased over the past 10 years, considerable variations - up to 10% - can be observed between countries (30). Recent data show that this variation goes from 18–19% of the population in low prevalence obesity level countries to high prevalence levels of 28–30% (Figure 5).

Figure 5: Trend of obesity prevalence of adults in Europe. Source: WHO.

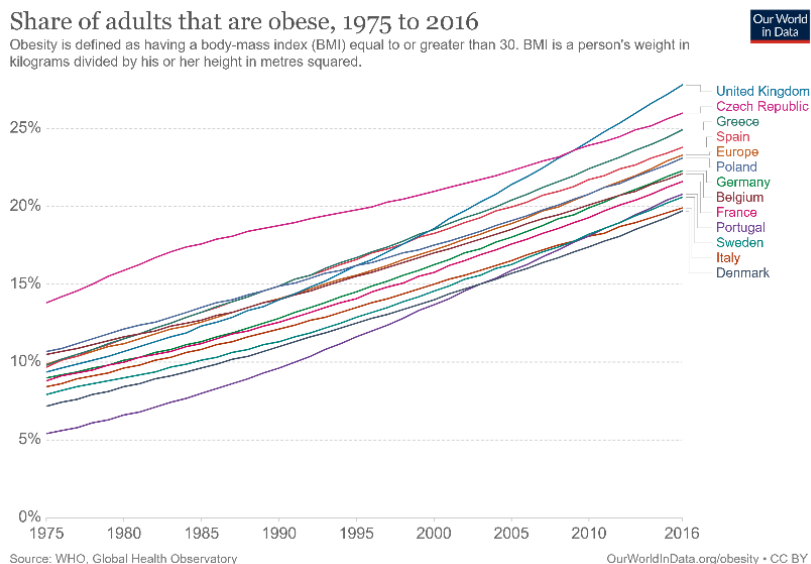This obesity pandemic situation is accompanied by higher public spending by governments for diseases associated with obesity. More specifically, obesity was estimated to account for between 0.7% and 2.8% of a country's total healthcare expenditures worldwide(31).

In Spain, different studies have evaluated the prevalence of obesity in adults and children in the last 20 years. According to the Spanish Society of Obesity (SEEDO 2003), the percentage of obese people in the Spanish population was 14.5% (32). The ENRICA and Di@betes studies estimated prevalence of obesity, in the period between 2008 and 2010, of 22.9% and 28.2%, respectively (33). The most recent data correspond to the period 2014-2015 and are provided by the ENPE study: 21.6% among adults between 25 and 64 years of age (22.8% among men and 20.5% among women) (Figure 6).
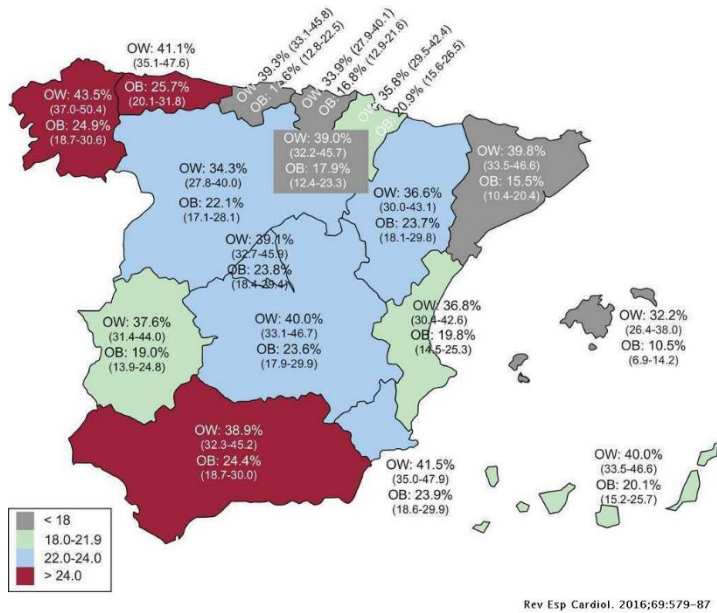
Figure 6: Map of obesity prevalence in the adult population (aged 25-64 years) in Spain. Age-adjusted rates. 95% confidence interval between parenthese0s (34)

In Spain, the latest ENPE study results included population aged 3 to 24 years (n=1601), and considered 3 different standardized international protocols to define overweight and obesity (the IOTF, WHO and Orbegozo 2011 criteria), concluding that the prevalence of excess overweight was estimated at 34.1% and obesity at 10.3% (35). The prevalence of weight excess in 6 to 9 year-old school children in Spain has decreased by 3.2% since 2011, due to the decrease in overweight, whilst obesity remained stable according to the latest results from the ALADINO study (36).

Approximately 1 in 4 obese children and adolescents have a cluster of cardiovascular risk factors contributing to the "metabolic syndrome" (37), and this, in the mid-term, appears to increase the risk of developing heart disease by ten compared to healthy children (38).

Additionally, obese individuals were found to have medical costs that were approximately 30% greater than people with normal weight (31).

If obesity could be addressed early in life, it could have a substantial impact on healthcare costs. Wang et al 2010 reported that if the number of individuals aged 16 and 17, who are overweight or obese, could be reduced by 1%, this would result in a decrease of life-time medical costs of around 480 million euro (39).

1.3. Intervention strategies

Over the last 50 years, dietary patterns in high-income countries have shifted, because of the increased availability and low cost of highly processed foods, towards non-healthy diets. These diets are characterized by a higher intake of energy-dense foods, which are rich in saturated fats and sugars, and an increased intake of ω-6 PUFA-s, favoring a high ω-6/ω-3 ratio and promoting inflammation (40). These changes in diet come hand in hand with an increase in sedentary lifestyles associated with the nature of many forms of work, the change in transport modes, and the increase in urban development (41).

There is a growing body of evidence of an inverse association between socioeconomic status and child obesity in developed countries as the prevalence of overweight and obesity is high in all age groups in many countries, but especially worrying in children and adolescents in developed countries and economies in transition (42).

However, many low- and middle-income countries are now facing a "double burden" of malnutrition. While these countries continue to deal with the problems of infectious diseases and undernutrition, they are also experiencing a rapid upsurge in noncommunicable disease risk factors such as obesity and overweight, particularly in urban settings. Increased obesity rates in these low socioeconomic groups are associated with higher unemployment, lower education levels, unhealthy dietary habits, and low physical activity (43).

Although many of the causes of obesity are preventable and reversable, there appears to be a complex relationship between biological, psychosocial, and behavioral factors (44). Due to this complexity, most successful strategies for the treatment and prevention of obesity have focused on the psychological-behavioral and social components in order to modify dietary habits, but all this complexity represents potential barriers to reducing weight and maintaining healthy long-term lifestyles.

As obesity occurs due to excessive energy intake, food consumption is the cornerstone of the cause and treatment of this condition (45). Low-fat diets, to reduce calory intake, have been the most recommended strategy for people with obesity in past decades (46). Moreover, there is not enough evidence from randomized control trials supporting beneficial effects of low-fat diets over other dietary interventions for long-term weight loss (46). A comparison of a low-carbohydrate diet with a standard low-fat diet produced no statistically significant differences in terms of BMI or BMI z-score reduction (47). Actually, in mice, it has been observed that a balanced fatty acid intake induces less lipogenic and inflammatory effects than a high carbohydrate diet (48).

Besides, recent scientific evidence has shown that low fat diets reduce LDL and HDL cholesterol and increase triglycerides. Further, the replacement of saturated fatty acids (SFA) with monounsaturated fatty acids (MUFA) has been proposed as an appropriate strategy to reduce obesity, since substituting SFA with MUFAs raises HDL-cholesterol levels, improves insulin sensitivity, and lowers LDL-cholesterol levels (49-51).

Other dietary plans have been proposed including low-carbohydrate diets, high-protein diets, very low-caloric diets with meal replacements, and diets with intermittent energy restrictions. However, controversial results have been reported evidencing that a successful diet must be healthy, balanced and without nutritional deficiencies (52, 53).

These different diet plans are strategies mainly used for adults with obesity, while in children, the strategies focus mainly on lifestyle modification due to the lack of evidence from nutritional or pharmacological treatments on severe obesity in children. As children tend to overeat and not to do enough physical exercise, the actions should focus on increasing the consumption of healthy foods, physical exercise, improving nutritional literacy in schools, weight management, monitoring, and evaluation (54).

The Mediterranean dietary pattern is being widely studied because of the multiple associated health benefits, such as lower risk of cardiovascular diseases, a decreased risk of developing type II diabetes, and prevention of some types of cancer (55-58). The main characteristics of the Mediterranean diet pattern are an abundant consumption of plant foods (whole grains, fruit, vegetables, legumes, nuts and seeds), olive oil as the main source of fat, a moderate/high consumption of fish and shellfish, a moderate consumption of eggs, poultry and dairy products, and a low consumption of red meat. Moreover, the consumption of fresh, local, and seasonal food, rich in biodiversity, must be considered as this also represents a sustainable lifestyle model (59).

In any case, most of the nutritional strategies to manage weight loss, include general dietary recommendations rather than specific dietary plans based on individual metabolism (52), and no differentiation between adults and children has been evaluated in previous studies, in order to identify metabolic and behavioral differences that may be affecting the success of interventions (60).

However, obesity prevalence in both child and adult populations continues to increase worldwide, as observed in Figure 3 and Figure 5, so, it is time to seek new approaches for effective interventions. Consequently, public health policies should focus on preventive strategies starting at early ages to prevent obesity. To be effective, these preventive strategies require defining reliable tools to determine such

parameters as the cutoff value or weight category to be considered for early diagnosis (61), and other biomarkers that can better represent the risks associated with obesity.

Childhood obesity generally continues into adulthood (62-64), therefore it is extremely important for obesity prevention to begin in children. Estimates have shown that the risk of adult obesity was ≥2 times greater for children with obesity than for nonobese children (65). Childhood obesity is not only considered the main predictor of obesity in adulthood (66), but also, adults who have been obese since childhood, have a worse perspective for developing diabetes or cardiovascular diseases (67, 68).

Most successful programs developed to reduce or prevent overweight and obesity during infancy, use school and family environments to implement interventions in order to change dietary behavior and lifestyles (54, 69-71). Experts agree that different community members, including families, schools, health professionals and local policymakers must collaborate in obesity prevention strategies (72). Some strategies include different digital tools to improve nutritional literacy through mobile apps, including gamified activities for children to stimulate and incentive them (73). Few nutritional intervention studies have been developed to study the effect of some specific diets to control obesity in children.

There is a high need for the young population to follow a healthy diet adapted to their needs, metabolism, preferences, and lifestyles. Additionally, there is a lack of personalized strategies focused on youth population with the aim of motivating them, and to provide precise information about which type of food and physical activity is more appropriate to their profile.

1.4. Metabolically healthy obesity (MHO)

Even though the obesity prevalence continues to increase, in both adults and children (Figure 3 and Figure 5), with all the associated risks - described above - that this

entails, there is emerging evidence that some people with obesity do not display the traditionally obesity-associated metabolic disorders, such as insulin resistance, arterial hypertension or dyslipidemia, among others. Several studies have shown that MHO patients have a reduced risk of cardiovascular disease and mortality (74). Although these "metabolically healthy obese" individuals (MHO) were described, for the first time, twenty years ago (75), the factors and mechanisms underlying these protective effects remain unknown. This is the first study to be presented that correlates the group of MHO with normal visceral adipose tissue, lower triglycerides, and increased high-density lipoprotein (HDL)-cholesterol compared with the rest of the patients with obesity. Over the years, several studies have established that the prevalence of the MHO phenotype between 10-35% (76-78) is more frequent in women than in men and that it decreases, regardless of gender, with age (79). One of the reasons for this variation in prevalence is that there is no single standardized criterion to define MHO, either in adults or in children. In most of the studies in adults, a BMI ≥ 30 kg/m^2 with no indication of associated metabolic disorders, such as type II diabetes or dyslipidemia, has been used to classify MHO individuals. Some studies focus on body fat content (more than 25% for men and more than 30% for women), instead of BMI to define MHO (80), while others highlight the importance of insulin resistance together with the body fat percentage to categorize MHO (81). In pediatric populations, several criteria have also been used to define MHO. For example, Prince et al based their definition of MHO on insulin resistance and cardiometabolic risk factors, such as serum lipids, blood pressure and glucose (82), while Vukovic et al. focused on insulin sensitivity, classifying MHO as the lower quartile of HOMA-IR (≤ 2.75) (83). However, fewer studies have been carried out in children than in adults, so there is an added difficulty in defining a unified MHO criterion.

Recently, greater interest in including inflammatory markers in the definition of MHO has emerged. There is increasing evidence suggesting that subclinical

inflammation could be the underlying mechanism that determines whether an obese individual is metabolically healthy or not (84). This subclinical inflammation is associated with insulin resistance and some inflammatory markers, such as the white blood cell count, have emerged as adequate predictors of (vascular) inflammation, cardiovascular disease and the metabolic syndrome (85). White blood cell count has the advantage that it correlates with metabolic syndrome markers in children, too (86).

Other inflammatory markers such as circulating cytokines, number of free fatty acids, and activation status of peripheral leukocytes, have been studied as representative sites of adipose tissue and systemic inflammatory status, showing that MHO individuals present a lower degree of inflammation compared with non-healthy individuals with obesity (87).

Philips et al, measured a wide range of circulating inflammatory markers, such as proinflammatory cytokines, adipokines and acute-phase response proteins, correlating a lower degree of inflammation of the MHO compared with non-healthy individuals with obesity (88).

In addition, it has been observed that MHO individuals have lower visceral, liver, and muscle fat content compared to non-healthy individuals with obesity, suggesting that the MHO phenotype is associated with a better ability to trap free fatty acids in adipose tissue. The latter is linked to the increasing importance that an additional marker, such as liver fat content, is attaining in the context of the MHO phenotype, since the prevalence of non-alcoholic fatty liver disease (NAFLD) seems to be significantly lower in patients with MHO compared to metabolically non-healthy individuals (89).

These inflammatory markers can be crucial to understand the mechanisms involved in the development of obesity and its associated comorbidities, and can also be used to design new nutritional intervention strategies based on this approach.

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1.5.Omic Sciences and Nutrition

In this sense, personalized intervention strategies could provide precise nutritional guidance and contribute to successful long-term interventions (90). Even though dietary guidelines for macronutrient intake in adults and children are established (91, 92), according to the different scientific evidence on requirements of both population groups, especially from energy intake, interventions to control obesity in children and adults are not specific or differentiated, regarding the intake of food groups or specific nutrients. For that reason, the optimal macronutrient distribution of the diet to improve weight status is unclear (47).

The use of molecular tools can provide new scientific evidence related to the characterization of different obesity phenotypes together with the impact of diet on metabolism (93). This can be useful to personalize therapy, and contribute to providing more precise nutritional recommendations, mainly for an adequate fat intake for different age groups and health conditions (94-96).

In the past two decades, our ability to study cellular and molecular systems has been transformed through the development of omic sciences. Omic sciences aim at the collective characterization and quantification of pools of biological molecules that translate into the structure, function, and dynamics of an organism or organisms (97).

Omics sciences can be separated into four major blocks consisting of genomics, transcriptomics, proteomics and metabolomics. Genomics represent the study of the structure, function, evolution, and mapping of genomes, and aim at the characterization and quantification of genes, which direct the production of proteins with the assistance of enzymes and messenger molecules. Transcriptomics is the set of all messenger RNA molecules in one cell, tissue, or organism. It includes the

amount or concentration of each RNA molecule in addition to the molecular identities (97).

Proteomics is the science that studies the biochemical properties and functional roles of all the proteins in a cell, tissue, or organism, and how their quantities, modifications, and structures change during growth and in response to internal and external stimuli. Proteomics have been applied in chronic diseases with a known nutritional component, such as obesity (98), or that involve a dietary intervention, for example in an study in which serum changes were measured after fish oil supplementation (99).

The metabolome refers to the collection of all metabolites in a biological cell, tissue, organ, or organism, which are the end products of cellular processes. Metabolomics is the science that studies all chemical processes involving metabolites. More specifically, metabolomics is the study of chemical fingerprints that specific cellular processes establish during their activity; it is the study of all small molecule metabolite profiles.

In clinical practice, one of the greatest difficulties lies in the ability to design effective nutritional strategies that include nutritional and supplement recommendations to control or prevent metabolic diseases. In this sense, omic sciences are being applied in medicine and precision nutrition, although with certain barriers given the difficulty in integrating the different biological knowledge to characterize the specific needs of individuals and population groups in order to provide a precise and personalized nutritional recommendation (100).

Precision nutrition aims to develop more complete and dynamic nutritional recommendations based on changing parameters, interacting with an individual's internal and external environment. The scientific community in general indicates that the future of precision nutrition should not just be based on nutrigenetics but also

include other factors, such as eating habits, eating behavior, physical activity, microbiota and metabolome (101, 102).

Apart from this characterization of biological molecules using the molecular tools mentioned above, the measurement of the interaction between diet and these biomarkers is of great interest to give precise nutritional recommendations. For example, nutrigenetics characterize the interaction of gene variants with specific nutrients associated with different diseases, such as obesity (103). These interactions occur both ways, as genes can determine the effect of diet on health and, on the other hand, nutrients can modify the gene expression through epigenetic variations (104). In this way, personalized nutritional recommendations can be designed according to an individual's genetic profile, to improve different metabolic states (105). For example, Loria-Kohen et al. demonstrated single nucleotide polymorphisms, in genes involved in lipid metabolism, and associated with dairy food consumption and susceptibility to developing cardiovascular disease (106).

The intricate diet-genetics interaction suggests that some biomarkers could only be valuable for particular segments of a population depending on their specific genetic characteristics. This represents the general use of nutritional biomarkers to properly assess nutritional status or to monitor the effect of nutritional intervention studies in non-targeted populations (107).

Regarding metabolomics, metabolites represent the real endpoints of the metabolism, and of underlying physiological regulatory processes. Individual metabolites, such as cholesterol, glucose, fatty acids and others are considered markers for health or disease status (108). In nutrition, metabolomics can be applied to characterize metabolite profiles, which define the molecular mechanism of bioactive compounds, discover status biomarkers, and allow the monitorization of dietary interventions studies (109, 110).

However, we need a better understanding of interindividual variability in response to diet in order to design nutritional strategies adapted to the age, type of pathology, and food preferences of each individual. In the era of nutrition and precision medicine, the search for new biomarkers is directed towards integrative markers that reflect not only nutritional status but also connect with metabolism, and therefore reflect the connection between nutrition and health status (111). The central problem is that appropriate biomarkers are not yet available for many health disorders, and those that are available require highly invasive or complex methodologies and are, therefore, costly from an economic point of view.

In this sense, the use of biomarkers that can be measured by these omic sciences and that have a direct interaction with the diet are of great interest when designing nutritional interventions, since they allow personalized recommendations, depending on the characterized needs.

1.6. Lipidomics

Lipidomics was first described by Han and Gross (112) as a branch of metabolomics that consists of a qualitative and quantitative analysis of the lipidome, which refers to whole lipids in cells. Apart from identifying and quantifying all lipids, lipidomics focuses on the characterization of their interactions with other lipids and proteins, along with protein expression associated with lipid metabolism and function, and gene regulation in response to a stimulation or disturbance. At the same time, it shows a strong correlation with diet as measured lipid levels will be affected by both metabolism and dietary intake.

The analyses of these metabolites are important in order to obtain more data that will integrate cellular function at a molecular level, and in this way, define the phenotype of each cell, or tissue, in response to environmental or genetic modifications (113).

Within the study field of lipidomics, how lipids influence membrane structure and respond to environmental changes due to physiological processes, as well as their response to diets, is studied. These studies correlate lipids with metabolites and/or metabolic pathways, together with the metabolic health status, and try to establish relationships between changes in the regulation of lipid metabolism and pathologic processes (114).

Many individual lipid molecular species have been closely involved in the processes of different diseases. Several studies have demonstrated that lipid dysregulation caused different diseases, and can be potential biomarkers of diabetes (115), metabolic syndrome and obesity, aging, cardiovascular disease, or cancer, shedding some light on the metabolic pathways involved (113).

Technological advances, including mass spectrometry (MS)-based shotgun lipidomics and MS, coupled with separation technique-based lipidomics, can be applied to characterize and discover the role of lipids in cellular functions and disease biomarkers in several pathologies that are related to lipids (116). These advances are not trivial, since they have opened the doors to a robust analysis of different lipids, facilitating a better understanding of lipid metabolism.

These biochemical experiments for the study of lipid functions, begin with the extraction of lipids from tissues or cells ( ) (117). The complex lipid mixture, either in
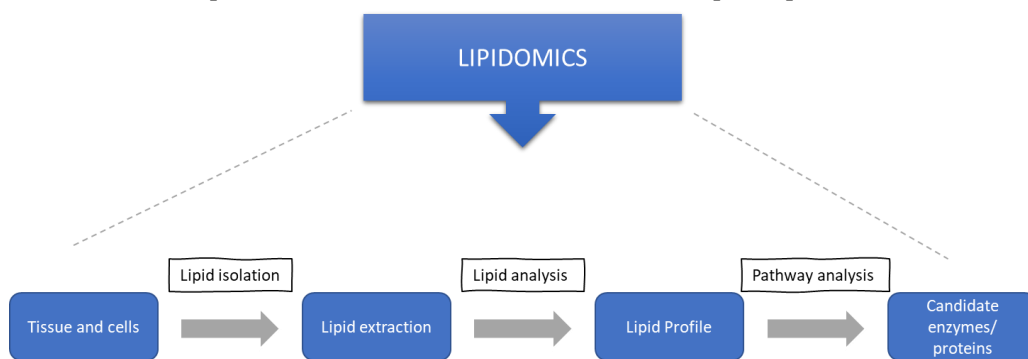

Figure 7: Lipidomics-systems-level scale analysis of lipids and their interactors. Modified from Wenk et al (2004)(1)

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its unprocessed form or after sample modification, is then analyzed by one or more analytical techniques to obtain a 'lipid profile', which contains information on the lipid composition and abundance of individual lipids present in the starting material. Typically, such experimental results are compared with a control condition to elucidate distinct metabolites whose levels change upon perturbation of the biological system under investigation (117).

Lipidomics will include profiling lipid extracts in order to identify metabolic pathways and enzymes that are affected by the perturbation of interest ( ). A major advantage of such lipidomics-based discovery is that, together with our relatively good understanding of many biosynthetic and metabolic pathways, it will lead to the identification of pathways and enzymes (and enzyme modulators).

Integration of the lipid profile through the use of multivariate statistics, can be helpful to discover potential biomarkers, by understanding disease pathology, and the mechanisms of lipid-mediated disease (118). As metabolites are considered direct products of biochemical processes, they are easily correlated with the studied phenotype.

At the same times, analysis of a lipid profile allows for the evaluation of a disease onset and its progression, enabling, by measuring the quantification of the lipidome alterations, the personalization and monitorization of the treatment.

Of all lipids that are present in the body, the lipid components of biological membranes have long been considered passive bystanders with a purely structural function. Although it is true that the shape of the lipid assemblages is determined, at least in part, by the geometric properties of the individual lipid molecules, the discovery that membrane lipids can act as precursors for second messengers has opened up a range of possibilities for lipid metabolism study. In addition to acting as precursors, many membrane lipids themselves act as signaling components, taking part in many cellular processes (1, 119).

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This correlation between lipids and the variations that occur in pathological states and the respective exogenous influences that act on them, are based on the fact that these variations are reflected on the metabolism, since lipids regulate the membrane lipid structure and membrane-related signaling events. In turn, the diet-metabolism interaction can be studied, as membrane lipid composition will be driven by both factors.

One of the practical applications of lipidomics, in this sense, could be the membrane lipid therapy, a new perspective based on the development of molecules that regulate membrane lipids in order to treat different diseases such as cancer, neurological pathologies, metabolic disorders or cardiovascular diseases (120). All these factors make the measurement of the lipids that constitute the cell membrane a major point of interest.

1.7.Cellular Membrane

The cell theory establishes that the minimum living autonomous system is a cell, and that all organisms are made up of several of them (121). The nature of the biological entity that defines cells is the plasma membrane, which, in addition to acting as a border with the cell exterior, regulates molecular traffic between internal and external spaces.

On the other hand, in eukaryotic cells there are membranes within the cells themselves, which form organelles that perform essential functions such as energy conservation, development of reaction sequences, and intra- and intercellular communication (122).

All membranes have a series of physical properties that allow them to perform a wide variety of functions in which they are involved. Their flexibility allows them to adapt to changes in shape derived from cell growth and movement; they are self-sealing, which favors them being able to carry out exocytosis and endocytosis; and they are

selectively permeable to compounds and ions, allowing them to incorporate and excrete only those that are necessary (123).

Cellular membranes are highly organized structures composed of 3 main components: the polar lipids, the proteins, and the carbohydrates. The relative composition of each of them varies depending on the cell type, but they share some common characteristics.

The glycerophospholipids are the building blocks of cellular membranes. They are characterized by an amphipathic structure with a hydrophobic tail composed of two fatty acyl chains linked by an ether bond to positions 1 and 2 (sn-1 and -2) of a glycerol backbone, and a hydrophilic head group consisting of a phosphate group linked to position 3 (sn-3) of the glycerol with a phosphodiester bond (124).

Hydrophobic chains of membrane glycerophospholipids are commonly divided into two subclasses depending on whether they are derived from SFAs, characterized by a straight hydrocarbon chain commonly containing an even number of carbon atoms, or from MUFA or polyunsaturated fatty acids (PUFA) containing one or several double bonds (125, 126). The physicochemical properties of FAs and FA-derived lipids depend on both the chain length and the saturation level of the acyl chains.

On the other hand, cell membranes incorporate a series of specialized proteins into their structure that allow different cellular processes to occur, such as the transport of compounds, reception and transmission of signals, and communication and union with neighboring cells, forming superior structures such as tissues. Finally, the composition of the membrane itself also varies depending on the cell type, thus developing different processes (127).

The membranes also contain carbohydrates which, together with proteins and phospholipids, form a dynamic structure known as the fluid mosaic model (Figure 8). In this structure, most of the interactions between the components occur through

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non-covalent bonds, which allows freedom of movement between lipids and proteins. In addition, cells change membrane fluidity by modifying the concentration of cholesterol and the proportions of FA. Another of the properties that characterizes the membrane is the asymmetry of the bilayer that allows it to have different functions on one side and the other (122).
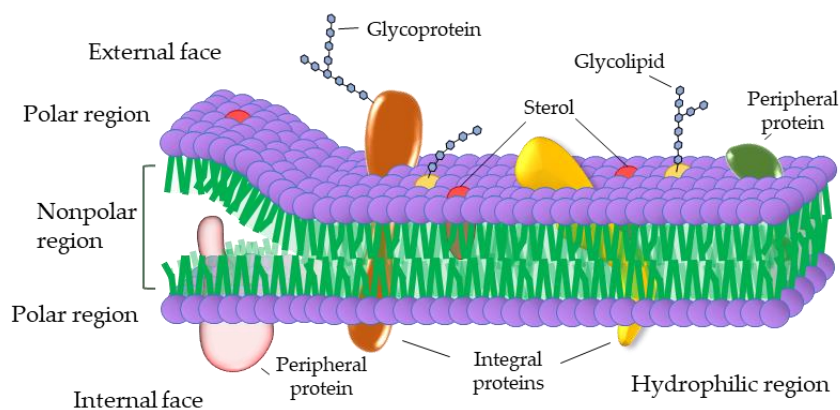

Figure 8: Fluid mosaic model of the lipid bilayer. Modified from Nelson, et al. (2017)(128)

In this research work we will focus on the FA profile of the phospholipids of the membranes, as the study of the great variety of proteins, lipids, and carbohydrates that we can observe in a membrane, both from the point of view of the composition and the functions they exert, represent a huge field of study.

1.8. Erythrocyte Membrane

As described above, cell membranes can act as key reporters of processes that occur in certain cellular environments and can provide a link between metabolism and dietary intake. It must be considered that the compositions of the membranes of the different cells adapt to the function that they must perform in each case. That is why there is great interest in the use of biomarkers, that facilitate obtaining information that leads to the conception of a global scenario of the metabolism. In this context, the

use of mature erythrocytes emerges as an ideal candidate due to some unique characteristics.

It is an easy sample to obtain as it only requires a blood draw.

It is a cell that circulates throughout the body and interacts with all types of tissues, sharing the fatty acids of its membrane with the rest of the tissues. Previous studies described in literature correlate the fatty acids of the erythrocyte membrane with the membranes of other tissues (129-131).

It is a cell devoid of organelles, a fact that facilitates the analysis of membrane lipids by not having to separate them from those of the mitochondrial membrane. In addition, it does not have genetic material that can influences its composition.

Its composition is representative of the general condition of the rest of the tissues and is subject to the metabolic modifications that may occur in them. However, it is not affected by variations of short periods of time, such as sporadic changes in diet. In this way it differs from plasma, which is much more influenced by these sporadic alterations.

All the fatty acid families (saturated, monounsaturated, polyunsaturated ω-6 and ω-3) are present in its membrane composition, which allows establishing relationships with the composition of the different tissues of an organism.

It presents a life cycle of about 120 days, which permits collecting information about the membrane composition changes that take place throughout the body, from the previous 4 months. It also presents changes in density depending on its age. In this way, the variation in the membrane associated with the life of the cell can be avoided, since it is possible to always study those cells that have similar age ranges (132).

For a new effect to be reflected in the erythrocyte membrane, it must be applied constantly for about 3-4 months, a complete life cycle of these cells.

1.9.Non-essential fatty acids

1.9.1. Saturated fatty acids (SFA)

SFAs are chains of carbon atoms organized in pairs (2 + 2), forming a linear structure (Figure 9). These fatty acids are synthesized by the body from the acetyl-CoA formed during glycolysis, and by

Figure 9: Stearic acid (C18:0).

adding carbon pairs until reaching palmitic acid (C16: 0). This process is known as *de novo* synthesis of FA and can be carried out thanks to the fatty acid synthase (FAS) enzyme. Its activity is necessary for cell proliferation, as it can generate the simplest unit of SFA that will be used in the formation of membranes.

Once C16: 0 is formed, cells use another series of enzymes that allow the synthesis of more complex fatty acids, reaching compounds of up to 26 carbon atoms. However, fatty acids with longer chains (> 20 carbons) have a higher affinity for desaturase enzymes, responsible for introducing unsaturations, so they do not accumulate in large quantities (133). In any case, these FAs are synthesized according to cell demand, which can be very different in each type of cell. The elongases (ELOVL) are the enzymes responsible for this elongation and can act on SFAs, monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA).

The function of SFAs in the membrane is highly relevant since it is related to structural aspects. Due to their lack of unsaturation, they are linear chains that link together with relative ease, providing rigidity and hydrophobicity. In addition, they are involved in transport processes, as they serve as anchors to different membrane proteins (122). In nature, it is relatively easy to find sources of SFAs, since they are very common in animal fats and in some vegetables, such as palm and coconut oils. Because of the ease with which SFAs can be obtained through diet, one might think that FAS tends

to be frequently inactivated. Indeed, the enzyme responds in this way to the presence of exogenous SFA, but, on many occasions (such as during a cell proliferation process), it responds positively to other cellular signals, remaining active, which can lead to excess SFA by combining both exogenous and endogenous sources (134). Taking into account both the metabolic and nutritional considerations, the WHO recommends not to exceed the consumption of SFAs by more than the 10% of the caloric intake (135).

1.9.2. Monounsaturated fatty acids (MUFA)

MUFAs are structurally very similar to SFAs, with the difference that they have a double bond between two carbon atoms (Figure 10). Like the previous group, they can also be synthesized by the body but, in this case, the simplest are formed from C16:0 and stearic acid (C18:0) (Figure 9) through the action of the desaturase enzymes,

Delta-9-desaturase (Δ9D) and Delta-6-desaturase (Δ6D) to form palmitoleic acid (C16:1; 9c), oleic acid (OA, C18:1; 9c), and sapienic acid (C16:1; 6c) (Figure 11).
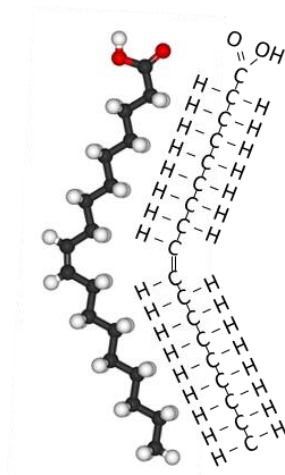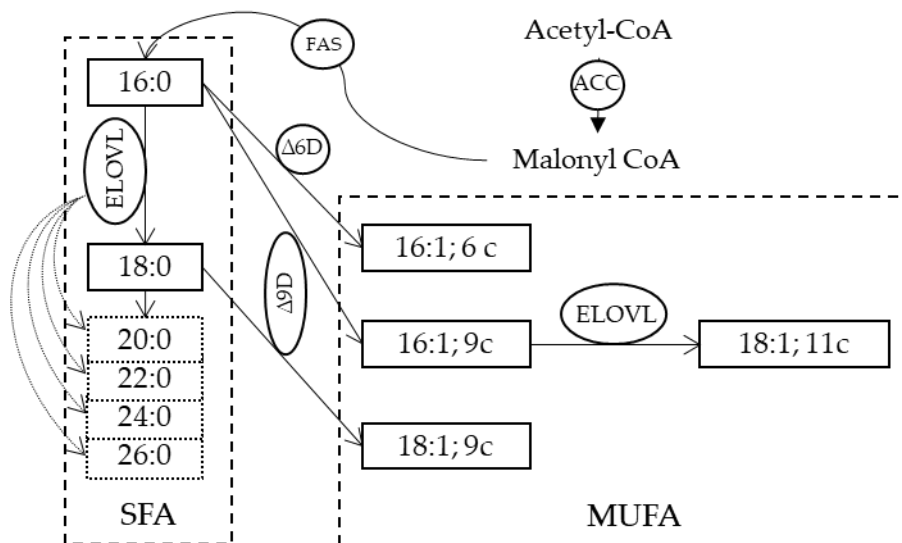

Figure 10: Oleic acid (C18:1; 9c).

Figure 11: Metabolic pathway for the synthesis of fatty acids. Malonyl CoA is formed from acetyl-CoA by acetyl-CoA carboxylase. Then, by the action of the fatty acid synthase (FAS), pairs of carbon atoms are added to form palmitic acid (C16:0). Once formed, C16:0 can stay on the SFA path by lengthening its chain through elongases (ELOVL), or form MUFAs through desaturases (Δ6D or Δ9D) that incorporate unsaturation in one of their bonds.

Depending on the type of MUFA, it will fulfil different roles, but in general the structural function also stands out since, having an unsaturated bond, the chain of atoms does not maintain its linear shape, and it will be more difficult for it to be able to bond with adjacent FAs, as occurred with SFAs. It thereby gives fluidity properties to the membrane, while it maintains the hydrophobicity.

The natural sources of MUFA usually come from vegetable oils, such as olive oil, seeds, and nuts. In this case, although they can be synthesized endogenously, the European Food Safety Authority (EFSA) does not establish an indication of maximum consumption, but around 10-15% of the caloric intake is indicated (136).

1.10.   Essential fatty acids

1.10.1. Polyunsaturated fatty acids (PUFA)

These fatty acids have more than one unsaturation in the bonds of their carbons (Figure 12). PUFAs are essential fatty acids that must be obtained from the diet because humans and other mammals lack endogenous enzymes to synthesize them. Although mammalian cells cannot synthesize PUFAs, they can metabolize them into more physiologically active compounds. These FAs fulfill an important function both at structural level and in cell signaling.
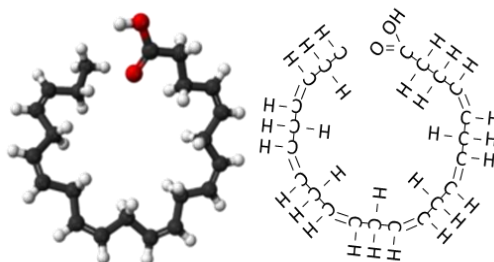
Figure 12: Docosahexaenoic acid (DHA, C22:6; ω-3).

In this group of FAs, we have two differentiated families, classified as omega-6 (ω-6) and omega-3 (ω-3), depending on where they have their last unsaturation, at six carbons from the final group or at three. The two precursors from which all the rest of metabolic compounds of both families can be synthesized are linoleic acid (LA), for the ω-6 family, and alpha- linolenic acid (ALA), for the ω-3 family. Since several enzymes are shared between the metabolic pathways of ω-3 and ω-6 PUFAs, ω-3 and ω-6 substrates compete to access these enzymes. Both precursors can be metabolized into more physiologically active compounds by a set of desaturating enzymes via Δ5 and Δ6 desaturases and by lengthening the acyl chain via elongases (137) (Figure 13).
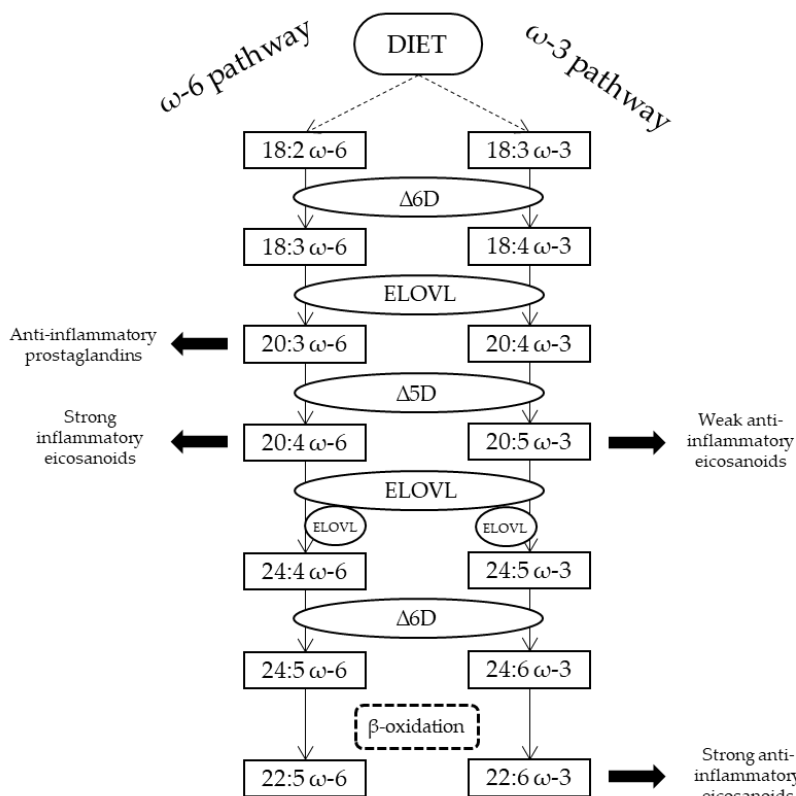
Figure 13: Metabolic pathways of ω-6 and ω-3. The cascade begins with the essential FAs, linoleic acid (C18: 2 ω-6) and α-linolenic acid (C18: 3 ω-6) to form, through desaturase and elongase enzymes, the rest of the semi-essential FA. C20: 4 ω-6 is a precursor of inflammation processes and C20: 5 ω-3 and C22: 6 ω-3, of anti-inflammation. Δ6D: Delta-6 desaturase. ELOVL: elongase. Δ5D: Delta-5 desaturase.

1.10.2. The ω-6 pathway

Once the metabolic cascade has started, γ-linolenic acid (GLA)(C18:3 ω-6) is formed from LA due to the action of Δ6D, and is subsequently metabolized into γ-linolenic dihomo (DGLA) (C20:3 ω-6) through elongation. This fatty acid develops decisive functions in the cell, as it can be converted by inflammatory cells into 15-(S)-hydroxy-8,11,13-eicosatrienoic acid and prostaglandin E1 , which possess anti-inflammatory and anti-proliferative properties (138). It also regulates the action of the phospholipase A2 enzyme (responsible for the extraction of FA from the phospholipids of the membrane), and is a key element in the maturation of lymphocytes and, therefore, the functioning of the immune system. (139).

