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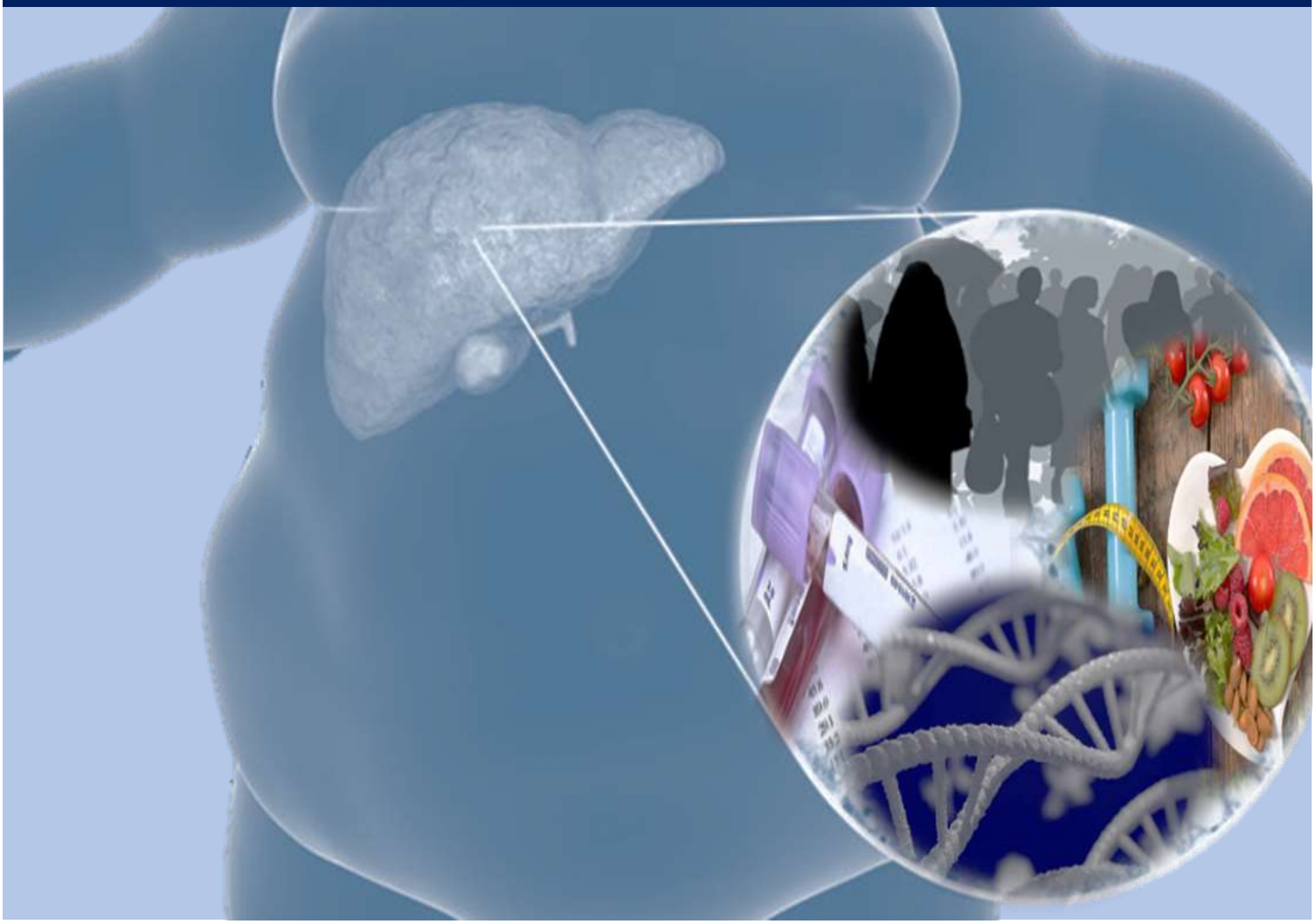
DOCTORAL PROGRAME IN HEALTH SCIENCES

IDENTIFICATION OF SOCIODEMOGRAPHIC, BIOCHEMICAL AND EPIGENETIC BIOMARKERS OF HEPATIC STEATOSIS IN CHILDREN WITH OVERWEIGHT OR OBESITY

Author **Maddi Oses Recalde** | Director **Idoia Labayen Goñi**



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INTERNATIONAL DOCTORAL THESIS

IDENTIFICATION OF SOCIODEMOGRAPHIC, BIOCHEMICAL AND EPIGENETIC BIOMARKERS OF HEPATIC STEATOSIS IN CHILDREN WITH OVERWEIGHT OR OBESITY

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PUBLIC UNIVERSITY OF NAVARRA

DEPARTMENT OF HEALTH SCIENCES

TESIS DOCTORAL CON MENCIÓN INTERNACIONAL

IDENTIFICACIÓN DE BIOMARCADORES SOCIODEMOGRÁFICOS, BIOQUÍMICOS Y EPIGENÉTICOS DE LA ESTEATOSIS HEPÁTICA EN NIÑOS/AS CON SOBREPESO U OBESIDAD

Autora: Maddi Oses Recalde

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UNIVERSIDAD PÚBLICA DE NAVARRA

DEPARTAMENTO DE CIENCIAS DE LA SALUD

The current international Doctoral Thesis is presented as a compendium of four studies. The references of these four studies included in this work are the following:

La siguiente Tesis Internacional está compuesta por el compendio de cuatro artículos. Las referencias de dichos artículos son las siguientes:

Study I: Oses M**,** Medrano M, Galbete A, Arenaza L, Ruiz JR, Sánchez-Valverde F, Ortega FB, Labayen I. A sociodemographic, anthropometric and lifestyle-based prediction score for screening children with overweight and obesity for hepatic steatosis: The HEPAKID index. Pediatric Obesity. 2021; 16

Study II: Oses M, Cadenas-Sanchez C, Medrano M, Galbete A, Miranda-Ferrua E, Ruiz JR, Sánchez-Valverde F, Ortega FB, Cabeza R, Villanueva A, Idoate F, Labayen I. Development of a prediction protocol for the screening of metabolic associated fatty liver disease in children with overweight or obesity. Status: Accepted in Pediatric Obesity

Study III: Oses M, Sanchez J, Portillo M, Aguilera C, Labayen I. Circulating miRNAs as Biomarkers of Obesity and Obesity-Associated Comorbidities in Children and Adolescents: A Systematic Review. Nutrients. 2019; 11(12): e2890.

Study IV: Oses M, Medrano M, Sanchez J, Portillo M, Aguilera C, Altmäe S, Labayen I. Peripheral blood mononuclear cells-expressed miRNA profiles derived from children with metabolic associated fatty liver disease and insulin resistance. Status: Submitted to Pediatric Obesity

The author of the current International Doctoral Thesis **Ms**. **Maddi Oses Recalde** was able to perform this project thanks to the predoctoral grant she obtained from the Spanish Ministry of Economy and Competitiveness (BES-2017-080770). This is an International Thesis as Maddi Oses Recalde conducted a three-month international research stay at the Rudbeck Laboratory, molecular biology laboratory of Uppsala University of Sweeden.

La doctoranda **Dña. Maddi Oses Recalde** ha realizado la presente Tesis Doctoral Internacional como beneficiaria de una beca-contrato Ayuda de Formación de Personal Investigador del Ministerio español de Economía y Competitividad (BES-2017-080770). La doctoranda Dña. Maddi Oses Recalde realizó la estancia predoctoral de tres meses de duración, que le permite optar a la mención de Tesis Doctoral Internacional, en el Laboratorio Rudbeck de biología molecular, en la Universidad de Uppsala, Suecia.

RESEARCH PROJECTS / PROYECTOS DE INVESTIGACIÓN

This International Doctoral Thesis includes data from the following research projects:

La presente Tesis Doctoral Internacional se realizó principalmente como resultado de los siguientes proyectos de investigación:

Study title: **Influencia de la diversidad y la composición de la microbiota en el desarrollo de hígado graso pediátrico: MICROKID Study**

Funding support: Gobierno de Navarra

Main Researcher: Idoia Labayen Goñi

Date: December 2020 – May 2022

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Study title: **Efectos de un programa de intervención multidisciplinar en el riesgo de diabetes de niños y niñas preadolescentes con sobrepeso: PREDIKID Study**

Funding support: MINECO. Ministerio de Economía y Competitividad

Main Researcher: Idoia Labayen Goñi

Date: 2016-2019

Funding: 108.900 €

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dentro y fuera de la ciencia, un ejemplo de científica, pero mucho más ejemplo de persona, y mira qué es difícil. Sin duda el trabajo de campo o producción científica no tendría sentido si no la pudiese compartir con personas como tú, si no se pudiesen compartir la producción de figuras o las tarde-noches de resonancias magnéticas.

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CONTENTS / ÍNDICE

LIST OF TABLES

STUDY I

STUDY II

STUDY III

STUDY IV

LIST OF FIGURES

STUDY I

STUDY II

STUDY III

STUDY IV

ABSTRACT

Childhood obesity is one of the 21st century's most serious public health challenges, becoming in the most prevalent cardiometabolic disease. In the last two decades, in parallel with the childhood obesity epidemic, several associated comorbidities such as cardiovascular diseases, type 2 diabetes (T2D) and metabolic associated fatty liver disease (MAFLD) have raised in paediatric population. MAFLD has emerged as one of the most serious comorbidities of childhood obesity, and the most common cause of chronic liver disease among children and young adults worldwide. MAFLD encompasses a spectrum of diseases ranging from steatosis to steatohepatitis, fibrosis and eventually cirrhosis, worsening and complicating the stage and the reversibility of the disease.

The asymptomatic evolution of MAFLD, together with its high prevalence and costly and/or invasive diagnosis methods (liver biopsy and/or magnetic resonance imaging) make difficult the early identification of children with the disease.

Paediatric MAFLD development and progression mechanisms are complex and multifactorial. Specific genetic polymorphisms and epigenetic modifications, sociodemographic and lifestyle factors have been associated with the development of the disease. The identification of risk factors for paediatric MAFLD development and the study of potential biomarkers for its diagnosis is crucial for its early prevention and treatment. Thus, there is agreement among scientific/medical associations on the need to develop useful screening tools to detect paediatric MAFLD. Therefore, the overall objectives of the present International Doctoral Thesis were: i) to develop a minimally invasive screening protocol with high predictive potential for the identification of children with overweight or obesity candidates to confirmatory diagnosis of MAFLD that can be useful in clinical practice ii) to systematically analyse the biomarker role of circulating miRNAs in the early onset of obesity and obesity associated co-morbidities through the examination of available circulating miRNA profile data in children and adolescents with obesity, and in obesity-associated metabolic abnormalities, and iii) to identify potential miRNA biomarkers of early MAFLD and/or IR in preadolescent children, and to test their associations with cardiometabolic risk factors. In order to achieve these objectives, data from the EFIGRO, PREDIKID and MICROKID investigation projects were considered.

The conclusions from the current Thesis are: i) sociodemographic and lifestyle factors such as ethnic minority, prematurity at birth, elevated waist to height circumference ratio, sugar sweetened consumption, screen time and low cardiorespiratory fitness are consistently associated with the presence of hepatic steatosis in children with overweight or obesity, ii) at present, available screening methods for MAFLD identification in children have limited accuracy and applicability, iii) the HEPAKID index pre-screening tool is the first sociodemographic, lifestyle and anthropometric data-based screening method for identifying children with overweight or obesity whit elevated risk to suffer hepatic steatosis, iv) biochemical parameters such as, plasma TG, insulin, HOMA-IR, AST, ALT, GGT and ferritin levels, as well as the presence of risk alleles of *PPARG*rs13081389, *PPARG*rs1801282, *HFE*rs1800562 and *PNLPLA3*rs4823173 polymorphisms, are consistently associated with the presence of hepatic steatosis in children with overweight or obesity. However, their prediction capacity is not enough for the screening of MAFLD, v) the HEPAKID prediction protocol identifies with high sensitivity, specificity and accuracy, as well as low time-consuming and economic cost, children with overweight or obesity who likely suffer from MAFLD, and who should be referred for confirmatory diagnosis, vi) circulating miRNAs could be promising diagnostic biomarkers of obesity-associated diseases, such as MAFLD and T2D, already in childhood. However, it was not possible to identify a concrete miRNA profile in children with obesity in the literature, vii) circulating miR-660-5p seems to be a biomarker of the presence of MAFLD in preadolescent children, regardless of weight status, and viii) circulating miR-320a, miR-142-3p, miR-190a-5p, miR-374a-5p and let-7 family miRNAs could serve as potential biomarkers of IR in children.

The findings from the present Thesis clear have clinical applications. The HEPAKID prediction protocol may be a helpful tool to detect those children with high risk of MAFLD in primary care, improving the early diagnosis and, treatment of the disease in paediatric population. However, it should be validated in larger paediatric multi-ethnic cohorts of children in order to test its reliability.

RESUMEN

La obesidad infantil se ha convertido en unos de los problemas más graves en materia de salud pública del siglo XXI. En las últimas dos décadas, y de forma paralela a la epidemia de la obesidad infantil, diferentes comorbilidades asociadas como las enfermedades cardiovasculares, la diabetes mellitus de tipo 2 y la enfermedad metabólica asociada al hígado graso (en inglés MAFLD; *metabolic associated fatty liver disease*) han aumentado su prevalencia en la población infantil. La MAFLD se ha convertido en la comorbilidad más frecuente de la obesidad infantil, siendo hoy en día a nivel mundial la primera causa de enfermedad hepática crónica en población pediátrica. La MAFLD puede progresar a esteatohepatitis y cirrosis, complicando su pronóstico y la reversibilidad de la patología. Sin embargo, su evolución asintomática, junto con la dificultad de su diagnóstico por tratarse de métodos costosos y/o invasivos (biopsia hepática y/o resonancia magnética), impiden su identificación y diagnóstico precoz.

La evolución y progresión de la MAFLD pediátrica es compleja, multifactorial y no está del todo esclarecida. Ciertos polimorfismos genéticos y modificaciones epigenéticas, así como factores sociodemográficos y de estilo de vida se han relacionado con el desarrollo de esta enfermedad. Así, la identificación de los factores de riesgo de desarrollo de MAFLD, así como el estudio de biomarcadores de la enfermedad es crucial para su prevención y tratamiento temprano.

En este sentido, asociaciones médicas y científicas subrayan la necesidad de desarrollar métodos de cribado útiles para la detección de la MAFLD pediátrica. Por ello, los objetivos de la presente Tesis Doctoral Internacional son: i) desarrollar un protocolo mínimamente invasivo y con alta capacidad predictiva, para la identificación de esteatosis hepática en niños/as con sobrepeso u obesidad, ii) analizar el papel de los miRNAs como biomarcadores de la obesidad infantil y de sus comorbilidades mediante un análisis sistematizado de los estudios publicados que identifican perfiles de miRNAs en la obesidad pediátrica y/o en sus comorbilidades asociadas, y iii) identificar miRNAs potencialmente marcadores de MAFLD y/o resistencia a la insulina en niños/as preadolescentes y estudiar su asociación con factores de riesgo metabólico.

Para dar respuesta a estos objetivos se llevaron a cabo cuatro estudios en el contexto de tres proyectos de investigación: EFIGRO, PREDIKID y MICROKID.

Las conclusiones de esta Tesis Doctoral son: i) los factores sociodemográficos y de estilos de vida como la pertenencia a una etnia minoritaria, la prematuridad, un elevado índice cintura-talla, el consumo de bebidas azucaradas, el tiempo de visualización de pantallas, y la baja capacidad cardiorrespiratoria están consistentemente asociados con la presencia de esteatosis hepática en niños/as con sobrepeso u obesidad, ii) los métodos de cribado de MAFLD pediátrica disponibles muestran una limitada precisión y aplicabilidad, iii) el índice HEPAKID es la primera herramienta de cribado basada en datos antropométricos, sociodemográficos y de estilos de vida capaz de identificar a niños/as con sobrepeso u obesidad con elevado riesgo de padecer esteatosis hepática, iv) los niveles elevados de triglicéridos (TG), HOMA-IR, alanina aminotransferasa (ALT) , aspartato aminotransferasa (AST), gamma-glutamil transferasa (GGT) y ferritina en plasma, así como la presencia de alelos de riesgo de las variantes genéticas *PPARG*rs13081389, *PPARG*rs1801282, *HFE*rs1800562 y *PNLPLA3*rs4823173 se asocian consistentemente con la presencia de esteatosis hepática en niños/as con sobrepeso u obesidad. Sin embargo, su capacidad predictiva es baja por lo que no son suficientes para el cribado de la MAFLD, v) el protocolo de predicción HEPAKID muestra una alta sensibilidad, especificidad y capacidad discriminatoria, así como con una mínima inversión de tiempo y de recursos económicos, para identificar la MAFLD en niños/as con sobrepeso u obesidad y que deben ser derivados a unidades especializadas para la confirmación del diagnóstico, vi) los miRNAs circulantes son biomarcadores prometedores de enfermedades asociadas a la obesidad como la MAFLD y la diabetes mellitus tipo 2. Sin embargo, no ha sido posible identificar un perfil de miRNAs concreto asociado con las comorbilidades mencionadas en niños/as con obesidad en la literatura científica actual, vii) el miRNA circulante miR-660-5p parece ser un biomarcador predictivo de la presencia de MAFLD en niños/as preadolescentes, independientemente de su peso corporal y viii) los miRNAs circulantes miR-320a, miR 142-3p, miR-190a-5p, miR-374a-5p y los de la familia let-7 podrían servir como potenciales biomarcadores de la resistencia a la insulina en población pediátrica.