Then, DGLA is metabolized into Arachidonic Acid (AA), through the action of the delta-5 desaturase (Δ5D). This FA is a main character of the metabolic pathway as it is the precursor to several potent pro-inflammatory mediators such as series 2 prostaglandins and series 4 leukotrienes (140).

Finally, it must be pointed out that the activity of the enzymes involved in the metabolic pathway can be affected by some factors, such as the absence of several co-factors and vitamins, and the presence of SFAs and trans FAs that inhibit their activity for Δ6D and for Δ5D. Apart from the absence of co-factors and vitamins, the presence of insulin can stimulate their activation (141).

As essential FAs that must be obtained from diet, the most common sources of ω-6 PUFA are some vegetable oils, such as sunflower, corn and soy, which are rich in LA, and egg yolk and lean meats, which are rich in AA. Currently, it is easier to find ω-6 than ω-3 in the foods that surround us, as they are widely used in a wide variety of products. For this family of PUFA, the WHO recommends that the intake should be between 2.5 - 9% of total caloric intake (135).

1.10.3. The ω-3 pathway

Alpha-linolenic acid (ALA) is the precursor of the metabolic pathway for ω-3 FA and can be found in green leafy vegetables and in some seeds (flax, rape, chia, perilla, and walnuts). The same enzymes that, in the ω-6 pathway, converted the LA into DGLA and AA, will metabolize the ALA in eicosapentaenoic acid (EPA). This FA is a precursor of prostaglandins, leukotrienes and resolvins, which have an anti-inflammatory response. EPA acts as a competitive inhibitor for proinflammatory AA on cyclooxygenase (COX), which produces proinflammatory eicosanoids (142) (Figure 14).
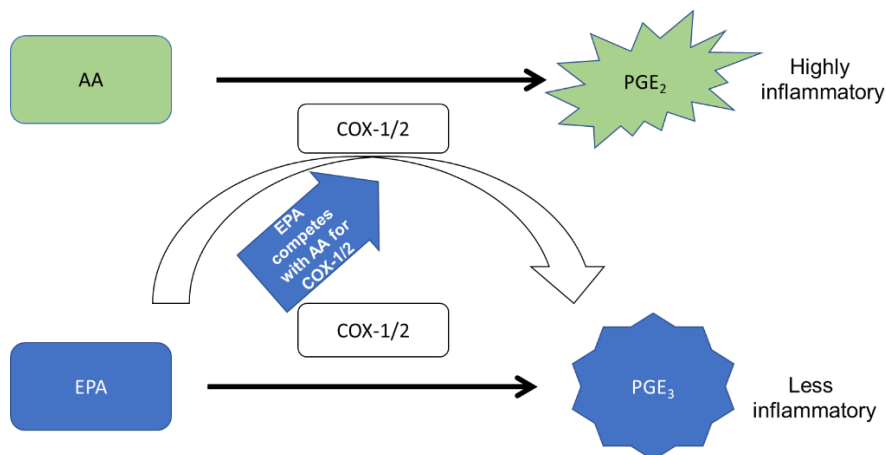
Figure 14: EPA competitively inhibits PGE2 formation by COX-1 and COX-2. EPA competitively binds with COX-1 and COX-2 and produces less-inflammatory PGE3 while suppressing AA binding which produces highly inflammatory PGE2. AA, arachidonic acid; COX, cyclooxygenase; EPA, eicosapentaenoic acid; PGE2/3, prostaglandin E2/3. Calder (2009) was used as reference for the design(143).

Then, elongations and unsaturations occur again until docosahexaenoic acid (DHA), a precursor of D-series resolvins, is formed, which, in combination with the products formed from EPA, generate a strong anti-inflammatory response. In addition, DHA is also highly involved in the central nervous system and in nerve cells, as it is one of the main components of the membrane that guarantees its correct cellular functioning (144).

Although EPA and DHA can be synthesized from shorter plant-derived ω-3 FA precursors such as ALA, this metabolic pathway is not efficient in humans. Only about 8-20% of ALA is converted to EPA in humans, while conversion of ALA to DHA is estimated to be around 0.5-9% (145, 146).

Apart from these conversions of ALA, these FA can be obtained through diet as they are present in some marine algae, rich in EPA and DHA and, within the animal kingdom, particularly in oily fish.

EFSA establishes that the total caloric intake of ω-3 PUFAs should be around 0.5-2%, with a minimum daily consumption of 250mg of EPA+DHA (136).

On the other hand, we must consider the fact that, due to globalization, the nutritional habits of many populations have changed, prioritizing the consumption of ω-6 PUFAs over ω-3 PUFAs, causing an imbalance in the ω-6/ω-3 ratio of around 15:1 in western diets, while the optimal ratio of PUFA intake should be between 1:1-4:1 (147). Considering that both metabolic pathways compete for the same set of enzymes, an adequate proportion of both PUFAs should be ingested in order to maintain an optimal balance.

1.10.4. Trans fatty acids (TFA)

These fatty acids contain at least one double bond, but, unlike the previous FA, this bond is in *trans* isomerism. Despite the unsaturations, the shape that TFAs acquire in space is closer to that of SFAs, since their configuration is linear (Figure 15).
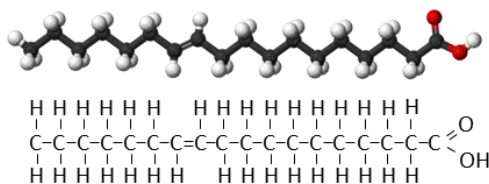

Figure 15: Structure of a FA with double bond in trans isomerism (C18: 1; 11t).

Some industrial processes can produce changes in the structure of FAs that undergo isomerization reactions of the double bonds, transforming the cis bonds into trans. Margarines are an example of this isomerization reaction as they go through hydrogenation processes, or fish oil-based supplements that also suffer from isomerization, due to deodorization processes and high temperatures. These fatty acids have harmful effects on health, since they can reduce the availability to metabolize PUFAs by inhibiting the activity of Δ5D, Δ6D and elongases (148). It should be noted that there are no enzymes capable of transforming a *trans* bond to a

cis one, so they can only take part as a structural component, providing a similar effect of rigidity to the membrane as SFA.

Endogenously, the formation of TFAs is also possible through the reaction between a free radical that may appear under conditions of cellular stress and a *cis* double bond of lipids. The isomerization of the double bonds is one of the strategies to neutralize free radicals, thus preventing them from damaging other cell structures (133).

1.11. Precision Nutrition: Personalized diet and dietary supplements to control obesity.

Modern western diets contain excessive levels of ω-6 PUFAs but very low levels of ω-3 PUFAs, leading to an unhealthy ω-6/ω-3 ratio of 20:1, instead of the 1:1 proportion that occurred during human evolution (149). As an illustrative example, around 70% of dietary calories currently consumed in the modern western diet did not exist in hunter-gatherer diets (150). Technological advances in food production and processing have provided high-caloric foods with a high content in sugars, refined grains and oils (151). Concerning FAs, because of the advice given to reduce SFAs, to lower cholesterol levels (152), a higher consumption of vegetable oils, such as soybean, corn or canola oil, rich in LA and poor in ω-3 PUFAs and MUFAs, has occurred. This increased consumption of vegetable oils has occurred to an extent of more than 1000-fold in the last century, representing around 7% of daily caloric intake (153).

Thus, an unbalanced ω-6/ω-3 ratio in favor of ω-6 PUFAs is highly proinflammatory, as described above, contributing to the prevalence of different diseases related to chronic inflammation such as atherosclerosis, obesity, or diabetes.

Because of this imbalance towards ω-6 PUFAs, reaching optimal levels of ω-3 PUFAs becomes vital for the metabolism, in order to restore the optimal equilibrium between the two FA families. As explained above, the ω-6/ω-3 ratio of Western diets has clearly

shifted towards ω-6 PUFAs, so even if the best option to obtain the necessary ω-3 levels comes through dietary intake, sometimes this is hard to achieve solely with diet.

The EAT-Lancet Commission "healthy reference diet" (154) shows that the leading dietary risk factors for mortality are diets high in sodium, low in whole grains, low in fruit, low in nuts and seeds, low in vegetables, and low in ω-3 fatty acids; each accounting for more than 2% of global deaths. An optimum daily consumption of fish of 28g/day can provide the recommended daily intake of essential ω-3 fatty acids (200-300 mg of EPA+DHA/day) that is associated with reduced risk of cardiovascular disease and total mortality rate (Figure 16).
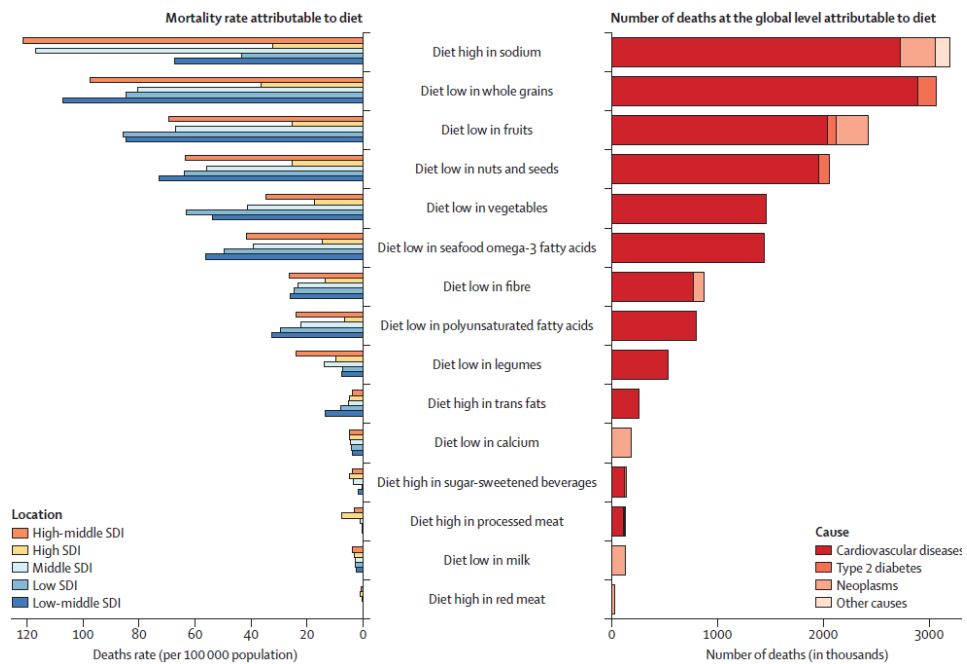

Figure 16: Number of deaths attributable to individual dietary risks at the global level in 2017 (Lancet 2019; 393: 1958–72).

It is in this sense where the use of specific ω-3 supplements arises as an adequate option to increase the consumption of ω-3 PUFAs.

These supplements have experimented an important growth during recent decades (155), as nowadays, supplements use 20 to 25% of world fish oil, up from just 5%, which was used in 1990 (156). For example, the use of ω-3 supplements among adults in the U.S. has increased from 4.8% in 2007 to 7.8% in 2012 (157).

Supplements containing these ω-3 FA oils, come from certain plant, marine or algae sources. Fish oils are sold as ω-3 PUFA supplements or in a concentrated form as ethyl esters or acylglycerols, whereas algal, fungal, and single-cell oils have recently become popular as novel and renewable sources of ω-3 PUFAs. In addition, krill oil containing both triacylglycerol and phospholipid forms containing EPA and DHA has been successfully marketed. Researchers have also incorporated ω-3 PUFAs into different oils such as borage oil and evening primrose oil to provide a better balance of PUFA components.

Even if ω-3 supplements are becoming more and more popular among consumers, most of them take ω-3 dietary supplements without the recommendation or supervision of a healthcare provider (158). Added to this lack of supervision, the countless options available in the market make it difficult for consumers to choose an appropriate option.

Many intervention trials with ω-3 supplements have been described in literature, but, even if the beneficial health properties of ω-3 FAs are well described in literature (159), these intervention trials show controversial results (160, 161). Multiple issues seem to be responsible for these results. The intervention duration and dose, the balance of the ω-6/ω-3 ratio obtained from diet during the intervention, the measurement of the baseline levels to design an optimal supplementation intervention, the stability of the ω-3 supplements, and different content proportions of specific FAs in ω-3 supplements, among other factors, seem to be crucial for an effective treatment (161).

It is in this sense where omic sciences, and above all, lipidomics, acquire a vital role in monitoring these type of intervention trials. Membrane lipidomics emerges as an effective tool to evaluate the content and effects of lipids, together with their interaction with other metabolites, allowing a membrane-based strategy for personalization of the diet and supplements (162). Lipidomics reflect the relationship between nutrition and membranes that can have a cascading effect on other relevant metabolic and signaling processes. Through the evaluation of membrane status and need for specific FAs to overcome membrane imbalance, lipidomic monitoring becomes a suitable method to assign FAs according to personal needs, facilitating an adequate use of ω-3 supplements (162).

2.Hypotheses and Objectives

Monitorization of the RBC FA profile in childhood obesity through lipidomics, will determine the relationship between the FA composition of the membrane and the nutritional status. It will allow the detection of specific metabolic needs related to the diet and the metabolism of each individual and from there, innovative nutritional strategies would be designed to improve health status.

The objective of this doctoral thesis is to characterize the fatty acid profile of the mature erythrocyte membrane of children with obesity, and establish the specific needs of FAs, and its relationship with nutritional and metabolic status. Once these differences have been established, they will form the basis for tailoring dietary interventions based on specific FA needs.

To achieve this objective, the following specific objectives were established:

Definition of the childhood obesity profile: characterization of the mature erythrocyte membrane lipid profile of children with obesity, and its relationship with nutritional and metabolic status compared to normal weight and overweight children.

Characterization of the differences in membrane lipid profile between children and adults with obesity, to differentiate effective nutritional strategies/interventions between both populations, based on specific FA needs.

Clustering of children with obesity, to determine the MHO presented by a FA profile, similar to children with normal weight, in order to personalize more dietary interventions.

The use of cell membrane lipidomic for precision nutrition together with personalized diet and ω-3 supplements, as a strategy to obtain the optimal levels of specific individual FAs.

3.Materials and Methods

3.1. Study Design

The study design of the present work consists of two phases that are described below. All children participants were recruited from pediatric endocrinology unit at the Hospital Universitario Cruces (Barakaldo, Spain) and adult participants, in the endocrinology department at the Hospital Universitario Cruces (Barakaldo, Spain). The characteristics of each population group included in the different studies of this thesis work are described further in each chapter.

Body weight (kg) and height (cm) were measured by standardized methods (22). Body mass index (BMI) was calculated as weight (kg) divided by the square of the height (m2). Anthropometric parameters, as well as blood sampling, were collected by specialized personal during the participant's visit to the Hospital.

Children were classified according to body mass index (BMI), using age and sex-specific pediatric z-scores from Orbegozo tables (23). The BMI was taken as a reference to define the different categories, defining normal weight when the standard deviation (SD) of BMI was -1 <SD ≤ +1, overweight when +1<SD ≤ +2, and obesity when SD> +2.

For adults, BMI>30 was taken as reference to classify obesity and 18.5<BMI<25 for the group with normal weight.

In all the conducted studies, subjects were excluded if they presented any kind of acute or chronic diseases, were taking medications, had any presence of metabolic syndrome symptoms or obesity associated to any type of pathology.

3.2. Food Habits and Nutrient Intakes

Estimations of food consumption, including dietary diversity and variety, were measured using a quantitative food frequency questionnaire (FFQ) on-line completed

by the parents of the children, except in those cases of adolescents, which were encouraged to complete it themselves, or by each adult volunteer.

For our study, an adapted FFQ was used, which was previously validated with the portion sizes and food groups for the Spanish juvenile population and for adults (163, 164). These questionnaires were then analyzed using the DIAL® software (UCM & Alce Ingeniería S.A. Madrid. Spain) (V 3.4.0.10) to translate the intake of specific foods into their corresponding energy and nutrient values.

3.3. Red Blood Cell (RBC) Membrane Fatty Acid Analysis

The fatty acid composition of mature RBC membrane phospholipids was obtained from blood samples (approximately 2 mL) collected in vacutainer tubes containing ethylenediaminetetraacetic acid (EDTA). Samples were shipped to the Lipidomic Laboratory and upon arrival underwent quality control for the absence of hemolysis. During the blood work-up, before lipid extraction and lipid transesterification to fatty acid methyl esters (FAMEs), the automated protocol includes the selection of mature RBCs, as reported previously (165-168).

 First, the whole blood in EDTA was centrifuged (4000 rpm for 5 min at 4ºC), and the mature cell fraction was isolated based on the higher density of the aged cells (169) and controlled by the use of cell counter (Scepter 2.0, EMD Millipore, Darmstadt, Germany).

All the subsequent steps were automated and included cell lysis, isolation of the membrane pellets, phospholipid extraction from pellets using the Bligh and Dyer method (170), transesterification to FAMEs by treatment with a potassium hydroxide (KOH)/methyl alcohol (MeOH) solution (0.5 mol/L) for 10 min at room temperature, and extraction using hexane (2 mL).

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The FAMEs were analysed using capillary column gas chromatography (GC). GC analysis was run on the Agilent 6850 Network GC System, equipped with a fused silica capillary column Agilent DB23 (60 m x 0.25 mm x 0.25 µm) and a flame ionization detector (FID). Optimal separation of all fatty acids and their geometrical and positional isomers was achieved. Identification of each fatty acid was made by comparison of commercially available standards. The amount of each FA was calculated as a percentage of the total FA content (relative %), as described in section 2.5, being more than 97% of the GC peaks recognized with appropriate standards.

3.4. Erythrocyte Membrane Lipid Profile

The same 12 FAs, previously stablished by Ferreri and Chatgilialoglu (171) were analysed in all the studies, as representative cluster of the main building blocks of the RBC membrane glycerophospholipids and of the three FA families (Table 1).

Table 1: Selection of fatty acids from the erythrocyte membrane

Families	Common name	Abbreviation	IUPAC
Saturated (SFA)	Palmitic acid		C16:0
	Stearic acid		C18:0
Monounsaturated (MUFA)	Palmitoleic acid		C16:1; 9c
	Oleic acid	OA	C18:1; 9c
	cis-Vaccenic acid		C18:1; 11c
Poliunsaturated (PUFA) ω-6	Linoleic acid	LA	C18:2; ω-6
	Dihomo γ-linoleic acid	DGLA	C20:3; ω-6
	Arachidonic acid	AA	C20:4; ω-6
Poliunsaturated ω-3	Eicosapentaenoic acid	EPA	C20:5; ω-3
	Docosahexaenoic acid	DHA	C22:5; ω-3
Trans	Elaic acid		C18:1; 9t
	trans Arachidonic acid		C20:4; t

Additionally, the enzymatic indexes of elongase and desaturase enzymes, the two classes of enzymes of the MUFA and PUFA biosynthetic pathways, were determined by calculating the product/precursor ratio of the involved FAs (Table 2).

Table 2: Indexes established for different combinations of fatty acids.

Indexes	Formula
FA indexes	
Saturation	SFA / MUFA
Unsaturation	(MUFAx1)+(LAx2)+(DGLAx3)+(AAx4)+(EPAx5)+(DHAx6)
Peroxidation	(MUFAx0.025)+(LAx1)+(DGLAx2)+(AAx4)+(EPAx6)+(DHAx8)
Inflammatory risk	ω-6 / ω-3
PUFA Balance	(EPA+DHA)/PUFAx100
Omega-3 Index (172)	EPA + DHA
De Novo Lipogenesis (173)	Palmitic acid/LA
Enzymatic Indexes	
Δ5D	AA / DGLA
Δ6D + ELO	DGLA / LA
Δ9D 16	C16:1; 9c / C16:0
Δ9D 18	C18:1; 9c / C18:0

This selection of FA-s and Indexes provides varied information.

Both saturated and monounsaturated FA, together with their indexes, allow the determination of the optimal balance between these components with opposite effects. From a structural point of view, SFA-s provide rigidity to the membrane, while MUFA-s provide fluidity. It should not be forgotten that, although both FA-s are present in the diet, they can also be synthesized endogenously. In fact, the palmitoleic acid levels provides information about the activation degree of the Δ9D enzyme, since it is a FA with little presence in the diet, it allows to determine if the synthesis of SFA or MUFA is favoured.

Regarding PUFA-s, the ω-6 and ω-3 concentrations together with their related index levels, allow us to know the balance between both families and its influence on the different aspects related to cell signalling, especially inflammation, thanks to the analysis of AA as an inflammatory precursor, and DGLA, EPA and DHA, as anti-inflammatories. On the other hand, ω-3 concentrations are being related to beneficial effects in a wide variety of pathologies, as explained before. As these FA-s have to be

obtained through diet, their actual levels can provide information whether their intake is being adequate or not.

Two *trans* FA-s are also measured, such as elaidic acid and *trans* arachidonic acid. Elaidic acid levels provide information on its contribution in the diet and *trans* arachidonic acid, reports on its endogenous synthesis and the degree of response to free radicals.

The estimated enzymatic indices indicate the degree of activation of various enzymes, allowing us to know if there is a greater activation of any of the routes that may have an impact on health.

4.General Discussion

The development of this research thesis has always been aligned with the scientific-technological objectives of a research center such as AZTI. This has allowed for a global and multidisciplinary vision, enabling the future application of the results, with the subsequent impact on society.

This thesis contributes to the search for new molecular biomarkers of obesity, and more specifically, biomarkers that reflect the nutritional and metabolic status associated with obesity.

The literature correlating membrane lipid profile with obesity is sparse compared to the large number of studies that have correlated obesity with plasma lipids and lipoproteins. Pan and collaborators (174) related dietary fat to membrane lipid metabolism, observing an increase in Δ9-desaturase activity and a decrease in Δ5-desaturase activity in obese people. Other studies correlated plasmatic FA composition with the metabolic syndrome and low-grade inflammation, establishing higher levels of SFA in overweight adolescents, and a protective effect of the intake of ω-3 PUFA (175). Another study described modifications in plasma FA composition, which were associated with the reduction of adiposity, and an improvement in cardiometabolic profiles in adolescents (176). Other studies described a correlation between an altered FA profile and estimated desaturase activities, consisting in high levels of SFA, SCD and D6D, and low levels of PUFA and D5D, with an increased probability to develop metabolic syndrome (177). Pietilainen et al. (178) established that, despite low dietary intakes of polyunsaturated fat, obese individuals had high palmitoleic and arachidonic acid ratios.

In summary, the biochemical studies carried out to date are important because they confirm the feasibility and validity of our experimental approach. Furthermore, they point to possible changes, as yet not well defined, in the

physiological activity of membranes and their associated enzymes, induced by changes in the lipid composition of the membrane, which in turn are caused by obesity, and the impact that diet can have on it.

The fatty acid composition of each tissue is specific, as is that of the erythrocyte membrane (179). That is, the proportion of fatty acids present in the erythrocyte membrane depends on the metabolic and environmental conditions of the cell at any given time. A dynamic view of phospholipid metabolism and, in particular, fatty acid transformations combined with nutritional aspects and their impact on an individual's health status, can provide important information for molecular medicine and nutrition (162). RBCs can serve as a surrogate marker for estimating major organ levels of EPA, DHA and AA. The extent to which the RBC can be used as an accurate marker of organ levels of these ω-3 FA levels is dependent on the target tissue of interest (140, 180).

To our knowledge, this is the first time that a characterization of the FA profile of RBC membranes in obese and overweight children, in comparison to normal-weight children, has been made. This study design allows us to establish optimal levels of the measured FAs using the RBC FA levels of children with normal weight as the control group. This aspect is important in nutrition intervention strategies when establishing nutritional recommendations based on FA levels, due to the importance of knowing the optimal levels of RBC FAs associated with a healthy condition compared to a diseased metabolism. The application of mature RBC membrane lipidomics in a huge population group will permit a more robust and effective characterization of obesity, and consequently, a better definition of the optimal levels of each fatty acid.

After comparing the three studied groups, an altered metabolism was observed in children with obesity. Obesity is supposed to be a *silent* chronical

inflammatory state (181) and this fact is reflected in the mature RBC FA profile of children with obesity, as they present higher levels of AA, DGLA and total ω-6 FAs.

These ω-6 FAs, especially the AA, are described in literature as precursors of proinflammatory mediators (182, 183) that act through different mechanisms on inflammatory processes. At the same time, unlike the other ω-6 FAs, children with obesity showed lower LA levels compared with children from the normal weight group. Higher RBC LA levels have been linked with improved body composition, insulin resistance, and lower levels of inflammatory markers in previous studies (184).

This imbalance of PUFA metabolism towards ω-6 FAs has also been described in literature, as it seems to contribute to excessive adipose tissue development, representing an emerging risk factor for obesity (185, 186). In this sense, the measurement in future studies of inflammation markers such as lipokines, cytokines or prostaglandins, among others, together with the measurement of RBC membrane FAs, would be of great interest, in order to confirm that high levels of the precursors of these inflammatory molecules positively correlate with inflammatory metabolites, and perfectly describe the state of silent inflammation that appears in obesity.

Together with this imbalance of PUFA levels, an altered ratio of SFA/MUFA in children with obesity has been determined. This mainly occurs due to lower levels of oleic acid, and higher levels of stearic acid. This imbalance in the SFA/MUFA ratio affects the rigidity of cell membranes, as both FA families have opposing effects. SFAs provide the membrane with a more rigid packing, whereas MUFAs possess a bent structure with a fluid effect on the membrane assembly (162).

The enzymatic activity of the Δ-9-desaturase, measured indirectly by the ratio between oleic and stearic acids, was observed to be less for the group with obesity. The overall picture that comes from examining the FA remodeling that takes place in obesity highlights the role of the *de novo* lipogenesis, with formation of SFAs and their enzymatic transformation into MUFAs, linked to the functioning of Δ-9 desaturase, and the respective gene expression (SCD1, Stearoyl CoA Desaturase).

In turn, the direct measurement of enzymatic activity would be interesting to confirm the measured differences between children with obesity and children with normal weight, since it would permit a better description of the metabolic processes that occur in obesity. In this study we correlated the enzymatic activity by means of the ratio between product and precursor, which, although its use is common in the literature, is still an indirect measure and therefore, of less precision.

Our work has allowed us to identify a typical childhood obesity RBC profile and compare it to an optimal profile in a normal-weight population. This will allow us to design an optimal diet for the prevention and control of childhood obesity, highlighting the need to reduce, increase or include new functional foods that provide the necessary nutrients and the most appropriate fats, according to the metabolic status of each individual. Our results reveal the need to increase sources rich in ω-3 fats, both to increase their levels and to reduce the levels of ω-6 observed in erythrocytes, as well as the need for a higher intake of foods rich in MUFA fats to reduce the SFA/MUFA ratio.

Regarding the type of nutritional strategies that are currently used to combat obesity, there is no clear differentiation between children and adults, although macronutrient and micronutrient requirements are defined according to the type of population (91, 187). In this sense, as described in chapter 2, the

characterization of RBC FA membrane profiles in children compared to the adult population revealed very different lipid profiles. Children with obesity presented higher LA, DGLA and total ω-6 values, along with lower DHA and total ω-3 values, compared to adults with obesity. At the same time, children with obesity presented lower levels of palmitic acid and a lower value of the *de novo* lipogenesis index compared to adults with obesity. These differences must be considered to provide more specific food group recommendations based on individual FA needs, rather than giving general recommendations for the population with obesity, as a whole and regardless of age.

Based on these differences, future nutritional intervention studies should be developed, in order to evaluate the power of RBC membrane FA analysis as a molecular tool applied to design a precision nutrition strategy in obesity. Although some intervention studies have already attempted to demonstrate the effect of diets rich in MUFA and ω-3, the use of lipidomics allows us to identify the baseline level and needs at the level of individual FA, so that the design of the strategy will be much more precise and will ensure the success of the intervention. However, a larger number of interventional clinical trials are needed to demonstrate the efficacy of a lipidomics-based intervention.

During the course of this thesis, we realized that some children with childhood obesity presented profiles that did not fit the typical profile of an obese child. Using statistical clustering techniques based on membrane fatty acid levels, we were able to identify a subgroup of obese children whose lipid profile was similar to the group of normal-weight children, who did not present an inflammatory profile.

Such statistical techniques applied to the fatty acid values obtained by membrane lipidomics of mature erythrocytes allow the differentiation of this MHO subgroup, and therefore enable us to design different intervention

strategies for this group compared to the rest of obese children, who do present a clear inflammatory profile.

At the same time, these types of statistical tools and RBC lipidomic analysis can be applied to isolate and characterize those children with normal weight that present an inflammatory profile, even if they are not overweight. The existence of this subgroup of individuals displaying metabolic outcomes, traditionally related to obesity, was first proposed in 1981 when patients with type II diabetes, hypertension, and hypertriglyceridemia, who were not obese but who responded positively to caloric restriction, were correlated (188). They defined this subgroup as metabolically obese normal weight (MONW) individuals. This phenomenon has been described in literature (189, 190) with different names, but as occurs with the MHO individuals, described in the third chapter of this thesis, there is no unified criterium related to the use of adequate biomarkers to define metabolically unhealthy individuals with normal weight.

Anyway, previous studies described in literature measure the risk that these MONW individuals have of suffering negative health outcomes compared to healthy normal weight individuals. An example of this can be found in a meta-analysis of 14 prospective cohort studies which compared MONW individuals with healthy individuals with normal weight, observing, in the former group, an increased risk of suffering cardiovascular diseases and all-cause death (191).

The characterization of these metabolically unhealthy patients, particularly in pediatric populations, is of great interest when studying the negative metabolic outcomes that may result in the long-term. The application of RBC membrane lipidomics to characterize those MONW individuals, as in the case of MHO, can be a good point of study due to all the advantages that its analysis presents. These advantages have already been described previously in this document, such as, for instance, being a good biomarker to define inflammatory profiles.

Therefore, studying MHO and MONW individuals could provide important insights into the inter-relationships between inflammation, metabolic health, and obesity (88).

However, we are aware that the results of this thesis set the starting point for future research in the field of precision nutrition, as nutritional intervention trials using mature erythrocyte membrane lipidomics are needed to design an optimal nutritional strategy adapted to the metabolic needs of each individual. This will demonstrate the efficacy of a personalized diet (food and supplements of certain fatty acids if necessary) to restore the balance and functionality of the membrane, and therefore have an effect on the individual's health.

In the different chapters of this thesis, the need to increase the intake of ω-3 fatty acids has been highlighted as a possible strategy to counteract the inflammatory effects caused by the presence of high levels of ω-6 (AA and DGLA), observed in obese individuals. In this sense, one of the nutritional strategies could be to include or increase foods rich in ω-3 fats containing, in particular, EPA and DHA (oily fish, algae, or flax seeds and oil), but the supplementation of fatty acids through nutraceuticals would permit rebalancing the erythrocyte membrane in a precise and effective way. As described in chapter 4, ω-3 supplementation is becoming increasingly popular, but the effectiveness of ω-3 supplementation will always depend on the measurement of the patient's baseline status. Defining their metabolic needs will influence what dose and type of fat should be supplemented to restore the optimal balance of RBC membrane FAs. The over-intake of these FAs can have potentially harmful effects (192) so its dosage should also be addressed with molecular tools such as lipidomics.

However, it is important to determine not only an individual's lipidomic profile, but we also need to generate new knowledge at molecular level,

integrating different omics tools, in order to design precise nutritional strategies, monitor the effect of the nutritional intervention, or as a way to define new early biomarkers of the studied disease (193).

In the increasingly important field of precision nutrition, the final target is to design tailored nutritional recommendations to treat or prevent metabolic disorders (194). More specifically, precision nutrition seeks to develop unique nutrition guidelines for each individual, combining genetic, environmental and lifestyle factors to develop effective approaches (194). For that purpose, precision nutrition approaches include different omic sciences such as genetics, metabolomics or microbiomics together with other factors such as dietary habits, food behavior and physical activity (Figure 17) (102).
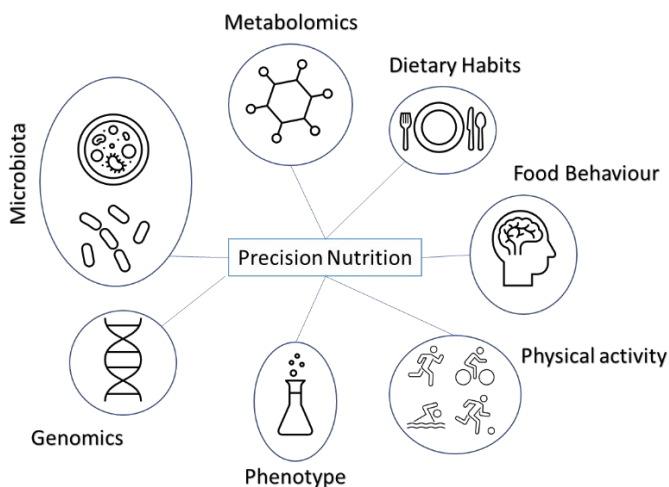

Figure 17: A representation of the main factors worth considering when addressing Precision Nutrition. Modified from de Toro. et al (102)

In this sense, while numerous genes and polymorphisms have already been identified as relevant factors in this heterogeneous response to nutrient intake (195-198), clinical evidence supporting these statistical relationships is currently too weak to establish personalized nutritional interventions in most cases (199). Thus, most of the findings on this topic are still relatively far from

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giving their fully expected potential in terms of translation and application of this knowledge to precision nutrition (200). Regarding the obesity and metabolic syndrome, recent published studies focusing on gene-environment interactions have revealed important insights into the impact of macronutrient intake in the association of genetic markers with metabolic health, fat mass accumulation or body composition. This is relevant for precision nutrition, since results from these studies, focusing on macronutrient intake, open the door to efficiently tailor diets based on the individual genetic composition. In this regard, a recent work by Goni et al. (201) has analyzed the impact of macronutrient intake in the genetic risk on obesity prediction, while other studies, which correlated BMI to single nucleotide polymorphisms (SNPs), have revealed that a high intake of sugar-sweetened beverages (202, 203), fried foods (204) or SFA-s (205) can also modulate the risk to develop obesity.

Together with this nutrigenomic profiling, the examination of gut microbiome is generating great interest in nutritional interventions, and the impact of specific dietary factors on the diversity of the gut microbiome is currently the subject of many ongoing investigations (102). The development of nutritional interventions, based on individual profiles, focuses on optimizing gut microbial composition, both in terms of richness and diversity, and emerging evidence suggests that gut microbiota profiling should be included as a key feature of precision nutrition (206).

In fact, both the composition and diversity of gut microbiota have been identified as potential risk factors for the development of several metabolic disorders including the metabolic syndrome, type II diabetes and CVD (207). In this regard, studies have described how gut microbiome profiling could represent an efficient tool, providing an accurate glucose response prediction after a meal (208).

Like RBC lipidomics, monitoring the gut microbiome during a nutritional intervention in obesity is necessary to provide more evidence regarding the effect of specific dietary patterns or nutrients on obesity metabolism. This will help to understand the biological function of gut microbiome better, as well as the association between microbiome modulation and progression of disease in order to define precise nutritional interventions.

To date, the PREDIMED study (209, 210) and the Food4Me project (211) could be considered as state-of-the-art trials in the field of precision nutrition, and two of the most stimulating wide-scale approaches in this field, which will hopefully provide guidance about how precision nutrition could be used to successfully prevent and manage cardiometabolic disorders. As already mentioned, such integrated approaches have the potential to improve dietary behaviors in an individualized or in a group-based manner, and to generate new and innovative tools, methods, and procedures.

The application of these results in future studies will focus on analyzing the association or correlation between membrane lipidomics and biochemical parameters, and the microbiota profile in obese children, as they will shed light on the effectiveness of a specific diet for the prevention or control of obesity.

The integration of genomics, microbiome, and other omics, with the lipidomics already described in this study, will allow a more global vision of the problem of childhood obesity, as well as a more specific and, therefore, more effective response to this disease.

In addition to the molecular profile, it is essential to take into account other behavioral aspects and preferences of an individual, in order to design a strategy that will be successful in the long term. In this sense, an individual's motivation, food preferences and behavior, as well as other socioeconomic factors will influence the design of a personalized strategy.

Any of the proposed lines of study will provide novel insights into childhood obesity, and investigating the correlation between them will raise new questions, and undoubtedly lead to further research. It will also lay the foundations for future studies on the impact of diet on other aspects of health, such as food intolerances, digestive system disorders, or severe diseases, such as cardiovascular diseases, cancer, or neurodegenerative diseases.

It is therefore expected that future research with the application of lipidomics will have an impact at a socioeconomic level, as new products (kits) can be developed for diagnostic, prognostic and intervention purposes, as well as new foods and supplements for use in children with obesity.

Likewise, the review of specific diets and the knowledge of the impact of the intake of different foods will allow the food sector to adapt its products to prevent obesity, and to design novel formulas and supplements for this purpose, opening up new market niches.

5.Bibliography

1.   Wenk MR, De Camilli P. Protein-lipid interactions and phosphoinositide metabolism in membrane traffic: insights from vesicle recycling in nerve terminals. Proc Natl Acad Sci U S A. 2004;101(22):8262-9.

2.   Smith SC, Jr. Multiple risk factors for cardiovascular disease and diabetes mellitus. Am J Med. 2007;120(3 Suppl 1):S3-S11.

3.   Hubert HB, Feinleib M, McNamara PM, Castelli WP. Obesity as an independent risk factor for cardiovascular disease: a 26-year follow-up of participants in the Framingham Heart Study. Circulation. 1983;67(5):968-77.

4.   Kêkê LM, Samouda H, Jacobs J, di Pompeo C, Lemdani M, Hubert H, et al. Body mass index and childhood obesity classification systems: A comparison of the French, International Obesity Task Force (IOTF) and World Health Organization (WHO) references. Revue d'Épidémiologie et de Santé Publique. 2015;63(3):173-82.

5.   Casazza K, Fontaine KR, Astrup A, Birch LL, Brown AW, Bohan Brown MM, et al. Myths, Presumptions, and Facts about Obesity. New England Journal of Medicine. 2013;368(5):446-54.

6.   Wilson PW, D'Agostino RB, Sullivan L, Parise H, Kannel WB. Overweight and obesity as determinants of cardiovascular risk: the Framingham experience. Arch Intern Med. 2002;162(16):1867-72.

7.   Capodaglio P, Liuzzi A. Obesity: a disabling disease or a condition favoring disability?: Eur J Phys Rehabil Med. 2013 Jun;49(3):395-8.

8.   Bhaskaran K, Douglas I, Forbes H, dos-Santos-Silva I, Leon DA, Smeeth L. Body-mass index and risk of 22 specific cancers: a population-based cohort study of 5·24 million UK adults. Lancet. 2014;384(9945):755-65.

9.   Bosello O, Donataccio MP. Obesity paradox. Eating and Weight Disorders - Studies on Anorexia, Bulimia and Obesity. 2013;18(4):447-8.

10.   Weir CB, Jan A. BMI Classification Percentile And Cut Off Points.

11.   Neovius M, Linné Y, Barkeling B, Rössner S. Discrepancies between classification systems of childhood obesity. Obesity reviews : an official journal of the International Association for the Study of Obesity. 2004;5(2):105-14.

12.   Wang Y, Wang JQ. Standard definition of child overweight and obesity worldwide. Authors' standard compares well with WHO standard. BMJ (Clinical research ed). 2000;321(7269):1158-.

13.   Ogden C, Flegal K. Changes in Terminology for Childhood Overweight and Obesity. National health statistics reports. 2010;25:1-5.

14.   de Onis M, Onyango AW, Borghi E, Siyam A, Nishida C, Siekmann J. Development of a WHO growth reference for school-aged children and adolescents. Bull World Health Organ. 2007;85(9):660-7.

15.   Li K, Haynie D, Palla H, Lipsky L, Iannotti RJ, Simons-Morton B. Assessment of adolescent weight status: Similarities and differences between

CDC, IOTF, and WHO references. Preventive medicine. 2016;87:151-4. Epub 2016/02/26.

16.   Reilly JJ, Dorosty AR, Emmett PM. Prevalence of overweight and obesity in British children: cohort study. BMJ (Clinical research ed). 1999;319(7216):1039-.