La aplicación clínica más relevante de esta Tesis Doctoral es el desarrollo de una herramienta para la identificación de MAFLD en niños/as con sobrepeso u obesidad. Este protocolo puede ser de gran ayuda en atención primaria para identificar a los niños/as con elevado riesgo de padecer MAFLD y así mejorar su diagnóstico y, en consecuencia, su tratamiento temprano. Sin embargo, se precisan estudios de validación en otras cohortes pediátricas de origen multiétnico y de mayor tamaño muestral para analizar su fiabilidad.

LIST OF ABBREVIATIONS

ALT, Alanine aminotransferase

AST, Aspartate aminotransferase

BMI, Body mass index

COSI, European Childhood Obesity Surveillance Initiative

CRF, cardiorespiratory fitness

CT, Computed tomography

CVD, Cardiovascular diseases

DNL, hepatic de novo lipogénesis

ESPGHAN, European Society of Paediatric Gastroenterology, Hepatology and Nutrition

GGT, Gamma-glutamyl transferase

HOMA-IR, Homeostatic Model Assessment for Insulin Resistance

HDL, high-density lipoprotein

IR, Insulin resistance

LDL, low-density lipoprotein

MAFLD, Metabolic associated fatty liver disease

MiRNA, microRNA

MRI, magnetic resonance imaging

MUFA, monounsaturated fatty acids

NASPGHAN, North American Society of Pediatric Gastroenterology, Hepatology and Nutrition

NASH, steatohepatitis

PNPLA3, Patatin-like phospholipase 3

PUFA, Polyunsaturated fatty acids

SNP, Single nucleotide polymorphisms

T2D, Type 2 diabetes

TC, total cholesterol

TG, triglycerides

WHO, World Health Organization

WtHR, Waist circumference and waist to height ratio

1. GENERAL INTRODUCTION

1.1. Childhood overweight and obesity

1.1.1. Prevalence

The prevalence of overweight and obesity among children and adolescents has risen dramatically in the last few decades, doubling the prevalence in more than 70 countries worldwide since 1980 (1). Thus, the last reports of the World Health Organization (WHO) estimated that over 340 million children and adolescents aged 5-19 (2016), and 38.2 million children under the age of 5 years (2019) were overweight or obese, all over the world (2).

In European region, in the 2015-2017 period, the WHO European Childhood Obesity Surveillance Initiative (COSI) estimated that in children aged between 7 and 9 years the prevalence of overweight or obesity, ranged from 9 to 43% in boys and from 5 to 43% in girls (3). The highest prevalence of overweight was observed in Mediterranean countries, such as Cyprus, Spain, Grece and Italy (between 38% and 43%), while central Asian countries such as Tajikistan, Kyrgyzstan and Turkmenistan showed the lowest prevalence (between 5% and 11%). These data placed Spain in the second position of the highest rates of children with overweight (including obesity) in Europe after Cyprus, and in the sixth position of highest rates of children with obesity.

In Spain, the ALADINO study estimated that the 40.6% of school children (aged between 6 and 9 years suffer from excess of body weight, the 23.3% had overweight and the 17.3% obesity (4). While the overweight status was more prevalent in girls (24.7%) than in boys (21.9%), the obesity status including severe obesity, was more prevalent in boys than in girls (19.4% vs. 15.5% obesity and 6% vs 2.4% severe obesity for boys and girls respectively) (4).

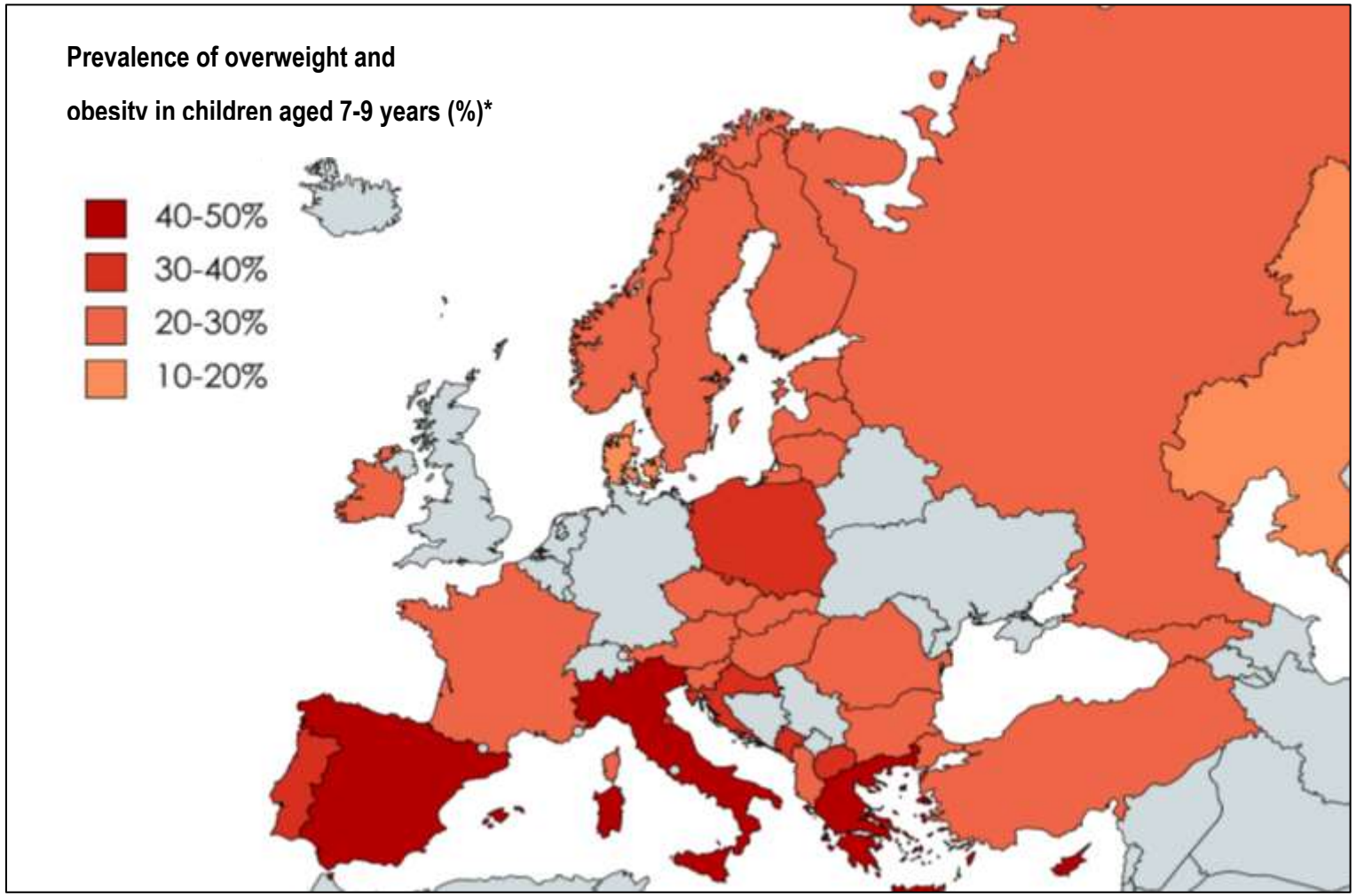

Illustration 1. Prevalence of overweight (including obesity – WHO definition) in children aged 7–9 years (%)*. Data of presentation map were obtained from WHO European Childhood Obesity Surveillance Initiative (COSI) 2015–2017 report where data of 36 countries of the WHO European Region are recollected.*Data relate to: (i) 7-year-olds in Bulgaria, Czechia, Denmark, Estonia, Finland, Georgia, Greece, Hungary, Ireland, Kyrgyzstan, Lithuania, Latvia, Malta, Montenegro, Portugal, North Macedonia, Russian Federation (Moscow only), Serbia, Slovakia, Slovenia, Spain, Tajikistan, Turkmenistan and Turkey; (ii) 8-year-olds in Albania, Austria, Croatia, France, Italy, Norway, Poland, Romania, San Marino and Sweden; and (iii) 9-year-olds in Cyprus and Kazakhstan.

1.1.2. Comorbidities related to childhood obesity

Childhood obesity is one of the 21st century's most serious public health challenges, becoming in the most prevalent cardiometabolic disease (5). In the last two decades, in parallel with the childhood obesity epidemic, several associated comorbidities have raised in paediatric population (5,6).

Children with overweight or obesity are more likely to be obese into the adulthood and to suffer from physical and psychological consequences already in childhood. In this way, cardiovascular or metabolic disorders such as high blood pressure, dyslipidaemia, hepatic steatosis, insulin resistance (IR) or type 2 diabetes (T2D) have increased their incidence among children (6). The Global Burden of Disease estimates that by 2025, 268 and 91 million of children aged 5-17 years will present overweight and obesity respectively, 38 million will suffer hepatic steatosis, 27 million hypertension, 12 million impaired glucose tolerance and 4 million of children will have T2D (7).

Musculoskeletal problems, asthma, systemic inflammation, certain types of cancer as well as psychological problems such as depression or anxiety are also common alterations associated with childhood obesity (6,8–10). In the long term, overweight or obesity during childhood increase the risk of developing cardiovascular diseases (CVD), diabetes and some cancers into the adulthood, which can lead to higher morbidity and mortality in midlife (11–13). In this context, the understanding of the pathophysiological mechanisms implicated in the development of these comorbidities in children with overweight or obesity is essential (14).

1.2. Paediatric metabolic associated fatty liver disease (MAFLD)

1.2.1. Definition and prevalence

Metabolic associated fatty liver disease (MAFLD) is considered the hepatic manifestation of metabolic syndrome and systemic insulin resistance (15). Paediatric MAFLD is considered a major risk factor for T2D and CVD (16,17). In adults, it is estimated that those patients with MAFLD had 1.5 to 5 times higher risk of T2D, CVD, and CVD-related mortality (18). Paediatric MAFLD is also associated with the increase of mortality from all causes and from some cancers, cardiometabolic and liver diseases (19).

The MAFLD term has been recently agreed among different expert groups in order to reflect more accurately the current knowledge of fatty liver disease associated with metabolic dysfunction (20,21). The definition of paediatric MAFLD is based on the evidence of intrahepatic fat accumulation in addition to one of the following three criteria: excess overall adiposity, presence of prediabetes or T2D, or as evidence of metabolic dysregulation defined as the presence of at least two cardiometabolic risks according to sex and age percentiles (increased waist circumference, hypertension, high triglycerides (TG), low serum high density lipoprotein (HDL), TG to HDL ratio of more than 2.25 or impaired fasting glucose as a proxy of IR) (21).

MAFLD has emerged as one of the most common comorbidities of childhood obesity, and it is the most common cause of chronic liver disease among children and young adults worldwide, affecting to an estimated 3-10% of general paediatric population and 30-80% of children with overweigh or obesity (22–24).The elevated prevalence of MAFLD and its association with the development of some chronic diseases, involve an important health and economic cost. Consequently, MAFLD represents an important economic burden in Europe, with an annual cost of about €35 billion (354-1.163€ per patient) (25).

1.2.2. Etiology and physiopathology

Paediatric MAFLD development and progression mechanisms are complex and multifactorial, and they have not been entirely elucidated (26). Specific genetic polymorphisms and epigenetic modifications, sociodemographic factors and environmental features such as diet (e.g., excessive fat and fructose) and lack of physical activity are directly associated with the development of MAFLD (24,26–29). The excess of adiposity and IR (28), dysregulation of adipokines (30), lipotoxicity (31), dysbiosis of the gut microbiota (32) and endocrine disruptors (33) are also involved in the development and progression of this disease.

The accumulation of TG within the hepatocytes, named hepatic steatosis, is the first step of MAFLD. Although, the metabolic pathways involved in hepatic steatosis are not completely elucidated, the hepatic uptake of plasma free acids, hepatic de novo lipogenesis (DNL), hepatic fatty acid secretion in very low-density lipoprotein (VLDT)-TG are the main mechanisms involved in the disease (34). In this line, hyperglycaemia and hyperinsulinemia are involved in a vicious circle promoting de novo lipogenesis (35). In adults, IR has been identified as one of the inductors of MAFLD, increasing hepatic de novo lipogenesis and impairing insulin-mediated suppression of adipose tissue lipolysis by inducing the free fatty acids flux into the liver (16,36–38). In this line, MAFLD has also been directly associated with the aggravation of IR (37,38).

MAFLD encompasses a spectrum of diseases ranging from steatosis to steatohepatitis (NASH), which is characterized by hepatocellular inflammation and injury, to fibrosis and eventually cirrhosis (39,40). In fact, it is estimated that the 23% of children with hepatic steatosis have NASH (24), and that among them, 9% develop fibrosis or cirrhosis (22), worsening and complicating the stage and the reversibility of the disease.

1.2.3. Diagnosis

The presence of MAFLD is determined as the evidence of intrahepatic fat accumulation (steatosis) in addition to one of the three following criteria: excess adiposity, presence of prediabetes or T2D, or evidence of metabolic dysregulation (21). The percentage of hepatic fat to define hepatic steatosis varies in the scientific literature from 4.85% to 6% (41–43).

Nowadays, the proposed criteria for the diagnosis of paediatric MAFLD are based on liver histology (biopsy sample), medical imaging methodologies, or blood biomarkers (21). Liver biopsy is the gold standard in the diagnosis of MAFLD and the only single test that can reliably distinguish simple steatosis from MAFLD and NASH. However, the applicability of liver biopsy is limited because it is a costly and invasive technique which has been associated with life-threatening (44,45).

Medical imaging techniques such as ultrasonography, computed tomography (CT) or magnetic resonance imaging (MRI) can be used to quantify hepatic fat. MRI shows the better utility and accuracy for the assessment of liver fat in children (46) with an estimated sensitivity of 100% and specificity of 90.4% compared with liver biopsy diagnosis (47). However, its high elevated cost limits its applicability in routine explorations. In this context, ultrasonography is the most commonly used imaging modality for the detection of MAFLD; it is inexpensive, widely available, simple and easy to use, and hepatic fat determined by liver ultrasonography is strongly correlated with the grade of steatosis on liver biopsy. However, it has very low sensitivity to diagnose MAFLD when the liver contains <30% fat (48,49); thus, its accuracy is limited showing a sensitivity of 60% and a specificity of 84% which determines low capacity as MAFLD diagnosis method (50,51).

The asymptomatic evolution of MAFLD, together with its high prevalence and costly and/or invasive diagnosis methods make difficult the early identification and diagnosis of children with the disease. There is agreement among scientific/medical associations on the need to develop useful screening tools and guidelines for treating MAFLD in children, but no consensus strategies have been produced. Currently, the detection of increased liver enzymes levels is the most used tool in the screening of MAFLD in clinical practice, suspecting of the disease when patients have overweight or obesity and high levels of alanine aminotransferase (ALT). For example, the American Academy of Pediatrics recommends the measurement of ALT levels in all children with

obesity, or with overweight plus any cardiometabolic risk factor (52). In the same way, the recommendation of the North American Society of Pediatric Gastroenterology, Hepatology and Nutrition (NASPGHAN) is to refer for further test children with ALT levels ≥44 IU/L, in girls, or ≥52 IU/L, in boys (42). Similarly, the recommendation of the European Society of Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) (50) is to assess ALT levels and perform abdominal ultrasound in all children >3 years with overweight or obesity, suggesting a cut point of >35 IU/L ALT to detecting hepatic steatosis. In this line, other authors such as, Schwimmer et al. suggest a lower cut-off to determine hepatic steatosis in children: >22 IU/L girls and >25 IU/L boys (53).

Considering the elevated prevalence of paediatric overweight and obesity, these complementary tests would be a large strain on public health systems. In addition, the majority of studies in children with biopsy-proven or MRI-diagnosed hepatic steatosis report that ALT levels and ultrasound techniques only show moderate diagnostic accuracy in terms of detecting hepatic steatosis, and that combining them leads to no improvement in sensitivity (54,55). Similarly, these ALT cut-off points proposed for the paediatric population by different authors or associations showed high specificity (between 90-100%), but very low sensitivity (between 5-48%) (56–59).

Recently, several scores have been developed for detecting the disease in adults (60–62), but only one for its use with children (63). However, the accuracy and clinical usefulness of these tools remain controversial, and they are yet to be validated (54). The ped-NAFLD score, based on anthropometric measurements and biochemical information such as ALT and homeostatic Model Assessment for Insulin Resistance (HOMA-IR), showed high accuracy in the study sample (63) with 89% (77%–100%) of sensitivity and 76% (60%–82%) of specificity, but limited accuracy in validations samples with a sensitivity between 33% and 75% and specificity between 68% and 95% (54,59). In addition, biochemical biomarkers are required for its calculation, and blood sampling is not routinely asked in apparently healthy children, even if they have overweight or mild obesity. Therefore, a simple and effective non-invasive screening tool is needed for its use in the routine primary care setting, becoming a priority line in MAFLD research the study and identification of potential biomarkers.

1.3. Early identification and prevention of MAFLD

The etiology of childhood obesity and its related comorbidities is complex, and it is influenced by genetics, lifestyle factors (diet and physical activity) and social and physical environment factors (64). The understanding of paediatric MAFLD risk factors is needed for the prevention of the disease and the study of potential biomarkers. The proper identification of children with MAFLD, particularly in the early stages of the disease is, therefore, of great public health interest, especially since lifestyle-based treatments are effective.

1.3.1. Sociodemographic and lifestyle factors

Sociodemographic factors such as age, gender, ethnic, socioeconomical status and maternal gestation and perinatal period have been studied in the developed of MAFLD. Hepatic steatosis seems to be more prevalent in older children; thus, while the prevalence in children aged 2-4 years old is 0.7%, the prevalence in adolescent aged 15-19 years is 17.3% (22). According to the literature, the prevalence of MAFLD is higher in boys than in girls (22), although there is a considerable heterogeneity in the results between studies (23,65).

Hispanic or Asian populations are more likely to suffer hepatic steatosis (10-11%) compared with white (8.6%) or black (1.5%) populations (22). In fact, in the United States, the prevalence of hepatic steatosis was higher in Hispanic, than in non-Hispanic children (22) or adults (66). The reason of this ethnic predisposition has been associated with a genetic variant associated with the increased risk of developing MAFLD that is more prevalent in Hispanic population (67,68).

Childhood obesity related comorbidities are more prevalent in low socioeconomic status subgroup, showing health disadvantages in the same high-income country, but different socioeconomic status (69,70). Among high-income countries, a high prevalence of obesity and obesogenic behaviours is observed in children and adults from ethnic minorities or socioeconomically disadvantaged backgrounds (65,70). In this line, socioeconomic status is emergently thought to be an influencing factor for MAFLD, affecting individual lifestyles and living environments and consequently in the development of the disease (71). Previous studies analysed the low incomes and parental education influence in dietary patterns, physical activity and sedentary behaviours (72–74), becoming children with low socioeconomic status family more susceptible to suffer metabolic diseases (69).

The maternal pre-pregnancy obesity, (pre)gestational diabetes, breastfeeding, and birth anthropometrics or preterm birth have been studied on the development of MAFLD in children and adolescents (75). The MAFLD risk is higher in children and adolescents born to mothers with overweight or obesity (19,76) and breastfeeding seems to be a protective factor for the development of MAFLD (75). Previous studies also showed that children with premature birth and low birth weight or high birth weight have increased risk for developing MAFLD (75,77). Although, some studies showed higher levels of hepatic fat in children born to mothers with gestational diabetes, this association is unclear yet (75).

Lifestyle factors such as sugar rich diets, low physical activity and sedentary behaviours are the strongest risk factors for the development and progression of childhood obesity and its related comorbidities. There is evidence of the adverse effects of high sugar intake, particularly fructose, on paediatric MAFLD because of the stimulation of hepatic *de novo* lipogenesis (78–80). In this line, physical inactivity is also consistently associated with IR (81), cardiometabolic risk factors (82) and hepatic fat (83) in children and adolescent. Similarly, poor cardiorespiratory fitness (CRF) has been associated with increased IR and higher levels of hepatic enzymes in children and adolescents (83,84). Thus, sedentary behaviours such as screen time are associated with the increased risk of obesity and metabolic diseases (85–87). The association between screen time and fatty liver was determined in different studies (59,87), becoming more susceptible of suffering MAFLD those children with sedentary and inactive lifestyle.

1.3.2. Clinical markers

Clinical markers such as anthropometric measurements and biochemical parameters have been studied in the development and progression of MAFLD. These are the most studied markers for the identification of MAFLD because they are easily measures and minimally invasive.

1.3.2.1. Anthropometric measurements

Anthropometric measurements such as body mass index (BMI) and waist circumference have been correlated with MAFLD and its progression (88,89). Children with overweight or obesity have 13 times more risk to suffer MAFLD compared with normal weight participants; likewise, the increase in BMI category elevates 5 times the risk of MAFLD (23).

Higher waist circumference and waist to height ratio (WtHR) are the most studied measurements as indicators of MAFLD (90,91). WtHR is associated with an increased risk of MAFLD in children with obesity and seems to be a better predictor of the disease than BMI (92). However, it is important to highlight that there are children with light or mild overweight with elevated percentage of hepatic fat.

1.3.2.2. Biochemical parameters

Children with overweight and MAFLD have higher levels of ALT, aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT) and HOMA-IR compared to obese controls (93). ALT is the most used biochemical marker of MAFLD and its severity, but its prediction capacity is controversial because it has high specificity, but very limited sensitivity. Liver enzymes and HOMA-IR are the most studies biochemical parameters as predictors of hepatic steatosis. The lipid profile is also altered in children with MAFLD, showing higher levels of low-density lipoprotein (LDL), total cholesterol (TC) and TG, and lower levels of HDL (94,95). High TG levels are the most associated lipid dysregulation with fatty liver and it has also been proposed as potential predictor of MAFLD (96).

Other biochemical parameters such as ferritin and serum uric acid are also significantly elevated in fatty liver disease (97–99). Both parameters are implicated in the oxidative stress, IR and metabolic alterations and some studies have reported mutual relationships and synergistic actions between them suggesting its potential use as predictors of MAFLD (97,98).

Other metabolites have also been associated with paediatric MAFLD. For example, osteocalcin and osteoprotegerin are decreased in children with fatty liver, affecting the osteoblast differentiation (93), and leptin levels significantly higher (100,101) in children with MAFLD, independently of weight status. These adipokines have been involved in fatty liver disease progression and severity Inflammatory markers such as TNF-α and IL-6 levels are also increased in children with obesity and MAFLD compared to obese controls, suggesting an interaction of systemic inflammation and hepatic steatosis (102,103).

1.3.3. Genetic and epigenetic markers

Since the advent of genome wide association studies, multiple genes and epigenetic modifications have been proposed to influence the development of MAFLD opening a new perspective on the pathogenesis, diagnosis and treatment of the disease (28).

Genetic variants, mainly in the form of single nucleotide polymorphisms (SNP), have been associated with hepatic FFA flux and metabolic alterations (104). The Patatin-like phospholipase 3 (*PNPLA3*) gene has been implicated in lipid metabolism processes and lipolytic activity on TG (68). Several genetic variants of the *PNPLA3* have been proposed as biomarkers of MAFLD in children (105,106) and in adults (107,108). The *PNPLA3* rs738409 increased the risk of NAFLD and NASH In adults, other SNPs in different genes such as, *NCAN, GCKR* and *LYPLAL1* were associated with histological steatosis and lobular inflammation (104), while genetic variants of the *TM6SF2, FTO* and *LIPA* genes were related to hepatic fat content and/or associated metabolic diseases (108). In paediatric MAFLD, of the most studied SNPs as predictors of MAFLD are those affecting to *PNPLA3*, *GCKR* and *TM6SF2* genes (109,110).

Epigenetic modifications are stable changes at transcriptional level, such as microRNAs (miRNAs), DNA methylation and histone modifications, which do not alter the basic DNA sequences (111). MiRNAs are one of the major forms of epigenetic modulation. MiRNAs are short noncoding RNA molecules (21-23 nucleotides) that suppress protein synthesis by inhibiting mRNA translation or leading to mRNA degradation (112). MiRNAs are responsible for a variety of crucial regulatory functions related to cell growth, development, differentiation, apoptosis and immune responses, and therefore are implicated in the development, progression and treatment of different diseases (113–117). In this line, several miRNAs that have been proposed as potential biomarkers and therapeutic targets for MAFLD (113,118,119) and T2D (114,120). However, in children, there are still few studies examining the miRNAs expression (121–123), all of them performed a specific search of miRNAs associated with hepatic fat in adults showed significantly higher levels of miR-122 and miR-34a-5p in children with MAFLD.

1.3.4. Treatment

MAFLD is reversible and easily treatable in its early stages and there is evidence that lifestyle-based treatments are effective in reducing hepatic fat in overweight children (124–126). These interventions are cornerstone in the prevention and treatment of paediatric MAFLD, but there are still insufficient data to recommend any particular dietary recommendation or exercise program (124). The Mediterranean diet has been proposed as a possible effective dietary treatment of MAFLD because its beneficial effects on cardiometabolic profile, and its elevated content of monounsaturated fatty acids (MUFA) and omega-3 polyunsaturated fatty acids (PUFA) rich foods, and low processed and sugar rich foods (127). Previous studies showed that the supplementation with antioxidants, PUFA and/or probiotics improved paediatric hepatic steatosis and steatohepatitis, but more studies are needed (124). Resistance and aerobic exercise have been proposed for the improvement of MAFLD (128). Interestingly, the addition of exercise to a lifestyle intervention program reduced 20% of hepatic fat and increased the rate of responders for hepatic steatosis in children with overweight/obesity (129).

2. HYPOTHESIS AND OBJECTIVES / HIPOTESIS Y OBJETIVOS

2.1. Hypothesis

The hypothesis of the current International Doctoral Thesis is that the identification of sociodemographic, biochemical and epigenetic biomarkers consistently associated with the development of hepatic steatosis in children with overweight or obesity may be useful for the design of a screening tool for the early diagnosis of this disease.

2.2. Objectives

The objectives of the present Thesis are the following:

1) To develop a minimally invasive screening protocol with high predictive potential for the identification of children with overweight or obesity candidates to confirmatory diagnosis of MAFLD that can be useful in clinical practice (Study I and Study II)

1.1) To test the prediction capacity of previously published paediatric screening tools for hepatic steatosis (Study I)

1.2) To develop a pre-screening tool for the identification of children with high risk to suffer hepatic steatosis, based on the recording of anthropometric, sociodemographic and lifestyle factors. (Study I)

1.3) To test the capacity of genetic and/or biochemical risk scores for the detection of MAFLD in children with overweight or obesity (Study II)

1.4) To develop an algorithm combining elevated sensitivity and specificity to identify MAFLD in children with overweight or obesity. (Study II)

2) To systematically analyse the biomarker role of circulating miRNAs in the early onset of obesity and obesity associated co-morbidities through the examination of available circulating miRNA profile data in children and adolescents with obesity, and in obesity-associated metabolic abnormalities. (Study III)

3) To identify potential miRNA biomarkers of early MAFLD and/or IR in preadolescent children and to test their associations with cardiometabolic risk factors, in preadolescent children. (Study IV)

2.1. Hipótesis

La hipótesis de esta Tesis Doctoral Internacional es que la identificación de marcadores sociodemográficos, bioquímicos y epigenéticos relacionados con la esteatosis hepática en niños/as con sobrepeso u obesidad puede ser de ayuda para desarrollar herramientas de cribado para la detección precoz de esta enfermedad.

2.2. Objetivos

Los objetivos de esta Tesis Doctoral Internacional son:

1) Desarrollar un protocolo mínimamente invasivo, y con alta capacidad predictiva, para la identificación de esteatosis hepática en niños/as con sobrepeso u obesidad (Estudio I y Estudio II)

1.1) Examinar la capacidad predictiva de los métodos de cribado de esteatosis hepática disponibles para población pediátrica. (Estudio I)

1.2) Desarrollar una herramienta de screening basada en información antropométrica, sociodemográfica y de estilos de vida, para identificar a niños/as con un elevado riesgo de padecer esteatosis hepática. (Estudio I)

1.3) Examinar la capacidad predictiva de variables genéticas y/o bioquímicas del riesgo de padecer MAFLD en niños/as con sobrepeso u obesidad. (Estudio II)

1.4) Desarrollar un algoritmo de identificación de niños/as con alto riesgo de MAFLD que combine elevada sensibilidad y especificidad (Estudio II)

2) Analizar el papel de los miRNAs como biomarcadores de la obesidad infantil y de sus comorbilidades mediante un análisis sistematizado de los estudios publicados que identifican perfiles de miRNAs en la obesidad pediátrica y/o en sus comorbilidades asociadas. (Estudio III)

3) Identificar miRNA potencialmente marcadores de MAFLD y/o resistencia a la insulina en niños/as preadolescentes y su asociación con factores de riesgo metabólico. (Estudio IV)

3. RESULTS

3.1 Study I

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ORIGINAL RESEARCH

Pediatric OBESITY WILEY

A sociodemographic, anthropometric and lifestyle-based prediction score for screening children with overweight and obesity for hepatic steatosis: The HEPAKID index

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ABSTRACT

Hepatic steatosis (HS) is currently the most prevalent hepatic disease in pediatric population and a major risk factor for type 2 diabetes and cardiovascular diseases. The proper identification of children with HS is therefore of great public health interest. The aim of this work was to develop a new prediction score (the HEPAKID index) using anthropometric, sociodemographic and lifestyle factors to identify children with HS. Previously published biochemical pediatric screening tools were validated in the same cohort. A total of 115 pre-adolescent children aged 8-12 years with overweight/obesity, recruited at hospital pediatric units were enrolled in this cross-sectional study. HS (≥5.5% hepatic fat) was assessed by MRI. Anthropometric, sociodemographic and lifestyle variables were collected by validated tests/questionnaires. Forty-one children had MRI-diagnosed HS (35.6%, 49% girls). These children had ($p<0.01$) a higher waist-height ratio, a lower cardiorespiratory fitness, a younger gestational age, and consumed more sugar-sweetened beverages than their HS-free peers. Children with HS were more likely to belong to an ethnic minority ($p<0.01$) and to spend longer viewing screens than recommended ($p<0.05$). The addition of these variables to the multivariate logistic regression model afforded a HEPAKID index with high discriminatory capacity (AUC-ROC: 0.808, 95% CI 0.715-0.901), and score of ≥25.0 was associated with high sensitivity (82%, 95% CI 68-96%). Biochemical biomarker-based pediatric tools for identifying HS showed only moderate discriminatory capacity and low sensitivity (5-41%) in this cohort. The HEPAKID index is the first simple, non-invasive, sensitive, inexpensive, and easy-to-perform screening that can identify children with overweight or obesity who have HS.

Key words: Pediatric obesity, hepatic steatosis, screening tool, lifestyle behaviors, Primary care

Abbreviations: HS, hepatic steatosis; ALT, alanine aminotransferase; MRI, Magnetic resonance imaging; WHtR, the waist to height ratio; BMI, body mass index; SSB, Sugar-sweetened beverage; CRF, cardiorespiratory fitness; 20mSRT, 20 m shuttle run test; YAP, youth activity profile questionnaire; TV, television; WHO, world health organization; CI, confidence intervals; AUC-ROC, area under the receiver-operating characteristic curve; SPSS, statistical package for social sciences.

INTRODUCTION

According to World Health Organization (WHO) childhood obesity is one of the 21st century's most serious public health challenges. In the last two decades, and in parallel with the childhood obesity epidemic, a number of associated comorbidities, such as hepatic steatosis (HS), have become more prevalent. Indeed, pediatric HS now affects some 8% of the general child population, and 34% of children with overweight or obesity (1). It is estimated that by 2025, 38 million children and adolescents will have HS (2). Depending on the diagnostic criteria and methodology used, however, prevalence rates for HS in children with overweight/obesity population can range as widely as 5-83% (1).

Unfortunately, HS can progress to cirrhosis and end-stage liver disease even at a young age (3,4) and is a major risk factor for type 2 diabetes and cardiovascular diseases (5,6). In children it is may be more severe than in adults and has a poorer prognosis (7), with some 15% of patients presenting with at least stage 3 fibrosis at diagnosis (8). The proper identification of children with HS, particularly in the early stages of the disease, is therefore of great public health interest, especially since lifestyle-based treatments are effective in reducing hepatic fat and even reversing the disease before fibrosis develops (9,10).

Pediatric HS is generally a silent liver disease; it can be present but cause no symptoms and give no warning signs (4). Liver biopsy is still the gold standard for its diagnosis (4,11), although other diagnostic procedures, including magnetic resonance imaging (MRI) are available (12). However, these methods are invasive and/or costly. HS may be suspected when patients have high levels of alanine aminotransferase (ALT), but ALT is not a sensitive marker of this disease or its severity (13). In fact, in children, the full spectrum of histological HS may be present even though they have an entirely normal blood ALT result (13,14).

Non-invasive biomarkers or screening tools for the early identification of children with HS are much needed (15). Several scores have been developed for detecting the disease in adults (16–18), but only one exists for use with children (19). However, the accuracy and clinical usefulness of these tools remain controversial, and they are yet to be validated (20). In addition, biochemical biomarkers are required if they are to be used, and blood sampling is not routine in apparently healthy children, even if they have overweight or mild obesity. A simple and effective non-invasive screening tool is therefore needed that can be used by clinicians in the routine primary care setting. Children suspected of having HS could then be referred for a confirmatory diagnosis. The aim of the present work was to develop such a tool - the HEPAKID index - based on the recording

of anthropometric, sociodemographic and lifestyle factors. Previously published pediatric screening tools were subjected to validation in the same cohort used to develop the new tool, and the results compared.