17.   Guillaume M. Defining obesity in childhood: current practice. The American journal of clinical nutrition. 1999;70(1).

18.   Cole TJ, Bellizzi MC, Flegal KM, Dietz WH. Establishing a standard definition for child overweight and obesity worldwide: international survey. BMJ. 2000;320(7244):1240.

19.   Dietz WH. Health consequences of obesity in youth: childhood predictors of adult disease. Pediatrics. 1998;101(3 Pt 2):518-25.

20.   Morrison JA, Barton BA, Biro FM, Daniels SR, Sprecher DL. Overweight, fat patterning, and cardiovascular disease risk factors in black and white boys. J Pediatr. 1999;135(4):451-7.

21.   Reilly JJ, Wilson ML, Summerbell CD, Wilson DC. Obesity: diagnosis, prevention, and treatment; evidence based answers to common questions. Arch Dis Child. 2002;86(6):392-4.

22.   Carrascosa A, Fernández JM, Fernández C, Ferrández A, López-Siguero JP, Sánchez E, et al. Estudios españoles de crecimiento 2008. Nuevos patrones antropométricos. Endocrinología y Nutrición. 2008;55(10):484-506.

23.   Sánchez González E, Carrascosa Lezcano A, Fernández García JM, Ferrández Longás A, López de Lara D, López-Siguero JP. Estudios españoles de crecimiento: situación actual, utilidad y recomendaciones de uso. Anales de Pediatría. 2011;74(3):193.e1-.e16.

24.   Organization WH. Obesity and overweight. 2016; Available from: https://www.who.int/en/news-room/fact-sheets/detail/obesity-and-overweight.

25.   Hales CM, Carroll MD, Fryar CD, Ogden CL. Prevalence of Obesity Among Adults and Youth: United States, 2015-2016. NCHS Data Brief. 2017;288:1-8.

26.   Global, regional, and national comparative risk assessment of 84 behavioural, environmental and occupational, and metabolic risks or clusters of risks for 195 countries and territories, 1990-2017: a systematic analysis for the Global Burden of Disease Study 2017. Lancet. 2018;392(10159):1923-94.

27.   Colditz GA, Willett WC, Rotnitzky A, Manson JE. Weight gain as a risk factor for clinical diabetes mellitus in women. Ann Intern Med. 1995;122(7):481-6.

28.   Manson JE, Nathan DM, Krolewski AS, Stampfer MJ, Willett WC, Hennekens CH. A prospective study of exercise and incidence of diabetes among US male physicians. Jama. 1992;268(1):63-7.

29.   Greven KM, Corn BW. Endometrial cancer. Curr Probl Cancer. 1997;21(2):65-127.

30.   Blundell JE, Baker JL, Boyland E, Blaak E, Charzewska J, de Henauw S, et al. Variations in the Prevalence of Obesity Among European Countries, and a Consideration of Possible Causes. Obes Facts. 2017;10(1):25-37. Epub 2017/02/11.

31.   Withrow D, Alter DA. The economic burden of obesity worldwide: a systematic review of the direct costs of obesity. Obesity reviews : an official journal of the International Association for the Study of Obesity. 2011;12(2):131-41.

32.   Aranceta J, Pérez Rodrigo C, Majem LS, Barba LR, Izquierdo JQ, Vioque J, et al. Prevalencia de la obesidad en España: resultados del estudio SEEDO 2000. Medicina Clínica. 2003;120(16):608-12.

33.   Gutiérrez-Fisac JL, Guallar-Castillón P, León-Muñoz LM, Graciani A, Banegas JR, Rodríguez-Artalejo F. Prevalence of general and abdominal obesity in the adult population of Spain, 2008–2010: the ENRICA study. Obesity Reviews. 2012;13(4):388-92.

34.   Aranceta-Bartrina J, Pérez-Rodrigo C, Alberdi-Aresti G, Ramos-Carrera N, Lázaro-Masedo S. Prevalence of General Obesity and Abdominal Obesity in the Spanish Adult Population (Aged 25-64 Years) 2014-2015: The ENPE Study. Rev Esp Cardiol. 2016;69(6):579-87.

35.   Aranceta-Bartrina J, Gianzo-Citores M, Pérez-Rodrigo C. Prevalence of overweight, obesity and abdominal obesity in the Spanish population aged 3 to 24 years. The ENPE study. Rev Esp Cardiol. 2020;73(4):290-9.

36.   García-Solano M, Gutiérrez-González E, López-Sobaler AM, Dal Re Saavedra M, Robledo de Dios T, Villar-Villalba C, et al. [Weight status in the 6 to 9 year-old school population in Spain: Results of the ALADINO 2015 Study]. An Pediatr. 2020;13(20):026.

37.   Sabin MA, Ford AL, Holly JMP, Hunt LP, Crowne EC, Shield JPH. Characterisation of morbidity in a UK, hospital based, obesity clinic. Archives of disease in childhood. 2006;91(2):126-30. Epub 2005/10/24.

38.   Morrison JA, Friedman LA, Gray-McGuire C. Metabolic syndrome in childhood predicts adult cardiovascular disease 25 years later: the Princeton Lipid Research Clinics Follow-up Study. Pediatrics. 2007;120(2):340-5.

39.   Wang LY, Denniston M, Lee S, Galuska D, Lowry R. Long-term Health and Economic Impact of Preventing and Reducing Overweight and Obesity in Adolescence. Journal of Adolescent Health. 2010;46(5):467-73.

40.   Simopoulos AP. An Increase in the Omega-6/Omega-3 Fatty Acid Ratio Increases the Risk for Obesity. Nutrients. 2016;8(3).

41.   Mozaffarian D. Dietary and Policy Priorities for Cardiovascular Disease, Diabetes, and Obesity: A Comprehensive Review. Circulation. 2016;133(2):187-225.

42.   Pérez Rodrigo C. Current mapping of obesity. Nutr Hosp. 2013;5:21-31.

43.   Żukiewicz-Sobczak W, Wróblewska P, Zwoliński J, Chmielewska-Badora J, Adamczuk P, Krasowska E, et al. Obesity and poverty paradox in developed countries. Ann Agric Environ Med. 2014;21(3):590-4.

44.   Apovian CM. Obesity: definition, comorbidities, causes, and burden. Am J Manag Care. 2016;22(7 Suppl):s176-85.

45.   Hoey H. Management of obesity in children differs from that of adults. Proc Nutr Soc. 2014;73(4):519-25.

46.   Tobias DK, Chen M, Manson JE, Ludwig DS, Willett W, Hu FB. Effect of low-fat diet interventions versus other diet interventions on long-term weight change in adults: a systematic review and meta-analysis. Lancet Diabetes Endocrinol. 2015;3(12):968-79.

47.   Gow ML, Ho M, Burrows TL, Baur LA, Stewart L, Hutchesson MJ, et al. Impact of dietary macronutrient distribution on BMI and cardiometabolic outcomes in overweight and obese children and adolescents: a systematic review. Nutr Rev. 2014;72(7):453-70.

48.   da Silva-Santi LG, Antunes MM, Caparroz-Assef SM, Carbonera F, Masi LN, Curi R, et al. Liver Fatty Acid Composition and Inflammation in Mice Fed with High-Carbohydrate Diet or High-Fat Diet. Nutrients. 2016;8(11).

49.   Mensink RP, Zock PL, Kester AD, Katan MB. Effects of dietary fatty acids and carbohydrates on the ratio of serum total to HDL cholesterol and on serum lipids and apolipoproteins: a meta-analysis of 60 controlled trials. The American journal of clinical nutrition. 2003;77(5):1146-55.

50.   Sacks FM, Katan M. Randomized clinical trials on the effects of dietary fat and carbohydrate on plasma lipoproteins and cardiovascular disease. Am J Med. 2002;30(113):00987-1.

51.   Jakobsen MU, O'Reilly EJ, Heitmann BL, Pereira MA, Bälter K, Fraser GE, et al. Major types of dietary fat and risk of coronary heart disease: a pooled analysis of 11 cohort studies. The American journal of clinical nutrition. 2009;89(5):1425-32.

52.   Koliaki C, Spinos T, Spinou M, Brinia M-E, Mitsopoulou D, Katsilambros N. Defining the Optimal Dietary Approach for Safe, Effective and Sustainable Weight Loss in Overweight and Obese Adults. Healthcare (Basel). 2018;6(3):73.

53.   Mansoor N, Vinknes KJ, Veierød MB, Retterstøl K. Effects of low-carbohydrate diets v. low-fat diets on body weight and cardiovascular risk factors: a meta-analysis of randomised controlled trials. Br J Nutr. 2016;115(3):466-79.

54.   Nittari G, Scuri S, Petrelli F, Pirillo I, di Luca NM, Grappasonni I. Fighting obesity in children from European World Health Organization member states. Epidemiological data, medical-social aspects, and prevention programs. Clin Ter. 2019;170(3):e223-e30.

55.   Martini D. Health Benefits of Mediterranean Diet. Nutrients. 2019;11(8).

56.   Schwingshackl L, Schwedhelm C, Galbete C, Hoffmann G. Adherence to Mediterranean Diet and Risk of Cancer: An Updated Systematic Review and Meta-Analysis. Nutrients. 2017;9(10).

57.   Estruch R, Ros E, Salas-Salvadó J, Covas M-I, Corella D, Arós F, et al. Primary Prevention of Cardiovascular Disease with a Mediterranean Diet Supplemented with Extra-Virgin Olive Oil or Nuts. New England Journal of Medicine. 2018;378(25):e34.

58.   Papadaki A, Nolen-Doerr E, Mantzoros CS. The Effect of the Mediterranean Diet on Metabolic Health: A Systematic Review and Meta-Analysis of Controlled Trials in Adults. Nutrients. 2020;12(11).

59.   Dernini S, Berry EM, Serra-Majem L, La Vecchia C, Capone R, Medina FX, et al. Med Diet 4.0: the Mediterranean diet with four sustainable benefits. Public health nutrition. 2017;20(7):1322-30.

60.   Rajjo T, Mohammed K, Alsawas M, Ahmed AT, Farah W, Asi N, et al. Treatment of Pediatric Obesity: An Umbrella Systematic Review. The Journal of clinical endocrinology and metabolism. 2017;102(3):763-75.

61.   Kêkê LM, Samouda H, Jacobs J, di Pompeo C, Lemdani M, Hubert H, et al. Body mass index and childhood obesity classification systems: A comparison of the French, International Obesity Task Force (IOTF) and World Health Organization (WHO) references. Rev Epidemiol Sante Publique. 2015;63(3):173-82.

62.   Guo SS, Chumlea WC, Roche AF, Siervogel RM. Age- and maturity-related changes in body composition during adolescence into adulthood: the Fels Longitudinal Study. Int J Obes Relat Metab Disord. 1997;21(12):1167-75.

63.   Rolland-Cachera M-F, Deheeger M, Guilloud-Bataille M, Avons P, Patois E, Sempé M. Tracking the development of adiposity from one month of age to adulthood. Annals of Human Biology. 1987;14(3):219-29.

64.   Guo SS, Roche AF, Chumlea WC, Gardner JD, Siervogel RM. The predictive value of childhood body mass index values for overweight at age 35 y. The American journal of clinical nutrition. 1994;59(4):810-9.

65.   Serdula MK, Ivery D, Coates RJ, Freedman DS, Williamson DF, Byers T. Do obese children become obese adults? A review of the literature. Prev Med. 1993;22(2):167-77.

66.   Sinha A, Kling S. A review of adolescent obesity: prevalence, etiology, and treatment. Obes Surg. 2009;19(1):113-20.

67.   Dart AB, Martens PJ, Rigatto C, Brownell MD, Dean HJ, Sellers EA. Earlier onset of complications in youth with type 2 diabetes. Diabetes Care. 2014;37(2):436-43.

68.   Pietrobelli A, Malavolti M, Battistini NC, Fuiano N. Metabolic syndrome: a child is not a small adult. Int J Pediatr Obes. 2008;1:67-71.

69.   Van Cauwenberghe E, Maes L, Spittaels H, van Lenthe FJ, Brug J, Oppert JM, et al. Effectiveness of school-based interventions in Europe to

promote healthy nutrition in children and adolescents: systematic review of published and 'grey' literature. Br J Nutr. 2010;103(6):781-97.

70.   Verbestel V, Henauw SD, Maes L, Haerens L, Mårild S, Eiben G, et al. Using the intervention mapping protocol to develop a community-based intervention for the prevention of childhood obesity in a multi-centre European project: the IDEFICS intervention. International Journal of Behavioral Nutrition and Physical Activity. 2011;8(1):82.

71.   Allender S, Millar L, Hovmand P, Bell C, Moodie M, Carter R, et al. Whole of Systems Trial of Prevention Strategies for Childhood Obesity: WHO STOPS Childhood Obesity. Int J Environ Res Public Health. 2016;13(11).

72.   Simmons A, Mavoa HM, Bell AC, De Courten M, Schaaf D, Schultz J, et al. Creating community action plans for obesity prevention using the ANGELO (Analysis Grid for Elements Linked to Obesity) Framework. Health Promotion International. 2009;24(4):311-24.

73.   Godino JG, Merchant G, Norman GJ, Donohue MC, Marshall SJ, Fowler JH, et al. Using social and mobile tools for weight loss in overweight and obese young adults (Project SMART): a 2 year, parallel-group, randomised, controlled trial. The lancet Diabetes & endocrinology. 2016;4(9):747-55. Epub 2016/07/14.

74.   Hamer M, Stamatakis E. Metabolically healthy obesity and risk of all-cause and cardiovascular disease mortality. The Journal of clinical endocrinology and metabolism. 2012;97(7):2482-8.

75.   Sims EAH. Are there persons who are obese, but metabolically healthy? Metabolism. 2001;50(12):1499-504.

76.   Pajunen P, Kotronen A, Korpi-Hyövälti E, Keinänen-Kiukaanniemi S, Oksa H, Niskanen L, et al. Metabolically healthy and unhealthy obesity phenotypes in the general population: the FIN-D2D Survey. BMC Public Health. 2011;11(1):754.

77.   Wildman RP, Muntner P, Reynolds K, McGinn AP, Rajpathak S, Wylie-Rosett J, et al. The Obese Without Cardiometabolic Risk Factor Clustering and the Normal Weight With Cardiometabolic Risk Factor Clustering: Prevalence and Correlates of 2 Phenotypes Among the US Population (NHANES 1999-2004). Archives of internal medicine. 2008;168(15):1617-24.

78.   Shea JL, Randell EW, Sun G. The Prevalence of Metabolically Healthy Obese Subjects Defined by BMI and Dual-Energy X-Ray Absorptiometry. Obesity. 2011;19(3):624-30.

79.   van Vliet-Ostaptchouk JV, Nuotio M-L, Slagter SN, Doiron D, Fischer K, Foco L, et al. The prevalence of metabolic syndrome and metabolically healthy obesity in Europe: a collaborative analysis of ten large cohort studies. BMC Endocrine Disorders. 2014;14(1):9.

80.   Roberson LL, Aneni EC, Maziak W, Agatston A, Feldman T, Rouseff M, et al. Beyond BMI: The "Metabolically healthy obese" phenotype & its

association with clinical/subclinical cardiovascular disease and all-cause mortality -- a systematic review. BMC Public Health. 2014;14(14):1471-2458.

81.   Blüher M. The distinction of metabolically 'healthy' from 'unhealthy' obese individuals. Curr Opin Lipidol. 2010;21(1):38-43.

82.   Prince RL, Kuk JL, Ambler KA, Dhaliwal J, Ball GD. Predictors of metabolically healthy obesity in children. Diabetes Care. 2014;37(5):1462-8.

83.   Vukovic R, Mitrovic K, Milenkovic T, Todorovic S, Soldatovic I, Sipetic-Grujicic S, et al. Insulin-sensitive obese children display a favorable metabolic profile. European Journal of Pediatrics. 2013;172(2):201-6.

84.   Karelis AD, Rabasa-Lhoret R. Obesity: Can inflammatory status define metabolic health?: Nat Rev Endocrinol. 2013 Dec;9(12):694-5. doi: 10.1038/nrendo.2013.198. Epub 2013 Oct 15.

85.   Sutherland J, McKinley B, Eckel R. Sutherland JP, McKinley B, Eckel RH. The metabolic syndrome and inflammation. Metab Syndr Relat Disord 2: 82-104. Metabolic syndrome and related disorders. 2004;2:82-104.

86.   DeBoer MD. Obesity, systemic inflammation, and increased risk for cardiovascular disease and diabetes among adolescents: A need for screening tools to target interventions. Nutrition. 2013;29(2):379-86.

87.   van Beek L, Lips MA, Visser A, Pijl H, Ioan-Facsinay A, Toes R, et al. Increased systemic and adipose tissue inflammation differentiates obese women with T2DM from obese women with normal glucose tolerance. Metabolism. 2014;63(4):492-501.

88.   Phillips CM, Perry IJ. Does inflammation determine metabolic health status in obese and nonobese adults? The Journal of clinical endocrinology and metabolism. 2013;98(10):2013-38.

89.   Stefan N, Kantartzis K, Machann J, Schick F, Thamer C, Rittig K, et al. Identification and characterization of metabolically benign obesity in humans. Arch Intern Med. 2008;168(15):1609-16.

90.   Teixeira PJ, Carraça EV, Marques MM, Rutter H, Oppert JM, De Bourdeaudhuij I, et al. Successful behavior change in obesity interventions in adults: a systematic review of self-regulation mediators. BMC Med. 2015;13(84):015-0323.

91.   EFSA Panel on Dietetic Products N, Allergies. Scientific Opinion on Dietary Reference Values for fats, including saturated fatty acids, polyunsaturated fatty acids, monounsaturated fatty acids, trans fatty acids, and cholesterol. EFSA Journal. 2010;8(3):1461.

92.   Fao. Fats and fatty acids in human nutrition: Report of an expert consultation2010.

93.   Camilleri M, Staiano A. Insights on Obesity in Children and Adults: Individualizing Management. Trends in Endocrinology & Metabolism. 2019;30(10):724-34.

94.   Abbott SK, Else PL, Atkins TA, Hulbert AJ. Fatty acid composition of membrane bilayers: Importance of diet polyunsaturated fat balance. Biochimica et Biophysica Acta (BBA) - Biomembranes. 2012;1818(5):1309-17.

95.   Perona JS. Membrane lipid alterations in the metabolic syndrome and the role of dietary oils. Biochimica et Biophysica Acta (BBA) - Biomembranes. 2017;1859(9, Part B):1690-703.

96.   Escribá PV. Membrane-lipid therapy: A historical perspective of membrane-targeted therapies — From lipid bilayer structure to the pathophysiological regulation of cells. Biochimica et Biophysica Acta (BBA) - Biomembranes. 2017;1859(9, Part B):1493-506.

97.   Vailati-Riboni M, Palombo V, Loor JJ. What Are Omics Sciences? In: Ametaj BN, editor. Periparturient Diseases of Dairy Cows: A Systems Biology Approach. Cham: Springer International Publishing; 2017. p. 1-7.

98.   Boden G. Endoplasmic reticulum stress: another link between obesity and insulin resistance/inflammation? Diabetes. 2009;58(3):518-9.

99.   de Roos B, Geelen A, Ross K, Rucklidge G, Reid M, Duncan G, et al. Identification of potential serum biomarkers of inflammation and lipid modulation that are altered by fish oil supplementation in healthy volunteers. Proteomics. 2008;8(10):1965-74.

100.   Boja ES, Kinsinger CR, Rodriguez H, Srinivas P, on behalf of Omics Integration Workshop P. Integration of omics sciences to advance biology and medicine. Clin Proteomics. 2014;11(1):45.

101.   Ferguson LR, De Caterina R, Görman U, Allayee H, Kohlmeier M, Prasad C, et al. Guide and Position of the International Society of Nutrigenetics/Nutrigenomics on Personalised Nutrition: Part 1 - Fields of Precision Nutrition. J Nutrigenet Nutrigenomics. 2016;9(1):12-27.

102.   de Toro-Martín J, Arsenault BJ, Després JP, Vohl MC. Precision Nutrition: A Review of Personalized Nutritional Approaches for the Prevention and Management of Metabolic Syndrome. Nutrients. 2017;9(8).

103.   Barrea L, Annunziata G, Bordoni L, Muscogiuri G, Colao A, Savastano S. Nutrigenetics-personalized nutrition in obesity and cardiovascular diseases. Int J Obes Suppl. 2020;10(1):1-13.

104.   Fenech M. The Genome Health Clinic and Genome Health Nutrigenomics concepts: diagnosis and nutritional treatment of genome and epigenome damage on an individual basis. Mutagenesis. 2005;20(4):255-69.

105.   Comerford KB, Pasin G. Gene-Dairy Food Interactions and Health Outcomes: A Review of Nutrigenetic Studies. Nutrients. 2017;9(7):710.

106.   Loria-Kohen V, Espinosa-Salinas I, Ramirez de Molina A, Casas-Agustench P, Herranz J, Molina S, et al. A genetic variant of PPARA modulates cardiovascular risk biomarkers after milk consumption. Nutrition. 2014;30(10):1144-50.

107.   Odriozola L, Corrales FJ. Discovery of nutritional biomarkers: future directions based on omics technologies. International journal of food sciences and nutrition. 2015;66(1):1038224.

108.   Frishman WH. Biologic markers as predictors of cardiovascular disease. Am J Med. 1998;104(6A):00184-3.

109.   Brennan L. Metabolomics in nutrition research: current status and perspectives. Biochem Soc Trans. 2013;41(2):670-3.

110.   Kinross J, Li JV, Muirhead LJ, Nicholson J. Nutritional modulation of the metabonome: applications of metabolic phenotyping in translational nutritional research. Curr Opin Gastroenterol. 2014;30(2):196-207.

111.   Picó C, Serra F, Rodríguez AM, Keijer J, Palou A. Biomarkers of Nutrition and Health: New Tools for New Approaches. Nutrients. 2019;11(5).

112.   Han X, Gross RW. Global analyses of cellular lipidomes directly from crude extracts of biological samples by ESI mass spectrometry: a bridge to lipidomics. Journal of lipid research. 2003;44(6):1071-9.

113.   Rolim AEH, Henrique-Araújo R, Ferraz EG, de Araújo Alves Dultra FK, Fernandez LG. Lipidomics in the study of lipid metabolism: Current perspectives in the omic sciences. Gene. 2015;554(2):131-9.

114.   Navas-Iglesias N, Carrasco-Pancorbo A, Cuadros-Rodríguez L. From lipids analysis towards lipidomics, a new challenge for the analytical chemistry of the 21st century. Part II: Analytical lipidomics. TrAC Trends in Analytical Chemistry. 2009;28(4):393-403.

115.   Meikle PJ, Wong G, Barlow CK, Kingwell BA. Lipidomics: Potential role in risk prediction and therapeutic monitoring for diabetes and cardiovascular disease. Pharmacology & therapeutics. 2014;143(1):12-23.

116.   Zhao Y-Y, Miao H, Cheng X-L, Wei F. Lipidomics: Novel insight into the biochemical mechanism of lipid metabolism and dysregulation-associated disease. Chemico-biological interactions. 2015;240:220-38.

117.   Wenk MR. The emerging field of lipidomics. Nature reviews Drug discovery. 2005;4(7):594-610.

118.   Hu C, van der Heijden R, Wang M, van der Greef J, Hankemeier T, Xu G. Analytical strategies in lipidomics and applications in disease biomarker discovery. Journal of Chromatography B. 2009;877(26):2836-46.

119.   Takenawa T, Itoh T. Phosphoinositides, key molecules for regulation of actin cytoskeletal organization and membrane traffic from the plasma membrane. Biochimica et biophysica acta. 2001;31(3):190-206.

120.   Escribá PV. Membrane-lipid therapy: a new approach in molecular medicine. Trends in Molecular Medicine. 2006;12(1):34-43.

121.   Schwann T. Microscopical Researches into the Accordance in the Structure and Growth of Animals and Plants: Рипол Классик; 1847.

122.   Nelson DL, Cox MM, Lehninger AL. Lehninger Principles of biochemistry. 7th ed. ed. New York: W. H. Freeman; 2017.

123.   Ferreri C, Chatgilialoglu C. Membrane Lipidomics for Personalized Health. 2015. p. 87-8.

124.   Ladha S. The lipid organisation of the cell membrane. Grasas y Aceites. 2000;51.

125.   Fahy E, Subramaniam S, Brown HA, Glass CK, Merrill AH, Jr., Murphy RC, et al. A comprehensive classification system for lipids. Journal of lipid research. 2005;46(5):839-61.

126.   Fahy E, Subramaniam S, Murphy RC, Nishijima M, Raetz CR, Shimizu T, et al. Update of the LIPID MAPS comprehensive classification system for lipids. Journal of lipid research. 2009;50(14):19.

127.   Lauritzen L, Hansen HS, Jorgensen MH, Michaelsen KF. The essentiality of long chain n-3 fatty acids in relation to development and function of the brain and retina. Prog Lipid Res. 2001;40(1-2):1-94.

128.   Lehninger AL, Nelson DL, Cox MM, Freeman WH, Company, Macmillan P. Lehninger principles of biochemistry. New York; Basingstoke: W. H. Freeman and Company : Macmillan Higher Education; 2017.

129.   Makrides M, Neumann MA, Byard RW, Simmer K, Gibson RA. Fatty acid composition of brain, retina, and erythrocytes in breast- and formula-fed infants. Am J Clin Nutr. 1994;60(2):189-94.

130.   Baur LA, O'Connor J, Pan DA, Wu BJ, O'Connor MJ, Storlien LH. Relationships between the fatty acid composition of muscle and erythrocyte membrane phospholipid in young children and the effect of type of infant feeding. Lipids. 2000;35(1):77-82.

131.   Ogura T, Takada H, Okuno M, Kitade H, Matsuura T, Kwon M, et al. Fatty acid composition of plasma, erythrocytes and adipose: their correlations and effects of age and sex. Lipids. 2010;45(2):137-44.

132.   Piomelli S, Seaman C. Mechanism of red blood cell aging: relationship of cell density and cell age. Am J Hematol. 1993;42(1):46-52.

133.   Ferreri C, Chatgilialoglu C. Membrane lipidomics for personalized health. New York: Wiley; 2015. p. p.

134.   Mylonis I, Simos G, Paraskeva E. Hypoxia-Inducible Factors and the Regulation of Lipid Metabolism. Cells. 2019;8(3).

135.   Organization WH. Healthy diet. Genova: World Health Organization; 2018 [cited 2019]; Available from: https://www.who.int/news-room/fact-sheets/detail/healthy-diet.

136.   Scientific Opinion on Dietary Reference Values for fats, including saturated fatty acids, polyunsaturated fatty acids, monounsaturated fatty acids, trans fatty acids, and cholesterol. EFSA Journal. 2010;8(3).

137.   Wall R, Ross RP, Fitzgerald GF, Stanton C. Fatty acids from fish: the anti-inflammatory potential of long-chain omega-3 fatty acids. Nutr Rev. 2010;68(5):280-9.

138.   Wang X, Lin H, Gu Y. Multiple roles of dihomo-γ-linolenic acid against proliferation diseases. Lipids in Health and Disease. 2012;11:25-.

139.   Wang X, Lin H, Gu Y. Multiple roles of dihomo-gamma-linolenic acid against proliferation diseases. Lipids in health and disease. 2012;11(25):11-25.

140.   Innes JK, Calder PC. Omega-6 fatty acids and inflammation. Prostaglandins, leukotrienes, and essential fatty acids. 2018;132:41-8.

141.   Brenner RR. Hormonal modulation of delta6 and delta5 desaturases: case of diabetes. Prostaglandins Leukot Essent Fatty Acids. 2003;68(2):151-62.

142.   Siriwardhana N, Kalupahana NS, Moustaid-Moussa N. Health benefits of n-3 polyunsaturated fatty acids: eicosapentaenoic acid and docosahexaenoic acid. Adv Food Nutr Res. 2012;65:211-22.

143.   Calder PC. Polyunsaturated fatty acids and inflammatory processes: New twists in an old tale. Biochimie. 2009;91(6):791-5.

144.   Guesnet P, Alessandri J-M. Docosahexaenoic acid (DHA) and the developing central nervous system (CNS) – Implications for dietary recommendations. Biochimie. 2011;93(1):7-12.

145.   Burdge G. Alpha-linolenic acid metabolism in men and women: nutritional and biological implications. Curr Opin Clin Nutr Metab Care. 2004;7(2):137-44.

146.   Brenna JT, Salem N, Jr., Sinclair AJ, Cunnane SC. alpha-Linolenic acid supplementation and conversion to n-3 long-chain polyunsaturated fatty acids in humans. Prostaglandins Leukot Essent Fatty Acids. 2009;80(2-3):85-91.

147.   Simopoulos AP. The omega-6/omega-3 fatty acid ratio: health implications. OCL. 2010;17(5):267-75.

148.   Sébédio J-L, Christie WW. Chapter 7 - Metabolism of trans fatty acid isomers. In: Destaillats F, Sébédio J-L, Dionisi F, Chardigny J-M, editors. Trans Fatty Acids in Human Nutrition (Second Edition): Woodhead Publishing; 2012. p. 163-94.

149.   Simopoulos AP. An Increase in the Omega-6/Omega-3 Fatty Acid Ratio Increases the Risk for Obesity. Nutrients. 2016;8(3):128-.

150.   Cordain L, Eaton SB, Sebastian A, Mann N, Lindeberg S, Watkins BA, et al. Origins and evolution of the Western diet: health implications for the 21st century. The American journal of clinical nutrition. 2005;81(2):341-54.

151.   Chilton FH, Dutta R, Reynolds LM, Sergeant S, Mathias RA, Seeds MC. Precision Nutrition and Omega-3 Polyunsaturated Fatty Acids: A Case for Personalized Supplementation Approaches for the Prevention and Management of Human Diseases. Nutrients. 2017;9(11).

152.   Miller M, Stone NJ, Ballantyne C, Bittner V, Criqui MH, Ginsberg HN, et al. Triglycerides and cardiovascular disease: a scientific statement from the American Heart Association. Circulation. 2011;123(20):2292-333.

153.   Blasbalg TL, Hibbeln JR, Ramsden CE, Majchrzak SF, Rawlings RR. Changes in consumption of omega-3 and omega-6 fatty acids in the United States during the 20th century. The American journal of clinical nutrition. 2011;93(5):950-62.

154.   Health effects of dietary risks in 195 countries, 1990-2017: a systematic analysis for the Global Burden of Disease Study 2017. Lancet. 2019;393(10184):1958-72.

155.   Kantor ED, Rehm CD, Du M, White E, Giovannucci EL. Trends in Dietary Supplement Use Among US Adults From 1999-2012. Jama. 2016;316(14):1464-74.

156.   Misund B, Oglend A, Pincinato RBM. The rise of fish oil: From feed to human nutritional supplement. Aquaculture Economics & Management. 2017;21(2):185-210.

157.   Clarke TC, Black LI, Stussman BJ, Barnes PM, Nahin RL. Trends in the use of complementary health approaches among adults: United States, 2002-2012. Natl Health Stat Report. 2015;10(79):1-16.

158.   Hilleman DE, Teply R, Packard KA. Knowledge, Perceptions, and Patterns of Fish Oil Use in Cardiac Patients. J Pharm Pract. 2020;33(5):580-5.

159.   Shahidi F, Ambigaipalan P. Omega-3 Polyunsaturated Fatty Acids and Their Health Benefits. Annu Rev Food Sci Technol. 2018;9:345-81.

160.   Flock MR, Skulas-Ray AC, Harris WS, Gaugler TL, Fleming JA, Kris-Etherton PM. Effects of supplemental long-chain omega-3 fatty acids and erythrocyte membrane fatty acid content on circulating inflammatory markers in a randomized controlled trial of healthy adults. Prostaglandins, leukotrienes, and essential fatty acids. 2014;91(4):161-8.

161.   Radcliffe JE, Thomas J, Bramley AL, Kouris-Blazos A, Radford BE, Scholey AB, et al. Controversies in omega-3 efficacy and novel concepts for application. Journal of Nutrition & Intermediary Metabolism. 2016;5:11-22.

162.   Ferreri C, Chatgilialoglu, C. Membrane Lipidomics for Personalized Health. Chichester, UK: John Wiley & Sons; 2015.

163.   Madrigal C, Soto-Mendez MJ, Hernandez-Ruiz A, Ruiz E, Valero T, Avila JM, et al. Dietary and Lifestyle Patterns in the Spanish Pediatric Population (One to <10 Years Old): Design, Protocol, and Methodology of the EsNuPI Study. Nutrients. 2019;11(12).

164.   Fernandez-Ballart JD, Pinol JL, Zazpe I, Corella D, Carrasco P, Toledo E, et al. Relative validity of a semi-quantitative food-frequency questionnaire in an elderly Mediterranean population of Spain. Br J Nutr. 2010;103(12):1808-16.

165.   Ghezzo A, Visconti P, Abruzzo PM, Bolotta A, Ferreri C, Gobbi G, et al. Oxidative Stress and Erythrocyte Membrane Alterations in Children with Autism: Correlation with Clinical Features. PloS one. 2013;8(6).

166.   Sansone A, Tolika E, Louka M, Sunda V, Deplano S, Melchiorre M, et al. Hexadecenoic Fatty Acid Isomers in Human Blood Lipids and Their Relevance for the Interpretation of Lipidomic Profiles. PloS one. 2016;11(4).

167.   Giacometti G, Ferreri C, Sansone A, Chatgilialoglu C, Marzetti C, Spyratou E, et al. High predictive values of RBC membrane-based diagnostics

by biophotonics in an integrated approach for Autism Spectrum Disorders. Scientific reports. 2017;7(1):017-10361.

168.   Amezaga J, Arranz S, Urruticoechea A, Ugartemendia G, Larraioz A, Louka M, et al. Altered Red Blood Cell Membrane Fatty Acid Profile in Cancer Patients. Nutrients. 2018;10(12). Epub 2018/12/06.

169.   Van Der Vegt SGL, Ruben AMT, Werre JM, Palsma DMH, Verhoef CW, de Gier J, et al. Counterflow centrifugation of red cell populations: a cell age related separation technique. British Journal of Haematology. 1985;61(3):393-403.

170.   Breil C, Abert Vian M, Zemb T, Kunz W, Chemat F. "Bligh and Dyer" and Folch Methods for Solid-Liquid-Liquid Extraction of Lipids from Microorganisms. Comprehension of Solvatation Mechanisms and towards Substitution with Alternative Solvents. International journal of molecular sciences. 2017;18(4).

171.   Ferreri C, Chatgilialoglu C. Role of fatty acid-based functional lipidomics in the development of molecular diagnostic tools. Expert Rev Mol Diagn. 2012;12(7):767-80.

172.   Harris WS, Von Schacky C. The Omega-3 Index: a new risk factor for death from coronary heart disease? Prev Med. 2004;39(1):212-20.

173.   Jacobs S, Jäger S, Jansen E, Peter A, Stefan N, Boeing H, et al. Associations of Erythrocyte Fatty Acids in the De Novo Lipogenesis Pathway with Proxies of Liver Fat Accumulation in the EPIC-Potsdam Study. PloS one. 2015;10(5).

174.   Pan DA, Hulbert AJ, Storlien LH. Dietary fats, membrane phospholipids and obesity. J Nutr. 1994;124(9):1555-65.

175.   Klein-Platat C, Drai J, Oujaa M, Schlienger JL, Simon C. Plasma fatty acid composition is associated with the metabolic syndrome and low-grade inflammation in overweight adolescents. The American journal of clinical nutrition. 2005;82(6):1178-84.

176.   Guerendiain M, Montes R, López-Belmonte G, Martín-Matillas M, Castellote AI, Martín-Bautista E, et al. Changes in plasma fatty acid composition are associated with improvements in obesity and related metabolic disorders: A therapeutic approach to overweight adolescents. Clin Nutr. 2018;37(1):149-56.

177.   Mayneris-Perxachs J, Guerendiain M, Castellote AI, Estruch R, Covas MI, Fitó M, et al. Plasma fatty acid composition, estimated desaturase activities, and their relation with the metabolic syndrome in a population at high risk of cardiovascular disease. Clin Nutr. 2014;33(1):90-7.

178.   Pietiläinen KH, Róg T, Seppänen-Laakso T, Virtue S, Gopalacharyulu P, Tang J, et al. Association of lipidome remodeling in the adipocyte membrane with acquired obesity in humans. PLoS biology. 2011;9(6):e1000623-e. Epub 2011/06/07.

179.   Harayama T, Riezman H. Understanding the diversity of membrane lipid composition. Nat Rev Mol Cell Biol. 2018;19(5):281-96.

180.   Gurzell EA, Wiesinger JA, Morkam C, Hemmrich S, Harris WS, Fenton JI. Is the omega-3 index a valid marker of intestinal membrane phospholipid EPA+DHA content? Prostaglandins, leukotrienes, and essential fatty acids. 2014;91(3):87-96. Epub 2014/04/24.

181.   Cox AJ, West NP, Cripps AW. Obesity, inflammation, and the gut microbiota. Lancet Diabetes Endocrinol. 2015;3(3):207-15.

182.   Sonnweber T, Pizzini A, Nairz M, Weiss G, Tancevski I. Arachidonic Acid Metabolites in Cardiovascular and Metabolic Diseases. International journal of molecular sciences. 2018;19(11).

183.   Burns JL, Nakamura MT, Ma DWL. Differentiating the biological effects of linoleic acid from arachidonic acid in health and disease. Prostaglandins, leukotrienes, and essential fatty acids. 2018;135:1-4.

184.   Belury MA, Cole RM, Bailey BE, Ke J-Y, Andridge RR, Kiecolt-Glaser JK. Erythrocyte linoleic acid, but not oleic acid, is associated with improvements in body composition in men and women. Molecular nutrition & food research. 2016;60(5):1206-12.

185.   Ailhaud G, Guesnet P, Cunnane SC. An emerging risk factor for obesity: does disequilibrium of polyunsaturated fatty acid metabolism contribute to excessive adipose tissue development? Br J Nutr. 2008;100(3):461-70.

186.   Muhlhausler BS, Ailhaud GP. Omega-6 polyunsaturated fatty acids and the early origins of obesity. Curr Opin Endocrinol Diabetes Obes. 2013;20(1):56-61.

187.   Fats and fatty acids in human nutrition. Report of an expert consultation. FAO Food Nutr Pap. 2010;91:1-166.

188.   Ruderman NB, Schneider SH, Berchtold P. The "metabolically-obese," normal-weight individual. The American journal of clinical nutrition. 1981;34(8):1617-21.

189.   Aung K, Lorenzo C, Hinojosa MA, Haffner SM. Risk of Developing Diabetes and Cardiovascular Disease in Metabolically Unhealthy Normal-Weight and Metabolically Healthy Obese Individuals. The Journal of Clinical Endocrinology & Metabolism. 2014;99(2):462-8.

190.   Yoo HJ, Hwang SY, Hong HC, Choi HY, Seo JA, Kim SG, et al. Association of metabolically abnormal but normal weight (MANW) and metabolically healthy but obese (MHO) individuals with arterial stiffness and carotid atherosclerosis. Atherosclerosis. 2014;234(1):218-23.

191.   Fan J, Song Y, Chen Y, Hui R, Zhang W. Combined effect of obesity and cardio-metabolic abnormality on the risk of cardiovascular disease: a meta-analysis of prospective cohort studies. Int J Cardiol. 2013;168(5):4761-8.

192.   Klaus WL, Yukiko N, Alexander MG, Shiming L. Are there serious adverse effects of omega-3 polyunsaturated fatty acid supplements? Journal of Food Bioactives. 2019;7(0).

193.   Corella D, Ordovás JM. [The role of omics in precision nutrition: strengths and weaknesses]. Nutr Hosp. 2018;35(Spec No4):10-8.