MATERIAL AND METHODS

Design, participants and data collection

This cross-sectional study made use of data collected during the EFIGRO project (ClinicalTrials.gov ID: NCT02258126), the overall aim of which was to examine the effect of exercise on percentage hepatic fat in children with overweight/obesity. In that trial, which was conducted from September 2014 to June 2017 in Vitoria-Gasteiz, Spain, all subjects participated in a 22-week family-based program involving lifestyle and psychological education. Details of sample calculation, randomization, the characteristics of the study subjects, the design of that work, its methods and the measurements taken are available elsewhere (21). For the present work, the baseline data of 115 pre-adolescent children with overweight/obesity (22), and aged between 8.5 and 12.0 years, were analyzed. Overweight and obesity status was defined according to the body mass index (BMI) international age- and sex-specific cut-off values provided by the World Obesity Federation (26). Having other hepatic disease or/and any other disease accompanied with elevated blood transaminase levels, such as viral hepatitis, toxic hepatitis or autoimmune diseases were exclusion criteria.

The Euskadi Clinical Research Ethics Committee approved the study protocol (PI2014045), which complies with the ethical guidelines of the Declaration of Helsinki (2013 revision). Subjects were recruited at the Pediatric Endocrinology Unit of the University Hospital of Araba, and at primary care clinics. The parents or legal guardians of the children provided informed consent for their charges to be enrolled in the study. The present study followed the Transparent Reporting of a Multivariable Prediction Model for Individual Prognosis or Diagnosis (TRIPOD) guidelines (23).

Percentage hepatic fat was assessed by MRI using a Magnetom Avanto system (Siemens Healthcare, Erlangen, Germany) as previously described (21). Thereafter, children were divided into those with and without HS (≥5.5% or <5.5% hepatic fat respectively (24).

Body mass (kg), height (m), and waist circumference (cm) were measured in duplicate following standard protocols; the body mass index (BMI) (kg/m2) and the waist to height ratio (WHtR) were then calculated (25).

The educational level and country of origin of the children's mothers were obtained via questionnaire. Belonging to an ethnic minority was defined as having a foreign-born mother from a low or middle income country (Supplemental table 1) or belonging to recognized ethnic minority for Spain (i.e., Roma) according to the categories provided by the European Commission for Spain (26). Perinatal variables such as gestational age at birth (weeks), birth weight (g) and duration of breastfeeding (weeks), and any family history of obesity and diabetes were collected via a questionnaire and from clinical records.

Dietary intake was assessed by two non-consecutive 24-hour recalls within a period of seven days. Sugar-sweetened beverage (SSB) consumption was determined as the ingestion of soft drinks, sweetened juices, and energetic drinks (27) in g/day. Children were also categorized as consumers or non-consumers of SSB. Adherence to the Mediterranean dietary pattern was evaluated using the Mediterranean Diet Quality Index for children and teenagers (KIDMED) questionnaire (28).

Cardiorespiratory fitness (CRF) was estimated from the number of laps completed in the 20 m shuttle run test (20mSRT) (29), and the children classified as fit (>20th percentile) or unfit (≤20th percentile) according to the sex- and age-specific percentiles of Tomkinson et al. (30). This is a validated test used to assess CRF in schools (31).

Physical activity (counts per min), sedentary and sleep time (min per day) were measured by accelerometry, as reported elsewhere (32). A self-reported sedentary behavior questionnaire (33) was completed in order to determine the frequency of specified sedentary behaviors such as watching TV, playing on-screen games, and surfing the Internet; the children were then categorized as meeting (<2h/day) or not-meeting (≥2h/day) WHO recommendations regarding screen time for children (34).

A brief questionnaire to collect the required information to calculate the HEPAKID index. The calculator is available on https://bit.ly/37WXV0j. Additionally, as the CRF assessment may not be available in clinical settings, a second model was generated excluding this variable (https://bit.ly/2AQTUPa).

In addition to developing the proposed tool, three previously published ALT cut-off points and a score for diagnosing HS in pediatric populations were validated in the present study population: 1) the ALT

concentrations of >22 IU/L in girls and >25 IU/L in boys, according to the criteria proposed by Schwimmer et al. (35), 2) the ALT cut-offs of ≥44 IU/L in girls and ≥52 IU/L in boys proposed by the North American Society For Pediatric Gastroenterology, Hepatology & Nutrition (NASPGHAN) (36), 3) the ALT cut-off point of >35 IU/L proposed by the European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) (37), and 4) the pediatric NAFLD (non-alcoholic fatty liver disease) score (ped-NAFLD score), determined as:

$$\text{ped} - \text{NAFLD score} = \frac{e^{[-13.83 + (0.16 \times WHtR) + (0.07 \times ALT) + (0.78 \times HOMA)]}}{1 + e^{[-13.83 + (0.16 \times WHtR) + (0.07 \times ALT) + (0.78 \times HOMA)]}}$$

for which the proposed cut-off point is 0.39 (19).

The plasma concentrations of ALT and HOMA variables included in the ped-NAFLD score were measured in the present sample of children using standard protocols (21).

Statistical analysis

Differences in sociodemographic, anthropometric and lifestyle characteristics between children with or without MRI-diagnosed HS were analyzed using the independent t-test (continuous variables) or $\chi 2$ test (categorical variables).

No missing data imputation was performed. All variables potentially associated with the presence of HS were included as candidates in a multivariate logistic regression model forming the base of the HEPAKID index. Those independent variables that showed collinearity, and those whose effect was negligible, were removed from the final model. The probability of having HS was determined from the model, multiplying by 100 to obtain the "sociodemographic, lifestyle and anthropometric data based pediatric hepatic steatosis index (HEPAKID index)", which therefore has a 0-100 score range.

The discriminatory capacity of the HEPAKID index was examined by calculating the area under the receiver-operating characteristic curve (AUC-ROC, with 95% confidence intervals [CI]). The calibration of the model was examined using a calibration plot (plotting the expected probabilities against observed event proportions and smoothing via the Loess method) and the Hosmer-Lemeshow test. Cross validation with 150 samples was performed as an internal validation and to provide an optimism-corrected AUC-ROC.

In the external validation of the pre-existing tools, the discrimination of the ALT tests and ped-NAFLD score were assessed by AUC-ROC analysis, and the calibration of the ped-NAFLD score was evaluated using a calibration plot.

The Youden index (38) was used to identify the optimal cut-off point for the HEPAKID index, prioritizing high sensitivity (≥80%). The performance of the proposed index and the already published tools was expressed as sensitivity, specificity, positive predictive value, and negative predictive value (with their corresponding 95%CIs) for the proposed cut-off points. All analyses were performed for the sample as a whole and separately for boys and girls.

All calculations were performed using SPSS software v.23.0 (IBM, Armonk, NY, USA) and R statistical software v.3.6.3. Significance was set at α= 0.05.

RESULTS

Table 1 shows the sociodemographic, anthropometric, lifestyle and biochemical characteristics of the children with (36%) and without HS (64%) as determined by MRI. Children with HS had a higher WHtR ($p<0.001$), higher SSB consumption ($p<0.005$), a lower CRF ($p<0.01$) and a lower gestational age at birth ($p<0.01$) than those without HS. Children with HS were also more likely to belong to an ethnic minority ($p<0.01$) and not to meet recommendations regarding screen time ($p<0.05$). The plasma ALT, plasma insulin and the HOMA index were also higher in children with HS ($p<0.01$).

Model development and validation

Table 2 shows the multivariate logistic regression analysis based on sociodemographic, anthropometric, and lifestyle variables potentially associated with having HS. The HEPAKID index was defined using the regression coefficients (β) obtained in the multivariate logistic regression model (**Table 2, I**). Only those children with valid data on maternal country of origin (non-missing data) on duration of gestation (missing data n=12), anthropometry (no missing data), screen time (no missing data), dietary habits (no missing data), and CRF level (missing data n=5) were included in the model (n=99):

$$'HEPAKID\ index' = \frac{e^{me}}{1+e^{me}} \times 100$$

me = 2.801 + 1.583 x (ethnic minority1) + [(-0.230) x (gestational age2)] + 0.095 x (WHtR3) + 0.656 x (screen time ≥2h/day4) + 0.834 x (SSB5) + [(-0.028) x (CRF6)]

Table 1. Sociodemographic, anthropometric, and lifestyle characteristics of overweight or obese children with and without hepatic steatosis.

	Non-hepatic steatosis		Hepatic steatosis		*p*
	N	**Mean (SD)**	**N**	**Mean (SD)**	
Sociodemographic characteristics					
Age (years)	74	10.6 (1.1)	41	10.5 (1.1)	0.749
Girls (N, %)	74	42, 57	41	20, 49	0.077
Ethnic minority[1] (N, %)	74	6, 8	41	11, 26	**0.007**
Maternal educational level (University N, %)	74	57, 77	40	26, 60	0.168
Family history for obesity (N, %)	74	29,39	40	20,50	0.266
Family history for T2D (N, %)	74	5,7	40	3, 8	0.882
Gestational age (weeks)	68	39.1 (2.2)	35	37.4 (3.4)	**0.009**
Birth weight (g)	72	3226 (597)	39	3072 (714)	0.256
Breastfeeding duration (weeks)	72	11.6 (10.9)	38	13.2 (14.1)	0.545
Anthropometric characteristics					
Height (cm)	74	145 (8)	41	147 (8)	0.520
Weight (kg)	74	53.4 (10.2)	41	56.7 (10.7)	0.112
Body mass index (kg/m^2)	74	25.0 (3.2)	41	26.2 (3.3)	0.059
Waist to height ratio (x100)	74	52.9 (4.5)	41	56.2 (4.3)	**<0.001**
Hepatic fat (%)	74	3.7 (1.0)	41	9.2 (4.9)	**<0.001**
Dietary, physical activity fitness, and sleep patterns					
Physical activity (counts/min)	71	3792 (676)	39	3577 (623)	0.097
Cardiorespiratory fitness (laps)	70	24 (13)	40	17 (9)	**0.002**
MVPA (min/day)	71	97 (26)	39	92 (27)	0.319
Sedentary time (min/day)	71	511 (69)	39	522 (65)	0.421
Screen hours ≥2h/day (N, %)	72	36, 50	39	31, 79.5	**0.002**
Sleep time (min/day)	71	464 (34)	40	455 (38)	0.203
SSB consumption (g/day)	74	51 (90)	41	121 (172)	**0.019**
Fruits and vegetables intake (g/day)	74	224 (159)	41	259 (182)	0.297
KIDMED index	74	5.9 (2.2)	41	6.1 (1.9)	0.694
Biochemical variables:					
ALT (IU/L)	73	18 (5)	41	25 (11)	**<0.001**
Glucose (mg/dL)	73	84.7 (4.9)	40	86.7 (6.1)	0.086
Insulin (IU/ml)	73	11.1 (4.3)	41	13.9 (5.5)	**0.006**
HOMA-IR	73	2.34 (0.95)	40	3.01 (1.28)	**0.006**

Abbreviations: T2D: type 2 diabetes mellitus, MVPA: moderate to vigorous physical activity, SSB: sugar-sweetened beverage, KIDMED: questionnaire about adherence to the Mediterranean Diet in children and young; HOMA:[1]Ethnic minority: the category of ethnic minority includes non-Spanish origin of the mother (Economic migrants: Latin America N=12, Maghreb N=3 and Eastern Europe N=5) and belonging from and Spanish ethnic minority such as Roma (N=6)).

Table 2. Multiple logistic regression analysis showing the association of sociodemographic, anthropometric and lifestyle factors with hepatic steatosis (dependent variable) with (I) and without (II) cardiorespiratory fitness among predictors.

	Hepatic steatosis	
	OR (95% CI)	β
I (n=99)		
Constant	-	1.339
Ethnic minority[1]	4.94 (1.29-18.88)	1.597
Gestational age (weeks)	0.84 (0.70-1.02)	-0.170
Waist to height ratio (x100)	1.08 (0.95-1.22)	0.073
Screen time (≥2h/day)[1]	2.06 (0.69-6.16)	0.722
SSB consumption [1]	2.77 (0.96-8.01)	1.018
Cardiorespiratory fitness (laps)	0.97 (0.93-1.02)	-0.027
II (n=100)		
Constant	-	0.417
Ethnic minority[1]	5.63 (1.51-21.09)	1.729
Gestational age (weeks)	0.82 (0.68-0.99)	-0.196
Waist to height ratio (x100)	1.01 (0.97-1.25)	0.097
Screen time (≥2h/day)[1]	2.23 (0.77-6.44)	0.801
SSB consumption [1]	2.54 (0.90-7.21)	0.933

Abbreviations: β: standardized regression coefficient; OR: Odds ratio; CI: confidence interval; SSB: sugar-sweetened beverages. Only participants with no missing data were included into the model. Missing data: gestational age (n=12), cardiorespiratory fitness (n=5). [1]: Categorical variables. The category of ethnic minority includes non-Spanish origin of the mother (economic migrants; Latin America n=12, Maghreb n=3, and Eastern Europe n=5), and belonging from and Spanish ethnic minority such as Roma (n=6).

Equation to calculate the "HEPAKID Index". Me=model equation, e=exponential function constant. 1Ethnic minority=1 and non-ethnic minority=0, 2Gestational age at birth in weeks, 3WHtR: waist to height circumference ratio, 4Screen time ≥2h/day=1 and <2h/day=0, 5Consumer of sugar sweetened beverages=1, non-consumer=0, 6CRF: cardiorespiratory fitness (number of laps completed in 20mSRT test).

The model included six categorical or continues variables collected in a brief questionnaire: 1) belonging to an ethnic minority (categorical, yes or no), 2) duration of gestation in weeks (continuous), 3) the WHtR multiplied by 100 (continuous), 4) meeting or not meeting screen time recommendations (categorical, yes or no), 5) consumption of SSB (categorical, yes or no), and 6) cardiorespiratory fitness in laps (discrete variable).

The Hosmer-Lemeshow test (p=0.380) and the calibration plot (**Figure 1**) showed the HEPAKID index to be well calibrated. The AUC-ROC value of 0.808 (95%CI 0.715-0.901) showed the index to have strong discriminatory capacity for detecting HS in the study population (**Figure 1**). The optimism corrected AUC-ROC was 0.755 (**Figure 1**).

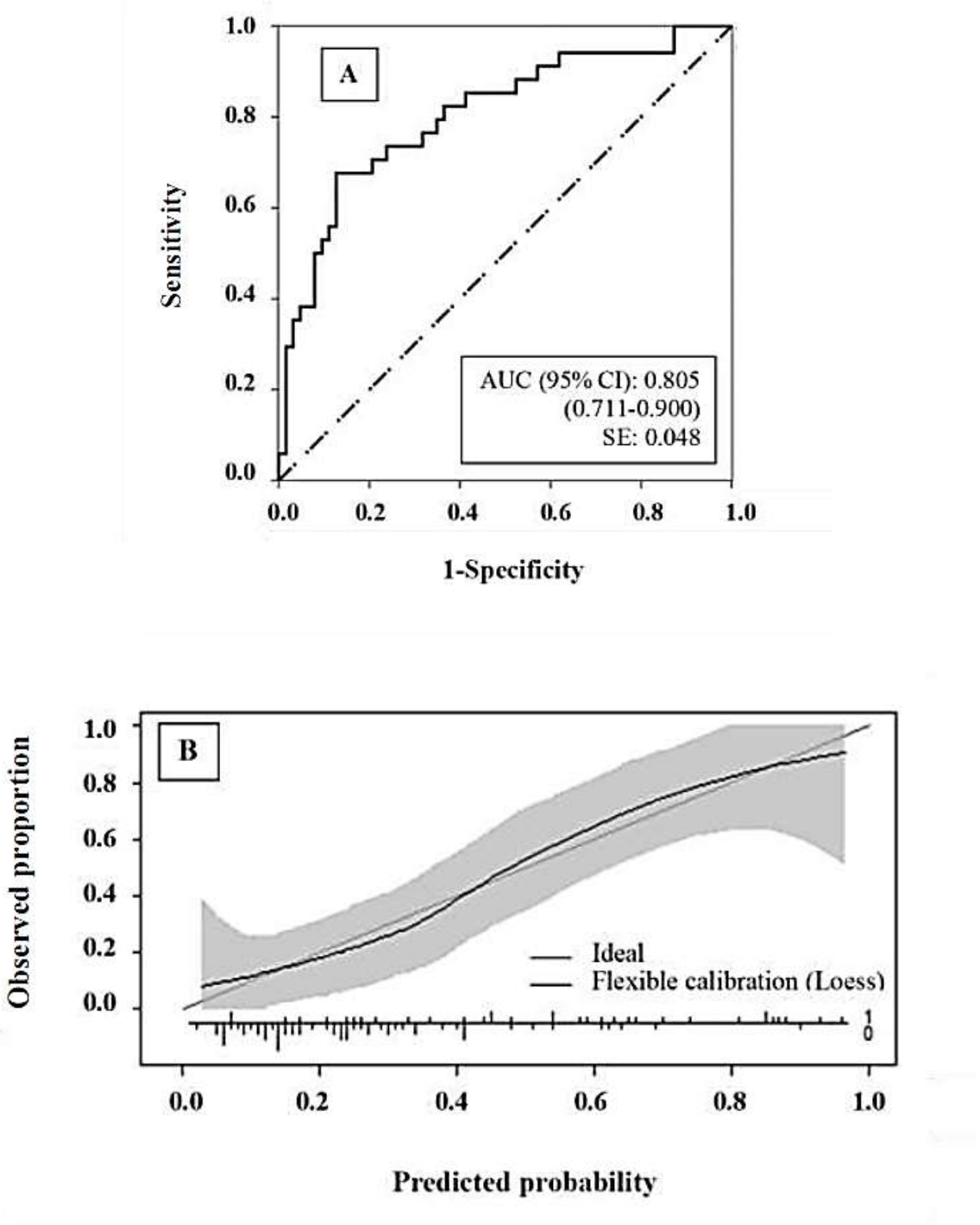

Figure 1. Receiver Operating Characteristics curve (panel A) and calibration (panel B) of the HEPAKID index (n=99). AUC-ROC: area under receiver operating characteristics curve; CI: confidence interval; SE: standard error.

Supplemental Table 2 shows the diagnostic performance of the HEPAKID index at different cut-off points. A value of 25.0 was selected as the optimum cut-off for HS (sensitivity 0.82, specificity 0.62).

A second model for the index was generated excluding CRF (**Table 2, II**). In this case the AUC-ROC was 0.793 (95%CI 0.694-0.893), the Hosmer-Lemeshow test (p=0.521), and the calibration plot again demonstrated good calibration (**Supplemental Figure 1** in the Supplement). The optimism corrected AUC-ROC was 0.754 (**Supplemental Figure 1** in the Supplement). The calculator is available on https://bit.ly/2AQTUPa.

Performance of the ped-NAFLD-score and ALT tests

The AUC-ROC value for both the ped-NAFLD score and the ALT levels (AUC=0.770, 95%CI 0.679-0.861, SE: 0.046 and AUC=0.751, 95%CI 0.657-0.845, SE: 0.048, respectively) were lower than for the HEPAKID index. In addition, the ped-NAFLD score showed poor calibration and detected far fewer cases of HS than did MRI (**Supplemental Figure 2**).

Table 3. Diagnostic performance of the HEPAKID-index and other pediatric prediction scores.

	SN, % (95% CI)	**SP,** % (95% CI)	**PPV,** % (95% CI)	**NPV,** % (95% CI)
HEPAKID index (≥25)				
Whole sample (n=99)	82 (68-96)	62 (49-75)	53 (39-68)	86 (76-98)
Girls (n=55)	82 (61-100)	61 (54-81)	48 (28-68)	88 (74-100)
Boys (n=44)	82 (61-100)	64 (43-85)	61 (39-83)	84 (65-100)
HEPAKID index (model without CRF)				
Whole sample (n=100)	79 (64-94)	58 (45-70)	49 (35-63)	84 (73-96)
Girls (n=57)	77 (53-99)	62 (46-79)	46 (26-67)	86 (72-100)
Boys (n=43)	81 (59-100)	48 (27-69)	48 (27-69)	81 (59-100)
High ALT tests				
Schwimmer et al. (>22 IU/L girls and >25 IU/L boys)[1]				
Whole sample (n=114)	41 (25-58)	90 (83-98)	71 (51-91)	73 (64-83)
Girls (n=62)	30 (7-53)	88 (77-99)	55 (21-89)	73 (59-86)
Boys (n=52)	52 (29-76)	93 (83-100)	85 (61-100)	74 (59-89)
NASPGHAN (≥44 IU/L girls and ≥52 IU/L boys)[2]				
Whole sample (n=114)	5 (0-13)	100 (99-100)	100 (75-100)	65 (56-74)
Girls (n=62)	-	-	-	-
Boys (n=52)	9 (0-24)	100 (98-100)	100 (75-100)	62 (48-76)
ESPGHAN (>35 IU/L)[3]				
Whole sample (n=114)	7 (0-17)	99 (95-100)	75 (20-100)	65 (56-75)
Girls (n=62)	-	-	-	-
Boys (n=52)	14 (0-31)	97 (89-100)	75 (20-100)	63 (48-77)
Ped-NAFLD score (≥0.39)[4]				
Whole sample (n=113)	33 (17-48)	95 (87-100)	76 (53-99)	72 (62-81)
Girls (n=62)	30 (7-53)	95 (88-100)	75 (39-100)	74 (61-87)
Boys (n=51)	35 (12-58)	94 (83-100)	78 (45-100)	69 (54-84)

Abbreviations: SN: sensitivity; SP: specificity; PPV: positive predictive value; NPV: negative predictive value; CRF: cardiorespiratory fitness.

Table 3 shows the diagnostic performance of the HEPAKID index (with and without CRF) compared to the ALT tests and the ped-NAFLD score using their optimum cut-offs. The HEPAKID index showed higher sensitivity (82% and 79% with and without CRF respectively) for identifying children with HS compared to the ped-NAFLD score (33%) and the ALT tests (between 5 and 41%) but showed lower specificity (62% vs. 90-100%). It is remarkable that the proposed cut-offs for the NASPGHAN and ESPGHAN ALT tests identified only 4 and 2 children (all boys) respectively among the 41 children with MRI-diagnosed HS (**Table 3**). The diagnostic accuracy of the HEPAKID index was similar in girls and boys, while the predictive capacity and accuracy of the ALT tests and the ped-NAFLD-score were lower in girls than in boys (**Table 3**).

DISCUSSION

The early, easy and rapid screening of children at increased risk of HS would allow pediatricians to identify those children who should be referred for confirmatory diagnosis. Early detection would also open the door to more effective treatment. The proposed HEPAKID index is a simple, non-invasive, sensitive, inexpensive and easy-to-perform screening method based on sociodemographic, lifestyle and anthropometric variables that can identify HS in pre-adolescent children with overweight or obesity. A HEPAKID index score of $\geqslant$25.0 shows high sensitivity and reasonable accuracy in identifying HS as detected by MRI. It could therefore be of great use in primary care clinics, allowing those children who need to be referred to pediatric gastrointestinal and hepatology specialists to be quickly identified.

The HEPAKID index includes anthropometric data (WHtR), sociodemographic factors (ethnic minority status and gestational age at birth), lifestyle variables (SSB consumption, screen time) and CRF (laps in 20mSRT test), all of which are easily measured or collected in a brief questionnaire (https://bit.ly/37WXV0j).

The most important contributor to the HEPAKID index was belonging to an ethnic minority group. Previous studies have reported that ethnicity and genetics play an important role in liver fat deposition. In fact, in the United Sates, the prevalence of hepatic steatosis was higher in Hispanic, than in non-Hispanic children (39) and adults (40). In addition, the accuracy of several predictive scores of NAFLD for adults was significantly influenced by ethnicity (41). Several genetic variants such as the PNPLA3 polymorphism have also been

associated with increased risk of developing NAFLD (24,42,43). Interestingly, this polymorphism is more prevalent in Hispanic than in non-Hispanic individuals, suggesting an ethnic predisposition for hepatic steatosis (42,43). In this line, ethnic minority was defined as belonging to a recognized ethnic minority for Spain or as having a foreign-born mother from a low- or middle-income country. Ethnic minority groups are different across countries; however, in our study this group shares social disadvantages more than a genetic or biological background. Social disadvantages such as low income and parental education, occupation, minimal social network, non-traditional family structure, migrant status or unemployment have been associated with obesogenic behaviors among children. Likewise, in high-income countries, there is an elevated prevalence of obesity and obesity-related comorbidities such as insulin resistance among racial and ethnic minority groups, as well as among individuals from disadvantaged socioeconomic backgrounds (44). Our results support these findings and extend to the presence of HS.

It should also be noted that a high WHtR is one of the most frequently used anthropometric measures for identifying abdominal adiposity and cardiometabolic risk in children (25), and that the lifestyle factors included in the HEPAKID index (screen time and SSB consumption) are also known to be strong determinants of pediatric obesity and/or HS (27,45,46).

In clinical practice, and particularly in primary care, the sensitivity of a screening tool is the main criterion for selecting it for use; the objective is to identify patients who warrant further, more invasive and/or expensive tests. The HEPAKID index can identify 82% (79% in the model without CFR) of children with overweight/obese who have developed HS (18% false negatives). In a sample of children and adolescents with severe obesity (N=119), a laboratory biomarker-based model (ALT, HOMA and leptin) returned a sensitivity of 77% (20). The latter authors also tested other previously published screening tools for adults (16–18) in a sample of white children with obesity (N=56) (19) and reported their sensitivity to be <70% in all cases. In the present work, the previously published ALT level tests (35–37) and the ped-NAFLD score (19) were tested in the current cohort, and their accuracy and sensitivity were found to be lower than those of the HEPAKID index. Indeed, the NASPGHAN (36) and ESPGHAN (37) ALT cut-off points failed to identify 93% of children with an MRI-diagnosed fatty liver as having HS. Indeed, even the revised ALT cut-off of Schwimmer et al. (35) failed to detect HS in 59% of MRI-diagnosed children, and the ped-NAFLD score failed to detect 67% of them. In addition, the

diagnostic accuracy of these scores was particularly low in girls, while the diagnostic performance of the HEPAKID index was similar in boys and girls. These findings highlight the usefulness and likely cost-effectiveness of the HEPAKID index.

The sensitivity of the HEPAKID index could be improved, but with the loss of specificity. Specificity was only 0.62 (0.59 in the model without CRF) for the ⩾25.0 cut-off point; thus, 38% of children without HS were identified as candidates for additional examination. The selected cut-point of ⩾25, however, represents the best trade-off between sensitivity and specificity.

While there is agreement among scientific/medical associations on the need to develop useful screening tools and guidelines for treating HS in children, no consensus strategies have been produced. For example, the American Academy of Pediatrics recommends ALT levels be measured in all children with obesity, or with overweight plus any cardiometabolic risk factor (46). The recommendation of the ESPGHAN (36) is to assess ALT levels and perform abdominal ultrasound in all children >3 years with overweight or obesity but given the elevated prevalence of pediatric overweight and obesity this would be a large strain on public health systems. In addition, the majority of studies in children with biopsy-proven or MRI-diagnosed HS report that ALT levels and ultrasound techniques only show moderate diagnostic accuracy in terms of detecting HS in children, and that combining them leads to no improvement in sensitivity (48,49). Other authors propose blood ALT concentrations of >35 IU/L as indicative of HS. However, while this cut-off has a high specificity (between 92-94%) it has only low sensitivity (between 24-48%) (50,51). Similarly, the ALT cut-off points proposed for the pediatric population by Schwimmer et al. (35), the ESPGHAN (36) and the NASPGHAN (36), all showed high specificity (90%-100%) in the present work, but very low sensitivity (5%-41%), particularly in girls (0%-30%), making them of little use as screening tools.

The 20mSRT test is a routine test used to measure CRF in schools; the results are reported to the children and their parents. However, in clinical settings this information may not be available. For this reason, a version of the HEPAKID index that does not take CRF into account was developed. This showed slightly lower sensitivity (79% vs. 82%) but is still useful as a screening tool for identifying children with HS. On the other hand, the HEPAKID index should be externally validated in other multiethnic, representative, large cohorts of preadolescent children with overweight/obesity before its implementation in clinical settings. In addition, in our

study, socioeconomic status and ethnic minority status were difficult to differentiate, so it is not possible to determinate their individual on the model. This should/will be further explored in future studies. Finally, the predictive capacity of the HEPAKID index has been tested in pre-adolescent children and may not apply to adolescent population at risk of HS.

In conclusion, the HEPAKID index is the first sociodemographic, lifestyle and anthropometric data-based screening tool for identifying HS in preadolescent children with overweight or obesity aged between 8.5 and 12.0 years. Pediatricians could easily use this index to identify children who should be referred for confirmatory diagnosis. The low cost of performing this screening, and the availability of the data required, render the HEPAKID index an ideal method for screening for HS in the pediatric primary care setting.

Conflict of interest: The authors have no conflicts of interest relevant to this article to disclose.

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Supplementary files:

Table S1. Ethnic minority groups in Spain*

National ethnic minorities	Gypsies
Economic migrants	Latin Americans (South Americans and Central Americans) Eastern European (i.e., Romania, Bulgaria) North Africans Sub-Saharan Africans Chinese Arabian

*according to the European Commission against Racism and Intolerance. ECRI Report on Spain (fifth monitoring cicle),2018)

Table S2. Prediction accuracy of the HEPAKID index.

Cut-off points	**Sensitivity (%)** (95% CI)	**Specificity (%)** (95% CI)	**PPV (%)** (95% CI)	**NPV (%)** (95% CI)	**LR+** (95% CI)	**LR-** (95% CI)
Youden index (0.55): ≥ 41.7	68 (50-85)	87 (78-96)	74 (57-91)	83 (74-93)	5.3 (2.7-10.6)	0.4 (0.2-0.6)
≥ 25	82 (68-96)	62 (49-75)	53 (39-68)	86 (76-98)	2.2 (1.5-3.1)	0.3 (0.1-0.6)
≥ 35	71 (54-87)	79 (69-90)	65 (48-81)	83 (73-94)	3.4 (2.0-5.8)	0.4 (0.2-0.6)
≥ 45	62 (44-80)	87 (78-96)	72 (54-90)	81 (71-91)	4.9 (2.4-9.8)	0.4 (0.3-0.7)
≥ 55	47 (29-65)	92 (85-99)	76 (56-97)	76 (66-86)	5.9 (2.4-14.8)	0.6 (0.4-0.8)
≥ 65	35 (18-53)	97 (92-100)	86 (64-100)	74 (63-84)	11.1 (2.6-46.8)	0.7 (0.5-0.9)
≥ 75	24 (8-39)	98 (95-100)	89 (63-100)	70 (60-81)	15.0 (2.0-113.0)	0.8 (0.6-0.9)

PPV: positive predictive value; NPV: negative predictive value; LR+: positive likelihood ratio; LR-: negative likelihood ratio

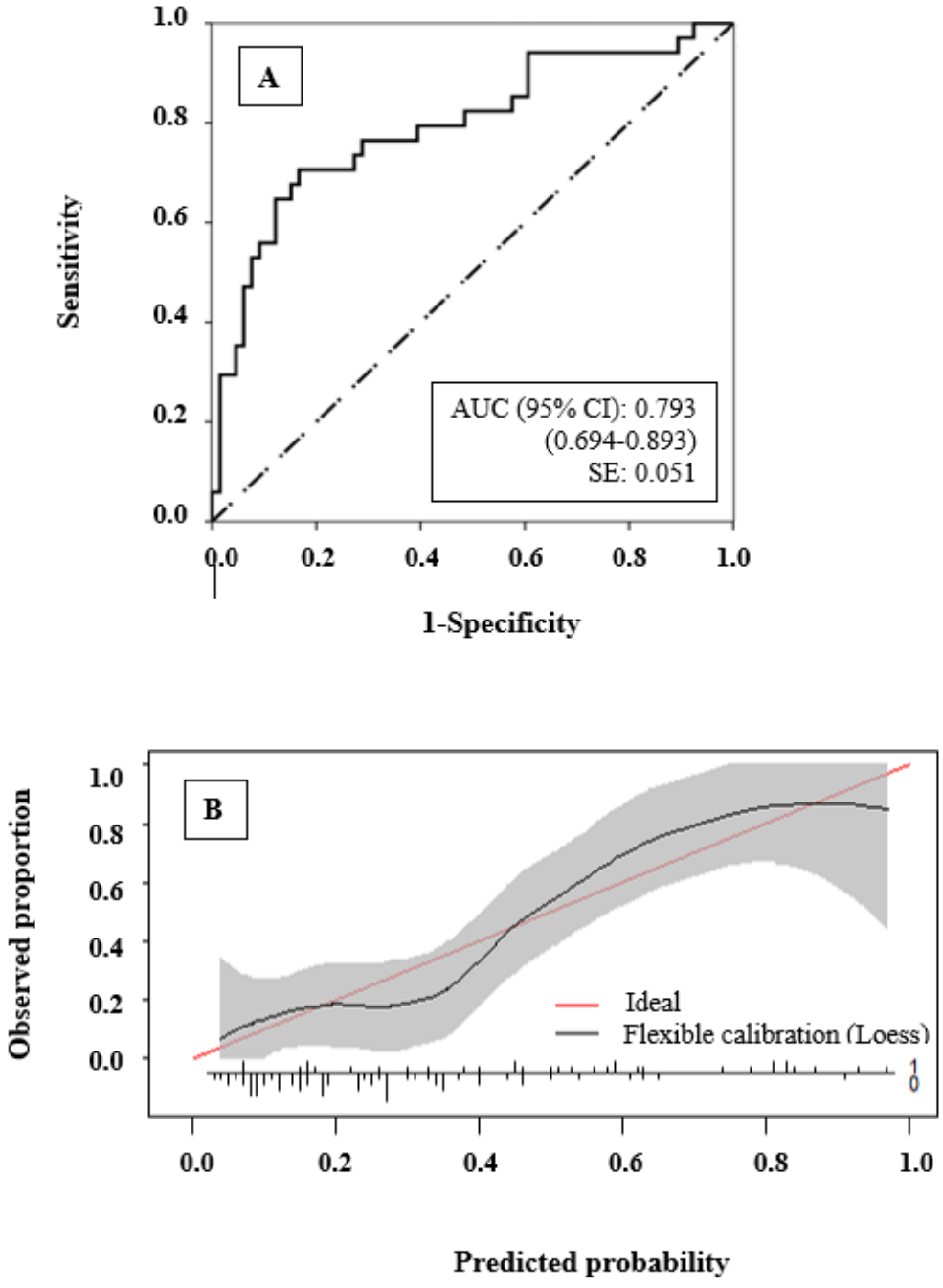

Figure S1. Receiver operating characteristics curve (panel A) and calibration (panel B) of the HEPAKID index model without cardiorespiratory fitness data. AUC-ROC: area under receiver operating characteristics curve; CI: confidence interval; SE: standard error.