194.   Betts JA, Gonzalez JT. Personalised nutrition: What makes you so special? Nutrition Bulletin. 2016;41(4):353-9.

195.   Rudkowska I, Pérusse L, Bellis C, Blangero J, Després JP, Bouchard C, et al. Interaction between Common Genetic Variants and Total Fat Intake on Low-Density Lipoprotein Peak Particle Diameter: A Genome-Wide Association Study. J Nutrigenet Nutrigenomics. 2015;8(1):44-53.

196.   Ouellette C, Rudkowska I, Lemieux S, Lamarche B, Couture P, Vohl MC. Gene-diet interactions with polymorphisms of the MGLL gene on plasma low-density lipoprotein cholesterol and size following an omega-3 polyunsaturated fatty acid supplementation: a clinical trial. Lipids Health Dis. 2014;13(86):13-86.

197.   Vallée Marcotte B, Cormier H, Guénard F, Rudkowska I, Lemieux S, Couture P, et al. Novel Genetic Loci Associated with the Plasma Triglyceride Response to an Omega-3 Fatty Acid Supplementation. J Nutrigenet Nutrigenomics. 2016;9(1):1-11.

198.   McMahon G, Taylor AE, Davey Smith G, Munafò MR. Phenotype refinement strengthens the association of AHR and CYP1A1 genotype with caffeine consumption. PloS one. 2014;9(7).

199.   Ahmadi KR, Andrew T. Opportunism: a panacea for implementation of whole-genome sequencing studies in nutrigenomics research? Genes Nutr. 2014;9(2):014-0387.

200.   Özdemir V, Kolker E. Precision Nutrition 4.0: A Big Data and Ethics Foresight Analysis--Convergence of Agrigenomics, Nutrigenomics, Nutriproteomics, and Nutrimetabolomics. Omics. 2016;20(2):69-75.

201.   Goni L, Cuervo M, Milagro FI, Martínez JA. A genetic risk tool for obesity predisposition assessment and personalized nutrition implementation based on macronutrient intake. Genes Nutr. 2015;10(1):014-0445.

202.   Qi Q, Chu AY, Kang JH, Jensen MK, Curhan GC, Pasquale LR, et al. Sugar-sweetened beverages and genetic risk of obesity. N Engl J Med. 2012;367(15):1387-96.

203.   Olsen NJ, Ängquist L, Larsen SC, Linneberg A, Skaaby T, Husemoen LL, et al. Interactions between genetic variants associated with adiposity traits and soft drinks in relation to longitudinal changes in body weight and waist circumference. The American journal of clinical nutrition. 2016;104(3):816-26.

204.   Qi Q, Chu AY, Kang JH, Huang J, Rose LM, Jensen MK, et al. Fried food consumption, genetic risk, and body mass index: gene-diet interaction analysis in three US cohort studies. BMJ. 2014;19(348).

205.   Casas-Agustench P, Arnett DK, Smith CE, Lai CQ, Parnell LD, Borecki IB, et al. Saturated fat intake modulates the association between an obesity genetic risk score and body mass index in two US populations. J Acad Nutr Diet. 2014;114(12):1954-66.

206.   Kang JX. Gut microbiota and personalized nutrition: J Nutrigenet Nutrigenomics. 2013;6(2):I-II. doi: 10.1159/000353144. Epub 2013 Jun 14.

207.   Ridaura VK, Faith JJ, Rey FE, Cheng J, Duncan AE, Kau AL, et al. Gut microbiota from twins discordant for obesity modulate metabolism in mice. Science. 2013;341(6150):1241214.

208.   Zeevi D, Korem T, Zmora N, Israeli D, Rothschild D, Weinberger A, et al. Personalized Nutrition by Prediction of Glycemic Responses. Cell. 2015;163(5):1079-94.

209.   Garcia-Aloy M, Llorach R, Urpi-Sarda M, Jáuregui O, Corella D, Ruiz-Canela M, et al. A metabolomics-driven approach to predict cocoa product consumption by designing a multimetabolite biomarker model in free-living subjects from the PREDIMED study. Molecular nutrition & food research. 2015;59(2):212-20.

210.   Ortega-Azorín C, Sorlí JV, Estruch R, Asensio EM, Coltell O, González JI, et al. Amino acid change in the carbohydrate response element binding protein is associated with lower triglycerides and myocardial infarction incidence depending on level of adherence to the Mediterranean diet in the PREDIMED trial. Circ Cardiovasc Genet. 2014;7(1):49-58.

211.   Ryan NM, O'Donovan CB, Forster H, Woolhead C, Walsh MC. New tools for personalised nutrition: The Food4Me project. Nutrition Bulletin. 2015;40(2):134-9.

212.   Juonala M, Magnussen CG, Berenson GS, Venn A, Burns TL, Sabin MA, et al. Childhood Adiposity, Adult Adiposity, and Cardiovascular Risk Factors. New England Journal of Medicine. 2011;365(20):1876-85.

213.   Blüher S, Schwarz P. Metabolically healthy obesity from childhood to adulthood - Does weight status alone matter? Metabolism. 2014;63(9):1084-92.

214.   Wildman R, Muntner P, Reynolds K, McGinn A, Rajpathak S, Wylie-Rosett J, et al. The Obese Without Cardiometabolic Risk Factor Clustering and the Normal Weight With Cardiometabolic Risk Factor Clustering: Prevalence and Correlates of 2 Phenotypes Among the US Population (NHANES 1999-2004). Archives of internal medicine. 2008;168:1617-24.

215.   Pajunen P, Kotronen A, Korpi-Hyövälti E, Keinänen-Kiukaanniemi S, Oksa H, Niskanen L, et al. Metabolically healthy and unhealthy obesity phenotypes in the general population: the FIN-D2D Survey. BMC Public Health. 2011;11(754):1471-2458.

216.   Karelis AD, St-Pierre DH, Conus F, Rabasa-Lhoret R, Poehlman ET. Metabolic and body composition factors in subgroups of obesity: what do we know? The Journal of clinical endocrinology and metabolism. 2004;89(6):2569-75.

217.   Sims EA. Are there persons who are obese, but metabolically healthy? Metabolism. 2001;50(12):1499-504.

218.   Brochu M. What Are the Physical Characteristics Associated with a Normal Metabolic Profile Despite a High Level of Obesity in Postmenopausal Women? Journal of Clinical Endocrinology & Metabolism. 2001;86:1020-5.

219.   Primeau V, Coderre L, Karelis AD, Brochu M, Lavoie ME, Messier V, et al. Characterizing the profile of obese patients who are metabolically healthy. Int J Obes. 2011;35(7):971-81.

220.   Hamer M, Stamatakis E. Metabolically healthy obesity and risk of all-cause and cardiovascular disease mortality. The Journal of clinical endocrinology and metabolism. 2012;97(7):2482-8. Epub 2012/04/16.

221.   Phillips C, Perry I. Does Inflammation Determine Metabolic Health Status in Obese and Nonobese Adults? The Journal of clinical endocrinology and metabolism. 2013;98.

222.   Damanhoury S, Newton AS, Rashid M, Hartling L, Byrne JLS, Ball GDC. Defining metabolically healthy obesity in children: a scoping review. Obesity reviews : an official journal of the International Association for the Study of Obesity. 2018;19(11):1476-91.

223.   Vukovic R, Dos Santos TJ, Ybarra M, Atar M. Children With Metabolically Healthy Obesity: A Review. Front Endocrinol. 2019;10(865).

224.   Innis SM. Dietary lipids in early development: relevance to obesity, immune and inflammatory disorders. Curr Opin Endocrinol Diabetes Obes. 2007;14(5):359-64.

225.   Genio G. Morbid Obesity is Associated to Altered Fatty Acid Profile of Erythrocyte Membranes. Journal of Diabetes & Metabolism. 2015;06.

226.   Wang X, Lin H, Gu Y. Multiple roles of dihomo-γ-linolenic acid against proliferation diseases. Lipids in Health and Disease. 2012;11(1):25.

227.   Jauregibeitia I, Portune K, Rica I, Tueros I, Velasco O, Grau G, et al. Fatty Acid Profile of Mature Red Blood Cell Membranes and Dietary Intake as a New Approach to Characterize Children with Overweight and Obesity. Nutrients. 2020;12(11).

228.   Giardina S, Sala-Vila A, Hernandez-Alonso P, Calvo C, Salas-Salvado J, Bullo M. Carbohydrate quality and quantity affects the composition of the red blood cell fatty acid membrane in overweight and obese individuals. Clin Nutr. 2018;37(2):481-7.

229.   Riezzo G, Ferreri C, Orlando A, Martulli M, D'Attoma B, Russo F. Lipidomic analysis of fatty acids in erythrocytes of coeliac patients before and after a gluten-free diet intervention: a comparison with healthy subjects. Br J Nutr. 2014;112(11):1787-96.

230.   Pérez-Farinós N, Villar-Villalba C, López Sobaler AM, Dal Re Saavedra M, Aparicio A, Santos Sanz S, et al. The relationship between hours of sleep, screen time and frequency of food and drink consumption in Spain in the 2011 and 2013 ALADINO: a cross-sectional study. BMC Public Health. 2017;17(1):016-3962.

231.   Stefan L, Prosoli R, Juranko D, Cule M, Milinovic I, Novak D, et al. The Reliability of the Mediterranean Diet Quality Index (KIDMED) Questionnaire. Nutrients. 2017;9(4).

232.   Serra-Majem L, Ribas L, Ngo J, Ortega RM, Garcia A, Perez-Rodrigo C, et al. Food, youth and the Mediterranean diet in Spain. Development of KIDMED, Mediterranean Diet Quality Index in children and adolescents. Public health nutrition. 2004;7(7):931-5.

233.   Ferreri C, Faraone Mennella MR, Formisano C, Landi L, Chatgilialoglu C. Arachidonate geometrical isomers generated by thiyl radicals: the relationship with trans lipids detected in biological samples. Free Radic Biol Med. 2002;33(11):1516-26.

234.   Plaza-Díaz J, Molina-Montes E, Soto-Méndez MJ, Madrigal C, Hernández-Ruiz Á, Valero T, et al. Clustering of Dietary Patterns and Lifestyles Among Spanish Children in the EsNuPI Study (†). Nutrients. 2020;12(9).

235.   Hierarchical Cluster Analysis. Wiley StatsRef: Statistics Reference Online. p. 1-13.

236.   Bobbioni-Harsch E, Pataky Z, Makoundou V, Laville M, Disse E, Anderwald C, et al. From Metabolic Normality to Cardiometabolic Risk Factors in Subjects With Obesity. Obesity. 2012;20(10):2063-9.

237.   Burns JL, Nakamura MT, Ma DWL. Differentiating the biological effects of linoleic acid from arachidonic acid in health and disease. Prostaglandins, Leukotrienes and Essential Fatty Acids. 2018;135:1-4.

238.   Martinez-Fernandez L, Laiglesia LM, Huerta AE, Martinez JA, Moreno-Aliaga MJ. Omega-3 fatty acids and adipose tissue function in obesity and metabolic syndrome. Prostaglandins & other lipid mediators. 2015;121(Pt A):24-41. Epub 2015/07/30.

239.   Oh DY, Talukdar S, Bae EJ, Imamura T, Morinaga H, Fan W, et al. GPR120 Is an Omega-3 Fatty Acid Receptor Mediating Potent Anti-inflammatory and Insulin-Sensitizing Effects. Cell. 2010;142(5):687-98.

240.   Kim H-K, Della-Fera M, Lin J, Baile CA. Docosahexaenoic Acid Inhibits Adipocyte Differentiation and Induces Apoptosis in 3T3-L1 Preadipocytes. The Journal of Nutrition. 2006;136(12):2965-9.

241.   De Caterina R. n–3 Fatty Acids in Cardiovascular Disease. New England Journal of Medicine. 2011;364(25):2439-50.

242.   Saravia L, Miguel-Berges ML, Iglesia I, Nascimento-Ferreira MV, Perdomo G, Bove I, et al. Relative validity of FFQ to assess food items, energy, macronutrient and micronutrient intake in children and adolescents: a systematic review with meta-analysis. Br J Nutr. 2020;18:1-27.

243.   Bertoia ML, Mukamal KJ, Cahill LE, Hou T, Ludwig DS, Mozaffarian D, et al. Changes in Intake of Fruits and Vegetables and Weight Change in United States Men and Women Followed for Up to 24 Years: Analysis from Three Prospective Cohort Studies. PLoS Med. 2015;12(9).

244.   Silbernagel G, Kovarova M, Cegan A, Machann J, Schick F, Lehmann R, et al. High Hepatic SCD1 Activity Is Associated with Low Liver Fat Content in Healthy Subjects under a Lipogenic Diet. The Journal of Clinical Endocrinology & Metabolism. 2012;97(12):E2288-E92.

245.   Riedl A, Gieger C, Hauner H, Daniel H, Linseisen J. Metabotyping and its application in targeted nutrition: an overview. Br J Nutr. 2017;117(12):1631-44.

246.   Zhang YY, Liu W, Zhao TY, Tian HM. Efficacy of Omega-3 Polyunsaturated Fatty Acids Supplementation in Managing Overweight and Obesity: A Meta-Analysis of Randomized Clinical Trials. J Nutr Health Aging. 2017;21(2):187-92.

247.   Del-Río-Navarro BE, Miranda-Lora AL, Huang F, Hall-Mondragon MS, Leija-Martínez JJ. Effect of supplementation with omega-3 fatty acids on hypertriglyceridemia in pediatric patients with obesity. J Pediatr Endocrinol Metab. 2019;32(8):811-9.

248.   Abdelhamid AS, Brown TJ, Brainard JS, Biswas P, Thorpe GC, Moore HJ, et al. Omega-3 fatty acids for the primary and secondary prevention of cardiovascular disease. Cochrane Database Syst Rev. 2018;7(7).

249.   Ulven SM, Kirkhus B, Lamglait A, Basu S, Elind E, Haider T, et al. Metabolic effects of krill oil are essentially similar to those of fish oil but at lower dose of EPA and DHA, in healthy volunteers. Lipids. 2011;46(1):37-46.

250.   Dyerberg J, Madsen P, Moller JM, Aardestrup I, Schmidt EB. Bioavailability of marine n-3 fatty acid formulations. Prostaglandins, leukotrienes, and essential fatty acids. 2010;83(3):137-41.

6.Conclusions

The main conclusions of this work are collected, which contribute to a greater knowledge about the characterization of the RBC membrane lipid profile of children with obesity:

1. An altered RBC FA profile of the pediatric population with obesity, characterized by two main features has been determined. An increased ω-6 FA levels, regardless of PUFA dietary intake, which can create a predisposition for unbalanced inflammatory signaling that departs from membranes, is presented in children with obesity. At the same time, higher SFA levels in RBC membranes, which can perturb membrane organization providing rigidity, are observed for children with obesity.

Virtually, no differences were observed in terms of dietary intake between children with obesity, overweight and normal weight.

2. Children and adults with obesity, presented differentiated RBC FA profiles. Children with obesity present higher LA, DGLA and total ω6 values in RBC membranes, along with lower DHA and total ω3 values, compared to adults with obesity, even after adjusting the values by their dietary intake. At the same time, children with obesity presented lower levels of palmitic acid and a lower value of the *de novo* lipogenesis index compared to adults with obesity. These differences, provided by obesity metabolism and age, must be considered to provide specific food groups recommendations based on individual FA needs, rather than giving general recommendations for population with obesity.

3. The RBC membrane FA profile can be used as a tool to identify MHO children from the rest of children with obesity, using a clustering method.

The MHO cluster presented similar FA levels than normal weight children and a differentiated profile compared to the rest of children with obesity. These differences in RBC membranes were characterized by:

- Lower stearic and total SFA levels.

- Higher oleic and total MUFA levels.

- Lower DGLA, AA and total ω-6 levels.

- Higher EPA, DHA and total ω-3 levels.

- Lower levels of ω-6/ω-3, SFA/MUFA and *Δ*9D *18:0* indexes.

- Higher *Δ*6D+*ELO* levels.

4. The analysis of the FAs of the mature erythrocyte membrane is a useful tool to evaluate the metabolic and nutritional status of children with obesity, and applicable to the design of personalized nutritional strategies.

7.Annex

7.1. Fatty Acid Profile of Mature Red Blood Cell Membranes and Dietary Intake as a New Approach to Characterize Children with Overweight and Obesity

Fatty Acid Profile of Mature Red Blood Cell Membranes and Dietary Intake as a New Approach to Characterize Children with Overweight and Obesity

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Abstract: Obesity is a chronic metabolic disease of high complexity and of multifactorial origin. Understanding the effects of nutrition on childhood obesity metabolism remains a challenge. The aim of this study was to determine the fatty acid (FA) profile of red blood cell (RBC) membranes as a comprehensive biomarker of children's obesity metabolism, together with the evaluation of their dietary intake. An observational study was carried out on 209 children (107 healthy controls, 41 who were overweight and 61 with obesity) between 6 and 16 years of age. Mature RBC membrane phospholipids were analyzed for FA composition by gas chromatography-mass spectrometry (GC-MS). Dietary habits were evaluated using validated food frequency questionnaires (FFQ) and the Mediterranean Diet Quality Index for children (KIDMED) test. Compared to children with normal weight, children with obesity showed an inflammatory profile in mature RBC FAs, evidenced by higher levels of ω-6 polyunsaturated FAs (mainly arachidonic acid, $p < 0.001$). Children who were overweight or obese presented lower levels of monounsaturated FA (MUFA) compared to children with normal weight ($p = 0.001$ and $p = 0.03$, respectively), resulting in an increased saturated fatty acid (SFA)/MUFA ratio. A lower intake of nuts was observed for children with obesity. A comprehensive membrane lipidomic profile approach in children with obesity will contribute to a better understanding of the metabolic differences present in these individuals.

Keywords: childhood obesity; inflammation; membrane lipidome; omega-6 fatty acids; red blood cell

1. Introduction

Interventions to control obesity have typically consisted of combined strategies including diet, exercise, and behavior therapy [1]. Despite efforts by governments, the food industry, and the science community, obesity and overweight rates keep increasing in both child and adult populations

worldwide [2,3], demonstrating a need for personalized strategies that guarantee the success of interventions in treating obesity.

The focus on early clinical markers for overweight/obesity onset is, nowadays, a clear research target [4]. A new trend to focus on fats, and specifically the quality of dietary lipids, is crucial for the prevention and treatment of obesity [5]. In this sense, a strong contribution from the molecular approach developed in the last two decades, characterizing fat accumulation, highlights different kinds of signaling occurring in this disease and leading to comorbidities [6]. Since fat accumulation is strictly connected with the quality and quantity of fatty acids (FAs) in human tissues, the lipidomic approach was found to have a key role in describing the scenario of molecular signaling, providing crucial information on the various phases of weight increase, from overweight to obesity [7].

Indeed, membrane fatty acid-based lipidomics has reached a high technology readiness level, developing simple (i.e., inexpensive and high-throughput) and robust analytics of high resolving power, as demonstrated by several applications to diseases [8,9].

Molecular information on membrane lipid composition is of great importance at least for two reasons: (i) the set of membrane fatty acid controls the fluidity and permeability properties as well as the thickness of the bilayer, which are all implicated in the receptor and channel responses [10], and (ii) lipid signaling departs from the fatty acid residues of the membrane phospholipids. Therefore, the fatty acid composition directly describes the cellular predisposition to respond to the various stimuli that arrive from the extracellular environment [11]. Therefore, the balance in the fatty acid composition of the cell membrane leads to the balance in the functions of each individual cell and, hence, of the tissues and the whole organism [12].

It is worth recalling that to measure the lipid composition of blood, different blood compartments have been targeted [13]. Plasma or serum FA levels have been widely analyzed because they reflect short-term dietary fat intake. However, analysis of lipid compositions from mature red blood cell (RBC) membranes offers an advantage over analysis of plasma because these cells last on average 120 days in the blood compared to 3 weeks for platelet or plasma lipids, reflecting better long-term dietary FA intake and tissue conditions [14]. Apart from this, RBCs maintain a more stable FA composition compared to plasma FA levels [15].

It is important to note that although future nutritional intervention studies are necessary to better understand the impact of personalized diet on lipid metabolism in children, lipidomics can help monitor the ω-6 fatty acid content involved in the inflammation pathways that can be accompanied by essential FA deficiencies in the diet, which can be connected to many diseases and tissue malfunctions. As a matter of fact, monitoring the RBC membrane FA profile at the individual level can be an excellent candidate biomarker as it can offer the possibility to follow up the optimal intake, membrane incorporation, and biochemical transformations in order to personalize dietary intervention designed to recover FA deficiencies to prevent or control disease. Fatty acids in phospholipids represent the combination between nutritional and metabolic factors, with a strong contribution of the individual metabolism and condition of the patients.

This study aims to generate knowledge on the importance of different fatty acid families (saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA)) for the cell membrane lipidome in a pediatric population. In this exploratory study, analysis of the RBC membrane shows its potential to provide indications of dietary and metabolic distinctions between children with overweight and obesity that can contribute, in the future, to designing more precise nutritional strategies that may be more effective at correcting the molecular imbalance in obesity.

2. Materials and Methods

2.1. Study Design

An observational case-control and retrospective study was conducted on 209 children (113 boys and 96 girls) between 6 and 16 years old, recruited from the pediatric endocrinology unit at the Hospital Universitario Cruces (Barakaldo, Spain). Children were classified according to body mass

index (BMI), using an age and sex-specific pediatric z-score from Orbegozo tables [16]. BMI was taken as a reference to define the different categories, defining normal weight when the standard deviation (SD) of BMI was −1 < SD ≤ +1, overweight when the +1 < SD ≤ +2, and obesity when SD > +2. Groups were homogeneously distributed by gender and age. Finally, 107 children with normal weight, 41 children with overweight, and 61 children with obesity were enrolled in the study.

Subjects were excluded if they presented any kind of acute or chronic diseases, were taking medications, or had any presence of metabolic syndrome symptoms or obesity associated to any type of pathology. A physical examination was performed by a pediatrician.

The study protocol was approved by the Euskadi Clinical Research Ethics Committee (permission number PI2016181) and accomplished according to the Helsinki Declaration in 1975, revised in 2013. Subjects under study were included after acceptance (of the parents) to participate in the study and signing of informed consent. All the informed consent documents were signed by their parents, and in the case of children between 12 and 16 years of age, the informed consent was also signed by themselves, according to the Euskadi Ethical Committee and sample biobank laws (Organic Law 3/2018, of December 5, on Protection of Personal Data and guarantee of digital rights; Law 14/2007 on Biomedical Research and RD 1716/2011 of Biobanks).

2.2. Anthropometric Measures

Body weight (kg) and height (cm) were measured by standardized methods [17]. Body mass index (BMI) was calculated as weight (kg) divided by the square of the height (m²). Anthropometric parameters as well as blood sampling were all conducted by pediatricians during the first visit to the Hospital Universitario Cruces.

2.3. Food Habits and Nutrient Intakes

During the first visit, a pediatrician interviewed the participants and collected personal data, including family medical history and information on the history of medication usage. Estimations of food consumption, including dietary diversity and variety, were measured using a quantitative food frequency questionnaire (FFQ), completed online by the parents of each volunteer, except in those cases of adolescents, who were encouraged to complete it themselves. For our study, an adapted FFQ was used, which was previously validated with portion sizes and food groups for the Spanish juvenile population [18–20]. Information about different food items collected from these questionnaires was then analyzed using DIAL® software to translate their intake into their corresponding energy and nutrient composition (UCM & Alce Ingeniería S.A, Madrid, Spain) (v3.4.0.10).

Dietary habits were also measured using the KIDMED test (Mediterranean Diet Quality Index), a validated questionnaire for the Spanish juvenile population that measures adherence to the Mediterranean diet, which is widely considered to be an optimally healthy diet for most populations [21,22]. According to the KIDMED index, a score of 0–3 reflects poor adherence to the Mediterranean diet, a score of 4–7 describes average adherence, and a score of 8–12 good adherence.

2.4. Red Blood Cell (RBC) Membrane Fatty Acid Analysis

The fatty acid composition of mature RBC membrane phospholipids was obtained from blood samples (approximately 2 mL) collected in vacutainer tubes containing ethylenediaminetetraacetic acid (EDTA). Samples were shipped to the Lipidomic Laboratory at a controlled temperature and, upon arrival, underwent quality control for the absence of hemolysis. During the blood work-up, before lipid extraction and lipid transesterification to fatty acid methyl esters (FAMEs), the automated protocol includes the selection of mature RBCs, as reported previously [9,23–25]. Briefly, the whole blood in EDTA was centrifuged (4000 revolutions per minute (rpm) for 5 min at 4 °C) and the mature cell fraction was isolated based on the higher density of the aged cells [26] and controlled by the use of a cell counter (Scepter 2.0 with Scepter™ Software Pro, EMD Millipore, Darmstadt, Germany). All the subsequent steps were automated and included cell lysis, isolation of the membrane pellets,

phospholipid extraction from pellets using the Bligh and Dyer method [27], transesterification to FAMEs by treatment with a potassium hydroxide (KOH)/methyl alcohol (MeOH) solution (0.5 mol/L) for 10 min at room temperature, and extraction using hexane (2 mL). The FAMEs were analyzed using capillary column gas chromatography (GC). GC analysis was run on the Agilent 6850 Network GC System (Agilent, USA), equipped with a fused silica capillary column Agilent DB23 (60 m × 0.25 mm × 0.25 µm) and a flame ionization detector. Optimal separation of all fatty acids and their geometrical and positional isomers was achieved. Identification of each fatty acid was made by comparison to commercially available standards and to a library of trans isomers of MUFAs and PUFAs. The amount of each FA was calculated as a percentage of the total FA content (relative %), as described in Section 2.5, being more than 97% of the GC peaks recognized with appropriate standards.

2.5. Red Blood Cell Membrane Fatty Acid Cluster

Twelve FAs were chosen as representative cluster of the main building blocks of the RBC membrane glycerophospholipids and of the three FA families (SFA, MUFA, and PUFA): for SFAs, palmitic acid (C16:0) and stearic acid (C18:0); for MUFAs, palmitoleic acid (C16:1; c9), and oleic acid (C18:1; c9), cis-vaccenic acid (C18:1; c11); for ω-3 PUFAs, eicosapentaenoic acid (EPA) (C20:5) and docosahexaenoic acid (DHA) (C22:6); for ω-6 PUFAs, linoleic acid (LA) (C18:2), dihomo-gamma-linolenic acid (DGLA) (C20:3), and arachidonic acid (AA) (C20:4); for geometrical trans fatty acids (TFAs): elaidic acid (C18:1 t9) and mono-trans arachidonic acid isomers (monotrans-C20:4; ω-6 recognized by standard references as previously described by Ferreri et al. [28]). Considering these fatty acids, different indexes previously reported in the literature [25] were calculated: Omega-3 Index: (%EPA + %DHA) an index suggested as a cardiovascular disease risk factor; (%SFA/%MUFA) index related with membrane rigidity; inflammatory risk index (% ω-6)/(% ω-3); PUFA balance (%EPA + %DHA)/total PUFA × 100; free radical stress index (sum of trans-18:1 + summary (Σ) of monotrans 20:4 isomers); unsaturation index (UI) (%MUFA) + (%LA/2) + (%DGLA/3) + (%AA/4) + (%EPA/5) + (%DHA/6); peroxidation index (PI) (%MUFA/0.025) + (%LA) + (%DGLA/2) + (%AA/4) + (%EPA/6) + (%DHA/8).

Additionally, the enzymatic indexes of elongase and desaturase enzymes, the two classes of enzymes of the MUFA and PUFA biosynthetic pathways, were inferred by calculating the product/precursor ratio of the involved FAs.

2.6. Statistical Analysis

A power calculation was performed using G*power software (v3.1.9.7., Heinrich-Heine- University, Düsseldorf, Germany), to determine the total sample size for analysis of covariance (ANCOVA) fixed effects, main effects, and interactions. A priori, we expected a medium effect size f = 0.25, (as the ratio of the variation among the group means to the average variation among subjects within each group as measured by their standard deviations), using an alpha = 0.05 as probability of the type I error, to have a 95% confidence for significative results, and beta = 0.2, as the acceptable probability of type II errors, concluding in a 0.8 power (where power is equal to 1-beta). It is estimated that a lower value would imply too great a risk of incurring a type II error. A higher value would imply excessively expanding the sample [29]. The total sample size required was 158, which corresponds with 53 participants for each group.

Differences between groups for nutrient intake, food group intake, and KIDMED test were determined by conducting a Kruskal-Wallis test because the data were not normally distributed. Normal data distribution was assessed by Shapiro-Wilk's test or/and the Kolmogorov-Smirnov test. Subsequently, Dunn's (1964) test was performed for post hoc comparisons. A Bonferroni correction for multiple comparisons was made, to correct for the increased risk of type I error.

An analysis of covariance test (ANCOVA) was run to determine the differences between RBC membrane fatty acids from children with normal weight, overweight, and obesity, after controlling for variables selected as potential confounders, such as age, gender, and dietary macro- and micronutrient intake. Post hoc analysis was performed with a Bonferroni adjustment for multiple

comparisons. First, a principal component analysis (PCA) was run on 18 dietary nutrient intake variables (individual FAs, families (SFA, MUFA, and PUFA), total lipids (%Energy), carbohydrates, fiber, proteins, and calories), obtained with DIAL software (v3.4.0.10, Department of Nutrition (UCM) & Alce Ingeniería, S.L., Madrid, Spain) after transforming the information about food items from FFQ questionnaires into micro- and macronutrient values, in order to reduce and simplify the dimensions of these variables and use the generated factors as diet covariates [25]. Kaiser-Meyer-Olkin (KMO) and Bartlett's test of sphericity were used to verify the sampling adequacy for the analysis. The PCA revealed three components that had eigenvalues greater than one and which explained 83.74% of the total variance. These components were included in the ANCOVA analysis as diet covariates. The level of significance was set at $p < 0.05$.

In order to establish correlations between the RBC membrane FA profile and the dietary intake and other parameters measured in this study, due to the non-normality of the data of most of the variables, Spearman's rank-order correlation was conducted. In those cases where all variables had a normal distribution, a Pearson product-moment correlation was run. All statistical analyses were performed using SPSS (IBM Corp. v24.0, Armonk, NY, USA).

3. Results

3.1. Dietary Intake

Table 1 shows dietary intake according to food categories calculated via food questionnaires. The diet of children with obesity was characterized by lower intake of cereals ($p = 0.04$), dairy products ($p = 0.05$), and nuts ($p = 0.01$), compared to children with normal weight. The overweight group only showed significant differences of lower intake of cereals compared with the group of children with normal weight ($p = 0.004$). Regarding diet quality, the KIDMED questionnaire was conducted to measure the adherence of different groups to the Mediterranean diet. As we can see in Table 1, only children with normal weight achieved good adherence (KIDMED score ≥8), while children with overweight and obesity showed mild adherence to the Mediterranean diet (less than 8 points and equal to or greater than 4 points) [21]. Even so, children with normal weight just had a difference of one point in the KIDMED scale compared with the other groups and no statistically significant differences were observed, ($p = 0.07$). KIDMED and BMI showed a statistically significant slight negative correlation (Pearson's Correlation coefficient r_s (98) = -0.198, $p = 0.004$). The KIDMED index did show some slight correlation with RBC membrane AA, EPA, and DHA levels (r_s (98) = −0.183, $p = 0.010$, r_s (98) = 0.195, $p = 0.006$, and r_s (98) = 0.227, $p = 0.001$, respectively).

Table 2 shows the differences among three groups in macronutrients and individual fatty acid daily intake expressed as % of Kcal. No differences among groups were observed for macronutrients, except for the group of children with normal weight that showed a lower intake of total lipids compared with the overweight group ($p = 0.01$). Considering the intake of specific fatty acids, the children with normal weight reported a higher intake of C16:0 and total SFAs compared to the groups with overweight and obesity, but no other differences were observed.

Table 1. Food group intakes.

Food Groups (g/day)	Children with Normal Weight (NO) n = 107 Med (Q1–Q3)	Children Who Are Overweight (OV) n = 41 Med (Q1–Q3)	Children with Obesity (OB) n = 61 Med (Q1–Q3)	Kruskal-Wallis H Test (p)	Post hoc Pairwise Comparison (p *)		
					NO:OV	NO:OB	OV:OB
Fruits	423 (297–532)	354 (273–509)	419 (272–603)	0.50			
Vegetables	159 (96–259)	154 (77–250)	141 (74–232)	0.49			
Cereals	161 (118–210)	127 (96–171)	139 (105–188)	0.01	0.01	0.11	0.95
Legumes	91 (54–102)	80 (51–102)	75 (48–96)	0.52			
Olive oil	15 (15–37)	15 (12–15)	15 (12–37)	0.26			
Dairy products	325 (255–512)	314 (226–329)	302 (207–358)	0.10			
Eggs	15 (15–34)	15 (15–34)	15 (15–34)	0.36			
Red meat	21 (21–21)	21 (21–50)	21 (21–50)	0.31			
White meat	50 (21–50)	50 (36–50)	50 (21–50)	0.15			
Dried fruits and nuts	2.1 (0–6.4)	1.1 (0–6.4)	1.1 (0–2.1)	0.03	0.73	0.03	0.96
Lean fish	27 (27–27)	27 (27–27)	27 (13–27)	0.613			
Oily fish and shellfish	27 (13–27)	27 (9–31)	27 (11–31)	0.55			
Sugary drinks	18 (0–45)	21 (0–54)	16 (0–54)	0.95			
Juices	80 (27–250)	107 (27–196)	80 (27–250)	0.95			
KIDMED score	8 (7–9)	7 (6–9)	7 (6–9)	0.07			

Data are expressed as medians and quartile 1 and quartile 3 (Med Q1-Q3). Not normally distributed variables. * Pairwise comparison conducted with a Bonferroni adjustment.

Table 2. Dietary daily intake expressed as % of energy (%E).

Variables	Children with Normal Weight (NO) n = 107 Med (Q1–Q3)	Children Who Are Overweight (OV) n = 41 Med (Q1–Q3)	Children with Obesity (OB) n = 61 Med (Q1–Q3)	Kruskal-Wallis H Test (*p*)	Post Hoc Pairwise Comparison (*p* *)		
					NO:OV	NO:OB	OV:OB
Macronutrients							
Calories (Kcal/day)	2058 (1749–2376)	1983 (1516–2335)	1916 (1709–2167)	0.18			
Proteins (%E)	16.3 (15.0–17.7)	16.8 (15.3–18.5)	16.4 (14.9–17.3)	0.42			
Carbohidrates (%E)	46.7 (43.2- 49.9)	48.1 (43.9–53.3)	46.7 (43.4–51.1)	0.19			
Simple sugars (%E)	20.9 (18.5–23.8)	20.9 (17.9–24.8)	21.8 (18.9–25.0)	0.42			
Lipids (%E)	33.7 (29.9–37.0)	31.1 (27.0–35.4)	32.9 (27.4–38.2)	0.05			
Individual FA (% E)							
C14:0	1.0 (0.8–1.2)	0.8 (0.6–1.1)	0.9 (0.7–1.1)	0.02	0.06	0.08	1.0
C16:0	6.3 (5.7–7.1)	5.8 (5.1–6.7)	5.8 (5.2–6.7)	0.01	0.04	0.03	1.0
C18:0	2.4 (2.1–2.7)	2.2 (1.9–2.5)	2.2 (2.0–2.6)	0.05			
Tot. SFA	9.7 (8.7–10.9)	9.0 (7.8–10.1)	9.0 (7.9–10.4)	0.004	0.02	0.04	1.0
C16:1	0.51 (0.46–0.58)	0.49 (0.42–0.54)	0.50 (0.42–0.60)	0.1			
C18:1	14.2 (11.4–16.5)	12.3 (10.1–14.8)	13.3 (11.0–17.3)	0.08			
Tot. MUFA	14.7 (11.9–17.1)	12.7 (10.5–15.2)	13.9 (11.4–17.8)	0.07			
C18:2	3.6 (3.2–4.2)	3.6 (3.1–3.9)	3.6 (2.9–4.3)	0.99			
C20:4	0.04 (0.03–0.05)	0.04 (0.03–0.06)	0.04 (0.03–0.05)	0.78			
Tot. ω6	3.5 (3.0–4.4)	3.7 (2.8–4.5)	3.4 (3.1–5.0)	0.97			
C18:3	0.52 (0.50–0.61)	0.50 (0.46–0.54)	0.52 (0.45–0.58)	0.44			
C20:5 (EPA)	0.07 (0.04–0.1)	0.07 (0.02–0.11)	0.07 (0.04–0.1)	0.88			
C22:5 (DPA)	0.017 (0.011–0.024)	0.017 (0.006–0.025)	0.016 (0.009–0.025)	0.59			
22:6 (DHA)	0.14 (0.09–0.19)	0.13 (0.05–0.20)	0.13 (0.09–0.19)	0.84			
Tot. ω3	0.8 (0.7–1.0)	0.8 (0.6–0.9)	0.8 (0.6–0.9)	0.36			
Tot. PUFA	4.3 (3.8–5.3)	4.5 (3.5–5.4)	4.3 (3.6–5.5)	0.96			
ω-6/ω-3	4.6 (4.0–5.4)	4.8 (3.7–6.8)	4.9 (4.0–6.7)	0.25			

Data are expressed as medians and quartile 1 and quartile 3 (Med Q1-Q3. Not normally distributed variables. SFA-saturated fatty acid; MUFA-monounsaturated fatty acid; PUFA-polyunsaturated fatty acid, (%E)-%Energy. * Pairwise comparison conducted with a Bonferroni adjustment.

3.2. RBC Membrane Fatty Acid Profile

In order to compare RBC FA profiles between groups, a one-way ANCOVA was conducted using age, sex, and dietary intake as covariates to adjust the error made by those confounding factors (Table 3). The group with obesity showed higher levels of stearic acid ($p = 0.03$) and total SFA ($p = 0.03$) than the normal weight group. Oleic acid and total MUFA levels in the groups with obesity and overweight were lower compared with the children with normal weight. Regarding ω-6 FA, linoleic acid was higher for the normal weight group compared to children with obesity ($p = 0.03$), but dihomo-γ-linolenic acid (DGLA) and arachidonic acid levels were higher for children with obesity compared with the children with normal weight ($p = 0.002$ and $p = 0.0003$, respectively). Individual and total ω-3 levels did not show significant differences. The SFA/MUFA ratio was higher for children with obesity and overweight compared with children with normal weight ($p = 0.001$ and $p = 0.03$, respectively), and for the ω-6/ω-3 ratio, children with obesity and overweight showed higher but not statistically significant values compared with the children with normal weight ($p = 0.09$ and $p = 0.1$, respectively).

With respect to enzymatic activity, Δ9D was lower for children with obesity, indicating the hypoactivity of this enzyme to convert stearic acid (C18:0) to oleic acid (9c C18:1).

In order to explore possible relationships between dietary intake components and RBC lipid profile, a Spearman's rank-order correlation was carried out. Only dietary EPA, DPA, and DHA showed mild correlations with levels of EPA in the RBC (r_s (98) = 0.33 $p < 0.0001$, r_s (98) = 0.363 $p < 0.0001$, and r_s (98) = 0.313 $p < 0.0001$, respectively).

At the same time, dairy products and cereal intake were higher for children with normal weight compared to children with obesity, but neither showed significant correlations with the RBC membrane FAs.

Table 3. Red blood cell (RBC) membrane fatty acid profile.