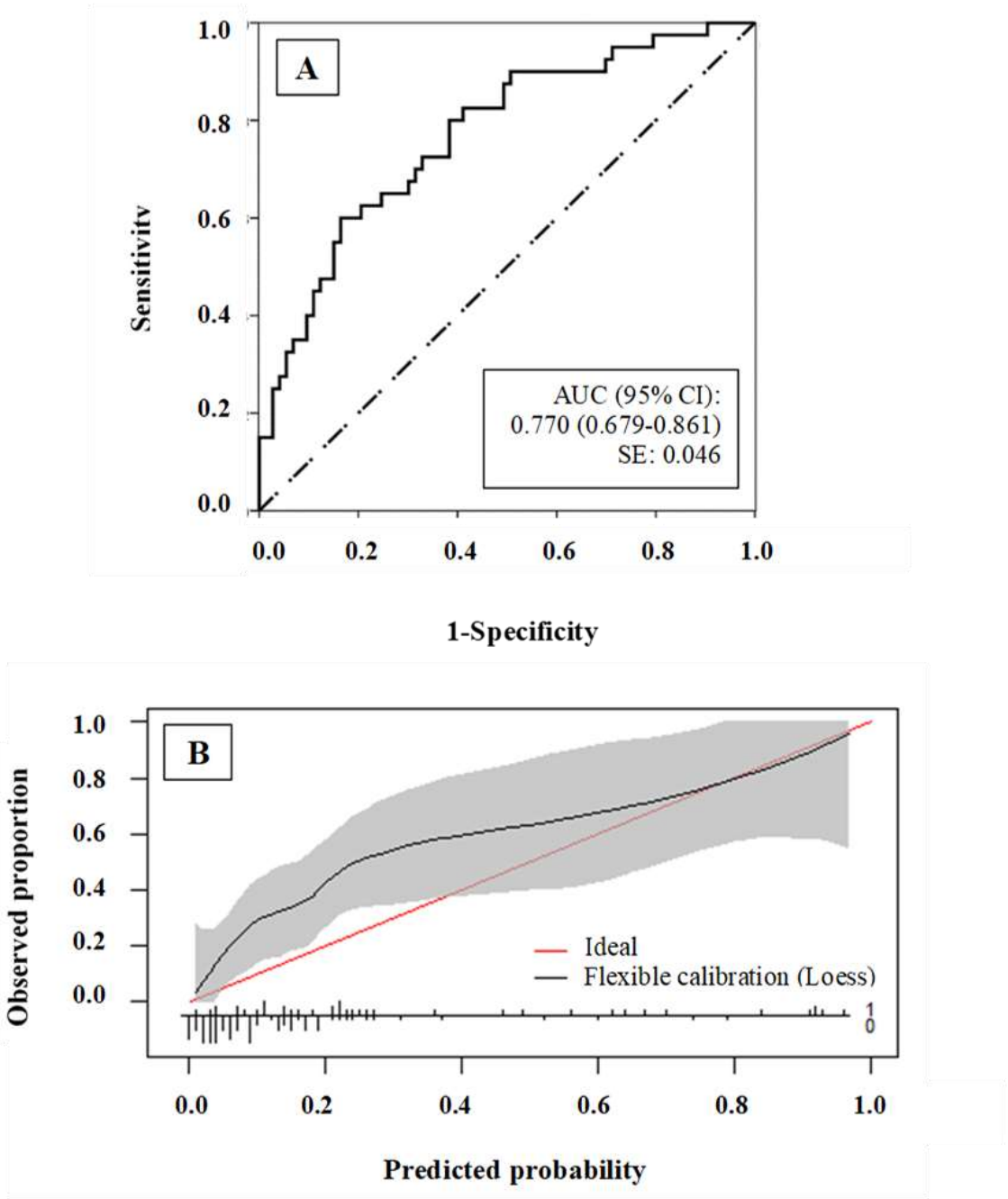

Figure S2. Receiver operating characteristics curve (panel A) and calibration (panel B) of the ped-NAFLD score. AUC-ROC: area under receiver operating characteristics curve; CI: confidence interval; SE: standard error.

3.2 Study II

Status: Accepted in Pediatric Obesity

Development of a prediction protocol for the screening of metabolic associated fatty liver disease in children with overweight or obesity

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ABSTRACT

Background: The early detection and management of children with metabolic associated fatty liver disease (MAFLD) is challenging. **Objective:** To develop a non-invasive and accurate prediction protocol for the identification of MAFLD among children with overweight/obesity candidates to confirmatory diagnosis. **Methods:** A total of 115 children aged 8-12 years with overweight/obesity, recruited at primary care, were enrolled in this cross-sectional study. The external validation was performed using a cohort of children with overweight/obesity (N=46) aged 8.5-14.0 years. MAFLD (≥5.5% hepatic fat) was diagnosed by magnetic resonance imaging (MRI). Fasting blood biochemical parameters were measured, and 25 candidate single nucleotide polymorphisms (SNP) were determined. Variables potentially associated with the presence of MAFLD were included in a multivariate logistic regression. **Results:** Children with MAFLD (36%) showed higher plasma triglycerides (TG), insulin, HOMA-IR, alanine aminotransferase (ALT), aspartate transaminase (AST), glutamyl-transferase (GGT) and ferritin ($p<0.05$). The distribution of the risk-alleles of PPARGrs13081389, PPARGrs1801282, HFErs1800562 and PNLPLA3rs4823173 was significantly different between children with and without MAFLD ($p<0.05$). Three biochemical and/or SNPs based predictive models were developed, showing strong discriminatory capacity (AUC-ROC: 0.708-0.888) but limited diagnostic performance (sensitivity 67-82% and specificity 63-69%). A prediction protocol with elevated sensitivity (72%) and specificity (84%) based on two consecutive steps was developed. The external validation showed similar results: sensitivity of 70% and specificity of 85%. **Conclusions:** The HEPAKID prediction protocol is an accurate, easy to implant, minimally invasive, and low economic cost tool useful for the early identification and management of pediatric MAFLD in primary care.

Key words: Fatty liver, Pediatric obesity, Metabolic diseases, Primary health care

Abbreviations: ALT: Alanine aminotransferase, AUC-ROC: Area under the receiver-operating characteristic curve, AST: Aspartate transaminase, BMI: Body mass index, GGT: Glutamyl-transferase, HOMA-IR: Homeostasis model assessment of insulin resistance, MAFLD: Metabolic associated fatty liver disease, MRI: Magnetic resonance imaging, SNP: Single nucleotide polymorphisms, SPSS: statistical package for social sciences, SSB: Sugar-sweetened beverage, T2D: Type 2 diabetes mellitus, TG: Triglycerides, WHtR: Waist to height ratio.

INTRODUCTION

Metabolic associated fatty liver disease (MAFLD) has become a global health burden with an increasingly prevalence in pediatric population (1). The term MAFLD has been recently agreed between expert groups in order to reflect more accurately the current knowledge of fatty liver diseases associated with metabolic dysfunction (2,3). The definition of pediatric MAFLD is based on evidence of intrahepatic fat accumulation in addition to one of the three criteria: excess adiposity, presence of prediabetes or type 2 diabetes, or evidence of metabolic dysregulation (presence of at least two metabolic risks according to sex and age percentiles increased waist circumference, hypertension, hypertrigliceridaemia, low serum HDL or impaired fasting glucose) (3).

MAFLD is considered a major risk factor for T2D and cardiovascular diseases, already in childhood (4). It is estimated that MAFLD is present in 3-10% of general pediatric population and this can be increase to 80% in obese/overweight children (5). MAFLD can progress to steatohepatitis, fibrosis and cirrhosis over time, and it is one of the most common chronic liver disease in the world that increase liver and non-liver related mortality (6).

Lifestyle-based treatments are effective in reducing hepatic fat in overweight children (7). Therefore, early detection and management of children with MAFLD is the most important step to prevent the progression of the disease (7,8). Unfortunately, MAFLD in childhood is often asymptomatic which, together with its high prevalence, long-term health risks and costly and/or invasive diagnostic methods, set up a challenge to clinicians and scientists for developing early diagnosis methods (9). Nowadays, the most widely used screening test in pediatric units is based on elevated alanine aminotransferase (ALT) levels (10–12); but, in children, this blood test shows very low sensitivity. Of note is that MAFLD may be present even with normal blood ALT results, leaving many children without further screening and clinical supervision (13). Therefore, the development of non-invasive, sensitive and accurate screening methods is of clinical interest.

Our group recently developed a simple, non-invasive, inexpensive and easy-to-perform pre-screening tool (the HEPAKID-index) to identify MAFLD among preadolescent children with

overweight/obesity (i.e., children at risk of MAFLD) (14). The HEPAKID-index does not require blood sampling; it is based on the recording of sociodemographic factors (ethnic minority status and gestational age at birth), anthropometric data (waist circumference and height) and lifestyle variables (sugar-sweetened beverage consumption (SSB) and screen time), and shows high sensitivity (82%) to identify children who should be referred for additional diagnostic tests, being appropriate for a pre-screening method (14). However, a second-step screening tool should improve its limited specificity (63%) before conducting very invasive (biopsy) or costly (magnetic resonance imaging, MRI) confirmatory diagnosis.

Several clinical biomarkers such as elevated levels of cholesterol, triglycerides (TG), aspartate aminotransferase (AST) and alanine aminotransferase, as well as hypertension or insulin resistance are associated with MAFLD (15–17). In addition, there is evidence that MAFLD is strongly associated with excess adiposity (16). Yet, MAFLD and obesity are not concomitant, and not every child with overweight/obesity develop the disease. Different ethnic groups display differences in MAFLD prevalence, indicating that genetics plays a role (18,19). In this line, several genetic polymorphisms have shown to confer higher susceptibility to MAFLD in children and adults (20).

The present study aims to develop a second-step screening tool gathering together elevated sensitivity (>80%) and specificity (>80%) with high predictive potential for identifying children at a high risk of MAFLD. To accomplish with this objective, the current work extends the search from sociodemographic, lifestyle and anthropometric data used in the HEPAKID-index (14) to biochemical and genetic variables potentially associated with MAFLD. Further, the present study seeks to develop a decision tree for the identification of children with overweight/obesity candidates to confirmatory diagnosis that can be useful in clinical practice.

METHODS

Study design and participants

This cross-sectional study uses baseline data from the EFIGRO project (ClinicalTrials.gov ID: NCT02258126) whose overall aim was to examine the effect of combined family-based lifestyle plus exercise program on hepatic fat in children with overweight or obesity. Details of sample calculation, randomization, characteristics of the study participants, methodological procedures, and the measurements taken are available elsewhere (21). The study protocol was approved by the Ethic Committee of Clinical Investigation of Euskadi (PI2014045) and complies with the ethical guidelines of the Declaration of Helsinki (2013 revision). Before being enrolled in the study, all parents/legal guardians signed an informed written consent, and all children gave their assent.

For the current purpose, the data of 115 preadolescent children with overweight or obesity, aged between 8.5 and 12.0 years were analyzed. Overweight and obesity status was defined according to the body mass index (BMI) international age- and sex-specific cut-off values provided by the World Obesity Federation (22). Having other hepatic disease or/and any other disease accompanied with elevated blood transaminase levels, such as viral hepatitis, toxic hepatitis, or autoimmune diseases were considered as exclusion criteria. The present study followed the Transparent Reporting of a Multivariable Prediction Model for Individual Prognosis or Diagnosis (TRIPOD) guidelines (23).

Measurements

Hepatic fat

Hepatic fat percentage was assessed by magnetic resonance imaging (MRI) using a Magnetom Avanto system (Siemens Healthcare, Erlangen, Germany). The details of the hepatic fat measurement protocol have been published elsewhere (21). Thereafter, children were categorized as having or not having MAFLD ($\geqslant$5.5% or <5.5% percentage hepatic fat, respectively) (24).

Sociodemographic, lifestyle and anthropometric characteristics

Body mass (SECA 760), height (SECA 220), and waist circumference (SECA 201) were measured in duplicate following standard protocols. Then, the BMI (kg/m2) and the waist to height ratio (WHtR) were then calculated (25).

The sociodemographic information was obtained via self-reported questionnaire. Belonging to an ethnic minority was defined as having a foreign-born mother from a low or middle income country or belonging to a recognized ethnic minority for Spain (i.e., Roma) according to the categories provided by the European Commission for Spain (26). Perinatal variables, such as gestational age at birth (weeks), were collected from clinical records.

Sugar-sweetened beverage (SSB) consumption was determined as the ingestion of soft drinks, sweetened juices, and energetic drinks (27) in g/day. Then, children were categorized as consumers or non-consumers of SSB. Dietary intake was assessed by two non-consecutive 24-hour recalls within a period of 7 days. A self-reported sedentary behavior questionnaire (28) was completed in order to determine the frequency of specified sedentary behaviors such as watching TV, playing on-screen games, and surfing the Internet; the children were then categorized as meeting (<2h/day) or not-meeting ($\geqslant$ 2h/day) the World Health Organization recommendations regarding screen time for children (29).

Biochemical and genetic variables

Blood extraction and collection details have been published elsewhere (21). Fasting serum concentrations of biochemical parameters such as total cholesterol, high - density lipoprotein (HDL), low - density lipoprotein (LDL), TG, glucose, insulin, ALT, AST, gamma - glutamyl transferase (GGT), and ferritin concentrations were measured as reported elsewhere (21). Thereafter, the homeostasis model assessment of insulin resistance [HOMA - IR=insulin (mU/L) × glucose (mmol/L)/22.5] was calculated (30). Genomic DNA was extracted from white blood cells using Maxwell® RSC Blood DNA Kit and Maxwell® RSC Instrument (Promega) equipment. The genotyping was done by an Illumina system (Illumina, Inc, San Diego, California) using the Golden-Gate technology (sampling procedure scheme,

Golden-Gate; Software, Inc, San Francisco, California). Candidate gene approach was the procedure used to select 25 single nucleotide polymorphisms (SNP) potentially associated with MAFLD (31,32) in the current study.

Statistical analysis

Differences in characteristics between children with or without MRI-diagnosed MAFLD were analyzed using the independent t-test (continuous variables) or $\chi 2$ test (categorical variables). Variables potentially associated with the presence of MAFLD were included as candidates in a multivariate logistic regression of each model. Those independent variables that showed collinearity and those whose effect was negligible were removed from the final model. Two different models were developed: 1) biochemical model (model I), 2) genetic variants model (model II) and 3) biochemical plus genetic variants model (model III). The probability of having MAFLD was determined from the models, multiplying by 100 to obtain the index of each model, which therefore has a score range from 0 to 100.

The discriminatory capacity of each model was analyzed by calculating the area under the receiver-operating characteristic curve (AUC-ROC, with 95% confidence intervals [CI]). The calibration of each model was examined using a calibration plot (plotting the expected probabilities against observed event proportions and smoothing via the Loess method) and the Hosmer-Lemeshow test. Bootstrap resampling with 150 samples was performed as an internal validation and to provide an optimism-corrected AUC-ROC.

The Youden index (33) was used to identify the optimal cut-off point for binary classification for the two models, prioritizing high sensitivity ($\geqslant$80%). The performance of the proposed models was expressed as sensitivity, specificity, positive and negative predictive values (with their corresponding 95% CIs) for the proposed cut-off points. All the analyses were performed for the sample as a whole, and separately for boys and girls.

All calculations were performed using SPSS software v.23.0 (IBM, Armonk, NY, USA) and R statistical software v.3.6.3. Significance was set at α= 0.05.

Development of the prediction protocol and external validation

Two steps algorithm was developed for the detection of MAFLD. The first step is based on a short questionnaire punctuation (HEPAKID-index) (14) which includes anthropometric data (WHtR), sociodemographic factors (ethnic minority status and gestational age at birth) and lifestyle variables (SSB consumption and screen time). The second step is based on biochemical screening: HOMA-IR, TG, ALT, AST, GGT and ferritin (model I equation).

Once the model was developed, the external validation was performed using the baseline data from the MICROKID project (ClinicalTrials.gov ID: NCT04575506) whose overall aim is the study of the influence of the diversity and composition of the microbiota in the development of MRI diagnosed MAFLD. A total of 46 preadolescent children with overweight/obesity (N= 20 girls), aged between 8.5 and 14.0 years were analyzed. The inclusion and exclusion criteria were the same than the original sample.

RESULTS

Table 1 shows the characteristics of participants with (36%) and without MAFLD (64%). Children with MAFLD showed higher plasma TG, insulin, HOMA-IR, AST, ALT, and GGT compared to those peers without MAFLD ($p<0.05$). **Table S1** shows the characteristic of the external validation sample with (28%) and without MAFLD (72%). This sample also showed higher plasma TG, insulin, HOMA-IR, AST and ALT in children with MAFLD compared to those peers without MAFLD ($p<0.05$).

From the 25 SNPs potentially associated with MAFLD (**Table S2**), four genetic variants (**Table S3**) were significantly associated with the presence of the disease. The distribution of carriers/non-carriers of the risk-alleles of the PPARG rs13081389, PPARG rs1801282, HFE rs1800562 and PNLPLA3 rs4823173 was significantly different between children with and without MAFLD ($p<0.05$, **Table 1**).

Table 1. Anthropometric and clinical characteristics of overweight/obese children with and without metabolic associated fatty liver disease in the exploratory sample of children.

	Non-MAFLD		MAFLD		
	N	**Mean (SD)**	**N**	**Mean (SD)**	***P***
Anthropometric and body composition characteristics					
Age (years)	74	10.6 (1.1)	41	10.5 (1.1)	0.749
Girls (N, %)	74	42. 57	41	20. 49	0.077
Body mass index (kg/m^2)	74	25.0 (3.2)	41	26.2 (3.3)	0.059
Hepatic fat (%)	74	3.67 (0.97)	41	9.18 (4.8)	**<0.001**
Biochemical parameters					
Cholesterol (mg/dL)	73	170.5 (28.0)	41	174.4 (29.0)	0.489
High-density lipoprotein (mg/dL)	73	51.8 (11.8)	41	49.2 (10.3)	0.232
Low-density lipoprotein (mg/dL)	73	104.1 (23.6)	41	105.5 (24.6)	0.768
Triglycerides (mg/dL)	73	73.5 (30.0)	41	98.2 (47.4)	**0.004**
Glucose (mg/dL)	73	84.7 (4.9)	40	86.8 (6.1)	0.086
Insulin (µU/ml)	73	11.1 (4.3)	41	13.9 (5.5)	**0.006**
HOMA-IR	73	2.34 (0.95)	40	3.01 (1.28)	**0.006**
Aspartate aminotransferase (U/L)	73	23.0 (4.3)	41	24.6 (3.9)	**0.045**
Alanine aminotransferase (U/L)	73	18.0 (5.3)	41	24.8 (11.2)	**0.001**
Gamma-glutamyl-transferase (U/L)	72	14.9 (3.6)	40	18.8 (5.4)	**<0.001**
Ferritin (ng/mL)	71	45.0 (22.2)	41	67.8 (64.8)	**0.035**
Genetic variants (% of risk allele carriers)					
PPARG rs13081389	51	4	24	21	**0.019**
PPARG rs1801282	51	12	24	33	**0.025**
HFE rs1800562	51	6	24	21	**0.050**
PNPLA3 rs4823173	51	22	24	46	**0.031**

SD: standard deviation, SSB: sugar-sweetened beverages, HOMA-IR: homeostasis model assessment of insulin resistance, MAFLD: Children with metabolic associated fatty liver disease, Non-MAFLD: Children without metabolic associated fatty liver disease.

Development of the models

Table 2 shows the multivariate logistic regression analysis of the three proposed models. The model I was based on six biochemical parameters potentially associated with having MAFLD. The model II was based on four SNPs potentially associated with having MAFLD. The model III was based on the six biochemical parameters plus the four SNPs. All models were defined using the standardized regression coefficients (β) obtained in the multivariate logistic regression analyses (**Table 2**). The calculators of each model are available on https://acortar.link/1yeEyY

The Hosmer-Lemeshow test (Model I, p=0.355, Model II, p=0.830 and Model III, p=0.299) and the calibration plots (**Figure S1**) showed the calibration of each model. The AUC-ROC values for the three

indexes were 0.824 (Model I), 0.708 (Model II) and 0.888 (Model III). Model I and model III showed strong discriminatory capacity for identifying MAFLD in the study population, while model II showed limited capacity (**Figure S1**). The optimism corrected AUC-ROC were 0.792 (panel A, Model I), (panel B, Model II) 0.665 and 0.812 (panel C, Model III).

Table 2. Multiple logistic regressions analysis showing the association of biochemical parameters with metabolic associated fatty liver disease in the exploratory sample of children (MAFLD, dependent variable).

	MAFLD		
	OR (95% CI)	**β**	**P**
Model I (n=109)			
Constant	-	-9.620	0.000
HOMA-IR	1.53 (0.93-2.52)	0.425	0.095
TG (mg/dL)	1.01 (0.99-1.02)	0.010	0.164
ALT (U/L)	1.03 (0.93-1.14)	0.030	0.558
AST (U/L)	1.14 (0.98-1.32)	0.133	0.078
GGT (U/L)	1.13 (0.98-1.30)	0.126	0.069
Ferritin (ng/mL)	1.02 (0.99-1.04)	0.020	0.070
Model II (n=75)			
Constant	-	-1.547	0.000
PPARG (rs13081389)	4.10 (0.39-42.65)	1.411	0.238
PPARG (rs1801282)	1.74 (0.33-9.36)	0.556	0.516
HFE (rs1800562)	4.47 (0.87-22.90)	1.498	0.072
PNPLA3 (rs4823173)	2.93 (0.95-9.02)	1.075	0.061
Model III (n=72)			
Constant	-	-10.940	0.002
HOMA-IR	1.38 (0.73-2,60)	0.320	0.326
TG (mg/dL)	1.01 (0.99-1.03)	0.009	0.415
ALT (U/L)	1.00 (0.81-1.23)	0.001	0.996
AST (U/L)	1.10 (0.89-1.37)	0.098	0.372
GGT (U/L)	1.35 (1.00-1.82)	0.301	0.050
Ferritin (ng/mL)	1.01 (0.98-1.04)	0.013	0.360
PPARG (rs13081389)	1.03 (0.05-23.30)	0.029	0.985
PPARG (rs1801282)	5.76 (0.65-51.10)	1.751	0.116
HFE (rs1800562)	10.96 (1.09-109.87)	2.394	0.042
PNPLA3 (rs4823173)	1.6 (0.282-9.08)	0.470	0.596

β: standardized regression coefficient; OR: Odds ratio; CI: confidence interval; HOMA-IR: homeostatic model assessment; TG: triglycerides; ALT: alanine transaminase; AST: aspartate transaminase; GGT: gamma-glutamyl-transferase; PPARG: Peroxisome Proliferator Activated Receptor Gamma; HFE: Homeostatic Iron Regulator; PNPLA3: Patatin Like Phospholipase Domain Containing 3. Only participants with no missing data were included into the model. Missing data model I: GGT (n=2), HOMA-IR (n=1), Ferritin (n=2). Missing data model II: GGT (n=2), HOMA-IR (n=1), Ferritin (n=2), SNPs analyzing (n=37). Missing data model III: SNPs analyzing (n=37).

Table S4 shows the diagnostic performance of the three developed models at different cut-off points. For models I, II and III the optimum cut-points were 25.0, 22.0 and 24.0, respectively. **Table 3** shows the diagnostic performance of the selected cut points of the three developed models for the whole sample, as well as separately for boys and girls. The models I and III showed high sensitivity (82%), but limited specificity (63-69%). The model II showed limited sensitivity (67%) and specificity (65%) in the whole sample; likewise, it showed large differences in diagnostic performance between girls and boys (sensitivity of 50% and 83%, specificity of 57% and 74%, respectively).

Table 3. Diagnostic performance of the two developed models for magnetic resonance imaging-diagnosed pediatric metabolic associated fatty liver disease identification in the exploratory sample of children.

Cut-off points	**SN,** % (95% CI)	**SP,** % (95% CI)	**PPV,** % (95% CI)	**NPV,** % (95% CI)
Model I				
Cut-point ≥25				
Whole sample (n=109)	82 (69-95)	63 (51-75)	55 (42-69)	86 (76-97)
Girls (n=61)	75 (54-96)	63 (47-79)	50 (30-70)	84 (69-98)
Boys (n=48)	89 (73-100)	62 (43-81)	61 (41-81)	90 (74-100)
Model II				
Cut-point ≥22				
Whole sample (n=75)	67 (46-88)	65 (51-79)	47 (29-65)	80 (67-94)
Girls (n=61)	50 (18-82)	57 (37-77)	33 (9-58)	73 (52-94)
Boys (n=48)	83 (58-100)	74 (54-94)	63 (36-89)	89 (73-100)
Model III				
Cut-point ≥24				
Whole sample (n=72)	82 (65-100)	69 (55-83)	56 (38-74)	89 (78-100)
Girls (n=40)	83 (58-100)	75 (57-93)	58 (32-85)	91 (78-100)
Boys (n=32)	82 (54-100)	62 (39-85)	53 (26-80)	87 (66-100)

SN: sensitivity; SP: specificity; PPV: positive predictive value; NPV: negative predictive value; CI: confidence interval.

Development of the prediction protocol and external validation

Figure 1. shows the developed decision protocol algorithm for the identification of children with overweight or obesity candidates to confirmatory diagnosis of MAFLD (HEPAKID prediction protocol). This algorithm includes two steps: 1st) pre-screening of children with high risk of having MAFLD using the short questionnaire of the HEPAKID index (available on https://bit.ly/2AQTUPa) (14), and 2nd) those children whose HEPAKID-index is ⩾25 are derived to a blood test to confirm their risk using the screening tool based on Model I equation (available on https://acortar.link/1yeEyY).

Figure S2. (panel A) shows the performance of the complete algorithm in the main sample. The complete algorithm showed high discriminatory capacity with 9 false negatives and 10 false positives in the original sample, reaching a sensitivity of 72% and a specificity of 84% (N=93).

The discriminatory capacity of the algorithm in the external validation sample can be found in the **Figure S2.** (panel B). The validation algorithm showed high discriminatory capacity (**Table S5**), with 4 false negatives and 5 false positives, reaching a sensitivity of 70% and a specificity of 85% (N=45).

Finally, the two steps comprised in the prediction protocol, the HEPAKID index and the biochemical screening, were also independently validated with the external sample and the results showed similar diagnostic performance in the two samples (**Table S5**).

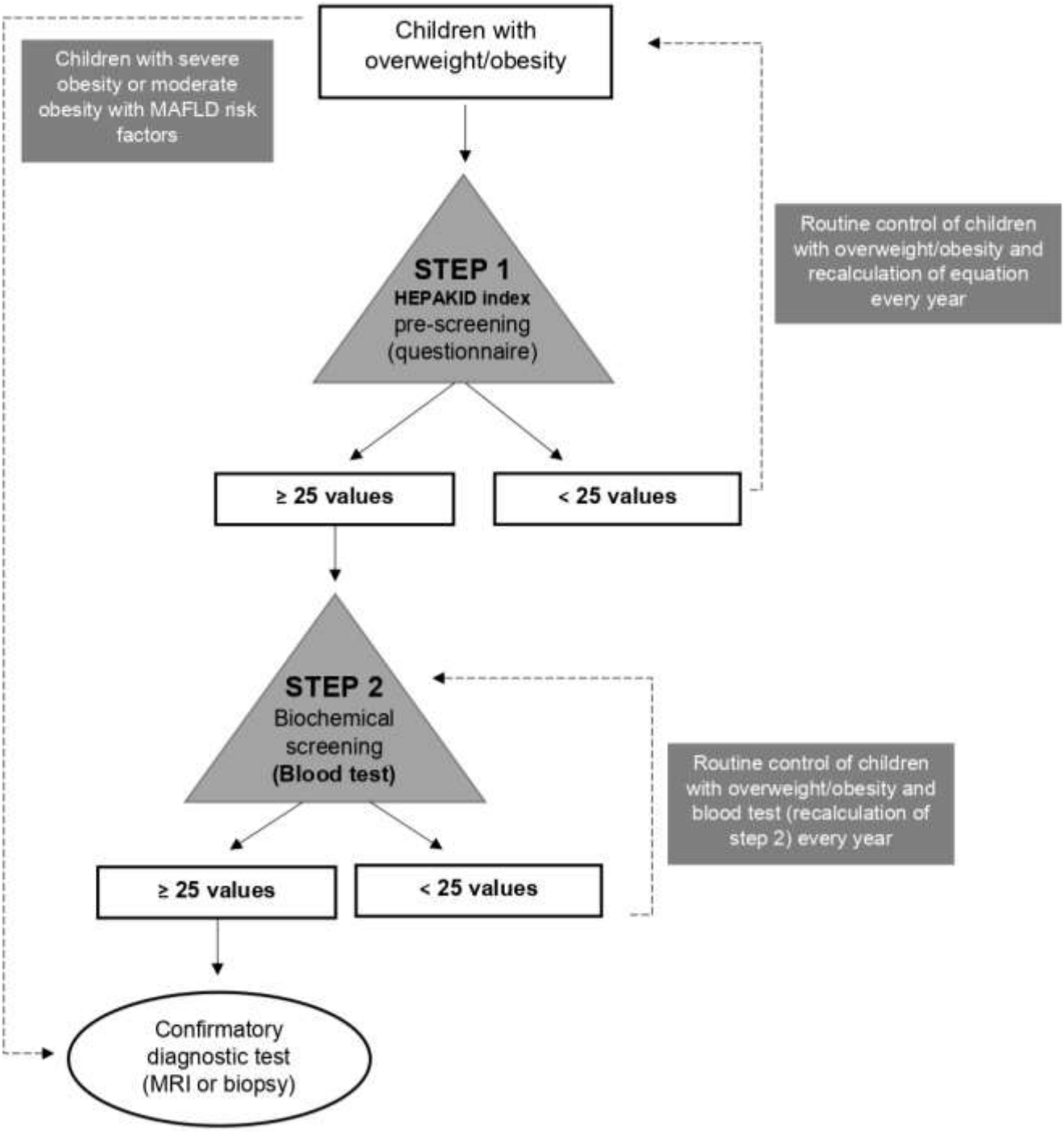

Figure 1. HEPAKID prediction protocol algorithm for screening pediatric metabolic associated fatty liver disease (MAFLD). MAFLD: Metabolic associated fatty liver disease; MRI: Magnetic resonance imagining.

DISCUSSION

The most important contribution of this study is the development of an easy to perform and minimally invasive prediction protocol for the identification of MAFLD among children with overweight/obesity, which encompasses elevated sensitivity, specificity and high accuracy. This algorithm, based on a short questionnaire and easy to measure biochemical parameters, may be useful in routine Primary Care clinical practice to identify early those children who should be referred to perform a confirmatory diagnosis.

We developed three different models in order to identify the most appropriate model to serve as a second-step screening tool for MAFLD in children with overweight/obesity. However, the exclusive application of these models, based on biochemical and/or SNPs data, showed moderate accuracy (sensitivity 67-82% and specificity 63-69%) to detect MAFLD.

These findings are in concordance with previous reports (12,14,34–36). In this way, the application of the model I, based exclusively on biochemical parameters, showed limited applicability. Thus, the prioritization of high sensitivity (82%) with a cut point of $\geqslant$25, showed low specificity (63%), while the prioritization of high specificity (94%) with a cut-point of $\geqslant$60, showed very low sensitivity (49%). Indeed, although biochemical parameters such as HOMA-IR, TG, ALT, AST, GGT, or ferritin levels are increased in children with MAFLD (37,38), their prediction capacity is not enough for the screening of MAFLD (12,13,35,39).

The genetic risk score (model II) based on a four SNPs associated with MAFLD (PPARG rs13081389, PPARG rs1801282, HFE rs1800562 and PNLPLA3 rs4823173) also showed limited discriminatory capacity (67% sensitivity and 65% specificity). In turn, the combination of the biochemical and genetic variables (model III) did not improve the accuracy enough (82% sensitivity and 69% specificity) in our study sample. Thus, considering the necessary technological resources for the analysis of the SNPs, the minimal specificity improvement of the prediction tool, and its high economic cost, this model becomes non eligible for the routine clinical practice. These results concur with previous reports in children and adults, where the addition of genetic information to clinical parameters in the calculations of the risk scores

resulted in minimal improvements of sensitivity and specificity (40,41). In a cohort of Italian obese children and adolescent aged 6-18 years, the addition of three, four or eleven SNPs only slightly improved the AUC-ROC from 0.77 to 0.80, 0.80 and 0.81, respectively (41). In older Chinese adults, the addition of genetic variants to different models improved their sensitivity, but worsened the specificity (40).

Genetic susceptibility seems to play a crucial role in the development and progression of MAFLD (42). Therefore, genetic variants have been proposed as potential biomarkers of MAFLD in adults (43) and children (20,44,45). However, MAFLD is a polygenetic disease where dynamic interactions between genes and environmental factors can modulate the development and progression of the disease (42). Therefore, we probably need more genetic information of MAFLD susceptible genes, as well as studies examining the genes-environmental factors interactions, rather than just several SNPs, to establish accurate predictive models. In addition, to date, genetic variables are not easily available in routine clinical practice, which limits its application as a massive screening tool. Thus, the model I is the most appropriate model to serve as a second-step of the proposed screening protocol.

This study adds to the current knowledge the development of an accurate, sensitive (72%), specific (84%), simple and minimally invasive screening protocol for the identification of MRI-diagnosed MAFLD among children with overweight/obesity: the HEPAKID prediction protocol. This algorithm combines two consecutive steps without genetic information and/or difficult to measure biochemical parameters in routine clinical practice. In the first step, children are classified as “at risk of having MAFLD” or “not” depending on the score achieved in the HEPAKID index pre-screening tool (14), which is derived from a questionnaire based on the recording of sociodemographic factors (ethnic minority status and gestational age at birth), anthropometric data (WHtR) and lifestyle variables (SSB consumption and screen time). In the second step, those children identified in the previous step as “at risk” (HEPAKID index $\geqslant$ 25) have to be referred for a blood test to perform a second screening using common blood biochemical parameters (glucose and insulin to calculate HOMA-IR, TG, ALT, AST, GGT, and ferritin). Those children with a score $\geqslant$25 in this second step should be sent to a medical specialist to confirm the diagnosis. In addition, the proposed protocol was validated in an external sample (N=45) showing similar results (sensitivity 70% and specificity 85%), which strengthens its prediction capacity.