Fatty Acids (%)	Children with Normal Weight (NO)		Children Who Are Overweight (OV)		Children with Obesity (OB)		ANCOVA	*p*-Value [a]		
	Mean	SE	Mean	SE	Mean	SE	*p*	NO:OV	NO:OB	OV:OB
Palmitic acid (C16:0)	22.44	0.10	22.54	0.16	22.49	0.13	0.86	1.00	1.00	1.00
Stearic acid (C18:0)	17.67	0.10	17.94	0.17	18.13	0.14	0.03	0.54	0.03	1.00
TOT. SFA	40.12	0.10	40.48	0.16	40.58	0.14	0.02	0.21	0.03	1.00
Palmitoleic acid (C16:1)	0.40	0.01	0.45	0.02	0.43	0.02	0.08	0.12	0.31	1.00
Oleic acid (9c C18:1) cis-	17.48	0.13	16.68	0.20	16.65	0.17	<0.001	<0.001	<0.001	1.00
Vaccenic acid (11c C18:1)	1.19	0.02	1.14	0.03	1.14	0.02	0.12	0.35	0.23	1.00
TOT. MUFA	19.09	0.13	18.27	0.22	18.27	0.18	<0.001	0.01	0.001	1.00
Linoleic acid (C18:2)	14.28	0.13	14.30	0.21	13.71	0.17	0.02	1.00	0.03	0.09
DGLA (C20:3) ARA	2.01	0.04	2.30	0.06	2.23	0.05	<0.001	<0.001	0.002	1.00
(C20:4) TOT. ω-6	18.76	0.13	19.23	0.21	19.66	0.18	<0.001	0.18	<0.001	0.37
EPA (C20:5)	35.06	0.15	35.83	0.25	35.65	0.21	0.12	0.03	0.08	1.00
DHA (C22:6) TOT.	0.60	0.02	0.49	0.03	0.54	0.03	0.01	0.02	0.35	0.66
ω-3	4.97	0.11	4.67	0.17	4.79	0.14	0.29	0.41	0.95	1.00
TOT. PUFA	5.57	0.12	5.16	0.19	5.34	0.16	0.16	0.21	0.73	1.00
Trans C18:1	40.63	0.14	40.99	0.22	40.98	0.18	0.21	0.51	0.39	1.00
Trans C20:4	0.08	0.01	0.09	0.01	0.09	0.01	0.88	1.00	1.00	1.00
	0.08	0.01	0.06	0.01	0.08	0.01	0.31	0.65	1.00	0.41
TOT. TRANS	0.17	0.01	0.15	0.01	0.17	0.01	0.47	0.71	1.00	0.72
Indexes										
ω-6/ω-3	6.59	0.18	7.33	0.28	7.23	0.24	0.09	0.09	0.10	1.00
Omega 3 Index	5.57	0.12	5.16	0.19	5.34	0.16	0.16	0.21	0.73	1.00
SFA/MUFA	2.12	0.02	2.21	0.03	2.24	0.02	<0.001	0.03	0.001	1.00
Δ6D + ELO 20:3/18:2 [b]	0.142	0.003	0.158	0.004	0.164	0.004	<0.001			
Δ5D 20:4/20:3	9.59	0.18	8.46	0.29	8.96	0.24	0.004	0.004	0.12	0.59
Δ9D 16:1/16:0	0.018	0.001	0.02	0.001	0.019	0.001	0.07	0.07	0.43	1
Δ9D 18:1/18:0	0.994	0.01	0.928	0.017	0.916	0.014	<0.001	0.004	<0.001	1
PUFA BALANCE	13.71	0.28	12.58	0.45	13.00	0.37	0.08	0.11	0.40	1.00
Peroxidation Index	137.18	0.81	136.62	1.31	138.00	1.10	0.71	1.00	1.00	1.00
Unsaturation Index	161.58	0.57	161.27	0.91	162.28	0.76	0.67	1.00	1.00	1.00

Data are presented as mean ± standard error (SE). Adjusted for age, sex, and dietary components, extracted from the principal component analysis of dietary nutrient intake (individual FAs, families (SFA, MUFA, and PUFA), total lipids (%Energy), carbohydrates, fiber, proteins, and calories). [a] Post hoc tests were conducted with a Bonferroni adjustment. [b] Levene's test of homogeneity of variance was not met.

4. Discussion

Lipidomic monitoring of mature RBC membranes evaluated in this study contributes to highlighting the importance of each fatty acid class as a molecular parameter to better understand the lipidomic pathways connected with childhood obesity. Our results indicate molecular inflammation derived from unbalanced levels of membrane fatty acids and dysregulation of desaturase enzymatic activity as key parameters for the metabolic outcome in obesity.

Different studies have been published reporting RBC membrane lipid profiles of adult populations with overweight and obesity [30,31] and a few, also, on child populations with obesity [32].

In a meta-analysis study published by Fekete et al. [33], the FA profiles from different blood fractions were analyzed in order to determine the long-chain PUFA status in obesity. In total plasma lipids and phospholipids, high variability in individual FA levels was observed throughout the different studies analyzed. All the studies agreed that differentiated lipid profiles were observed for subjects with overweight and obesity compared to subjects with normal weight, characterized by a greater alteration in ω-6 FA. In agreement with our results, all the biomarkers analyzed in this meta-analysis showed increased levels of DGLA and decreased LA for the population with overweight and/or obesity. This meta-analysis could not find any significant result for AA, despite increased levels of AA in adipose tissue, which has been previously associated with obesity [34–36]. However, our results revealed significant increased levels of AA in RBC membranes in children with obesity. This heterogeneity points towards a need for a more precise biomarker to characterize and compare different population groups, highlighting the advantages of choosing mature RBC membrane as a representative of nutritional and metabolic contributions [11].

Our results can be seen also in view of other studies that have also analyzed RBC membranes. A study on children with overweight and obesity with metabolic syndrome from 5 to 18 years of age in an Italian population showed similar FA levels of total ω-6 and total SFAs, whereas for all the other measured PUFAs and indexes, differences could be observed [32]. The variability in RBC FA between different countries [37] might be the reason, and this is an important point to emphasize in our approach, which proposes common features of an automatized procedure for cell sampling and membrane isolation and of a precise cluster of fatty acids to analyze.

To our knowledge, this is the first time that a systematic approach was employed that analyzes FAs from isolated mature RBC membranes in pediatric populations with overweight and obesity compared to children with normal weight in order to identify specific characteristics of the fatty acid profile for childhood obesity. Furthermore, as the composition of the RBC membrane is substantially affected by diet and metabolism, the elimination of the effect of diet as a confounding variable in our ANCOVA analysis allows a more robust and realistic examination of the effects of metabolic status of the obesity condition on the RBC FA profile.

According to data obtained in our study, the group with obesity is characterized by an increase in ω-6 fatty acids due to the higher levels of AA and, at the same time, of DGLA, but ω-3 mediated signaling also has to be balanced. Omega-6 FAs have been previously described in the literature as precursors of proinflammatory mediators [38,39] that act through different mechanisms on inflammatory processes. Unlike the other ω-6 FAs, LA showed lower levels for the group with obesity compared with the children with normal weight. Higher RBC LA levels have been linked with improved body composition, insulin resistance, and lower levels of inflammatory markers in previous studies. This disequilibrium of PUFA metabolism towards ω-6 FAs seems to contribute to excessive adipose tissue development and represents, itself, an emerging risk factor for obesity [40,41].

Previous studies have shown an inverse correlation between ω-3 intake and AA levels in RBCs that can be due the competition of the Δ6-desaturase [42]. Although we did not observe differences in the dietary intake of ω-3, nor in RBC ω-3 levels, but considering that AA levels appear to be enhanced in obesity, an increase in ω-3 consumption in the population with obesity can be a crucial dietary recommendation in order to counteract proinflammatory precursors linked with the disease.

Regarding SFA and MUFA levels, an altered ratio in the group with obesity can be observed, mainly due to lower levels of oleic acid and higher levels of stearic acid in the group with obesity compared with the children with normal weight. The enzymatic activity of Δ-9-desaturase or stearoyl-CoA desaturase-1 (SCD1), measured indirectly by the ratio between oleic and stearic acids, showed a lower activity for the group with obesity. The overall picture that comes from examination of the fatty acid remodeling occurring in obesity highlights the role of de novo lipogenesis with the formation of saturated fatty acids (SFA) and their enzymatic transformation to monounsaturated fatty acids (MUFA), connected with the functioning of delta-9 desaturase and the corresponding gene expression (SCD1, Stearoyl CoA Desaturase). As a consequence, the main fatty acid biomarkers of weight increase are MUFAs, such palmitoleic acid (9 cis-16:1) and oleic acid (9 cis-18:1), the former being considered for its role as a lipokine [43] and the latter being considered the main fatty acid accumulating in adipose tissue as triglycerides [44–48].

Although higher dietary intake of SFAs was observed in the normal weight group compared with the group with obesity, the SFA level in RBC membranes was slightly higher in the study population with obesity. Possible explanations for this result, as explained above, could be due to the greater activity of SCD1 in the children with normal weight, which may have converted higher proportions of SFAs to MUFAs, as reflected in higher oleic acid levels in this group. At the same time, the SFA/MUFA ratio, which is correlated with increased membrane rigidity, appears in higher levels in the group with obesity [12,49].

Precision nutrition based on molecular data considers that the assessment of dietary patterns provides a more reliable picture of real food intake compared to the assessment of individual macronutrient intake [50]. Links between dietary patterns and RBC lipid composition have been considered in our study to provide information that could be useful for more precise nutritional recommendations. Dietary patterns of children with normal weight were characterized by higher intake of nuts compared with the group with obesity and a higher intake of cereals compared with the overweight group, but neither food groups showed correlations with the RBC membrane FAs. However, different epidemiological and nutritional clinical trials conducted in adults have reported an inverse relationship between nut consumption and body mass index (BMI) [51–53], associated with several health benefits, such as antioxidant, hypocholesterolemic, cardioprotective, anticancer, anti-inflammatory, and antidiabetic benefits, among other functional properties [54,55]. Previously published results based on self-reported intakes using food frequency questionnaires pointed out that a high intake of grains could be protective against obesity [56,57]. Regarding diet quality, measured by the KIDMED test, statistically significant differences were not observed. Even so, a tendency of a higher adherence to Mediterranean diet in the children with normal weight, compared with the groups with overweight and obesity, can be observed ($p = 0.07$), and these results agree with other studies with pediatric populations with overweight and obesity [58].

In our opinion, there are several points to be considered for the identification of a fat biomarker in obesity, as well as in other physio-pathological states. The first focus is on the choice of the sample to examine: (i) mature red blood cells are representative for both functional and structural roles of the fatty acid residues which compose its membrane phospholipids; (ii) the fatty acids in phospholipids represent the combination between nutritional and metabolic factors, with strong contribution to the individual metabolism and condition of the patients.

The second focus is on the variations in the fatty acid residues of membrane phospholipids, which point to a differentiation between the overweight and obese status: (a) oleic acid is reduced in both subjects with overweight and those with obesity, which is interesting since, in adults, it is known that the decrease in this fatty acid is correlated with weight increase [31]. The role of the FFQ checked in our study clarified that there is no increase in SFA intake in children with obesity, thus shifting the attention to the metabolism of children with obesity and enzymatic functioning. It should be noted that in overweight and obesity, the decrease in desaturase delta-9 activity is significant, thus suggesting a shift in the enzyme functioning toward the SFA pathway, and (b) the role of the omega-6 pathway for the arachidonic acid increase is clearly shown in the comparison between children with normal weight and children with obesity, showing that in obesity, the increase in inflammatory

signals could be crucial to be controlled in order to treat metabolic deficiencies related to childhood obesity.

This is an exploratory study that highlights some aspects to be further assessed in larger populations but also gives an indication for dietary and metabolic distinctions between children with overweight and obesity, to be further explored with intervention studies. At the same time, the indirect measurement of enzyme activity by the ratio between product and precursors, although very popular, could be considered as a limitation of the study and should be measured directly to emphasize and reaffirm the conclusions obtained. The use of an FFQ is also a limitation of the study as this type of questionnaire usually underestimates dietary intake [59]. Regarding the sample size, the authors are aware that the overweight group did not achieve the expected sample size, which was attributed to difficulties in recruiting, typically observed in human studies and especially in recruiting children; therefore, there might not have been enough power to detect differences in the overweight group and this could have led to a type II error. Future work should be focused on increasing the study sample size to achieve a greater statistical power. In order to compare with other studies that use international tables for determining obesity, such as the International Obesity Task Force (IOTF) or the World Health Organization (WHO), using the Spanish tables described above can be seen as a limitation, even if they better describe our study population. We can summarize that the presented study showed an altered RBC FA profile of the pediatric population with obesity, characterized by two main features: (i) an increased ω-6 molecular contribution, although no differences in PUFA dietary intake between groups were observed, which can create a predisposition for unbalanced signaling that departs from membranes; (ii) higher SFAs in the RBC membranes of children with obesity, contrary to the SFA intake that was found to be higher in controls, which can perturb membrane organization. Both results highlight the crucial role of molecular diagnostics for precise evaluation of patient status. Indeed, the lipidomic analysis of mature RBCs provides a systematic, automatized approach for the characterization of the lipid composition in these cells from the pediatric population with obesity, which can provide molecular insights to assist further development of precise and personalized nutritional strategies. Restoration of the optimal levels of each individual fatty acid, families, and ratios appears to be an important strategy to be considered in the treatment of potentially metabolic deficiencies related to childhood obesity from a nutritional point of view. Future studies, mainly nutritional intervention studies with children, are needed to elucidate an adequate selection among the types of fats that must be ingested, with the crucial target of the recovery of the homeostatic levels of the cell membrane, for proper functioning, both of signaling and metabolic pathways.

Abbreviations

AA	Arachidonic Acid
ANCOVA	Analysis of Covariance Body
BMI	Mass Index Dihomo-Gamma-
DGLA	Linolenic Acid Docosahexaenoic
DHA	Acid Docosapentaenoic Acid
DPA	Ethylenediaminetetraacetic Acid
EDTA	Essential Fatty Acid
EFA	Eicosapentaenoic Acid
EPA	Fatty Acid
FA	Food Frequency Questionnaire
FFQ	Fatty Acid Methyl ester Kaiser-
FAME	Meyer-Olkin
KMO	Methyl Alcohol
MeOH	Monounsaturated Fatty Acids
MUFA	Potassium Hydroxide
KOH	Linoleic Acid
LA	Polyunsaturated Fatty Acid
PUFA	Red Blood Cell
RBC	Saturated Fatty Acid
SFA	Stearoyl-CoA Desaturase-1
SCD1	Standard Deviation
SD	Trans Fatty Acid
TFA	Unsaturation Index
UI	Peroxidation Index
PI	Principal Component Analysis
PCA	

References

1. Mozaffarian, D. Dietary and Policy Priorities for Cardiovascular Disease, Diabetes, and Obesity: A Comprehensive Review. *Circulation* **2016**, *133*, 187–225.

2. Ng, M.; Fleming, T.; Robinson, M.; Thomson, B.; Graetz, N.; Margono, C.; Mullany, E.C.; Biryukov, S.; Abbafati, C.; Abera, S.F.; et al. Global, regional, and national prevalence of overweight and obesity in children and adults during 1980–2013: A systematic analysis for the Global Burden of Disease Study 2013. *Lancet* **2014**, *384*, 766–781.

3. Murtagh, E. Worldwide trends in body-mass index, underweight, overweight, and obesity from 1975 to 2016: A pooled analysis of 2416 population-based measurement studies in 128.9 million children, adolescents, and adults. *Lancet* **2017**, *390*, 2627–2642.

4. Juonala, M.; Lau, T.; Wake, M.; Grobler, A.; Kerr, J.A.; Magnussen, C.G.; Sabin, M.A.; Burgner, D.P.; Lycett, K. Early clinical markers of overweight/obesity onset and resolution by adolescence. *Int. J. Obes.* **2020**, *44*, 82–93.

5. Beulen, Y.; Martinez-Gonzalez, M.A.; van de Rest, O.; Salas-Salvado, J.; Sorli, J.V.; Gomez-Gracia, E.; Fiol, M.; Estruch, R.; Santos-Lozano, J.M.; Schröder, H.; et al. Quality of Dietary Fat Intake and Body Weight and Obesity in a Mediterranean Population: Secondary Analyses within the PREDIMED Trial. *Nutrients* **2018**, *10*, 2011.

6. Zamanian-Azodi, M.; Vafaee, R.; Azodi, T.; Omidi, R.; Gilanchi, S.; Azizi-Jalilian, F.; Khodarahmi, R. Molecular approaches in obesity studies. *Gastroenterol. Hepatol. Bed Bench* **2013**, *6* (Suppl. 1), S23–S31.

7. Hodson, L.; Skeaff, C.M.; Fielding, B.A. Fatty acid composition of adipose tissue and blood in humans and its use as a biomarker of dietary intake. *Prog. Lipid Res.* **2008**, *47*, 348–380.

8. Viviani Anselmi, C.; Ferreri, C.; Novelli, V.; Roncarati, R.; Bronzini, R.; Marchese, G.; Somalvico, F.; Condorelli, G.; Montenero, A.S.; Puca, A.A. Fatty acid percentage in erythrocyte membranes of atrial flutter/fibrillation patients and controls. *J. Interv. Card. Electrophysiol.* **2010**, *27*, 95–99.

9. Ghezzo, A.; Visconti, P.; Abruzzo, P.M.; Bolotta, A.; Ferreri, C.; Gobbi, G.; Malisardi, G.; Manfredini, S.; Marini, M.; Nanetti, L. Oxidative Stress and Erythrocyte Membrane Alterations in Children with Autism: Correlation with Clinical Features. *PLoS ONE* **2013**, *8* e66418.

10. Van Meer, G.; Voelker, D.R.; Feigenson, G.W. Membrane lipids: Where they are and how they behave. *Nat. Rev. Mol. Cell Biol.* **2008**, *9*, 112–124.

11. Ferreri, C.; Masi, A.; Sansone, A.; Giacometti, G.; Larocca, A.V.; Menounou, G.; Scanferlato, R.; Tortorella, S.; Rota, D.; Conti, M.; et al. Fatty Acids in Membranes as Homeostatic, Metabolic and Nutritional Biomarkers: Recent Advancements in Analytics and Diagnostics. *Diagnostics* **2016**, *7*, 1.

12. Ferreri, C.; Chatgilialoglu, C. *Membrane Lipidomics for Personalized Health*; John Wiley and Sons, Incorporated: Hoboken, NJ, USA, 2015; pp. 87–88.

13. Risé, P.; Eligini, S.; Ghezzi, S.; Colli, S.; Galli, C. Fatty acid composition of plasma, blood cells and whole blood: Relevance for the assessment of the fatty acid status in humans. *Prostaglandins Leukot. Essent. Fat. Acids* **2007**, *76*, 363–369.

14. Sun, Q.; Ma, J.; Campos, H.; Hankinson, S.E.; Hu, F.B. Comparison between plasma and erythrocyte fatty acid content as biomarkers of fatty acid intake in US women. *Am. J. Clin. Nutr.* **2007**, *86*, 74–81.

15. Gunes, O.; Tascilar, E.; Sertoglu, E.; Tas, A.; Serdar, M.A.; Kaya, G.; Kayadibi, H.; Ozcan, O. Associations between erythrocyte membrane fatty acid compositions and insulin resistance in obese adolescents. *Chem. Phys. Lipids* **2014**, *184*, 69–75.

16. Sánchez González, E.; Carrascosa Lezcano, A.; Fernández García, J.M.; Ferrández Longás, A.; López de Lara, D.; López-Siguero, J.P. Estudios españoles de crecimiento: Situación actual, utilidad y recomendaciones de uso. *An. Pediatría* **2011**, *74*, 193.e1–e16.

17. Carrascosa, A.; Fernández, J.M.; Fernández, C.; Ferrández, A.; López-Siguero, J.P.; Sánchez, E.;& Yeste, D. Estudios españoles de crecimiento 2008. Nuevos patrones antropométricos. *Endocrinol. Nutr.* **2008**, *55*, 484–506.

18. Madrigal, C.; Soto-Mendez, M.J.; Hernandez-Ruiz, A.; Ruiz, E.; Valero, T.; Avila, J.M.; Lara-Villoslada, F.; Leis, R.; Martínez de Victoria, E.; Moreno, J.M.; et al. Dietary and Lifestyle Patterns in the Spanish Pediatric Population (One to <10 Years Old): Design, Protocol, and Methodology of the EsNuPI Study. *Nutrients* **2019**, *11*, 3050.

19. Fernandez-Ballart, J.D.; Pinol, J.L.; Zazpe, I.; Corella, D.; Carrasco, P.; Toledo, E.; Perez-Bauer, M.; Martínez-González, M.Á.; Salas-Salvadó, J.; Martín-Moreno, J.M. Relative validity of a semi-quantitative food-frequency questionnaire in an elderly Mediterranean population of Spain. *Br. J. Nutr.* **2010**, *103*, 1808–1816.

20. Pérez-Farinós, N.; Villar-Villalba, C.; López Sobaler, A.M.; Dal Re Saavedra, M.; Aparicio, A.; Santos Sanz, S.; de Dios, T.R.; Castrodeza-Sanz, J.J.; Anta, R.M. The relationship between hours of sleep, screen time and frequency of food and drink consumption in Spain in the 2011 and 2013 ALADINO: A cross-sectional study. *BMC Public Health* **2017**, *17*, 1-12.

21. Stefan, L.; Prosoli, R.; Juranko, D.; Cule, M.; Milinovic, I.; Novak, D.; Sporiš, G. The Reliability of the Mediterranean Diet Quality Index (KIDMED) Questionnaire. *Nutrients* **2017**, *9*, 419.

22. Serra-Majem, L.; Ribas, L.; Ngo, J.; Ortega, R.M.; Garcia, A.; Perez-Rodrigo, C.; Aranceta, J. Food, youth and the Mediterranean diet in Spain. Development of KIDMED, Mediterranean Diet Quality Index in children and adolescents. *Public Health Nutr.* **2004**, *7*, 931–935.

23. Sansone, A.; Tolika, E.; Louka, M.; Sunda, V.; Deplano, S.; Melchiorre, M.; Anagnostopoulos, D.; Chatgilialoglu, C.; Formisano, C.; Di Micco, R.; et al. Hexadecenoic Fatty Acid Isomers in Human Blood Lipids and Their Relevance for the Interpretation of Lipidomic Profiles. *PLoS ONE* **2016**, *11*, e0152378.

24. Giacometti, G.; Ferreri, C.; Sansone, A.; Chatgilialoglu, C.; Marzetti, C.; Spyratou, E.; Georgakilas, A.G.; Marini, M.; Abruzzo, P.M.; Bolotta, A.; et al. High predictive values of RBC membrane-based diagnostics by biophotonics in an integrated approach for Autism Spectrum Disorders. *Sci. Rep.* **2017**, *7*, 9854. Amezaga,

25. J.; Arranz, S.; Urruticoechea, A.; Ugartemendia, G.; Larraioz, A.; Louka, M.; Uriarte, M.; Ferreri, C.; Tueros, I. Altered Red Blood Cell Membrane Fatty Acid Profile in Cancer Patients. *Nutrients* **2018**, *10*, 1853.

26. Van Der Vegt, S.G.L.; Ruben, A.M.T.; Werre, J.M.; Palsma, D.M.H.; Verhoef, C.W.; de Gier, J.; Staal, G.E. Counterflow centrifugation of red cell populations: A cell age related separation technique. *Br. J. Haematol.* **1985**, *61*, 393–403.

27. Breil, C.; Abert Vian, M.; Zemb, T.; Kunz, W.; Chemat, F. "Bligh and Dyer" and Folch Methods for Solid-Liquid-Liquid Extraction of Lipids from Microorganisms. Comprehension of Solvatation Mechanisms and towards Substitution with Alternative Solvents. *Int. J. Mol. Sci.* **2017**, *18*, 708.

28. Ferreri, C.; Faraone Mennella, M.R.; Formisano, C.; Landi, L.; Chatgilialoglu, C. Arachidonate geometrical isomers generated by thiyl radicals: The relationship with trans lipids detected in biological samples. *Free Radic. Biol. Med.* **2002**, *33*, 1516–1526.

29. Cohen, J. Statistical Power Analysis. *Curr. Dir. Psychol. Sci.* **1992**, *1*, 98–101.

30. Choi, H.-N, Yim, J.-E. Effects of Erythrocyte Membrane Polyunsaturated Fatty Acids in Overweight, Obese, and Morbidly Obese Korean Women. *J. Cancer Prev.* **2017**, *22*, 182–188.

31. Genio, G. Morbid Obesity is Associated to Altered Fatty Acid Profile of Erythrocyte Membranes. *J. Diabetes Metab.* **2015**, *6*, 1–4.

32. Bonafini, S.; Tagetti, A.; Gaudino, R.; Cavarzere, P.; Montagnana, M.; Danese, E.; Benati, M.; Ramaroli, D.A.; Raimondi, S.; Giontella, A.; et al. Individual fatty acids in erythrocyte membranes are associated with several features of the metabolic syndrome in obese children. *Eur. J. Nutr.* **2019**, *58*, 731–742.

33. Fekete, K.; Györei, E.; Lohner, S.; Verduci, E.; Agostoni, C.; Decsi, T. Long-chain polyunsaturated fatty acid status in obesity: A systematic review and meta-analysis. *Obes. Rev. Off. J. Int. Assoc. Study Obes.* **2015**, *16*, 488–497.

34. Araya J, Rodrigo R, Videla LA, Thielemann L, Orellana M, Pettinelli P, et al. Increase in long-chain polyunsaturated fatty acid n − 6/n − 3 ratio in relation to hepatic steatosis in patients with non-alcoholic fatty liver disease. *Clin. Sci.* **2004**, *106*, 635–643.

35. Savva, S.C.; Chadjigeorgiou, C.; Hatzis, C.; Kyriakakis, M.; Tsimbinos, G.; Tornaritis, M.; Kafatos, A. Association of adipose tissue arachidonic acid content with BMI and overweight status in children from Cyprus and Crete. *Br. J. Nutr.* **2004**, *91*, 643–649.

36. Pietiläinen, K.H.; Róg, T.; Seppänen-Laakso, T.; Virtue, S.; Gopalacharyulu, P.; Tang, J.; Rodriguez-Cuenca, S.; Maciejewski, A.; Naukkarinen, J.; Ruskeepää, A.L.; et al. Association of lipidome remodeling in the adipocyte membrane with acquired obesity in humans. *PLoS Biol.* **2011**, *9*, 7.

37. Stark, K.D.; Van Elswyk, M.E.; Higgins, M.R.; Weatherford, C.A.; Salem, N. Global survey of the omega-3 fatty acids, docosahexaenoic acid and eicosapentaenoic acid in the blood stream of healthy adults. *Prog. Lipid Res.* **2016**, *63*, 132–152.

38. Sonnweber, T.; Pizzini, A.; Nairz, M.; Weiss, G.; Tancevski, I. Arachidonic Acid Metabolites in Cardiovascular and Metabolic Diseases. *Int. J. Mol. Sci.* **2018**, *19*, 3285.

39. Burns, J.L.; Nakamura, M.T.; Ma, D.W.L. Differentiating the biological effects of linoleic acid from arachidonic acid in health and disease. *Prostaglandins Leukot. Essent. Fat. Acids* **2018**, *135*, 1–4.

40. Ailhaud, G.; Guesnet, P.; Cunnane, S.C. An emerging risk factor for obesity: Does disequilibrium of polyunsaturated fatty acid metabolism contribute to excessive adipose tissue development? *Br. J. Nutr.* **2008**, *100*, 461–470.

41. Muhlhausler, B.S.; Ailhaud, G.P. Omega-6 polyunsaturated fatty acids and the early origins of obesity. *Curr. Opin. Endocrinol. Diabetes Obes.* **2013**, *20*, 56–61.

42. Simopoulos, A.P. An Increase in the Omega-6/Omega-3 Fatty Acid Ratio Increases the Risk for Obesity. *Nutrients* **2016**, *8*, 128.

43. Cao, H.; Gerhold, K.; Mayers, J.R.; Wiest, M.M.; Watkins, S.M.; Hotamisligil, G.S. Identification of a lipokine, a lipid hormone linking adipose tissue to systemic metabolism. *Cell* **2008**, *134*, 933–944.

44. Warensjo, E.; Ohrvall, M.; Vessby, B. Fatty acid composition and estimated desaturase activities are associated with obesity and lifestyle variables in men and women. *Nutr. Metab. Cardiovasc. Dis.* **2006**, *16*, 128–136.

45. Puri, P.; Wiest, M.M.; Cheung, O.; Mirshahi, F.; Sargeant, C.; Min, H.K.; Contos, M.J.; Sterling, R.K.; Fuchs, M.; Zhou, H.; et al. The plasma lipidomic signature of nonalcoholic steatohepatitis. *Hepatology* **2009**, *50*, 1827–1838.

46. Sjogren, P.; Sierra-Johnson, J.; Gertow, K.; Rosell, M.; Vessby, B.; de Faire, U.; Hamsten, A.; Hellenius, M.L.; Fisher, R.M. Fatty acid desaturases in human adipose tissue: Relationships between gene expression, desaturation indexes and insulin resistance. *Diabetologia* **2008**, *51*, 328–335.

47. Warensjo, E.; Riserus, U.; Vessby, B. Fatty acid composition of serum lipids predicts the development of the metabolic syndrome in men. *Diabetologia* **2005**, *48*, 1999–2005.

48. Notarnicola, M.; Caruso, M.G.; Tutino, V.; Bonfiglio, C.; Cozzolongo, R.; Giannuzzi, V.; De Nunzio, V.; De Leonardis, G.; Abbrescia, D.I.; Franco, I.; et al. Significant decrease of saturation index in erythrocytes membrane from subjects with non-alcoholic fatty liver disease (NAFLD). *Lipids Health Dis.* **2017**, *16*, 1-7.

49. Weijers, R. Membrane flexibility, free fatty acids, and the onset of vascular and neurological lesions in type 2 diabetes. *J. Diabetes Metab. Disord.* **2016**, *15*, 13.

50. De Toro-Martín, J.; Arsenault, B.J.; Després, J.P.; Vohl, M.C. Precision Nutrition: A Review of Personalized Nutritional Approaches for the Prevention and Management of Metabolic Syndrome. *Nutrients* **2017**, *9*, 913.

51. Abbaspour, N.; Roberts, T.; Hooshmand, S.; Kern, M.; Hong, M.Y. Mixed Nut Consumption May Improve Cardiovascular Disease Risk Factors in Overweight and Obese Adults. *Nutrients* **2019**, *11*, 1488.

52. Rock, C.L.; Flatt, S.W.; Barkai, H.S.; Pakiz, B.; Heath, D.D. Walnut consumption in a weight reduction intervention: Effects on body weight, biological measures, blood pressure and satiety. *Nutr. J.* **2017**, *16*, 76.

53. Razquin, C.; Sanchez-Tainta, A.; Salas-Salvado, J.; Buil-Cosiales, P.; Corella, D.; Fito, M.; Ros, E.; Estruch, R.; Arós, F.; Gómez-Gracia, E.; et al. Dietary energy density and body weight changes after 3 years in the PREDIMED study. *Int. J. Food Sci. Nutr.* **2017**, *68*, 865–872.

54. Kim, Y.; Keogh, J.B.; Clifton, P.M. Benefits of Nut Consumption on Insulin Resistance and Cardiovascular Risk Factors: Multiple Potential Mechanisms of Actions. *Nutrients* **2017**, *9*, 1271.

55. Ros, E. Health benefits of nut consumption. *Nutrients* **2010**, *2*, 652–682.

56. Iaccarino Idelson, P.; Scalfi, L.; Valerio, G. Adherence to the Mediterranean Diet in children and adolescents: A systematic review. *Nutr. Metab. Cardiovasc. Dis.* **2017**, *27*, 283–299.

57. Jennings, A.; Welch, A.; van Sluijs, E.M.; Griffin, S.J.; Cassidy, A. Diet quality is independently associated with weight status in children aged 9–10 years. *J. Nutr.* **2011**, *141*, 453–459.

58. Lydakis, C.; Stefanaki, E.; Stefanaki, S.; Thalassinos, E.; Kavousanaki, M.; Lydaki, D. Correlation of blood pressure, obesity, and adherence to the Mediterranean diet with indices of arterial stiffness in children. *Eur. J. Pediatr.* **2012**, *171*, 1373–1382.

59. Saravia, L.; Miguel-Berges, M.L.; Iglesia, I.; Nascimento-Ferreira, M.V.; Perdomo, G.; Bove, I.; Slater, B.; Moreno, L.A. Relative validity of FFQ to assess food items, energy, macronutrient and micronutrient intake in children and adolescents: A systematic review with meta-analysis. *Br. J. Nutr.* **2020**, *18*, 1–27.

7.2. Molecular differences based on erythrocyte fatty acid profile to personalize dietary strategies between adults and children with obesity.

MDPI

Article

Molecular Differences Based on Erythrocyte Fatty Acid Profile to Personalize Dietary Strategies between Adults and Children with Obesity

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Abstract: As the obesity epidemic continues to grow inexorably worldwide, the need to develop effective strategies to prevent and control obesity seems crucial. The use of molecular tools can be useful to characterize different obesity phenotypes to provide more precise nutritional recommendations. This study aimed to determine the fatty acid (FA) profile of red blood cell (RBC) membranes, together with the evaluation of their dietary intake and biochemical parameters, of children and adults with obesity. An observational study was carried out on 196 children (113 with normal weight and 83 with obesity) and 91 adults (30 with normal weight and 61 with obesity). Mature RBC membrane phospholipids were analyzed for FA composition by gas chromatography-mass spectrometry (GC-MS). Dietary habits were evaluated using validated food frequency questionnaires (FFQ). Children with obesity presented higher levels of ω-6 polyunsaturated FAs (mainly linoleic acid, $p = 0.01$) and lower values of ω-3 FAs (mainly DHA, $p < 0.001$) compared with adults. Regarding blood biochemical parameters, children with obesity presented lower levels of glucose, LDL cholesterol, and alanine aminotransferase compared with adults with obesity. These lipidomic differences could be considered to provide specific nutritional recommendations for different age groups, based on an adequate fat intake.

Keywords: lipid metabolism; mature erythrocyte; obesity; precision nutrition

1. Introduction

In the last decades, unhealthy dietary patterns are increasing and affecting the prevalence of noncommunicable diseases in the world. According to the World Health Organization (WHO), worldwide obesity has nearly tripled since 1975, indicating that, in 2016, 39% of adults aged 18 years and over, were overweight and 13% suffered from obesity [1]. In addition to affecting the adult population, obesity is becoming a rising problem affecting children and adolescents as well. As the WHO states, more than 340 million children and adolescents, around one in three from 5 to 19 years, were overweight or obese in 2016 and 38 million children under the age of 5 were overweight or obese in 2019 [1].

Obesity prevention and treatment strategies include lifestyle and behavioral interventions, focused on changes in diet and physical activity. Low-fat diets, aimed at reducing caloric intake, have been the most recommended strategy for people with obesity in the past decades [2]. Moreover, there is not enough evidence from randomized control trials supporting the beneficial effects of low-fat diets over other dietary interventions for long–term weight loss [2]. Besides, recent scientific evidence showed that low-fat diets reduced LDL and HDL cholesterol and increase triglycerides. Further, the replacement of saturated fatty acids (SFA) with monounsaturated fatty acids (MUFA) has been proposed as an appropriate strategy to reduce obesity, since substituting SFA with MUFAs raises HDL-cholesterol levels, improves insulin sensitivity, and lowers LDL-cholesterol levels [3–5]. Other dietary plans, as a strategy for obesity management, have been proposed including low–carbohydrate diets, high-protein diets, very-low-calorie diets with meal replacements, Mediterranean diet, and diets with intermittent energy restrictions, evidencing that a successful diet to reduce weight must be healthy, balanced and without nutritional deficiencies. In any case, most of them include general dietary recommendations rather than specific dietary plans based on individual metabolism [6].

However, obesity prevalence in both child and adult populations continues increasing worldwide, suggesting that, personalized intervention strategies could provide precise nutritional guidance and contribute to successful long-term interventions [7]. Even though dietary guidelines for macronutrients intake in adults and children are established [8,9], according to the different requirements of both population groups, especially from energy intake, interventions to control obesity in children and adults are not specific nor differentiated, regarding the intake of food groups or specific nutrients. For that reason, the optimal macronutrient distribution of the diet to improve weight status is unclear [10]. The use of molecular tools (metabolomic, nutrigenetic, metagenomic, etc.) can provide new scientific evidence related to the characterization of different obesity phenotypes together with the impact of diet on metabolism [11]. This can be useful to personalize therapy and contribute to providing more precise nutritional recommendations, mainly for an adequate fat intake for different age groups and health conditions [12–14].

The use of mature erythrocyte membrane as a representative site for all other body tissues in FA profiling is an established protocol for membrane-based molecular diagnos- tics [15–17]. The measure of the lipid profile at the cellular level, precisely at the membrane phospholipid level, provides not only information related to the nutritional status of an individual, but also information related to FA metabolism that is involved in the formation of the most important lipid building blocks for cell life, which are the phospholipids. This approach has a profound diagnostic meaning, not only from the biochemical point of view related to the lipid pathways, but also from the biophysical and biological consequences, since the balance reached by the FA components of the membrane phospholipids must respect the tissue type and, ultimately, satisfy the homeostatic requirement for the optimal cell functioning [18].

This study aimed to evaluate lipid profile differences in mature RBC membranes between children and adults with obesity, in relation to their nutrient intake. Defining these differences in RBC FA profiles, related to individual molecular and nutritional status, will allow the design of differentiated nutrition strategies for children and adults with obesity, giving relevance to the functional roles of different types of dietary fats.

2. Results

2.1. Descriptive Characteristics of the Participants

A total of 83 children with obesity (26 boys and 57 girls) between 6 to 16 years old and a group of 61 adults with obesity (19 males and 42 females) between 19 to 68 years old participated in the study (Table S1 in Supplementary Material). At the same time, control subjects, consisting of 113 children and 30 adults with normal weight and same age ranges were also included. A matched gender distribution was found for children and adults with obesity ($p = 0.83$) but for normal weight group, there was not a matched gender distribution

(p = 0.04). Obvious differences were observed for age between children and adults with obesity and children and adults with normal weight.

2.2. Red Blood Cell Membrane Fatty Acids Profile

The pediatric group with obesity showed lower levels of palmitic acid and cis–vaccenic acid (p = 0.01 and p = 0.05 respectively) compared to the adult group with obesity (Table 1). LA, DGLA, and total ω–6 FA levels in the pediatric group with obesity were higher compared with the adult group with obesity (p = 0.01, p = 0.04, and p < 0.01 respectively). DHA and total ω-3 FA levels were lower for the pediatric group with obesity (p < 0.01 and p < 0.01 respectively) and hence, the ω-6/ω-3 ratio was higher (p < 0.01). Regarding other indexes, PUFA balance, PI, and UI were lower for the pediatric group with obesity (p < 0.01, p = 0.01 and p = 0.04 respectively).

Table 1. Red blood cell (RBC) membrane fatty acid profile.

Fatty Acid (%)	Group with Obesity					Group with Normal Weight				
	Pediatric n = 83		Adult n = 61		Ancova	Pediatric n = 113		Adult n = 30		Ancova
	Mean	SE	Mean	SE	p *	Mean	SE	Mean	SE	p *
Palmitic acid (C16:0)	22.31	0.16	23.22	0.22	0.01	22.51	0.10	22.72	0.23	0.43
Stearic acid (C18:0)	18.22	0.18	17.54	0.24	0.06	17.68	0.10	17.66	0.23	0.94
TOTAL SFA	40.55	0.16	40.78	0.21	0.48	40.21	0.10	40.22	0.23	0.97
Palmitoleic acid (C16:1)	0.46	0.02	0.37	0.03	0.08	0.41	0.02	0.48	0.03	0.08
Oleic acid (9c C18:1)	16.55	0.20	17.08	0.27	0.20	17.46	0.12	17.79	0.27	0.29
cis–Vaccenic acid (11c C18:1)	1.17	0.04	1.34	0.06	0.05	1.22	0.02	1.32	0.05	0.07
TOTAL MUFA	18.19	0.21	18.78	0.29	0.18	19.10	0.13	19.57	0.28	0.17
Linoleic acid (C18:2)	14.00	0.27	12.39	0.37	0.01	14.22	0.12	13.12	0.27	<0.01
DGLA (C20:3)	2.35	0.07	2.07	0.09	0.04	2.05	0.04	1.81	0.08	0.02
ARA (C20:4)	19.73	0.21	19.39	0.29	0.44	18.75	0.14	18.32	0.32	0.26
TOTAL ω6	36.12	0.29	33.84	0.39	<0.01	35.02	0.16	33.27	0.35	<0.01
EPA (C20:5)	0.49	0.04	0.63	0.06	0.10	0.59	0.02	0.65	0.05	0.31
DHA (C22:6)	4.52	0.19	5.84	0.26	<0.01	4.93	0.10	5.83	0.23	<0.01
TOTAL ω3	5.01	0.21	6.47	0.29	<0.01	5.52	0.11	6.48	0.26	<0.01
TOTAL PUFA	41.12	0.24	40.31	0.32	0.10	40.54	0.15	39.88	0.33	0.09
Trans C18:1	0.08	0.01	0.07	0.01	0.65	0.08	0.01	0.10	0.02	0.34
Trans C20:4	0.07	0.01	0.08	0.01	0.59	0.07	0.01	0.10	0.02	0.14
TOTAL TRANS	0.15	0.01	0.15	0.02	0.98	0.16	0.01	0.14	0.02	0.46
Indexes										
ω6/ω3	7.55	0.27	5.51	0.37	<0.01	6.66	0.15	5.11	0.34	<0.01
SFA/MUFA	2.24	0.03	2.19	0.04	0.37	2.12	0.02	2.07	0.04	0.32
Omega–3 Index	5.01	0.21	6.47	0.29	<0.01	5.52	0.11	6.48	0.26	<0.01
Δ6D+ELO 20:3/18:2 [a]	0.17	0.006	0.17	0.008	0.96	0.14	0.003	0.14	0.006	–
Δ5D 20:4/20:3	8.60	0.31	9.51	0.41	0.15	9.45	0.21	10.55	0.47	0.05
Δ9D 16:1/16:0	0.02	0.001	0.016	0.001	0.06	0.018	0.001	0.02	0.001	–
Δ9D 18:1/18:0	0.91	0.02	0.98	0.02	0.07	0.99	0.01	1.00	0.01	0.67
DNL Index 16:0/18:2	1.62	0.03	1.85	0.05	<0.01	1.59	0.02	1.72	0.03	<0.01
PUFA BALANCE	12.13	0.52	16.09	0.70	<0.01	13.61	0.27	16.21	0.61	<0.01
Peroxidation Index	136.77	1.42	145.07	1.91	0.01	136.81	0.82	141.31	1.89	0.04
Unsaturation index	161.53	0.92	165.51	1.24	0.04	161.25	0.61	163.57	1.37	0.15

Data are presented as mean ± standard error (SE). * Adjusted for age, sex, and dietary components, extracted from the principal component analysis of dietary nutrient intake (individual FAs, families (SFA, MUFA, and PUFA), total lipids, carbohydrates, and proteins). Post hoc tests were conducted with a Bonferroni adjustment. [a] Levene's test of homogeneity of variance was not met.

After observing these differences, a sample of normoweight adults (30) and children (113) were analyzed to determine if these differences observed between the groups with obesity were due only to age differences or whether they could be attributed to metabolic differences. Similar patterns for LA, DGLA, DHA, total ω-3 FA levels, total ω–6 FA levels, and ω-6/ω-3 ratios were observed in the normoweight populations, but no differences for palmitic acid, cis–vaccenic acid, or UI were observed when comparing adult and child populations with normal weight. The DNL index [19], which is the ratio between C16:0/C18:2 ω-6 fatty acids, that correlates directly with the liver fat content, appears in higher levels for adults with normal weight and obesity when compared with the respective child populations ($p < 0.01$ for both).

2.3. Blood Biochemical Parameters

Blood biochemical parameters were determined in a subsample of the study (Table 2). Glucose levels were significantly higher in the adult populations with obesity ($p < 0.001$) but not for the groups with normal weight ($p = 0.38$). Alanine Aminotransferase (ALT/GPT) values were lower for children with obesity compared with adults with obesity ($p = 0.03$) but no differences were observed between groups with normal weight. Cholesterol and triglycerides were statistically higher for adults with obesity ($p < 0.01$ for both) compared to children with obesity, but no differences between groups with normal weight were observed.

Table 2. Biochemical values measured in plasma in a fraction of the observed groups.

	Group with Obesity			Group with Normal Weight		
	Pediatric n = 69	Adult n = 44	p *	Pediatric n = 34	Adult n = 30	p *
	Med (Q1–Q3)	Med (Q1–Q3)		Med (Q1–Q3)	Med (Q1–Q3)	
Glucose (mg/dL)	85 (79–89.25)	97 (90.5–107.5)	<0.01	84 (81–89)	85 (79.75–92)	0.38
Uric Acid (mg/dL)	4.95 (4.375–5.7)	5.6 (4.9–6.95)	<0.01	3.95 (3.37–4.62)	4.75 (3.8–5.22)	0.03
Total Cholesterol (mg/dL)	150 (132.7–172)	180 (158–211)	<0.01	165 (148.5–186.7)	176.5 (141.7–206.2)	0.46
Triglycerides (mg/dL)	76 (55.5–108.7)	123 (89.5–180.5)	<0.01	65.5 (46–86)	68 (58.75–84.75)	0.48
HDL cholesterol (mg/dL)	44.6 (40.0–54.25)	47 (41.75–56)	0.32	55 (48.5–64.5)	59 (48.5–71)	0.48
LDL cholesterol (mg/dL) AST/GOT	88.4 (71.25–98)	118 (95–141)	<0.01	95 (77–110)	102 (72.5–121)	0.35
(U/L) ALT/GPT	22 (19–26.25)	20 (16.5–26.5)	0.12	26 (22–27)	19 (16.75–23.5)	<0.01
(U/L) Bilirubin	18.5 (15–23.25)	23 (15–35.5)	0.03	16 (13.75–18)	17 (12.75–21.25)	0.59
(mg/dL)	0.4 (0.3–0.6)	0.4 (0.2–0.5)	0.5	0.6 (0.4–1)	0.4 (0–0.625)	0.01

Data expressed as medians and quartile 1 and quartile 3 (Med (Q1–Q3)). * Not normally distributed variables. A Mann-Whitney U test was carried out.

2.4. Food Groups

To compare the dietary pattern of each study group, we considered food groups as shown in Table S2 (Supplementary Material) and observed that both adults with obesity and with normal weight showed a higher intake of vegetables, olive oil, white meat, oily fish, sugary drinks and dried fruits and nuts ($p < 0.01$ for all) compared to a group of children. On the other hand, cereals, legumes, and juice intakes were higher for the pediatric population ($p < 0.01$ for both obese and normoweight). In any case, these results should be taken into account from the perspective that, adults, reported a higher intake of daily calories, so when comparing food groups in grams per day units, it is normal to observe differences.

2.5. Nutrient Intake

Regarding macronutrient intake shown in Table 3, as both population groups differ in terms of quantity requirements, to compare each other, variables were expressed in %

of the energy obtained from each macro-micronutrient. Differences between obese adults and children were observed for total calories (Kcal/day) and the intake of carbohydrates, simple sugars, and total lipids. Some of these differences, such as calorie differences ($p < 0.01$) can be related to different requirements depending on age. The distribution of the energy intake obtained from macronutrients differs between both populations for carbohydrates ($p < 0.01$), being higher for the pediatric population. Although adults showed, proportionally, a higher intake of total lipids than children ($p < 0.01$), the pediatric population showed a higher intake of stearic acid ($p = 0.04$). Oleic acid and total MUFA intake were higher for the adult population. Regarding PUFAs, total ω-6, corresponding ω-6 fatty acids (LA and AA), and total ω-3 dietary intake were higher for adults than for children. The ω-6/ω-3 ratio was lower for the pediatric population ($p < 0.01$).

Table 3. Macronutrients and individual fatty acids (FA) intake expressed as % energy (%*E*) in pediatric and adult groups with obesity.

	Pediatric Group with Obesity, n = 83		Adult Group with Obesity, n = 61		Mann-Whitney U Test
	Mean	**SD**	**Mean**	**SD**	**p**
Macronutrient					
Calories (Kcal/day)	2044.1	564.3	2480.1	794.2	<0.01
Proteins (%E)	16.5	2.1	16.3	3.2	0.54
Carbohydrates (%E)	46.7	5.3	36.3	6.6	<0.01 *
Simple sugars (%E)	21.7	4.9	19.1	6.4	<0.01
Lipids (%E)	33.6	6.3	42.6	6.3	<0.01 *
Individual Fatty Acids					
C14:0	1.0	0.5	0.8	0.3	0.08
C16:0	6.1	1.3	5.6	1.0	0.02 *
C18:0	2.3	0.6	2.3	0.5	0.93
Total SFA	10.8	2.9	11.2	2.2	0.09
C16:1	0.5	0.1	0.5	0.1	0.86
C18:1	14.1	3.5	18.9	4.1	<0.01 *
Total MUFA	15.0	3.6	19.9	4.2	<0.01 *
C18:2	4.1	1.7	6.9	2.5	<0.01
C20:4	0.04	0.01	0.08	0.04	<0.01
Total ω6	4.1	1.7	7.0	2.5	<0.01
C18:3	0.54	0.13	0.79	0.34	<0.01
C20:5 (EPA)	0.07	0.06	0.07	0.05	0.24
C22:5 (DPA)	0.02	0.01	0.02	0.01	0.48
22:6 (DHA)	0.15	0.09	0.14	0.08	0.62
Total ω3	0.79	0.22	1.03	0.39	<0.01
Total PUFA	5.1	1.8	8.2	2.7	<0.01
ω6/ω3	5.5	2.2	7.2	2.8	<0.01

Data presented as mean and standard deviation (SD). Not normally distributed variables. A Mann–Whitney U test was carried out. * Normally distributed variables, an independent-samples t-test was performed.

2.6. RBC FAs and Blood Biochemical Parameters Correlation

Different correlation profiles between RBC FAs and biochemical parameters were observed for children and adults with obesity (Figures 1 and 2, respectively).

For the pediatric population inverse correlations between LA and total cholesterol and LDL cholesterol were observed, as well as an inverse correlation between EPA and triglycerides.

For adults, other correlations were observed. DHA correlated positively with AST and ALT. At the same time, oleic acid showed an inverse correlation with LDL cholesterol, and stearic acid correlated inversely with HDL cholesterol.

2.7. RBC FAs and Food Groups Values Correlation

Different correlations between RBC FAs and food groups were observed for adults and children with obesity (see heatmaps in Supplementary Figures S1 and S2, respectively). Children showed a positive correlation between EPA in RBC and white fish intake, and DHA in RBC with oily fish. Read meat correlated positively with cis-vaccenic acid in RBC. For adults, only positive correlations between trans fatty acids in RBC and eggs were observed.
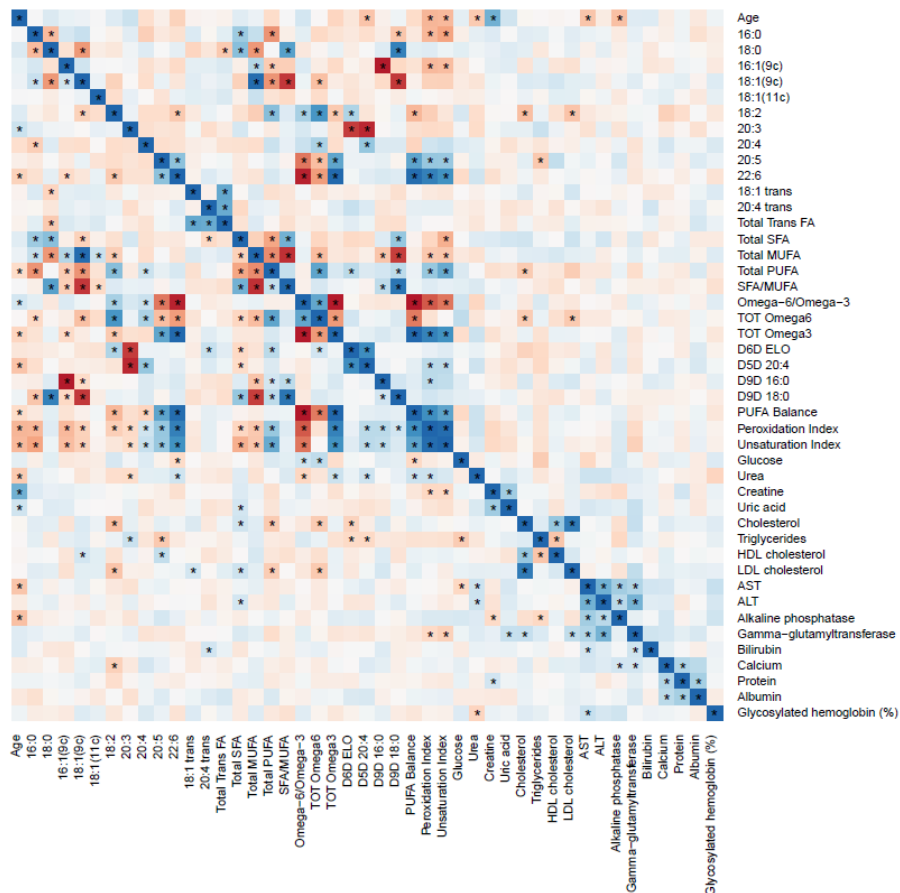

Figure 1. Heat map of the correlations between red blood cell membrane fatty acids and biochemical parameters for children with obesity. The color represents the Spearman correlation coefficient (ρ) (blue = positive; red = negative). * Represent significant correlations (*p* < 0.05) between variables. The pair variables that have an * above and below the diagonal line are significantly correlated after correction for multiple comparisons (*q* < 0.05).
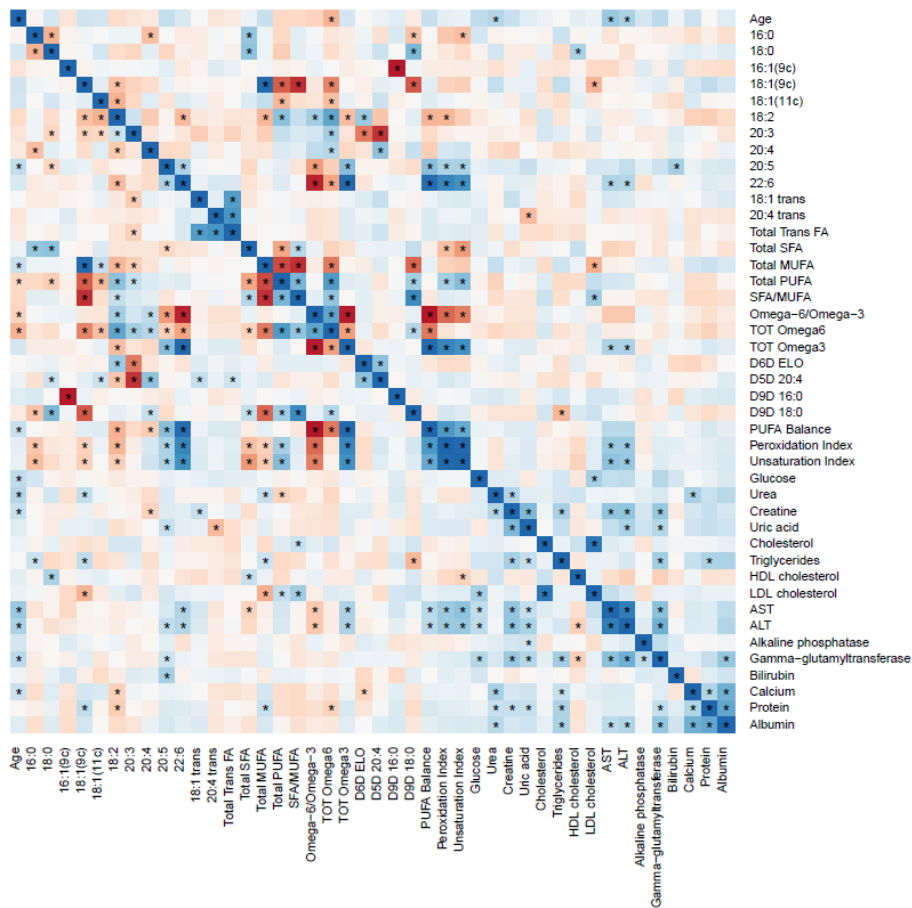
Figure 2. Heat map of the correlations between red blood cell membrane fatty acids and biochemical parameters for adults with obesity. The color represents the Spearman correlation coefficient (ρ) (blue = positive; red = negative). * Represent significant correlations ($p < 0.05$) between variables. The pair variables that have an * above and below the diagonal line are significantly correlated after correction for multiple comparisons ($q < 0.05$).

3. Discussion

To our knowledge, this is the first time that a comparison of RBC membrane FA com- position between adults and children with obesity has been made to determine metabolic differences, to establish dietary requirements that can contribute to design more precise nutritional strategies based on dietary fat quality to manage obesity at different age stages. The fact that the RBC membrane fatty acid composition is close to that of hepatocytes, having saturated (43% vs. 42%), monounsaturated (23.0% vs. 23.8%), polyunsaturated ω-6 (27.6 vs. 27.4%) and ω-3 (5.7% vs. 4.6%) fatty acid residues in almost similar quantities [20] is an important observation as the RBC examination avoids to run invasive investigations, especially in children.

Regarding individual nutrient intake, differences were observed between children and adults with obesity, taking into account that the nutritional recommendations for both population groups differ because metabolism requirements are different [21]. Two main reasons to measure dietary intake through a FFQ questionnaire were: to establish the eating pattern of each group and to consider dietary intake as a confounding factor in the Ancova analysis, of the study of metabolic differences, between the children and adults

with obesity [22]. The elimination of the variability generated by diet in the RBC FA profile, allows us to focus on the metabolic differences between adults and children with obesity. Concerning the dietary pattern of each group, the higher levels of ω6/ω3 intake for adults with obesity was an interesting result, showing its relationship with the quality and not with the quantity of lipids intake.

Measurement of RBC FAs revealed two differentiated profiles between children and adults with obesity, where not all the differences were attributable to age, as those results have been compared with a population of adults and children with normal weight and can be due to metabolic differences.

Related with age, differences in PUFA levels between pediatric and adult, for both group with normal weight and obesity, were observed. Adults with obesity showed a proportionally, higher intake of ω6 FAs than children with obesity, contributing to a higher intake ratio of ω6/ω3, but in the RBC membrane profile, adults with obesity showed lower levels of ω6/ω3 FA ratio. Even if the children´s intake of ω6 was lower compared with adults, a higher value of ω6/ω3 FA ratio in RBC membrane was determined for children with obesity. It can be observed, that for children the contribution to ω6 levels is given, in a significantly higher manner, by linoleic and DGLA acids, whereas for ω3 levels, is given by DHA, revealing a different metabolic fate of the dietary intakes. Certainly, the greater proportion of DHA needed for heart and brain tissues [23], could be responsible for a higher distribution of this FA in children compared to the adult group, because of the growth associated with that stage of life [24]. In our opinion, levels of DHA specifically determined in cell membranes, in particular in mature RBC membranes where PUFA ω-3 were found higher than in non-selected RBC [18], should be considered as important information of the bioavailability of essential or semi-essential FA for the fundamental building up of the membrane compartment. Formation of membranes is needed for living organisms [25] and must be combined with an appropriate composition of the FA pool to avoid critical imbalances. The fatty acid–based membrane lipidomics is a diagnostic tool that provides an important piece of information in the puzzle of the metabolic pathways of health and disease [18].

Similar connections with mediator formation can be inferred for the ω6 FAs in RBC, as LA and DGLA levels were higher for the pediatric population compared with the adult population, indistinctly for groups with obesity and normal weight. Higher values of DGLA in children compared to adults can indicate a metabolic connection with mediators for inflammatory, immune and defense processes, since this FA is a precursor of series 1 prostaglandins, like PGE1, connected with the cAMP activity and this can occur in a higher rate in children, regardless of the intake [26].

These metabolic differences appear as important factors to evaluate the fat quality and quantity especially in diseases which involve fats, such as obesity. The adequate nutritional strategies for each population group should be personalized, for example, reinforcing the ω3 FAs intake recommendation in the pediatric population compared to the adult population, because of their higher requirements [8]. Increasing dietary intake of ω3 sources such as oily fish and seafood, especially cold-water fatty fish (sardine, tuna, salmon, mackerel) and vegetable sources such as walnuts, chia seeds, flaxseed, or even with a personalized nutraceutical plan [27] could be a roadmap.

When comparing MUFA and SFA levels in RBC, adults with obesity showed higher levels of palmitic acid and cis–vaccenic acid compared with children with obesity, while these differences were not observed among the normoweight groups (Table 1). The % of energy obtained from palmitic acid in the diet, appears in a higher proportion for children with obesity than for adults with obesity, but contrary than expected adults with obesity, reflect a higher value of RBC palmitic acid in the membrane profile.

The higher value in the DNL index in adults with obesity compared with children with obesity, reflects a higher de novo synthesis of lipids, that can explain the higher levels of palmitic acid in adults with obesity [28]. Together with this higher DNL index, a tendency of a reduced activity of delta-9-desaturase ($p = 0.06$) for adults with obesity compared with

children with obesity can be observed. This reduced activity of the enzyme is correlated to factors that have been recalled several times in the SFA pathway, as: the absence of enzymatic cofactors, the inhibition of desaturase activity and liver impairment [29]. A high–carbohydrate diet can increase rates of DNL, that has been suggested to contribute to the pathogenesis of non–alcoholic fatty liver disease (NAFLD), linked in its turn to the development of type 2 diabetes mellitus [30].

As desaturase transformation prevents SFA accumulation and toxicity triggering hepatocellular apoptosis and liver damage [31], adults with obesity, that have suffered the accumulation of SFA for years, or at least for a longer time than children, might be a plausible reason that delta-9-desaturase presents a tendency of less activity in adults than in children with obesity.

In any case, recommendations should consider these differences between adults and children, highlighting the importance of not promoting de novo synthesis in adults by lowering the intake of SFA and simple carbohydrates. Moreover, increasing the intake of PUFAs has been associated to inhibitory effects on SFA and MUFA biosynthesis [32] and could be seen as a proper recommendation.

According to biochemical parameters, even if most of them are within the optimal ranges in both groups with obesity and normal weight, it is remarkable that glucose, total cholesterol, LDL cholesterol, triglycerides and ALT/GPT showed significative lower values for children with obesity compared with adults with obesity, fact that was not observed in the group with normal weight. Additionally, differentiated correlations between biochemical parameters and RBC FAs were observed. For children, higher levels of LA inversely correlated with LDL cholesterol and total cholesterol were determined (Supplementary Figure S1). This correlation has been previously reported also for both, circulating LA and RBC LA and cholesterol [33,34]. Food groups with higher content of LA, such as nuts, would be recommendable to lower LDL cholesterol levels in pediatric populations. On the other hand, adults showed an inverse correlation with oleic acid and LDL cholesterol, so higher consumption of food groups containing this FAs, such as olive oil, would be recommendable to reduce LDL cholesterol levels. SFA/MUFA ratio in adults, showed a positive correlation with LDL cholesterol, so the replacement of SFA with MUFAs in dietary intake would be advisable in order to reduce LDL cholesterol levels. For adults, DHA and total ω3 showed positive correlations with ALT and AST. Anyway, it has been reported in the literature that PUFAs can only decrease ALT and AST after long term supplementation in children [35].

As a limitation of the study, the uneven group distribution, the number of each group, should be noted even if in this type of observational studies, a perfect match is hard to achieve. At the same time, the indirect measurement of enzyme activity by the ratio between product and precursors, although very popular, could be considered as a limitation of the study and should be measured directly to emphasize and reaffirm the conclusions obtained. Another limitation of the study was the use of a population subgroup for the analysis of differences in biochemical parameters, which reduces the possibility of finding more significant differences and correlations.

In conclusion, the present study establishes the differences of RBC FA profiles, between children and adult with obesity, demonstrating that both groups have differentiated profiles. Children with obesity present higher LA, DGLA and total ω6 values, along with lower DHA and total ω3 values, compared to adults with obesity, even after adjusting the values by their dietary intake. At the same time, children with obesity presented lower levels of palmitic acid and a lower value of the de novo lipogenesis index compared to adults with obesity. These differences must be considered to provide more specific food group recommendations based on individual FA needs, rather than giving general recommendations for a population with obesity, as a whole and regardless of age.

4. Materials and Methods

4.1. Subjects and Study Design

An observational, case-control, and retrospective study was conducted on 83 children with obesity (26 boys and 57 girls) between 6 to 16 years old and a group of 61 adults with obesity (19 males and 42 females) between 19 to 68 years old, recruited from pe- diatric endocrinology and the endocrinology department at the Hospital Universitario Cruces (Barakaldo, Spain). Control subjects, consisting of 113 normoweight children and
30 normoweight adults, were also recruited from the same centers as patients with obesity. Children were classified according to body mass index (BMI), using age and sex-specific pediatric z-scores from Orbegozo tables [19]. The BMI was taken as a reference to define the different categories, defining normal weight when the standard deviation (SD) of BMI was $-1 < SD \leq +1$, overweight when $+1 < SD \leq +2$, and obesity when $SD > +2$. For adults, $BMI > 30 \ Kg/m^2$ was taken as a reference to classify obesity and $18.5 < BMI < 25 \ Kg/m^2$ for the normoweight group.

Subjects were excluded if they presented any kind of acute or chronic diseases, were tak- ing medications, had any presence of metabolic syndrome symptoms, or obesity-associated with any type of pathology. A physical examination was performed by an endocrinologist.

The study protocol was approved by the Euskadi Clinical Research Ethics Committee (permission number PI2016181) and carried out according to the Declaration of Helsinki Good Clinical Practice guidelines. Subjects under study were included after acceptance (by the parents in the case of the pediatric population) to participate in the study and signing of informed consent. In the case of children between 12–16 years of age the informed consent was also signed by themselves according to the Euskadi Ethical Committee and sample biobank laws (Organic Law 3∕2018, of December 5, on Protection of Personal Data and guarantee of digital rights; Law 14∕2007 on Biomedical Research and RD 1716∕2011 of Biobanks).

4.2. Anthropometric Measures

Bodyweight (kg) and height (cm) were measured by standardized methods [36]. Body mass index (BMI) was calculated as weight (kg) divided by the square of the height (m^2). Anthropometric parameters, as well as blood sampling, were all conducted by pediatricians and doctors during the participant's visit to the Hospital Universitario Cruces/IIS Biocruces Bizkaia.

4.3. Nutrient Intakes

During the participant's visit with the endocrinologist, the doctor interviewed the participants and collected personal data, including family medical history and information on the history of medication usage. Estimations of food consumption, including dietary diversity and variety, were measured using a quantitative food frequency questionnaire (FFQ) on-line completed by the parents of the children, except in those cases of adolescents, which were encouraged to complete it themselves, or by each adult volunteer. For our study, an adapted FFQ was used, which was previously validated with the portion sizes and food groups for the Spanish juvenile population and adults [37,38]. These questionnaires were then analyzed using the DIAL[®] software (UCM & Alce Ingeniería S.A., Madrid, Spain) (V 3.4.0.10) to translate the intake of specific foods into their corresponding energy and nutrient values.

4.4. Red Blood Cell (RBC) Membrane Fatty Acid Analysis

The fatty acid composition of mature RBC membrane phospholipids was obtained from blood samples (approximately 2 mL) collected in vacutainer tubes containing ethylene-diaminetetraacetic acid (EDTA). Samples were shipped to the Lipidomic Laboratory ap- proved for the method by the UNI CEI EN ISO/EIC 17025:2018 (#1836L belonging to the company Lipinutragen, Bologna, Italy) and upon arrival underwent the certified procedure MEM_LIP_1 according to the quality control guidelines. At first, the absence of hemolysis was checked upon arrival. From the blood, the protocol consists of the selection of ma-

ture RBCs by a robotic platform, as reported previously [17,22,39,40], followed by lipid extraction and lipid transesterification to fatty acid methyl esters (FAMEs). Briefly, the whole blood in EDTA was centrifuged (4000 rpm for 5 min at 4 °C), and the mature cell fraction was isolated by the robotic platform, based on the higher density of the aged cells [41], and checked by the use of cell counter (Scepter 2.0, EMD Millipore, Darmstadt, Germany). The automation included cell lysis, isolation of the membrane pellets, phospholipid extraction from pellets using the Bligh and Dyer method [42], transesterification to FAMEs by treatment with a potassium hydroxide (KOH)/methyl alcohol (MeOH) solution (0.5 mol/L) for 10 min at room temperature, and extraction using hexane (2 mL). The final FAME mixtures were analyzed using capillary column gas chromatography (GC). GC analysis was run on the Agilent 6850 Network GC System, equipped with a fused silica capillary column Agilent DB23 (60 m × 0.25 mm × 0.25 µm) and a flame ionization detector. Optimal separation of all fatty acids and their geometrical and positional isomers was achieved. Identification and quantification of each fatty acid were made by calibrated procedures that are part of the MEM_LIP_1 method. Commercially available standards and a library of trans isomers of MUFAs and polyunsaturated fatty acids (PUFA) were used as standards. The amount of each FA was calculated as a quantitative percentage of the total FA content (relative quantitative %), as described in Section 4.5, being more than 97% of the GC peaks recognized with appropriate standards. The use of mass spectrometry is only at the level of comparing the LIBRARY of fatty acid standard references mass data with the GC peaks and masses obtained from the samples.

4.5. Red Blood Cell Membrane Fatty Acid Cluster

12 FAs were selected as a representative cluster of the dominant glycerophospholipids present in the RBC membrane, as well as three FA families (SFA, MUFA and PUFA): for SFAs, palmitic acid (C16:0) and stearic acid (C18:0); for MUFAs, palmitoleic acid (C16:1;9c), oleic acid (C18:1; 9c), cis-vaccenic acid (C18:1; 11c); for ω-3 PUFAs, eicosapentaenoic acid (EPA) (C20:5), docosahexaenoic acid (DHA) (C22:6); for ω-6 PUFAs, linoleic acid (LA) (C18:2), dihomo–gamma–linolenic acid (DGLA) (C20:3) and arachidonic acid (AA) (C20:4); for geometrical trans fatty acids (TFA): elaidic acid (C18:1 9t) and mono–trans arachidonic acid isomers (monotrans-C20:4; ω-6 recognized by standard references as previously described by Ferreri et al. [43]. Considering these fatty acids, different indexes previously reported in the literature [22] were calculated: (%SFA/%MUFA) index related with membrane rigidity; Omega-3 index (DHA + EPA); Inflammatory risk index (%ω-6)/(%ω-3); PUFA balance [(%EPA + %DHA)/total PUFA × 100]; Free radical stress index (sum of trans-18:1 + Σ monotrans 20:4 isomers); Unsaturation Index (UI) [(%MUFA) + (%LA/2) + (%DGLA/3) + (%AA/4) + (% EPA/5) + (%DHA/6)]; Peroxidation Index (PI) [(%MUFA/0.025) + (%LA) + (%DGLA/2) + (%AA/4) + (% EPA/6) + (%DHA/8)]; De Novo Lipogenesis index (DNL) [(%Palmitic acid)/(%LA)] [44].

Additionally, the enzymatic indexes of elongase and desaturase enzymes, the two classes of enzymes of the MUFA and PUFA biosynthetic pathways, were inferred by calculating the product/precursor ratio of the FAs involved in these reactions.

4.6. Biochemical Parameters

Blood biochemical parameters were measured with standard laboratory assays after collecting venous blood samples performed in the morning in fasting state from a subgroup of the studied population (69 children vs. 44 adults with obesity and 34 pediatric vs. 30 adults with normal weight). Plasma concentrations of glucose, serum concentrations of total cholesterol (TC), high–density lipoprotein cholesterol (HDL-C), low–density lipoprotein cholesterol (LDL-C), triglycerides (TG), aspartate aminotransferase (AST), alanine aminotransferase (ALT), uric acid, and bilirubin were measured.

4.7. Statistical Analysis

Differences between groups for the nutrient intake and biochemical values were determined by using the Mann–Whitney U test for data that were not normally distributed and the t–student test for normally distributed variables. Normal data distribution was assessed by Shapiro–Wilk's test and Kolmogorov–Smirnov test.

An ANCOVA was run to determine the differences between RBC membrane fatty acids from children and adults with obesity, and also for normoweight children and normoweight adults, after controlling for variables selected as potential confounders, such as gender, BMI, and dietary intake [45]. Post hoc analysis was performed with a Bonferroni adjustment. First, a Principal Component Analysis (PCA) was run on 15 dietary nutrient intake variables (individual FAs, families (SFA, MUFA, and PUFA), total lipids, carbohydrates, and proteins) obtained with the DIAL software (v3.4.0.10, Department of Nutrition (UCM) & Alce Ingeniería, S.L., Madrid, Spain) after transforming the information about food items from FFQ questionnaires into micro and macronutrient values, to reduce and simplify the dimension of these variables and use the generated factors as diet covariates [22]. The Kaiser–Meyer–Olkin (KMO) and Bartlett's test of sphericity were used to verify the sampling adequacy for the analysis. PCA revealed four components that had eigenvalues greater than one and which explained 82.55% of the total variance. These components were included in the ANCOVA analysis as diet covariates. The level of significance was set at $p < 0.05$.

Correlations between the RBC membrane FA profile and dietary intake and FA profile and biochemical values were performed using Spearman's rank-order correlation coeffi- cients (ρ) and *p*-values were adjusted with the false discovery rate method for multiple comparisons. Correlation plots were visualized using the R heatmap.2() function. All other statistical analyses were performed using SPSS (IBM Corp. v24.0, Armonk, NY, USA).

Abbreviations

AA	arachidonic acid
ANCOVA	analysis of covariance body
BMI	mass index dihomo–gamma–
DGLA	linolenic acid docosahexaenoic
DHA	acid docosapentaenoic acid
DPA	ethylenediaminetetraacetic acid
EDTA	essential fatty acid
EFA	eicosapentaenoic acid
EPA	fatty acid
FA	food frequency questionnaire
FFQ	fatty acid methyl ester Kaiser–
FAME	Meyer–Olkin
KMO	methyl alcohol
MeOH	monounsaturated fatty acids
MUFA	potassium hydroxide
KOH	linoleic acid
LA	polyunsaturated fatty acids
PUFA	red blood cell
RBC	saturated fatty acids
SFA	stearoyl–CoA desaturase–1
SCD1	standard deviation
SD	trans fatty acids
TFA	unsaturation index
UI	peroxidation index
PI	principal component analysis
PCA	

References

1. World Health Organization. *Obesity and Overweight*; World Health Organization: Geneva, Switzerland, 2016; Available online: https://www.who.int/en/news-room/fact-sheets/detail/obesity-and-overweight (accessed on 1 April 2020).
2. Tobias, D.K.; Chen, M.; Manson, J.E.; Ludwig, D.S.; Willett, W.; Hu, F.B. Effect of low-fat diet interventions versus other diet interventions on long-term weight change in adults: A systematic review and meta-analysis. *Lancet Diabetes Endocrinol.* **2015**, *3*, 968–979. [CrossRef]
3. Mensink, R.P.; Zock, P.L.; Kester, A.D.; Katan, M.B. Effects of dietary fatty acids and carbohydrates on the ratio of serum total to HDL cholesterol and on serum lipids and apolipoproteins: A meta-analysis of 60 controlled trials. *Am. J. Clin. Nutr.* **2003**, *77*, 1146–1155. [CrossRef] [PubMed]
4. Sacks, F.M.; Katan, M. Randomized clinical trials on the effects of dietary fat and carbohydrate on plasma lipoproteins and cardiovascular disease. *Am. J. Med.* **2002**, *113* (Suppl. 9B), 13S–24S. [CrossRef]
5. Jakobsen, M.U.; O'Reilly, E.J.; Heitmann, B.L.; Pereira, M.A.; Bälter, K.; Fraser, G.E.; Goldbourt, U.; Hallmans, G.; Knekt, P.; Liu, S.; et al. Major types of dietary fat and risk of coronary heart disease: A pooled analysis of 11 cohort studies. *Am. J. Clin. Nutr.* **2009**, *89*, 1425–1432.
6. Koliaki, C.; Spinos, T.; Spinou, M.; Brinia, M.-E.; Mitsopoulou, D.; Katsilambros, N. Defining the Optimal Dietary Approach for Safe, Effective and Sustainable Weight Loss in Overweight and Obese Adults. *Healthcare* **2018**, *6*, 73. [CrossRef]

7. Teixeira, P.J.; Carraça, E.V.; Marques, M.M.; Rutter, H.; Oppert, J.M.; De Bourdeaudhuij, I.; Lakerveld, J.; Brug, J. Successful behavior change in obesity interventions in adults: A systematic review of self-regulation mediators. *BMC Med.* **2015**, *13*, 84. [CrossRef]

8. EFSA Panel on Dietetic Products, Nutrition, and Allergies. Scientific Opinion on Dietary Reference Values for fats, including saturated fatty acids, polyunsaturated fatty acids, monounsaturated fatty acids, trans fatty acids, and cholesterol. *EFSA J.* **2010**, *8*, 1461.

9. FAO. *Fats and Fatty Acids in Human Nutrition: Report of an Expert Consultation*; Food and Agriculture Organization of the United Nations: Rome, Italy, 2010.

10. Gow, M.L.; Ho, M.; Burrows, T.L.; Baur, L.A.; Stewart, L.; Hutchesson, M.J.; Cowell, C.T.; Collins, C.E.; Garnett, S.P. Impact of dietary macronutrient distribution on BMI and cardiometabolic outcomes in overweight and obese children and adolescents: A systematic review. *Nutr. Rev.* **2014**, *72*, 453–470. [CrossRef]

11. Camilleri, M.; Staiano, A. Insights on Obesity in Children and Adults: Individualizing Management. *Trends Endocrinol. Metab.* **2019**, *30*, 724–734. [CrossRef]

12. Abbott, S.K.; Else, P.L.; Atkins, T.A.; Hulbert, A.J. Fatty acid composition of membrane bilayers: Importance of diet polyunsaturated fat balance. *Biochim. Biophys. Acta Biomembr.* **2012**, *1818*, 1309–1317. [CrossRef]

13. Perona, J.S. Membrane lipid alterations in the metabolic syndrome and the role of dietary oils. *Biochim. Biophys. Acta Biomembr.* **2017**, *1859*, 1690–1703. [CrossRef] [PubMed]

14. Escribá, P.V. Membrane-lipid therapy: A historical perspective of membrane-targeted therapies—From lipid bilayer structure to the pathophysiological regulation of cells. *Biochim. Biophys. Acta Biomembr.* **2017**, *1859*, 1493–1506. [CrossRef] [PubMed]

15. Ferreri, C.; Chatgilialoglu, C. Role of fatty acid-based functional lipidomics in the development of molecular diagnostic tools. *Expert Rev. Mol. Diagn.* **2012**, *12*, 767–780. [CrossRef] [PubMed]

16. Genio, G. Morbid Obesity is Associated to Altered Fatty Acid Profile of Erythrocyte Membranes. *J. Diabetes Metab.* **2015**, *6*. Ghezzo,

17. A.; Visconti, P.; Abruzzo, P.M.; Bolotta, A.; Ferreri, C.; Gobbi, G.; Malisardi, G.; Manfredini, S.; Marini, M.; Nanetti, L.; et al. Oxidative Stress and Erythrocyte Membrane Alterations in Children with Autism: Correlation with Clinical Features. *PLoS ONE* **2013**, *8*, e66418. [CrossRef]

18. Ferreri, C.; Masi, A.; Sansone, A.; Giacometti, G.; Larocca, A.V.; Menounou, G.; Scanferlato, R.; Tortorella, S.; Rota, D.; Conti, M.; et al. Fatty Acids in Membranes as Homeostatic, Metabolic and Nutritional Biomarkers: Recent Advancements in Analytics and Diagnostics. *Diagnostics* **2016**, *7*, 1. [CrossRef]

19. Sánchez González, E.; Carrascosa Lezcano, A.; Fernández García, J.M.; Ferrández Longás, A.; López de Lara, D.; López-Siguero, J.P. Estudios españoles de crecimiento: Situación actual, utilidad y recomendaciones de uso. *Anal. Pediatr.* **2011**, *74*, 193.e1–193.e16. [CrossRef]

20. Lauritzen, L.; Hansen, H.S.; Jørgensen, M.H.; Michaelsen, K.F. The essentiality of long chain n-3 fatty acids in relation to development and function of the brain and retina. *Prog. Lipid. Res.* **2001**, *40*, 1–94. [CrossRef]

21. Khan, F.A.; Fisher, J.G.; Sparks, E.A.; Jaksic, T. Metabolism of Infants and Children. In *Pediatric Surgery: General Principles and Newborn Surgery*; Puri, P., Ed.; Springer: Berlin/Heidelberg, Germany, 2020; pp. 231–243.

22. Amezaga, J.; Arranz, S.; Urruticoechea, A.; Ugartemendia, G.; Larraioz, A.; Louka, M.; Uriarte, M.; Ferreri, C.; Tueros, I. Altered Red Blood Cell Membrane Fatty Acid Profile in Cancer Patients. *Nutrients* **2018**, *10*, 1853. [CrossRef] [PubMed]

23. Harayama, T.; Riezman, H. Understanding the diversity of membrane lipid composition. *Nat. Rev. Mol. Cell. Biol.* **2018**, *19*, 281–296. [CrossRef]

24. Weiser, M.J.; Butt, C.M.; Mohajeri, M.H. Docosahexaenoic Acid and Cognition throughout the Lifespan. *Nutrients* **2016**, *8*, 99. [CrossRef] [PubMed]

25. Sender, R.; Fuchs, S.; Milo, R. Revised Estimates for the Number of Human and Bacteria Cells in the Body. *PLoS Biol.* **2016**, *14*, e1002533. [CrossRef] [PubMed]

26. Kirtland, S.J. Prostaglandin E1: A review. *Prostaglandins Leukot. Essent. Fatty Acids* **1988**, *32*, 165–174. [CrossRef]

27. Jauregibeitia, I.; Portune, K.; Amezaga, J.; Tueros, I.; Arranz, S. A Journey through ?-3 Supplements: Future Perspectives for Precision Nutrition. *J. Food Nutr. Res.* **2020**, *8*, 556–560. [CrossRef]

28. Alves-Bezerra, M.; Cohen, D.E. Triglyceride Metabolism in the Liver. *Compr. Physiol.* **2017**, *8*, 1–8. [PubMed] Ferreri,

29. C.; Chatgilialoglu, C. *Membrane Lipidomics for Personalized Health*; John Wiley & Sons: Chichester, UK, 2015.

30. Sanders, F.W.; Griffin, J.L. De novo lipogenesis in the liver in health and disease: More than just a shunting yard for glucose. *Biol. Rev. Camb. Philos. Soc.* **2016**, *91*, 452–468. [CrossRef]

31. Silbernagel, G.; Kovarova, M.; Cegan, A.; Machann, J.; Schick, F.; Lehmann, R.; Häring, H.U.; Stefan, N.; Schleicher, E.; Fritsche, A.; et al. High Hepatic SCD1 Activity Is Associated with Low Liver Fat Content in Healthy Subjects under a Lipogenic Diet. *J. Clin. Endocrinol. Metab.* **2012**, *97*, E2288–E2292. [CrossRef]

32. Albracht-Schulte, K.; Kalupahana, N.S.; Ramalingam, L.; Wang, S.; Rahman, S.M.; Robert-McComb, J.; Moustaid-Moussa, N. Omega-3 fatty acids in obesity and metabolic syndrome: A mechanistic update. *J. Nutr. Biochem.* **2018**, *58*, 1–16. [CrossRef] Burns,

33. J.L.; Nakamura, M.T.; Ma, D.W.L. Differentiating the biological effects of linoleic acid from arachidonic acid in health and disease. *Prostaglandins Leukot. Essent. Fatty Acids* **2018**, *135*, 1–4. [CrossRef]

34. Ruiz-Núñez, B.; Kuipers, R.S.; Luxwolda, M.F.; De Graaf, D.J.; Breeuwsma, B.B.; Dijck-Brouwer, D.A.; Muskiet, F.A.J. Saturated fatty acid (SFA) status and SFA intake exhibit different relations with serum total cholesterol and lipoprotein cholesterol: A mechanistic explanation centered around lifestyle-induced low-grade inflammation. *J. Nutr. Biochem.* **2014**, *25*, 304–312. [CrossRef]
35. Chen, L.H.; Wang, Y.F.; Xu, Q.H.; Chen, S.S. Omega-3 fatty acids as a treatment for non-alcoholic fatty liver disease in children: A systematic review and meta-analysis of randomized controlled trials. *Clin. Nutr.* **2018**, *37*, 516–521. [CrossRef] [PubMed]
36. Carrascosa, A.; Fernández, J.M.; Fernández, C.; Ferrández, A.; López-Siguero, J.P.; Sánchez, E.; Sobradillo, B.; Yeste, D. Estudios españoles de crecimiento 2008. Nuevos patrones antropométricos. *Endocrinol. Nutr.* **2008**, *55*, 484–506. [CrossRef]
37. Madrigal, C.; Soto-Mendez, M.J.; Hernandez-Ruiz, A.; Ruiz, E.; Valero, T.; Avila, J.M.; Lara-Villoslada, F.; Leis, R.; Victoria, E.M.; Moreno, J.M.; et al. Dietary and Lifestyle Patterns in the Spanish Pediatric Population (One to < 10 Years Old): Design, Protocol, and Methodology of the EsNuPI Study. *Nutrients* **2019**, *11*, 3050.
38. Fernandez-Ballart, J.D.; Pinol, J.L.; Zazpe, I.; Corella, D.; Carrasco, P.; Toledo, E.; Perez-Bauer, M.; Martínez-González, M.A.; Salas-Salvadó, J.; Martín-Moreno, J.M. Relative validity of a semi-quantitative food-frequency questionnaire in an elderly Mediterranean population of Spain. *Br. J. Nutr.* **2010**, *103*, 1808–1816. [CrossRef]
39. Sansone, A.; Tolika, E.; Louka, M.; Sunda, V.; Deplano, S.; Melchiorre, M.; Anagnostopoulos, D.; Chatgilialoglu, C.; Formisano, C.; Di Micco, R.; et al. Hexadecenoic Fatty Acid Isomers in Human Blood Lipids and Their Relevance for the Interpretation of Lipidomic Profiles. *PLoS ONE* **2016**, *11*, e0152378. [CrossRef] [PubMed]
40. Giacometti, G.; Ferreri, C.; Sansone, A.; Chatgilialoglu, C.; Marzetti, C.; Spyratou, E.; Georgakilas, A.G.; Marini, M.; Abruzzo, P.M.; Bolotta, A.; et al. High predictive values of RBC membrane-based diagnostics by biophotonics in an integrated approach for Autism Spectrum Disorders. *Sci. Rep.* **2017**, *7*, 9854. [PubMed]
41. Van Der Vegt, S.G.L.; Ruben, A.M.T.; Were, J.M.; Palsma, D.M.H.; Verhoef, C.W.; de Gier, J.; Staal, G.E. Counterflow centrifugation of red cell populations: A cell age related separation technique. *Br. J. Haematol.* **1985**, *61*, 393–403. [CrossRef] [PubMed]
42. Breil, C.; Abert Vian, M.; Zemb, T.; Kunz, W.; Chemat, F. "Bligh and Dyer" and Folch Methods for Solid-Liquid-Liquid Extraction of Lipids from Microorganisms. Comprehension of Solvatation Mechanisms and towards Substitution with Alternative Solvents. *Int. J. Mol. Sci.* **2017**, *18*, 708. [CrossRef] [PubMed]
43. Ferreri, C.; Faraone Mennella, M.R.; Formisano, C.; Landi, L.; Chatgilialoglu, C. Arachidonate geometrical isomers generated by thiyl radicals: The relationship with trans lipids detected in biological samples. *Free Radic. Biol. Med.* **2002**, *33*, 1516–1526. [CrossRef]
44. Jacobs, S.; Jäger, S.; Jansen, E.; Peter, A.; Stefan, N.; Boeing, H.; Schulze, M.B.; Kröger, J. Associations of Erythrocyte Fatty Acids in the De Novo Lipogenesis Pathway with Proxies of Liver Fat Accumulation in the EPIC-Potsdam Study. *PLoS ONE* **2015**, *10*, e0127368. [CrossRef]
45. Jauregibeitia, I.P.K.; Rica, I.; Tueros, I.; Velasco, O.; Grau, G.; Trebolazabala, N.; Castaño, L.; Larocca, A.V.; Ferreri, C.; Arranz, S. Fatty Acid Profile of Mature Red Blood Cell Membranes and Dietary Intake as a New Approach to Characterize Children with Overweight and Obesity. *Nutrients* **2020**, *12*, 3446. [CrossRef]

7.2.1. Supplementary Material

Table S13: Characteristics of population groups

	GROUP WITH OBESITY					GROUP WITH NORMAL WEIGHT					COMPARISON BETWEEN GROUPS *	
	Pediatric		Adult			Pediatric		Adult			Pediatric	Adult
	Mean	SD	Mean	SD	p value	Mean	SD	Mean	SD	p value	p value	p value
AGE	11.0	2.3	46.4	10.5	<0.01	10.9	3.1	33.0	10.7	<0.01	0.78	<0.01
GENDER (% GIRLS)	68.3		70.0		0.83	45.5		66.7		0.04	<0.01	0.75
BMI	28.8	3.3	40.8	7.0	<0.01	18.0	2.5	22.4	1.2	<0.01	<0.01	<0.01

Data presented as mean and standard deviation (SD). Not normally distributed variables.

* A Mann-Whitney U test was carried out for p-values

Table S24: Food groups intake

Food Groups (g/day)	Group with obesity			Group with normal weight		
	Pediatric	Adult	p*	Pediatric	Adult	p*
	Med (Q1 - Q3)	Med (Q1 - Q3)		Med (Q1 - Q3)	Med (Q1 - Q3)	
Fruits	390 (264 - 577)	353 (231 - 682)	0.74	413 (297 - 531)	361 (210 - 691)	0.72
Vegetables	134 (76 - 237)	296 (158 - 442)	**<0.01**	160 (100 - 248)	313 (222 - 476)	**<0.01**
Cereals	132 (102 - 185)	98 (60 - 175)	**<0.01**	158 (117 - 210)	114 (94 - 224)	0.09
Legumes	80 (54 - 96)	43 (32 - 64)	**<0.01**	91 (50 - 102)	48 (40 - 86)	**<0.01**
Olive oil	15 (12 - 38)	38 (24 - 39)	**<0.01**	15 (15 - 38)	20 (16 - 38)	**0.04**
Dairy products	298 (207 - 374)	268 (163 - 509)	0.91	325 (254 - 513)	306 (175 - 436)	0.17
Eggs	15 (15 - 35)	30 (10 - 30)	0.45	15 (15 - 35)	30 (0 - 30)	0.21
Red meat	21 (21 - 50)	36 (18 - 63)	0.13	21 (21 - 21)	36 (18 - 47)	**<0.01**
White meat	50 (21 - 50)	98 (54 - 116)	**<0.01**	50 (21 - 50)	36 (0 - 112)	0.91
Dried Fruits and nuts	1 (0 - 3)	6 (0 - 21)	**<0.01**	2 (0 - 6)	7 (2 - 36)	**<0.01**
Lean fish	27 (27 - 27)	21 (11 - 64)	0.91	27 (27- 27)	21 (21 - 21)	**<0.01**
Oily fish and shellfish	27 (13 - 35)	61 (39 - 108)	**<0.01**	27 (13 - 27)	68 (47- 126)	**<0.01**
Sugary drinks	16 (0 - 54)	29 (14 - 86)	**<0.01**	18 (0 - 43)	14 (0 - 32)	0.18
Juices	80 (29 - 243)	14 (0 - 71)	**<0.01**	71 (27 - 250)	14 (0 - 89)	**<0.01**

Data expressed as medians and quartile 1 and quartile 3. *Not normally

distributed variables. A Mann-Whitney U test was carried out.

Supplementary Figure S1.
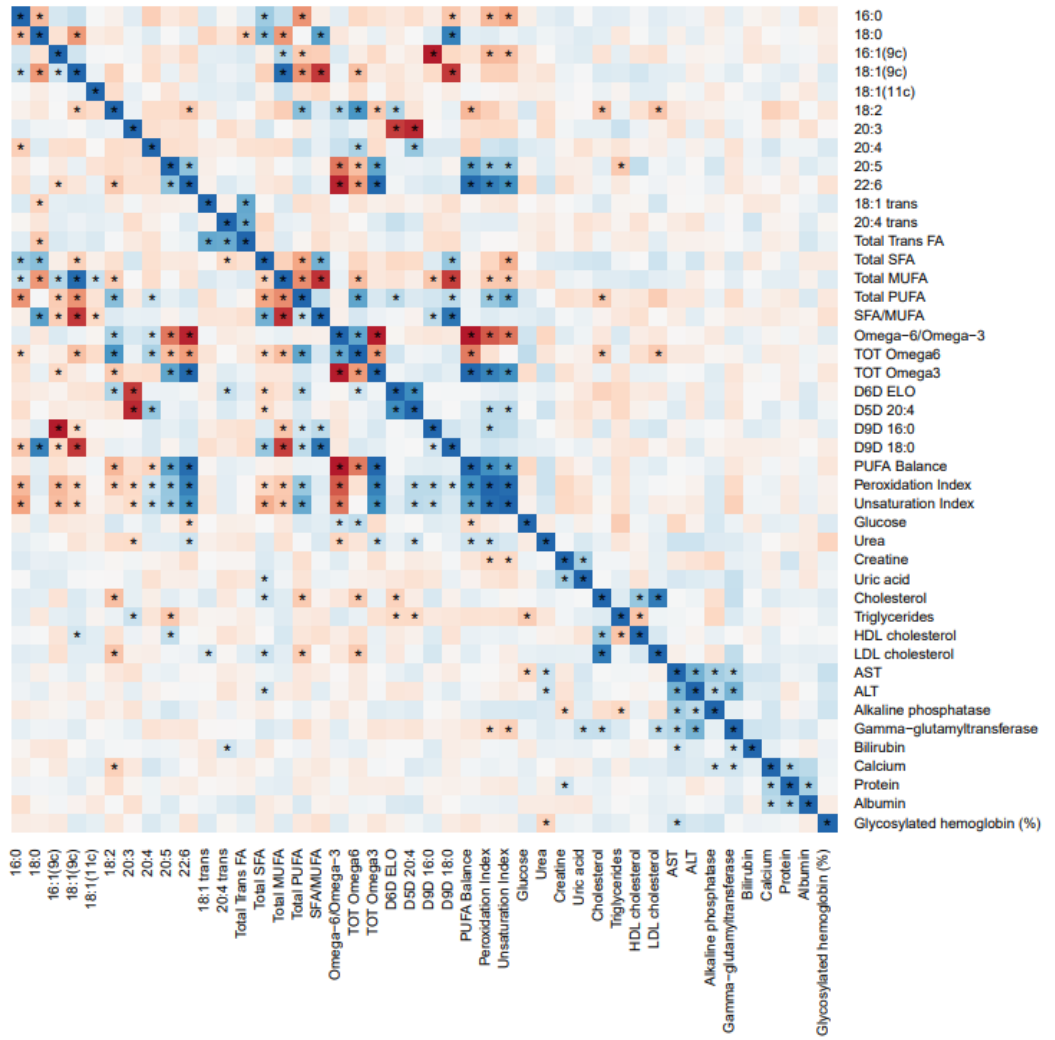
*Figure S1: Heat map of the correlations between red blood cell membrane fatty acids and food groups for children with obesity. The Key Colour represents the Spearman correlation coefficient (ρ) (blue = positive; red = negative). * Represent significant correlations (p <0.05) between variables. The pair variables that have an * above and below the diagonal line are significantly correlated after correction for multiple comparisons (q <0.05).*

Supplementary Figure S2.
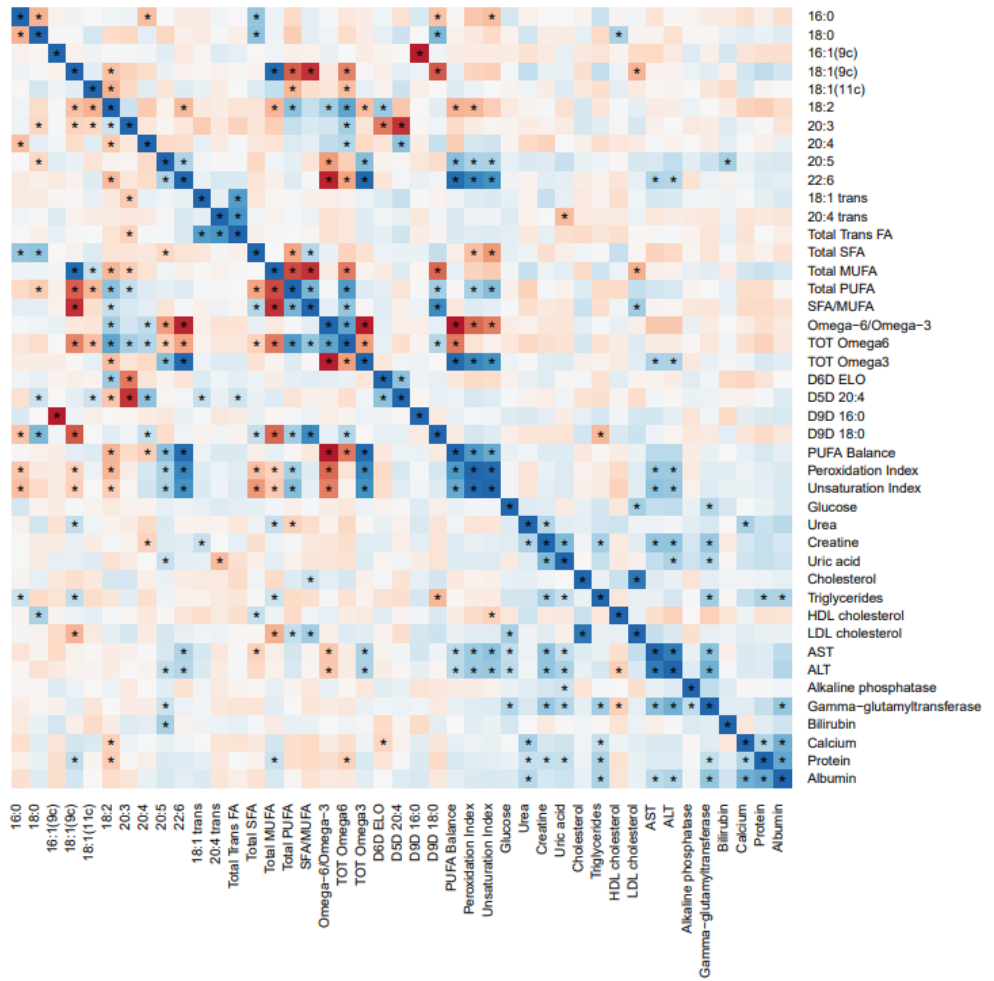
*Figure S2: Heat map of the correlations between red blood cell membrane fatty acids and food groups for adults with obesity. The Key Colour represents the Spearman correlation coefficient (ρ) (blue = positive; red = negative). * Represent significant correlations ($p < 0.05$) between variables. The pair variables that have an * above and below the diagonal line are significantly correlated after correction for multiple comparisons ($q < 0.05$).*

7.3. Potential of erythrocyte membrane lipid profile as a novel inflammatory biomarker to distinguish metabolically healthy obesity in children.

To be Submitted:

Iker Jauregibeitia, Kevin Portune, Itxaso Rica, Itziar Tueros, Olaia Velasco, Gema Grau, Luis Castaño, Federica Di Nolfo, Carla Ferreri and Sara Arranz

7.3.1. Abstract

Metabolically Healthy Obesity (MHO) has been described as BMI ≥ 30 kg/m2, without metabolic disorders traditionally associated with obesity. Beyond this definition a standardized criterion, for adults and children, has not been established yet to explain the absence of those metabolic disorders. In this context biomarkers of inflammation have been proposed as suitable candidates to describe MHO. The use of mature red blood cell fatty acid (RBC FA) profile is here proposed, since its membrane lipidome includes biomarkers of pro- and anti- inflammatory conditions with a strict relationship with metabolic and nutritional status that can differentiate MHO children. An observational study was carried out in 194 children (76 children with obesity and 118 children with normal weight) between 6 and 16 years old. RBC FA-s were analysed by gas chromatography-flame ionization detector (GC-FID). Dietary habits were evaluated using validated food frequency questionnaires (FFQ). An unsupervised hierarchical clustering method was conducted on children with obesity, based on the RBC FA profile, to isolate the MHO cluster. The MHO cluster showed FA levels similar to children with normal weight, characterised by lower values of arachidonic acid, total ω-6 FA, ω6/ω3 FA ratios and higher values for EPA, DHA and total ω-3 FA compared to the rest of the children with obesity (obese cluster). The MHO cluster also presented lipid indexes for higher desaturase enzymatic activity and lower SFA/MUFA ratio compared to the obese cluster. These differences are relevant for the follow-up of patients, also in view of personalized protocols providing tailored nutritional recommendations for the essential fatty acid intakes.

7.3.2. Introduction

Obesity is a growing health problem affecting children and adolescents. According to the WHO states, with more than 340 million children and adolescents, around one in three from 5 to 19 years old, in condition of

overweight or obesity in 2016, and 38 million children under the age of 5 overweight or obese in 2019 (24). Furthermore, children with obesity tend to be obese later in life, increasing their risk for morbidity and mortality (212).

Within this large patient cohort, there is an emerging evidence that not all of them display common obesity-associated metabolic disorders, such as insulin resistance, glucose intolerance, arterial hypertension, or dyslipidemia, suggesting that a paradox of metabolically healthy obesity (MHO) can exist, independent of fat accumulation (213). Indeed, although most people with obesity are metabolically unhealthy, a percentage varying from 10 to 30% of those with obesity (75, 78, 80, 214, 215), are considered MHO and do not present metabolic abnormalities (216).

Even though the existence of MHO has been known for decades (217), there is still a lack of a proper definition of metabolically healthy obese individuals. Some studies describe MHO as BMI ≥ 30 kg/m^2, without metabolic disorders (e.g. type 2 diabetes or dyslipidemia)(75, 213, 216) while others take into account their body fat percentage and insulin resistance (218). The MHO phenotype has been associated with a good metabolic profile characterized by high levels of insulin sensitivity, low prevalence of hypertension, and a favourable fasting glucose, lipid, and inflammatory profile (219). However, no consensus has been reached to define a standardized criterion to categorize MHO in adults. Recent investigations showed the importance of including inflammatory parameters as possible biomarkers to define MHO (84, 220) such as circulating blood proinflammatory cytokines, adipokines and acute-phase response proteins, that have been studied as possible underlying factors of inflammation (221).

Pediatric MHO was recently defined (222), as those children characterized by the absence of traditional cardiometabolic risk factors. However, recent studies also focused on considering other parameters such as hepatic steatosis,

inflammatory biomarkers, as well as the degree of visceral fat accumulation (223), as is considered in adult populations. Obesity in children has been also associated with circulating inflammatory mediators (224), where essential fats, such as ω-6 and ω-3 FAs, have a direct impact on the onset and control of metabolic pathways of inflammation (140).

Currently, there are only limited longitudinal studies on pediatric population evaluating MHO according to inflammatory biomarkers, and this is a limitation both in the clinical evaluation and in the personalization of the therapeutical approach, including nutritional intervention strategies for children with obesity, according to their metabolic health status.

In this regard, the use of mature erythrocyte as a representative site for all other body tissues, in particular determining the fatty acid-based membrane lipidomic profile, is an established protocol for membrane-based molecular diagnostics (165, 171, 225) and can help with monitoring the ω-6 and ω-3 FA contents in phospholipids that are directly linked with the inflammation mediators. Arachidonic acid, which is the main ω-6 FA in the RBC membrane, is released from membrane phospholipids, and exerts its activity in defence mechanisms, but the presence of a continuous inflammatory stimulus can induce an excess of inflammatory mediators thus triggering inflammation as a chronic status. The unbalance created by essential FA deficiency in diet, mainly ω-3 FA, is known to be connected to many symptoms and tissue malfunctions (162). On this basis, the membrane RBC the fatty acid profile can ultimately define the status of "silent inflammation" in obesity patients. It is worth adding the role of a key fatty acid in ω-6 pathway, namely the AA precursor, dihomo-gamma linolenic acid (DGLA), that is well known for its anti-inflammatory effects through the transformation into series 1 prostaglandins (226).

Moreover, a balance between ω-6 and ω-3 FA has been described as a good marker for inflammation status in a subject (40, 227).

In view of the intense research on molecular medicine and the set-up of nutritional intervention studies to better understand the impact of personalized diet on lipid metabolism in children, we were interested in understanding the role of mature RBC lipid profile to individuate metabolic differences in patients with obesity, extending previously published works on this subject (227-229).

In this study we used the mature RBC FA profile as a comprehensive biomarker to individuate the pro- and anti-inflammatory conditions and distinguish metabolically healthy obesity in children in relation with their nutritional status.

7.3.3. Materials and Methods

7.3.3.1. Subjects and study design

An observational study was conducted on 194 children (76 children with obesity and 118 children with normal weight) between 6 and 16 years old, recruited from the pediatric endocrinology unit at the Hospital Universitario Cruces (Barakaldo, Spain). Children were classified according to body mass index (BMI), using age- and sex-specific paediatric z-scores from Orbegozo table (23). BMI was taken as a reference to define the different categories, defining normal weight when the standard deviation (SD) of BMI was -1 <SD ≤ +1 and obesity when SD> +2. Groups were homogeneously distributed by age.

Subjects were excluded based on the following criteria: they presented any kind of acute or chronic diseases, were taking medications, had any presence of metabolic syndrome symptoms, or if their obesity condition was associated to any type of pathology. An anthropometric examination was performed by an endocrinologist.

The study protocol was approved by the Euskadi Clinical Research Ethics Committee (permission number PI2016181) and accomplished according to the

Helsinki Declaration in 1975, revised in 2013. Subjects under study were included after acceptance (by the parents of the individuals) to participate in the study and signing of informed consent. All the informed consent documents were signed by their parents and in the case of children between 12-16 years of age the informed consent was also signed by themselves according to the Euskadi Ethical Committee and sample biobank laws (Organic Law 3/2018, of December 5, on Protection of Personal Data and guarantee of digital rights; Law 14/2007 on Biomedical Research and RD 1716/2011 of Biobanks).

7.3.3.2. Anthropometric measures

Body weight (kg) and height (cm) were measured by standardized methods (22). Body mass index (BMI) was calculated as weight (kg) divided by the square of the height (m^2). Anthropometric parameters, as well as blood sampling were all conducted by pediatricians during the participant's visit to the Hospital Universitario Cruces.

7.3.3.3. Nutrient Intakes

During the first visit, a pediatrician interviewed the participants and collected personal data, including family medical history and information on the history of medication usage. Estimations of food consumption, including dietary diversity and variety, were measured using a quantitative food frequency questionnaire (FFQ) on-line completed by the parents of each volunteer except in those cases of adolescents, which were encouraged to complete it themselves. For this study, an adapted FFQ was used, which was previously validated with the portion sizes and food groups for the Spanish juvenile population (163, 164, 230). Information about different food items collected from these questionnaires were then analyzed using the DIAL® software to translate the intake into their corresponding energy and nutrient composition (UCM & Alce Ingeniería S.A, Madrid, Spain) (V 3.4.0.10).

Dietary habits were also measured using the KIDMED test (Mediterranean Diet Quality Index), a validated questionnaire for the Spanish juvenile population that measures the adherence to the Mediterranean diet, which is widely considered to be an optimally healthy diet for most populations (231, 232). According to the KIDMED index, a score of 0–3 reflects poor adherence to the Mediterranean diet, a score of 4–7 describes average adherence, and a score of 8–12 good adherence.

7.3.3.4. Red Blood Cell (RBC) Membrane Fatty Acid Analysis

The fatty acid composition of mature RBC membrane phospholipids was obtained from blood samples (approximately 2 mL) collected in vacutainer tubes containing ethylenediaminetetraacetic acid (EDTA). Samples were shipped to the company Lipinutragen (Bologna, Italy) and upon arrival underwent quality control for the absence of hemolysis. During the blood analysis, the automated protocol consists of selection of mature RBCs, as reported previously (165-168), followed by lipid extraction and lipid transesterification to fatty acid methyl esters (FAMEs). Briefly, the whole blood in EDTA was centrifuged (4000 rpm for 5 min at 4ºC), and the mature cell fraction was isolated based on the higher density of the aged cells (169) and controlled by the use of cell counter (Scepter 2.0, EMD Millipore, Darmstadt, Germany). All the subsequent steps were automated and included cell lysis, isolation of the membrane pellets, phospholipid extraction from pellets using the Bligh and Dyer method (170), transesterification to FAMEs by treatment with a potassium hydroxide (KOH)/methyl alcohol (MeOH) solution (0.5 mol/L) for 10 min at room temperature, and extraction using hexane (2 mL). The FAMEs were analyzed using capillary column gas chromatography (GC). GC analysis was run on the Agilent 6850 Network GC System, equipped with a fused silica capillary column Agilent DB23 (60 m x 0.25 mm x 0.25 μm) and a

flame ionization detector (FID). Optimal separation of all fatty acids and their geometrical and positional isomers was achieved. Identification of each fatty acid was made by comparison of commercially available standards and to a library of trans isomers of MUFAs and PUFAs. The amount of each FA was calculated as a percentage of the total FA content (relative %), as described in section 2.5, being more than 97% of the GC peaks recognized with appropriate standards.

7.3.3.5. Red Blood Cell Membrane Fatty Acid Profile

A pool of 12 FAs were selected as a representative profile of the dominant glycerophospholipids present in the RBC membrane, as well as three FA families (SFA, MUFA and PUFA): for SFAs, palmitic acid (C16:0) and stearic acid (C18:0); for MUFAs, palmitoleic acid (C16:1;9c), oleic acid (C18:1; 9c), cis-vaccenic acid (C18:1; 11c); for ω-3 PUFAs, eicosapentaenoic acid (EPA) (C20:5), docosahexaenoic acid (DHA) (C22:6); for ω-6 PUFAs, linoleic acid (LA) (C18:2), dihomo-gamma-linolenic acid (DGLA) (C20:3) and arachidonic acid (AA) (C20:4); for geometrical trans fatty acids (TFA): elaidic acid (C18:1 9t) and mono-trans arachidonic acid isomers (monotrans-C20:4; ω-6 recognized by standard references as previously described by Ferreri et al (233). Considering these fatty acids, different indexes previously reported in the literature (168) were calculated: (%SFA/%MUFA) index related with membrane rigidity; Omega-3 index (DHA + EPA); Inflammatory risk index (% ω-6)/(% ω-3); PUFA balance [(%EPA + %DHA)/total PUFA x 100]; Free radical stress index (sum of trans-18:1 + Σ monotrans 20:4 isomers); Unsaturation Index (UI) [(%MUFA) + (%LA/2) + (%DGLA/3) + (%AA/4) + (% EPA/5) + (%DHA/6)]; Peroxidation Index (PI) [(%MUFA/0.025) + (%LA) + (%DGLA/2) + (%AA/4) + (% EPA/6) + (%DHA/8)]; De Novo Lipogenesis index (DNL) [(%Palmitic acid)/(%LA)](173).

Additionally, the enzymatic indexes of elongase and desaturase enzymes, the two classes of enzymes of the MUFA and PUFA biosynthetic pathways,

were inferred by calculating the product/precursor ratio of the FAs involved in these reactions.

7.3.3.6. Statistical Analysis

In order to classify individuals based on metabolic similarities in their fatty acid profile, an unsupervised hierarchical clustering method was conducted on children with obesity, based on the RBC FA profile, using SPSS v.25 (IBM, Chicago, IL, USA). The idea of cluster analysis is to measure the distance between each pair of objects (participants) in terms of the variables suggested in the study (PLM FA levels), and then to group subjects which are close together. More specifically, based on the distance matrix, the clustering algorithm identifies the closest observations (i.e., subjects with similar RBC FA profile levels) and iteratively merged them within the same cluster until all clusters were merged together(234). The result is a hierarchical classification tree(235) (Figure 18).

The clustering was performed based on the method of Ward (1963), which was found to be most suitable as it creates a small number of clusters with relatively more participants. Additionally, the Ward method has proved to outperform other hierarchical methods (Punj and Stewart, 1983; Harrigan, 1985) in producing homogeneous and interpretable clusters.

Once the MHO cluster was isolated from the rest of the children with obesity (the rest of the obese clusters that were not the MHO cluster, were merged to form the obese cluster), statistical analyses was made between the MHO cluster, the obese cluster and the children with normal weight groups.

Differences between the MHO cluster, obese cluster and children with normal weight for nutrient intake, food group intake and KIDMED test were determined by conducting a Kruskal-Wallis test for the data that was not normally distributed. Normal data distribution was assessed by Shapiro-Wilk's

test or/and Kolmogorov-Smirnov test. Subsequently, Dunn's (1964) test was performed for post hoc comparisons. A Bonferroni correction for multiple comparisons was made, to correct for the increased risk of type I error. For normally distributed variables, a one-way ANOVA with Tukey post hoc analysis was conducted.

An Analysis of Covariance Test (ANCOVA) was run to determine the differences between RBC membrane fatty acids from the obese cluster, MHO cluster and children with normal weight, after controlling for variables selected as potential confounders, such as age, gender and dietary macro and micronutrient intake. Post hoc analysis was performed with a Bonferroni adjustment for multiple comparisons. First, a Principal Component Analysis (PCA) was run on 15 dietary nutrient intake variables (individual FAs, families (SFA, MUFA and PUFA), total lipids (%E), carbohydrates and proteins), obtained with the DIAL software after transforming the information about food items from FFQ questionnaires into micro and macronutrient values, in order to reduce and simplify the dimensions of these variables and use the generated factors as diet covariates (168). The Kaiser-Meyer-Olkin (KMO) and Bartlett's test of sphericity were used to verify the sampling adequacy for the analysis. PCA revealed four components that had eigenvalues greater than one and which explained 83.74% of the total variance. These components were included in the ANCOVA analysis as diet covariates. The level of significance was set at $p < 0.05$. All statistical analyses were performed using SPSS (IBM Corp. V 24.0, New York, USA).

7.3.4. Results

7.3.4.1. Clustering

A hierarchical clustering was performed using the squared euclidean distance and Ward's method to classify each subject based on the 12 FA measured in the RBC analysis. Five cluster where isolated (Figure 18), one of them presenting an RBC fatty acid profile similar to children with normal weight. Such cluster was determined as the MHO cluster and all the others were merged to form the obese cluster.
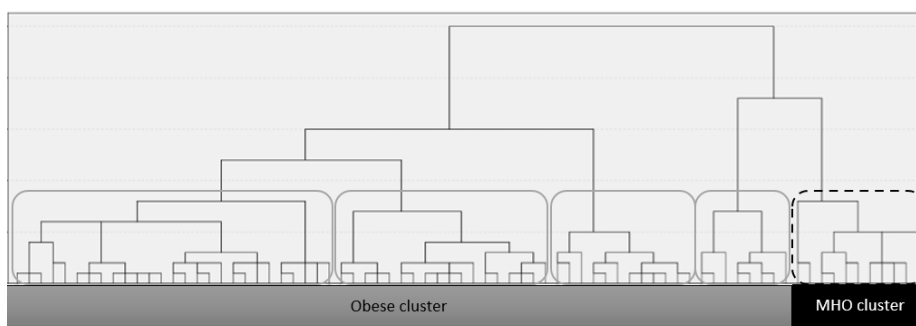
Figure 18: Hierarchical clustering classification tree

7.3.4.2. Descriptive characteristics of the clusters

A total of 194 children between 6 and 16 years old took part in the study sample (Table 5). The MHO cluster and the obese cluster together with normal weight group were included in a comparative analysis to describe their characteristics (Table 5). A matched gender distribution was found for the obese cluster and MHO but not for the normal weight group. Three groups presented similar age without statistically significative differences. No variation was observed for BMI between the obese cluster and the MHO cluster.

Table 5: General characteristics of the studied population

	Obese Clusters (G1) n=65		MHO Cluster (G2) n=11		Normoweight (G3) n=118		Kruskal-Wallis H test (p)	Post hoc Pairwise comparison(p)		
	Mean	SD	Mean	SD	Mean	SD		G1:G2	G1:G3	G2:G3
Age	11.0	0.3	10.8	0.7	10.9	0.3	0.94			
Gender(%girls)	68		72.7		46.5		**0.01**	1.00	**0.01**	0.26
BMI	28.7	0.4	29.0	1.23	18.4	0.3	**<0.001**	1.0	**<0.001**	**<0.001**

7.3.4.3. Red blood cell membrane fatty acids profile

In order to compare RBC FA profiles between groups, a one-way ANCOVA was conducted using age, sex and dietary intake as covariates to adjust the error made by those confounding factors (Table 10). No statistically significant difference was observed between the MHO cluster compared with the control group, apart from the 20:4 trans FA that showed higher levels for the MHO cluster (p=<0.001).

The obese cluster presented significant differences when it was compared with both MHO cluster and the control group. The obese cluster presented higher values for total SFA, AA, total ω-6, ω6/ω3, SFA/MUFA and D9D 18:0 and lower values for oleic acid, total MUFA, EPA, DHA, total ω-3, D6D+ELO and PUFA Balance. The obese cluster also had higher values of DGLA and stearic acid compared to the normoweight group (p=<0.001 for both), but not differences with the MHO cluster were observed (p=0.08 for both). The obese cluster presented lower levels of 20:4 trans FA compared to the MHO cluster (p=<0.001) but no differences with the normoweight group.

Table 6: RBC membrane fatty acid profile

Fatty acids (%)	Obese Cluster (G1) n=65		MHO Cluster (G2) n=11		Normoweight (G3) n=118		Ancova	Post hoc Pairwise comparison (p-value)		
	Mean	SE	Mean	SE	Mean	SE	p	G1:G2	G1:G3	G2:G3
16:0	22.38	0.13	22.62	0.32	22.50	0.09	0.64	1.00	1.00	1.00
18:0	18.35	0.13	17.59	0.32	17.72	0.10	**<0.001**	0.08	**<0.001**	1.00
TOT. SFA	40.72	0.12	39.84	0.32	40.13	0.09	**<0.001**	**0.03**	**<0.001**	1.00
16:1/9c	0.42	0.02	0.50	0.04	0.40	0.01	0.07	0.28	0.83	0.76
18:1/9c	16.34	0.15	17.34	0.37	17.45	0.11	**<0.001**	**0.04**	**<0.001**	1.00
18:1/11c [b]	1.13	0.03	1.32	0.07	1.19	0.02	**0.02**	-	-	-
TOT. MUFA	17.92	0.16	19.15	0.39	19.06	0.12	**<0.001**	**0.01**	**<0.001**	1.00
18:2	13.90	0.17	13.91	0.41	14.24	0.12	0.26	1.00	0.33	1.00
20:3	2.32	0.05	2.05	0.11	2.02	0.03	**<0.001**	0.08	**<0.001**	1.00
20:4	20.02	0.17	18.16	0.43	18.62	0.13	**<0.001**	**<0.001**	**<0.001**	0.93
TOT. ω6	36.23	0.20	34.12	0.49	34.94	0.15	**<0.001**	**<0.001**	**<0.001**	0.33
20:5	0.46	0.03	0.77	0.07	0.61	0.02	**<0.001**	**<0.001**	**<0.001**	0.09
22:6	4.52	0.13	5.57	0.33	5.01	0.10	**0.001**	**0.01**	**0.01**	0.33

TOT. ω3	4.92	0.15	6.33	0.36	5.63	0.11	**<0.001**	**<0.001**	**<0.001**	0.20
TOT. PUFA	41.20	0.18	40.45	0.46	40.57	0.14	**0.02**	0.38	**0.03**	1.00
18:1t	0.09	0.01	0.06	0.02	0.08	0.01	0.52	0.79	1.00	1.00
20:4t	0.06	0.01	0.13	0.02	0.07	0.01	**<0.01**	**<0.001**	0.72	**<0.001**
TOT. TRANS	0.14	0.01	0.19	0.03	0.16	0.01	0.21	0.27	1.00	0.61
Indexes										
ω6/ω3	7.65	0.21	5.58	0.51	6.47	0.15	**<0.001**	**<0.001**	**<0.001**	0.29
SFA/MUFA	2.28	0.02	2.11	0.06	2.13	0.02	**<0.001**	**0.01**	**<0.001**	1.00
Δ6D+ELO	6.09	0.16	6.91	0.38	7.20	0.11	**<0.001**	0.14	**<0.001**	1.00
Δ5D 20:4	8.87	0.24	9.06	0.59	9.43	0.18	0.18	1.00	0.20	1.00
Δ9D 16:0	58.21	2.49	51.42	6.17	59.11	1.86	0.50	0.91	1.00	0.72
Δ9D 18:0	1.13	0.01	1.01	0.03	1.02	0.01	**<0.001**	**<0.001**	**<0.001**	1.00
PUFA BALANCE	12.02	0.35	15.72	0.86	13.91	0.26	**<0.001**	**<0.001**	**<0.001**	0.14
Peroxidation Index	137.00	1.05	140.18	2.55	137.12	0.76	0.74	0.74	1.00	0.77
Unsaturation index	162.17	0.76	163.00	1.88	161.33	0.56	1.0	1.00	1.00	1.00
DNL Index	1.63	0.02	1.60	0.05	1.59	0.02	1.0	1.00	0.36	1.00

Data is presented as mean ± standard error. Adjusted for age, sex and dietary components extracted from the Principal Component Analysis of dietary nutrient intake (individual FAs, families (SFA, MUFA and PUFA), total lipids (%E), carbohydrates, proteins and calories).

[a] Post hoc tests were conducted with a Bonferroni adjustment. [b] Levene's test of homogeneity of variance, was not met.

7.3.4.4. Dietary intake

Table 11 shows the differences in macronutrients and individual fatty acid daily intake expressed as % of Kcal among the three groups. No statistically differences were observed for any of measured macro and micronutrients intake except for total PUFA, for which obese cluster showed lower intake compared to the normoweight group (*p=0.03*).

	Obese Clusters (G1) n=65		MHO Cluster (G2) n=11		Normoweight (G3) n=118		Kruskal-Wallis H test	Post hoc Pairwise comparison(p)		
Macronutrients										
	Mean	SD	Mean	SD	Mean	SD		G1:G2	G1:G3	G2:G3
Calories (Kcal/day)	2002.34	583.09	2320.70	371.52	2479.31	1811.90	0.09			
Proteins (%E)	16.54	2.16	16.34	1.89	16.37	2.68	0.96			
Carbohydrates (%E)	46.96	5.25	45.73	5.84	42.64	7.50	0.64*			
Simple sugars (%E)	21.74	5.25	21.72	2.28	20.38	5.84	0.90*			
Lipids (%E)	33.34	6.25	35.05	6.83	37.28	7.33	0.64			
Individual FA (% E)										
C14:0	0.95	0.43	1.17	0.73	0.91	0.39	0.31*			
C16:0	6.07	1.21	6.58	1.74	5.93	1.18	0.17			
C18:0	2.32	0.56	2.49	0.83	2.33	0.58	0.62*			
Tot. SFA	10.61	2.65	11.84	4.32	10.98	2.45	0.09			
C16:1	0.52	0.14	0.56	0.18	0.52	0.12	0.65			
C18:1	13.91	3.63	14.63	2.95	16.27	4.17	0.55*			
Tot. MUFA	14.86	3.72	15.66	3.09	17.22	4.22	0.50			

C18:2	4.19	1.78	3.63	1.30	5.00	2.44	0.33			
C20:4	0.53	0.12	0.60	0.14	0.65	0.27	0.82*			
Tot. ω-6	4.23	1.78	3.68	1.33	5.07	2.46	0.37			
C18:3	0.04	0.01	0.04	0.01	0.06	0.03	0.25			
C20:5 (EPA)	0.08	0.06	0.07	0.05	0.07	0.05	0.97			
C22:5 (DPA)	0.02	0.01	0.02	0.01	0.02	0.01	0.68			
22:6 (DHA)	0.15	0.10	0.14	0.08	0.15	0.09	0.96			
Tot. ω-3	0.78	0.22	0.82	0.24	0.90	0.33	0.46			
Tot. PUFA	5.14	1.84	4.67	1.48	6.11	2.67	**0.01**	1.00	**0.03**	0.12
ω6/ω3	5.70	2.34	4.57	1.19	6.00	3.20	0.05			

Data is presented as mean ± standard deviation. Not normally distributed variables. Pairwise comparison conducted with a Bonferroni adjustment. *ANOVA was conducted instead of Kruskal-Wallis. Post hoc pairwise comparisons are only shown for cases with a significant difference between FA using ANOVAs or Kruskal-Wallis tests.

7.3.4.5. Food Groups

Table 12 shows dietary intake according to food categories calculated via food frequency questionnaires. The MHO cluster showed a significantly higher consumption of fruits than the obese cluster (*p=0.01*) and the normoweight group (*p=0.02*). The obese cluster presented a lower intake of cereals compared with the normoweight group (*p=0.04*) and a lower score from the Kidmed test (*p=0.02*). No other differences were observed regarding food groups intake.

Table 8: Food groups intake

Food Groups (gr/day)	Obese Clusters (G1) n=65		MHO Cluster (G2) n=11		Normoweight (G3) n=118		Kruskal-Wallis H test (p)	Post hoc Pairwise comparison(p*)		
	Mean	SD	Mean	SD	Mean	SD		G1:G2	G1:G3	G2:G3
Fruits	434.7	39.5	611.3	56.4	445.9	23.8	**0.01**	**0.01**	0.77	**0.02**
Vegetables	166.4	16.3	180.0	27.6	192.1	13.4	0.3			
Cereals	142.9	7.0	173.8	20.8	172.9	8.5	**0.04**	0.45	**0.04**	1.0
Legumes	79.0	3.7	75.0	8.3	81.2	3.6	0.86			
Olive oil	19.5	1.6	27.3	3.5	21.1	1.1	0.06			
Dairy products	340.0	28.7	271.9	37.4	360.3	15.8	0.12			
Eggs	20.9	1.4	28.5	4.1	24.4	1.9	0.28			
Red meat	30.2	2.5	36.7	6.8	27.7	1.8	0.34			
White meat	40.3	2.3	47.4	2.6	41.2	2.6	0.25			
Dried Fruits and nuts	3.8	0.9	4.7	2.6	5.3	0.7	0.24			
Lean fish	29.0	2.5	41.8	7.8	31.6	1.8	0.16			
Oily fish and shellfish	28.4	2.9	32.5	6.6	26.1	2.1	0.27			
Sugary drinks	46.0	10.3	43.8	16.2	43.6	12.0	0.65			
Juices	123.8	15.0	148.5	28.4	134.9	15.2	0.68			
Kidmed	7.11	2.23	7.60	1.90	7.95	1.87	**0.02**	1.0	**0.02**	1.0

Data is presented as mean ± standard deviation. *Pairwise comparison conducted with a Bonferroni adjustment.

7.3.5. Discussion

To our knowledge, this is the first time that RBC FA membrane profile has been used as a biomarker to differentiate MHO in a cohort of obese patients, and especially in children, that present metabolic imbalances. Inflammation seems to play a key role in distinguishing metabolically healthy from metabolically unhealthy individuals with obesity(213), so here the analysis of a RBC FA profile as a comprehensive biomarker of the pro- and anti-inflammatory status, is assayed, that can be applied as a potential tool to distinguish MHO.

Previous studies in MHO adults and children have been focused on biochemical parameters such as insulin resistance, blood pressure, serum lipids and glucose (75, 78, 80, 214, 215), but it has been increasingly suggested to include circulatory inflammatory markers in the definition of MHO (84, 236).

The RBC FA profile characteristic of children with obesity has been previously reported in the literature(227), compared to children with normal weight, with a clear shift of the group with obesity towards a pro-inflammatory condition, mainly due to the higher levels of arachidonic acid, a well described precursor of inflammatory mediators(182, 237), accompanied by a higher SFA/MUFA ratio.

After conducting a hierarchical clustering analysis of our patient cohort, within those individuals with obesity, a MHO cluster was isolated, which showed a differentiated FA profile, compared to the rest of the children with obesity, and, similar profile to children with normal weight. Our MHO cluster, matched the previously published prevalence of 10 to 30% of the study population (14% in our study)(75, 78, 80, 214, 215).

Compared to children with obesity, the MHO cluster, showed lower levels of total ω-6 FA, mainly due to significant lower value of AA (p<0.001), a well-known precursor of proinflammatory mediators prostaglandins, thromboxane A2 and prostacyclins (182, 237). The MHO cluster does not present a shift to an inflammatory metabolism, as with other children with obesity do, and displays values of ω-6 FAs similar to children with normal weight.

Regarding ω-3 FAs, the MHO cluster presents higher values of EPA, DHA and total ω-3 FAs (p<0.001, p=0.001 and p<0.001 consecutively) compared to children with obesity withsimilar values compared to the normal weight children. This is an important feature as, ω-3 PUFAs can improve impaired metabolism in obesity by modulating main metabolic pathways(238), such as promoting anti-inflammatory response or insulin sensitivity(239), regulating the adipocyte apoptosis(240) or modulating membrane fluidity by altering lipid rafts(241). In fact, a balanced ω-6/ω-3 ratio is important in the prevention and management of obesity, as both metabolic pathways compete to bind the same enzymes and an unbalanced ratio towards the ω-6 PUFAs, appears remarkably enhanced in obesity(40).

Regarding SFA, the MHO cluster showed lower levels of total SFA in RBC membranes (p=0.03) and higher values of total MUFA (p=0.01), mainly due to higher levels of oleic acid (p=0.04). However, these differences were not associated to different dietary intake of SFA or MUFA, as could be considered a priori, because no differences were observed for any nutrient intake between the MHO cluster and the rest of children with obesity. The MHO cluster only showed lower intake for total PUFA intake (p=0.03), which could be associated to lower levels of total PUFA in RBC for MHO cluster (p=0.03). However, dietary variables that can act as confounder factors, have been used as covariates in the ANCOVA analysis, as explained in the experimental section, to observe differences in RBC profile between groups. Moreover, despite food

frequency questionnaires used in this study are validated and widely used, they have their limitations to describe accurately diet intake and this could be seen as a limitation of the study(242).

Regarding food groups intake, only a higher intake of fruits was observed (p=0.01) for MHO compared to other children with obesity, which can be considered a protective factor together with the consumption of vegetables due to a higher consumption of fiber and low glicemic load(243), since it can provide an antioxidant intake that exert their protection toward the FA in the lipid pools, especially PUFA. However, this could be enough explaining the FA composition in our cohort, considering that no differences were observed for the KIDMED score.

As practically no differences were seen in terms of reported intakes, our attention turned to evaluate enzymatic activity by using lipid indexes in order to explain the differentiated levels of RBC SFA and MUFA. The enzymatic activity of the Δ-9-desaturase (namely, stearoyl-CoA desaturase-1, SCD1) appeared higher for the MHO compared to the rest of the children with obesity (p<0.001) and indicated the higher proportions of SFAs converted to MUFAs, as reflected in higher oleic acid levels and almost statistically significant lower stearic acid levels (p=0.08) in this group. A reduced activity of the enzyme in the obese cluster is correlated to factors that have been recalled several times to explain the involvement of SFA pathway in metabolic derangements, such as: the absence of enzymatic cofactors, the inhibition of desaturase activity and liver impairment (162), since the desaturase transformation prevents SFA accumulation and toxicity triggering hepatocellular apoptosis and liver damage (244).

It is worth mentioning that, although very popular, the indirect measurement of enzyme activity, by partition between product and precursors,

could be considered as a limitation of the study whereas the direct measurement is needed to reaffirm the conclusions obtained.

The underlined differences between MHO cluster and the rest of children with obesity are connected with the importance of a personalised approach in obesity, in particular regarding nutritional recommendations based on the specific FA needs. Currently, general recommendations for individuals with obesity, such as low caloric diets with restriction on fats, are given(46), but not considering the quality of fats and specifically the types of FA needed. The membrane FA lipidome analysis should be included in the protocols for two reasons: i) to better define and differentiate the molecular status of the patients; ii) to envisage adequate nutritional strategies for each population group and to test their effects increasing the personalization of the treatments. According to our results, individuals in the obese cluster need a higher intake of ω-3 FA, to induce a better balance between ω-6 and ω-3 pathways and reduce the inflammatory precursors. This recommendation is not extended to all children with obesity, as the MHO cluster has optimal levels of both ω-3 and ω-6 FAs.

The capacity of the mature RCB FA profile can be used for larger population studies, to extend its validation as a biomarker to differentiate MHO in children and describe the characteristics of those children with obesity that do not display typically obesity-associated metabolic imbalances. At the same time, intervention studies, with personalised nutritional strategies, can be carried out keeping the optimal balance of the RBC FA profile as a molecular target to couple with clinical observations.

7.4.Future perspectives for precision nutrition

There is no doubt that diet has an impact on health, but its implication in well-being, as well as in different pathologies, is difficult to determine due to the individual molecular response. As the metabolic diversity leads to differences in nutrient requirements and responses to diet between individuals, recent research, is pointing out to the personalization of the nutritional recommendations (245).

The holistic study of lipid metabolism and the specific application of cell membrane lipidomic for precision nutrition builds a strong basis for understanding the biological significance of lipids on obesity which will allow establish a correct lifestyle and diet (probably enriched in certain fats), for comprehensive precision nutrition recommendations and personalized obesity management.

As we have evidenced in previous chapters of this thesis work, children with obesity requires of specific dietary fats, mainly ω-3, to establish an optimal balance between ω-6 and ω-3 pathways to restore the cell membrane structure and reduce inflammatory status. Moreover, dietary MUFA (especially oleic acid) are relevant dietary fats that must be increased in replacement of SFA to improve the action of the D9D enzyme.

Literature reported that ω-3 supplementation can be an adequate strategy to improve lipid metabolism in obesity and other associated risk factors such as CVD (246, 247). Although national and international guidelines recommend increasing ω-3-rich foods, and sometimes supplementation, there are some controversial results from recent trials (248).

In the following review we summarize the utility of ω-3 PUFA supplements as a personalized nutrition strategy to restore optimal levels of this ω-3 PUFA in the RBC membrane based on a precision nutrition approach.

7.4.1. A journey through ω-3 supplements: Future perspectives for precision nutrition

A Journey through ω-3 Supplements: Future Perspectives for Precision Nutrition

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Abstract Increasing evidence has shown that fatty acids play a key factor in nutrition and health. Despite the well described health benefits associated to the omega-3 fatty acids (ω-3 FA) intake from epidemiological and clinical trials, controversial results are found from some clinical trials regarding the effect of ω-3 FA supplementation to handle certain diseases. In this review, we provide orientation for the reader to understand the importance of a personalized recommendation of the ω-3 polyunsaturated fatty acid supplementation based on a precision nutrition approach. We begin by reviewing the metabolic relevance of ω-3 fatty acids and then discuss the current state of ω-3 fatty acid supplements regarding their indications, regulation, variety from brand to brand, adverse effects and the need to implement a personalized supplementation. We conclude with future perspectives for practitioners and general guidance on precision nutrition.

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1. Introduction

In the last decades, omega-3 fatty acids (ω-3 FA) supplements have experienced a growing increase in consumption by the general public, becoming an increasingly important part of the diet [1]. For example, the use of ω-3 supplements among adults in the U.S. has increased from 4.8% in 2007 to 7.8% in 2012 [2]. The factors that explain this increase in consumption, among others, are the more scientifically backed evidences on the beneficial health effects of ω-3 FA [3] and the fact that typical current dietary habits in western countries do not meet the recommended amount of ω-3 FA intake that should be used for optimal health conditions [4].

2. Metabolic Relevance of ω-3 Fatty Acids

ω-3 FA are essential fatty acids that must be obtained from the diet because humans and other mammals lack endogenous enzymes to synthesize them. Alpha-linolenic acid (ALA) is the precursor of the metabolic pathway for ω-3 FA and can be found in green leafy vegetables and in some seeds (flax, rape, chia, perilla and walnuts). Even if mammalian cells are not able to synthesize ALA, they can metabolize it into more

physiologically active compounds by a set of desaturating enzymes via Δ5 and Δ6 desaturases and by lengthening the acyl chain (elongation) via elongases, thus converting them into longer-chain fatty acids of 20 and 22 carbon atoms [5].

Metabolically speaking, the most important ω-3 FA are EPA (20:5 ω-3) and DHA (22:6 ω-3), which have been associated with numerous health benefits. Adequate consumption of ω-3 FA, mainly EPA and DHA fatty acids, has been proven to be vitally important for fetal and infant development [6], improved cardiovascular health [7], benefits in cancer by promoting tumor cell apoptosis [8], immune system [9], or a decreased inflammatory response to injury [10], among other benefits.

Although EPA and DHA can be synthesized from shorter plant-derived ω-3 FA precursors such as ALA, this metabolic pathway is not efficient in humans. Approximately only 8-20% of ALA is converted to EPA in humans, while conversion of ALA to DHA is estimated to be around 0.5-9% [11], although this rate may be affected by hormones [12], sex [13], genetics [14] and age [15]. In addition, since several enzymes are shared between the metabolic pathways of ω-3 and ω-6 PUFAs, ω-3 and ω-6 substrates compete for access to these enzymes and this competition is highly influenced by the relative ratios of each type of PUFA. Furthermore, the ratio of ω-6/ω-3 FA is highly affected by dietary intake and is found in excess of ω-6 in many Western countries (i.e.20:1 ω-6/ω-3 compared to the 1:1 ratio during evolution), due to increased consumption of the linoleic acid (LA), a precursor of the ω-6 metabolic pathway that is rich in vegetable oils [16]. For example, consumption of LA in the U.S. according to the U.S. Department of Agriculture has increased 8 times for men and 6 times for women from the beginning of the XIX century to nowadays [17].

An added problem lies in the fact that long chain ω-6 and ω-3 PUFAs synthesized from LA and ALA not only have different, but often opposing effects on immunity and inflammation. Arachidonic acid, which is the main long chain ω-6, metabolites promote acute and chronic inflammation acting as local hormones. In contrast, EPA and DHA can be metabolized to anti-inflammatory mediators [18].

This illustrates the importance of a sufficient dietary intake of EPA and DHA to provide enough levels of ω-3 FA for optimal human health.

3. Indications for ω-3 Fatty Acid Intake

Currently, different recommendations are given by associations and government organizations regarding the dosage of ω-3 FA for the maintenance of optimal health conditions or alleviation of possible disease states. For example, in the UK, the recommendation of ω-3 FA intake is at least two fish meals per week including at least one meal consisting of oily fish (i.e.salmon, tuna, anchovies, sardines etc..), in which translates to an EPA+DHA recommendation of around 450 mg/day [19]. In France, the official recommendation for ω-3 FA intake is 400–500 mg/day of EPA+DHA with at least 100–120 mg/day DHA [20]. The Superior Health Council of Belgium recommends a minimum of 1-2% energy from ω-3 FA for adults, with at least one oily fish per week to supply 250 mg/day of EPA+DHA [21]. The Health Council of the Netherlands establishes a weekly consumption of one serving of fish, preferably oily fish, for a target quantity of 225 mg/day of EPA+DHA [22]. The target intake for Australia and New Zealand is 160 mg/day EPA+DHA for men and 90 mg/day for women [23]. The European Food Safety Authority and the Dietary guideline for Americans, from the U.S. Department of Agriculture and the Department of Health and Human Services, point out that as the available data on ω-3 FA intake is insufficient to derive an average requirement, an intake of 250 mg per day of EPA+DHA appears to be sufficient for cardiovascular prevention in healthy subjects [24,25]. The World Health Organization recommends regular fish consumption (one to two servings per week; each serving should provide the equivalent of 200–500 mg of EPA+DHA) [26].

Differences in ω-3 FA intake recommendations are reflected in differences in the actual average intake of EPA and DHA between countries with western diets [27]. For example, a nationally representative cross-sectional survey collected by the National Center

for Health Statistics of the Centers for Disease Control and Prevention in the U.S. showed that the intake of DHA and EPA from foods and dietary supplements for adults was 72 ± 4 mg/d and 41 ± 4 mg/d, respectively [28]. The average consumption of ω-3 FA in Canada is around 177 mg/day, in Australia around 143 mg/day, and in many parts of Europe the daily intake of EPA + DHA by adults is <100 mg/d, since many never eat oily fish [29].

Regarding this, the European Food Safety Authority (EFSA) reported different health benefits associated to daily intake of ω-3 FA that can be included as health claims in manufactured food labeling [30].

A recommended dietary allowance for the optimum minimum intake of EPA and DHA ω-3 FA is therefore needed for the general population without considering any disease state or other specific requirement in special situations (i.e. pregnancy, sports performance) that could potentially alter the recommended amount of each respective fatty acid.

At the same time, there is not a current consensus regarding the tolerable upper intake level (UL) for ω-3 supplements due to insufficient data. Different recommendations have been established in different countries. For example, Australia, New Zealand, and The US Food and Drug Administration set a reference value for the UL of EPA+DHA at 3 g/d [26]. Meanwhile, EFSA states that long-term supplemental intakes of combined EPA and DHA up to 5 g/day do not appear to increase the risk of adverse side effects such as spontaneous bleeding episodes or bleeding complications, or lipid peroxidation among others [4].

4.   Regulation   of   ω-3 Supplements

Since dietary supplements are widely available to the general public (i.e. they are not over-the-counter drugs and are not regulated as such), their purity, chemical integrity, efficacy and safety remains unverified [31]. Some studies show that the concentration of ω-3 FA was lower than what it was stated in the supplemental labels [32] and contained higher oxidation levels than what was permitted by current legislation [33]. In this sense, lipid peroxides contribute to accelerate oxidation of other fatty acids leading to lipid membrane peroxidation, cell damage, and oxidative stress [34]. Endogenous membrane lipid peroxidation results in altered membrane fluidity, transport, and cell signaling [35].

At the same time, levels of trans-isomers have been measured in different concentrations [36] likely due to the high temperatures and pressures used during the manufacturing of concentrated supplements [37]. The dietary intake of trans-fatty acids (TFA) has adverse effects on blood lipid levels because they cause an increase on LDL-cholesterol and a decrease on HDL-cholesterol [38], both well-established markers of cardiovascular disease [39]. Similarly, cohort studies demonstrated that high intake of TFA is associated with an increased coronary heart disease and mortality rates [40,41].

Current product formulations of ω-3 supplements are offered in various options, ranging from soft gels (most common) to liquids, powders, and gummies, with oil sources from fish, krill, algae and plants. The quantities and prices of ω-3 FA found in dietary supplements are highly variable within and between brands. The dosage recommendations for ω-3 consumption by different manufacturing brands also largely differs, even for recommendations for the treatment of the same disease. For example, figure 1 shows a comparison of the recommendations of the daily intake of EPA, DHA   and

EPA+DHA for treating cholesterol from 10 different commercial ω-3 supplement products that authors have selected from available products in the European market. This comparison reveals large differences between the recommended doses of supplements from one brand to another, leading to an increased confusion in consumer choice for selecting the adequate supplemental dosages.

ω-3 FA in supplements can be found in different forms such as triglycerides, free fatty acids, ethyl esters and phospholipids. Bioavailability of ω-3 PUFAs as phospholipids (as it appears mainly in krill oil) is higher than other forms of ω-3s (249)], while triglycerides and free fatty acids have higher bioavailability than ethyl esters (250)]. This is another feature that must be considered when deciding between the appropriate supplement for personal use.
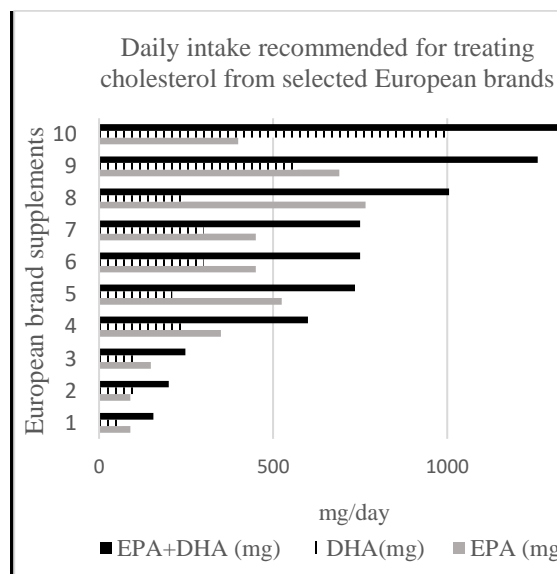
Figure 1. Daily EPA+DHA intake recommended for treating cholesterol from selected European brands.

5. Personalized Supplementation

Due to the controversy regarding specific recommendations of adequate intake of ω-3 for the general population as well as different population sub-groups (i.e. those with diet-related diseases), personalized ω-3 intake strategies should be adopted. Implementation of new omic tools, such as genomics, metabolomics, proteomics and transcriptomics, to the field of precision nutrition can facilitate detailed information on specific genotypes as well as the current levels of proteins and metabolites, thus enabling the identification of metabolic deficiencies or genetic variations within individuals. Hence, information obtained from these technologies can lead to a more precise personalized nutritional recommendation by providing information on different responses to diets, on dietary intake, and on new early biomarkers of certain diseases [44], all of which should be considered for recommendations of adequate dosage for each individual.

Examples of applying targeted-omic tools to asses ω-3 supplementation include studying the effect of PUFAs on genetic variation via epigenetic modifications [45] or the study of different metabolomic and transcriptomic

es of responders and non-responders of ω-3 ementation [46].

l membrane lipidomics, a specific sub- within metabolomics, offers analyses of erm food consumption and metabolism y: cell membrane composition reflects the tion efficiency of the ingested fatty acids, olism and distribution of the resulting ules [47,48]. Cell membrane lipidomic oring can also be applied to large ations allowing the molecular terization of specific diseases or health risk s, such as, diabetes mellitus [49], vascular disease [50], obesity/overweight, , allergies/intolerance [51]. By ining the actual levels of cell membrane cids of an individual, innovative nutritional ies to improve health status can be designed, including diet and specific supplements targeting different population groups to help consumers to properly choose the adequate fatty acid supplementation among commercially available products according to their specific needs.

Controversial results from ω-3 supplementation trials can be found in the literature for patients with cardiovascular disease [52]. One reason that ω-3 supplementation was not beneficial in these studies may be due to the fact that they did not measure the basal and endpoint ω-3 FA levels [53,54], therefore it is impossible to determine the actual changes in ω-3 levels in these patients. This further supports the application of lipidomic tools to these studies in order to assess the potential benefits of ω-3 FA supplementation.

6. Conclusions

It can be concluded that the intake of ω-3 supplements is a useful way to reach the recommended levels of ω-3 FA when the current dietary habits don´t meet these demands, especially in cases where higher levels may be required (i.e. disease, pregnancy, infant development). Nevertheless, more research should be carried out in the field to establish an adequate recommended dietary allowance for ω-3 FA in order to provide a clear message to the general public.

However, in the current situation where more research is leading to individualized health and nutritional recommendations, the use of omic technologies for a more precise and personalized supplementation appears to be crucial in order to achieve the expected health benefits and to allow consumers to make correct choices for their needs concerning dosage or different fatty acid supplements. This is not a trivial issue because the supplements are at the reach of any consumer, without any control, and when they are consumed in excess or for long-term, can negatively impact health.

In this sense, the application of cell membrane lipidomics provides a valid option to understand the structural and functional changes in fatty acid composition in both normal and pathological states. It also provides a measurement of the specific fatty acid needs for each individual based on their basal metabolic levels, as well as different metabolic changes that occur supplementation.

Future perspectives should focus as well on the effectiveness of different ω-3 FA sources for supplements (marine and vegetable oils) and how new industrial processes can affect the quality of these supplements.

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Competing Interests

None of the authors have any competing interest.

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List of Abbreviations

ALA: Alpha-linolenic Acid; **DHA:** Docosahexaenoic Acid; **EFSA:** European Food Safety Authority**; EPA:** Eicosapentaenoic Acid; **FA:** Fatty Acids; **LA:** Linoleic Acid; **MUFA:** Monounsaturated Fatty Acids; **PUFA:** Polyunsaturated Fatty Acids; **SFA:** Saturated Fatty Acids; **TFA:** Trans-Fatty Acids**; UL:** Upper intake Level; **ω-3:** Omega 3.

References

[1]   Bailey RL, Gahche JJ, Lentino CV, Dwyer JT, Engel JS, Thomas PR, et al. Dietary supplement use in the United States, 2003-2006. J Nutr. 2011; 141(2): 261-6.

[2]   Clarke TC, Black LI, Stussman BJ, Barnes PM, Nahin RL. Trends in the use of complementary health approaches among adults: United States, 2002-2012. Natl Health Stat Report. 2015; 10(79): 1-16.

[3]   Bailey RL, Gahche JJ, Miller PE, Thomas PR, Dwyer JT. Why US adults use dietary supplements. JAMA Intern Med. 2013; 173(5): 355-61.

[4]   Scientific Opinion on the Tolerable Upper Intake Level of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and docosapentaenoic acid (DPA). EFSA Journal. 2012; 10(7): 2815.

[5]   Wall R, Ross RP, Fitzgerald GF, Stanton C. Fatty acids from fish: the anti-inflammatory potential of long-chain omega-3 fatty acids. Nutr Rev. 2010; 68(5): 280-9.

[6]   Lauritzen L, Brambilla P, Mazzocchi A, Harslof LB, Ciappolino V, Agostoni C. DHA Effects in Brain Development and Function. Nutrients. 2016; 8(1).

[7]   Mori TA. Omega-3 fatty acids and cardiovascular disease: epidemiology and effects on cardiometabolic risk factors. Food Funct. 2014; 5(9): 2004-19.

[8]   Gleissman H, Johnsen JI, Kogner P. Omega-3 fatty acids in cancer, the protectors of good and the killers of evil? Exp Cell Res. 2010; 316(8): 1365-73.

[9]   Calder PC. n-3 fatty acids, inflammation and immunity: new mechanisms to explain old actions. Proc Nutr Soc. 2013; 72(3): 326-36.

[10]   Kiecolt-Glaser JK, Belury MA, Andridge R, Malarkey WB, Glaser R. Omega-3 supplementation lowers inflammation and anxiety in medical students: a randomized controlled trial. Brain Behav Immun. 2011; 25(8): 1725-34.

[11]   Burdge GC. Metabolism of alpha-linolenic acid in humans. Prostaglandins, leukotrienes, and essential fatty acids. 2006; 75(3): 161-8.

[12]   Vessby B, Gustafsson IB, Tengblad S, Boberg M, Andersson A. Desaturation and elongation of Fatty acids and insulin action. Ann N Y Acad Sci. 2002; 967: 183-95.

[13] Childs CE, Romeu-Nadal M, Burdge GC, Calder PC. Gender differences in the n-3 fatty acid content of tissues. Proc Nutr Soc. 2008; 67(1): 19-27.

[14] Koletzko B, Lattka E, Zeilinger S, Illig T, Steer C. Genetic variants of the fatty acid desaturase gene cluster predict amounts of red blood cell docosahexaenoic and other polyunsaturated fatty acids in pregnant women: findings from the Avon Longitudinal Study of Parents and Children. The American journal of clinical nutrition. 2011; 93(1): 211-9.

[15] Bolton-Smith C, Woodward M, Tavendale R. Evidence for age-related differences in the fatty acid composition of human adipose tissue, independent of diet. European journal of clinical nutrition. 1997; 51(9): 619-24.

[16] Simopoulos AP. An Increase in the Omega-6/Omega-3 Fatty Acid Ratio Increases the Risk for Obesity. Nutrients. 2016; 8(3).

[17] Jandacek RJ. Linoleic Acid: A Nutritional Quandary. Healthcare. 2017; 5(2).

[18] Chilton FH, Dutta R, Reynolds LM, Sergeant S, Mathias RA, Seeds MC. Precision Nutrition and Omega-3 Polyunsaturated Fatty Acids: A Case for Personalized Supplementation Approaches for the Prevention and Management of Human Diseases. Nutrients. 2017; 9(11).

[19] Levy L, Tedstone A. UK Dietary Policy for the Prevention of Cardiovascular Disease. Healthcare. 2017; 5(1).

[20] The omega-3 fatty acids and the cardiovascular system: Nutritional benefits and claims. French Agency for Food, Environmental and Occupational Health Safety, 2003.

[21] Belgium Superior Health Council. Dietary guidelines for the Belgian adult population 2019; Available from: https://www.health.belgium.be/en/advisory-report-9284-fbdg-2019#anchor-36076.

[22] Kromhout D, Spaaij CJ, de Goede J, Weggemans RM. The 2015 Dutch food-based dietary guidelines. European journal of clinical nutrition. 2016;70(8):869-78.

[23] Nutrient reference values for Australia and New Zealand including recommended dietary intakes. Australia and New Zealand National Health and Medical Research Council, 2006.

[24] Scientific Opinion on Dietary Reference Values for fats, including saturated fatty acids, polyunsaturated fatty acids, monounsaturated fatty acids, trans fatty acids, and cholesterol. EFSA Journal. 2010; 8(3): 1461.

[25] U.S. Department of Health and Human Services and U.S. Department of Agriculture. Dietary Guidelines for Americans. 2015-2020 [December 2015]; 8:[Available from: https://health.gov/dietaryguidelines/2015/resources/2015-2020_Dietary_Guidelines.pdf.

[26] Fats and fatty acids in human nutrition. Proceedings of the Joint FAO/WHO Expert Consultation. November 10-14, 2008. Geneva, Switzerland: Ann Nutr Metab. 2009; 55(1-3): 5-300.

[27] Patterson AC, Stark KD. Direct determinations of the fatty acid composition of daily dietary intakes incorporating nutraceuticals and functional food

strategies to increase n-3 highly unsaturated fatty acids. J Am Coll Nutr. 2008; 27(5): 538-46.

[28] Papanikolaou Y, Brooks J, Reider C, Fulgoni VL, 3rd. U.S. adults are not meeting recommended levels for fish and omega-3 fatty acid intake: results of an analysis using observational data from NHANES 2003-2008. Nutr J. 2014; 13(31): 1475-2891.

[29] Ian Givens D, Gibbs RA. Current intakes of EPA and DHA in European populations and the potential of animal-derived foods to increase them. Proc Nutr Soc. 2008; 67(3): 273-80.

[30] European Food Safe Authority EFSA. EU Register of nutrition and health claims made on foods. 2016.

[31] Hilleman D, Smer A. Prescription Omega-3 Fatty Acid Products and Dietary Supplements Are Not Interchangeable. Manag Care. 2016; 25(1): 46-52.

[32] Ritter JC, Budge SM, Jovica F. Quality analysis of commercial fish oil preparations. Journal of the science of food and agriculture. 2013; 93(8): 1935-9.

[33] Jackowski SA, Alvi AZ, Mirajkar A, Imani Z, Gamalevych Y, Shaikh NA, et al. Oxidation levels of North American over-the-counter n-3 (omega-3) supplements and the influence of supplement formulation and delivery form on evaluating oxidative safety. Journal of nutritional science. 2015; 4(4).

[34] Albert BB, Cameron-Smith D, Hofman PL, Cutfield WS. Oxidation of marine omega-3 supplements and human health. Biomed Res Int. 2013; 464921(10): 30.

[35] Shahidi F, Zhong Y. Lipid oxidation and improving the oxidative stability. Chemical Society Reviews. 2010; 39(11): 4067-79.

[36] Sciotto C, Mjos SA. Trans isomers of EPA and DHA in omega-3 products on the European market. Lipids. 2012; 47(7): 659-67. Epub 2012/05/09.

[37] Menounou G, Giacometti G, Scanferlato R, Dambruoso P, Sansone A, Tueros I, et al. Trans Lipid Library: Synthesis of Docosahexaenoic Acid (DHA) Monotrans Isomers and Regioisomer Identification in DHA-Containing Supplements. Chem Res Toxicol. 2018; 31(3): 191-200.

[38] Brouwer IA, Wanders AJ, Katan MB. Effect of animal and industrial trans fatty acids on HDL and LDL cholesterol levels in humans--a quantitative review. PloS one. 2010; 5(3): 0009434.

[39] Lewington S, Whitlock G, Clarke R, Sherliker P, Emberson J, Halsey J, et al. Blood cholesterol and vascular mortality by age, sex, and blood pressure: a meta-analysis of individual data from 61 prospective studies with 55,000 vascular deaths. Lancet. 2007; 370(9602): 1829-39.

[40] Bendsen NT, Christensen R, Bartels EM, Astrup A. Consumption of industrial and ruminant trans fatty acids and risk of coronary heart disease: a systematic review and meta-analysis of cohort studies. European journal of clinical nutrition. 2011; 65(7): 773-83.

[41] Brouwer I. Effect of trans-fatty acid intake on blood lipids and lipoproteins: a systematic review and meta-regression analysis. Geneva: World Health Organization; 2016.

[42] Ulven SM, Kirkhus B, Lamglait A, Basu S, Elind E, Haider T, et al. Metabolic effects of krill oil are essentially similar to those of fish oil but at lower dose

of EPA and DHA, in healthy volunteers. Lipids. 2011; 46(1): 37-46.

[43] Dyerberg J, Madsen P, Moller JM, Aardestrup I, Schmidt EB. Bioavailability of marine n-3 fatty acid formulations. Prostaglandins, leukotrienes, and essential fatty acids. 2010; 83(3): 137.

[44] Corella D, Ordovas JM. The role of omics in precision nutrition: strengths and weaknesses. Nutr Hosp. 2018; 35(Spec No4): 10-8.

[45] Hernando Boigues JF, Mach N. The effect of polyunsaturated fatty acids on obesity through epigenetic modifications. Endocrinol Nutr. 2015; 62(7): 338-49.

[46] Rudkowska I, Paradis A-M, Thifault E, Julien P, Barbier O, Couture P, et al. Differences in metabolomic and transcriptomic profiles between responders and non-responders to an n-3 polyunsaturated fatty acids (PUFAs) supplementation. Genes & Nutrition. 2013; 8(4): 411-23.

[47] Stanley EG, Jenkins BJ, Walker CG, Koulman A, Browning L, West AL, et al. Lipidomics Profiling of Human Adipose Tissue Identifies a Pattern of Lipids Associated with Fish Oil Supplementation. J Proteome Res. 2017; 16(9): 3168-79.

[48] Visioli F. Lipidomics to Assess Omega 3 Bioactivity. J Clin Med. 2015; 4(9): 1753-60.

[49] Rhee EP, Cheng S, Larson MG, Walford GA, Lewis GD, McCabe E, et al. Lipid profiling identifies a triacylglycerol signature of insulin resistance and improves diabetes prediction in humans. The Journal of clinical investigation. 2011; 121(4): 1402-11.

[50] Stegemann C, Pechlaner R, Willeit P, Langley SR, Mangino M, Mayr U, et al. Lipidomics profiling and risk of cardiovascular disease in the prospective population-based Bruneck study. Circulation. 2014; 129(18): 1821-31.

[51] Ferreri C, Chatgilialoglu, C. Membrane Lipidomics for Personalized Health. Chichester, UK: John Wiley & Sons; 2015.

[52] Abdelhamid AS, Brown TJ, Brainard JS, Biswas P, Thorpe GC, Moore HJ, et al. Omega-3 fatty acids for the primary and secondary prevention of cardiovascular disease. Cochrane Database Syst Rev. 2018; 18(7).

[53] Shaikh NA, Tappia PS. Why are there inconsistencies in the outcomes of some omega-3 fatty acid trials for the management of cardiovascular disease? Clinical Lipidology. 2015; 10(1): 27-32.

[54] Bowen KJ, Harris WS, Kris-Etherton PM. Omega-3 Fatty Acids and Cardiovascular Disease: Are There Benefits? Curr Treat Options Cardiovasc Med. 2016;18(11):016-048