In adults, several prediction scores showed the elevated capacity of anthropometric and clinical parameters to predict the risk of suffering fatty liver disease (46–48). Nevertheless, in children, these scores have very limited accuracy (AUC-ROC between 0.68 and 0.75) (34). Previously proposed prediction scores or algorithms for the screening of pediatric MAFLD (34,35,41) showed reasonable accuracy (between 0.81 and 0.88) and sensitivity (between 77% and 89%), but very limited application in external validations. For instance, the Ped-NAFLD score (35) was tested in a cohort of 119 children, showing 75% of sensitivity and 68% of specificity (34). In another study with 113 children, its sensitivity dropped to 33% while the specificity was 95% (14). However, these models include non-easy to measure parameters such as blood leptin and adiponectin (35) or genetic information (41) that limit their routine applicability. Similarly, the algorithms and the ALT-levels based cut-off points proposed by either NASPGHAN (10) or ESPGHAN (11) show high specificity (between 88% and 94%), but very low sensitivity (between 26% and 48%) compromising their utility as screening tools.

The combination of sociodemographic, anthropometric, lifestyle, and clinical information within the same algorithm seems to be the key to achieve high sensitivity (>70%), specificity (>80%) and elevated discriminatory capacity to identify children with MAFLD among those with overweight or obesity. Likewise, the high specificity achieved after performing the two steps, makes this tool useful for clinical practice avoiding unnecessary costly or invasive testing in patients without the disease, allowing its application in the entire child population with overweight or obesity. The proposed decision tree also contemplates the possibility of direct derivation to confirmatory diagnostic tests of children with moderate or severe obesity with MAFLD risk factors (such as family history of MAFLD, very high hepatic enzyme levels or hepatic symptomatology). Moreover, those children who maintain their overweight/obesity status, but who were not classified as children at risk of MAFLD in the first or in the second step, should be monitored and assessed yearly to avoid leaving any patient untreated in the future. A simple guide explaining the application of the proposed protocol can be found in **Table S6**.

The proposal of the current study complements our previous sensitive pre-screening tool (the HEPAKID index) (14), adding the necessary specificity of a medical screening tool, but maintaining its simplicity, easiness and low economic cost. In any case, although the results were consistent in the

validation sample, the proposed protocol should be externally validated in larger, multiethnic, and representative cohorts of children with overweight/obesity before its implementation in clinical settings. In this line, in our study, ethnic minority condition was defined as belonging to a recognized ethnic minority for Spain or as having a foreign-born mother from a low- or middle-income country. In Spain, these groups share social disadvantages, and the results were consistent in the external validation sample: However, it should be also tested in other multiethnic cohorts from other European and non-European countries.

In conclusion, the HEPAKID prediction protocol identifies with high sensitivity, specificity and accuracy, as wells as low time-consuming and economic cost children with overweight/obesity who likely suffer MAFLD, and who should be referred for confirmatory diagnosis.

Potentials Conflict of Interest: The authors have no conflicts of interest relevant to this article to disclose.

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Supplementary files:

Table S1. Sociodemographic, anthropometric, lifestyle and biochemical characteristics of the validation sample.

External validation sample (n=46)	**Non-MAFLD**		**MAFLD**		
	N	**Mean (SD)**	**N**	**Mean (SD)**	***p***
Sociodemographic, lifestyle and clinical characteristics					
Age (years)	33	10.93 (1.8)	13	11.18 (1.8)	0.688
Girls (N, %)	33	18, 55	13	2, 14	**0.016**
Hepatic fat (%)	32	3.8 (0.9)	13	14.2 (9.7)	**<0.001**
Ethnic minority (N, %)	33	12, 36	13	7, 54	0.225
Gestational age (weeks)	32	39.0 (1.8)	13	37.0 (4.2)	**0.032**
SSB consumption (N, %)	33	10, 30	13	4, 31	0.251
Screen hours ≥2h/day (N, %)	33	29, 88	13	13, 100	0.619
Body mass index (kg/m^2)	33	24.7 (2.9)	13	27.4 (3.3)	**0.018**
Waist to height ratio (x100)	33	48.8 (4.8)	13	54.9 (3.8)	**<0.001**
Biochemical parameters					
Cholesterol (mg/dL)	32	165.7 (31.2)	13	161.5 (36.4)	0.722
High-density lipoprotein (mg/dL)	32	54.1 (11.3)	13	46.77 (8.6)	**0.025**
Low-density lipoprotein (mg/dL)	32	97.8 (25.4)	13	93.8 (27.3)	0.648
Triglycerides (mg/dL)	32	69.3 (29.3)	13	106.0 (68.8)	**0.015**
Glucose (mg/dL)	32	91.5 (4.9)	13	91.1 (6.1)	0.857
Insulin (µl/ml)	32	12.0 (4.6)	13	19.6 (11.1)	**0.002**
HOMA-IR	32	2.7 (1.1)	13	4.3 (2.4)	**0.002**
Aspartate aminotransferase (U/L)	32	24.8 (9.8)	13	35.5 (16.1)	**0.009**
Alanine aminotransferase (U/L)	32	21.6 (18.5)	13	53.6 (42.8)	**0.001**
Gamma-glutamyl-transferase (U/L)	32	19.0 (19.7)	13	23.5 (10.3)	0.317
Ferritin (ng/mL)	32	33.0 (19.3)	13	57.23 (32.1)	**0.003**

SD: Standard deviation, SSB: Sugar-sweetened beverages, HOMA-IR: Homeostasis model assessment of insulin resistance. The category of ethnic minority includes non-Spanish origin of the mother (economic migrants; Latin America n=16 and Portugal n=1) and belonging from and Spanish ethnic minority such as Roma (n=2).

Table S2. Main characteristics of single nucleotide polymorphisms potentially associated with metabolic associated fatty liver disease in children with overweight/obesity.

Rs code	Nearest Gene	Alleles (Major/Minor)	MAF	Genotyping success rate	HWE	P*
rs12743824	*LPPR4*	C/A	0.428	98.7	0.44	**0.006**
rs12137855	*LYPLAL1*	C/T	0.154	100.0	0.16	0.927
rs13412852	*LPIN1*	C/T	0.333	100.0	0.33	0.907
rs780094	*GCKR*	C/T	0.409	98.7	0.40	0.630
rs13081389	*PPARG*	A/G	0.051	100.0	0.05	0.673
rs1801282	*PPARG*	C/G	0.103	100.0	0.10	0.737
rs1799945	*HFE*	C/G	0.250	100.0	0.26	0.228
rs1800562	*HFE*	G/A	0.058	100.0	0.06	0.583
rs1044498	*ENPP1*	A/C	0.199	100.0	0.20	0.977
rs4240624	*PPP1R3B*	A/G	0.026	98.7	0.06	**<0.001**
rs657152	*ABO*	C/A	0.346	100.0	0.36	0.838
rs116928232	*LIPA*	C/T	0.000	100.0	0	-
rs4237591	*CTN5*	T/C	0.333	100.0	0.34	0.957
rs2259816	*HNF1A*	G/T	0.417	100.0	0.41	0.655
rs7324845	*LCP1*	A/G	0.066	97.4	0.05	0.620
rs9939609	*FTO*	T/A	0.396	98.7	0.40	0.054
rs11864146	*SLC38A8*	A/G	0.104	98.7	0.11	0.858
rs11868035	*SREBF-1c*	G/A	0.250	100.0	0.24	0.351
rs641738	*MBOAT7*	C/T	0.058	100.0	0.05	0.628
rs2228603	*NCAN*	C/T	0.077	100.0	0.07	0.496
rs58542926	*TM6SF2*	C/T	0.577	100.0	0.57	0.605
rs738409	*PNPLA3*	C/G	0.250	97.4	0.25	0.140
rs4823173	*PNPLA3*	G/A	0.186	100.0	0.18	0.064
rs2294918	*PNPLA3*	G/A	0.397	100.0	0.40	0.716
rs1800206	*PPARα*	C/G	0.083	100.0	0.09	0.514

MAF: Minor allele frequency; HWE: Hardy-Weinberg Equilibrium; ABO: Alpha 1-3-N-Acetylgalactosaminyltransferase and alpha 1-3-Galactosyltransferase; CTN5: Conjugative transposons 5; ENPP1: Ectonucleotide pyrophosphatase/phosphodiesterase 1; FTO: Fat mass and obesity-associated gene; GCKR: Glucokinase regulator; HFE: Homeostatic Iron Regulator; HNF1A: HNF1 Homeobox A; LIPA: Lysosomal acid lipase; LCP1: Lymphocyte cytosolic protein 1; LPIN1: Lipin 1; LPPR4: Phospholipid Phosphatase Related 4; LYPLAL1: Lysophospholipase Like 1; MBOAT7: Membrane Bound O-Acyltransferase Domain Containing 7; NCAN: Neurocan; PNPLA3: Patatin Like Phospholipase Domain Containing 3; PPARα: Peroxisome Proliferator Activated Receptor Alpha; PPARG: Peroxisome Proliferator Activated Receptor Gamma; PPP1R3B: Protein Phosphatase 1 Regulatory Subunit 3B; SLC38A8: Solute Carrier Family 38 Member 8; SREBF-1c: Sterol Regulatory Element Binding Transcription Factor 1; TM6SF2: Transmembrane 6 Superfamily Member 2. P*: Hardy-Weinberg Equilibrium p value.

Table S3. Frequencies of single nucleotide polymorphisms (dominant model) in overweight/obese children with and without metabolic associated fatty liver disease.

SNP			Non-MAFLD			MAFLD			
Rs code	**Nearest Gene**	**Alleles (M/m)**	**N**	**Non-carriers (N)**	**Carriers (N)**	**N**	**Non-carriers (N)**	**Carriers (N)**	**P**
rs12137855	*LYPLAL1*	C/T	51	35	16	24	18	6	0.572
rs13412852	*LPIN1*	C/T	51	22	29	24	11	13	0.826
rs780094	*GCKR*	C/T	50	19	31	24	9	15	0.967
rs13081389	*PPARG*	A/G	51	49	2	24	19	5	**0.019**
rs1801282	*PPARG*	C/G	51	45	6	24	16	8	**0.025**
rs1799945	*HFE*	C/G	51	26	25	24	14	10	0.552
rs1800562	*HFE*	G/A	51	48	3	24	19	5	**0.050**
rs1044498	*ENPP1*	A/C	51	33	18	24	15	9	0.853
rs657152	*ABO*	C/A	51	24	27	24	8	16	0.262
rs116928232	*LIPA*	C/T	51	51	0	24	24	0	-
rs4237591	*CTN5*	T/C	51	20	31	24	12	12	0.378
rs2259816	*HNF1A*	G/T	51	17	34	24	10	14	0.483
rs7324845	*LCP1*	A/G	50	43	7	23	21	2	0.522
rs9939609	*FTO*	T/A	50	18	32	24	12	12	0.251
rs11864146	*SLC38A8*	A/G	51	42	9	23	17	6	0.403
rs11868035	*SREBF-1c*	G/A	51	32	19	24	12	12	0.296
rs641738	*MBOAT7*	C/T	51	46	5	24	21	3	0.724
rs2228603	*NCAN*	C/T	51	45	6	24	19	5	0.300
rs58542926	*TM6SF2*	C/T	51	10	41	24	5	19	0.901
rs738409	*PNPLA3*	C/G	50	32	18	23	12	11	0.337
rs4823173	*PNPLA3*	G/A	51	40	11	24	13	11	**0.031**
rs2294918	*PNPLA3*	G/A	51	18	33	24	9	15	0.853
rs1800206	*PPARα*	C/G	51	43	8	24	20	4	0.914

M: major; m: minor; MAF: Minor allele frequency; HWE: Hardy-Weinberg Equilibrium; ABO: Alpha 1-3-N-Acetylgalactosaminyltransferase and alpha 1-3-Galactosyltransferase; CTN5: Conjugative transposons 5; ENPP1: Ectonucleotide pyrophosphatase/phosphodiesterase 1; FTO: Fat mass and obesity-associated gene; GCKR: Glucokinase regulator; HFE: Homeostatic Iron Regulator; HNF1A: HNF1 Homeobox A; LIPA: Lysosomal acid lipase; LCP1: Lymphocyte cytosolic protein 1; LPIN1: Lipin 1; LPPR4: Phospholipid Phosphatase Related 4; LYPLAL1: Lysophospholipase Like 1; MBOAT7: Membrane Bound O-Acyltransferase Domain Containing 7; NCAN: Neurocan; PNPLA3: Patatin Like Phospholipase Domain Containing 3; PPARα: Peroxisome Proliferator Activated Receptor Alpha; PPARG: Peroxisome Proliferator Activated Receptor Gamma; PPP1R3B: Protein Phosphatase 1 Regulatory Subunit 3B; SLC38A8: Solute Carrier Family 38 Member 8; SREBF-1c: Sterol Regulatory Element Binding Transcription Factor 1; TM6SF2: Transmembrane 6 Superfamily Member 2.

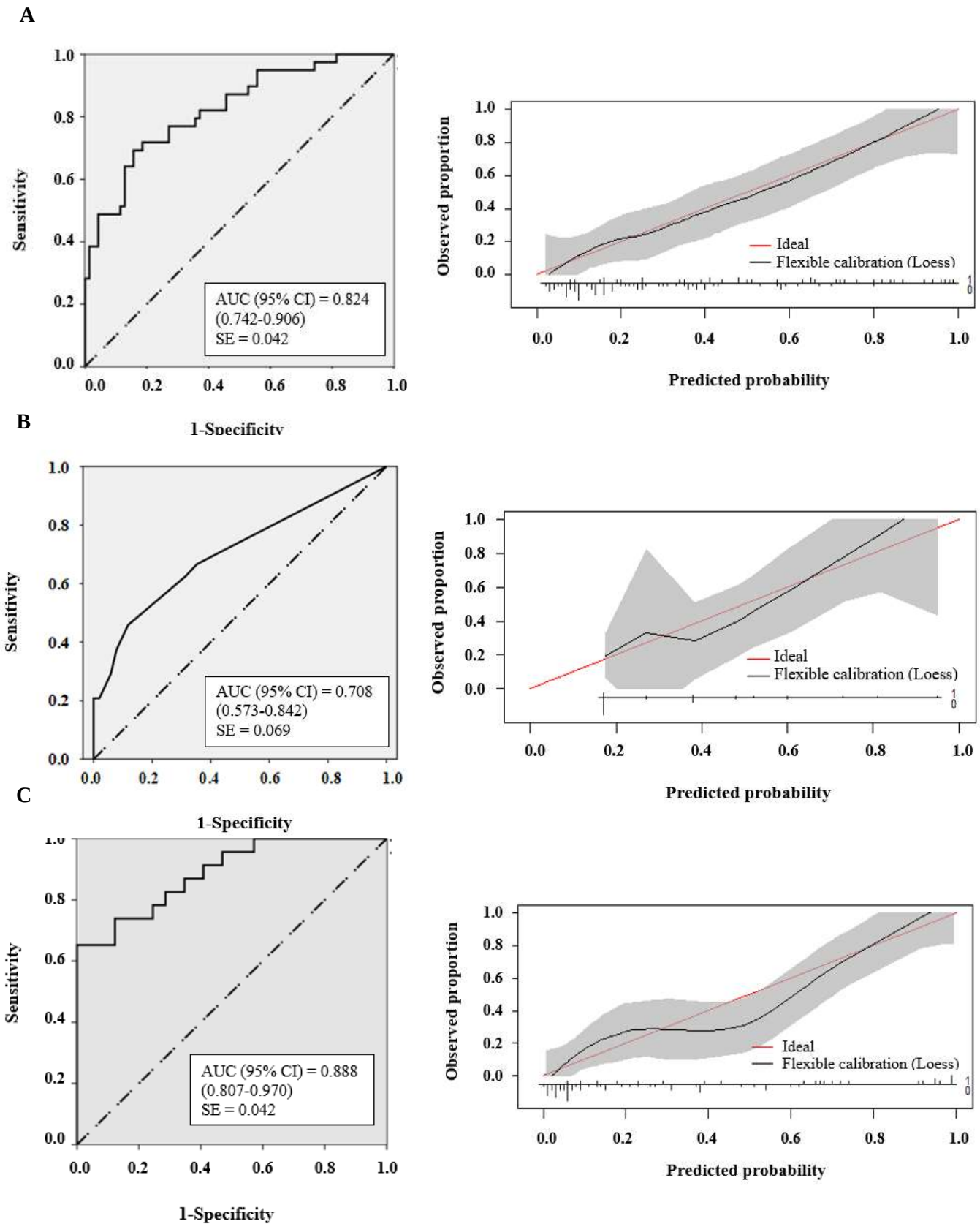

Figure S1. Receiver Operating Characteristics curve (left) and calibration (right) of the Model 1 (panel A), Model II (panel B) and Model III (panel C). AUC-ROC: Area under receiver operating characteristics curve; CI: Confidence interval; SE: Standard error.

Table S4. Diagnostic performance of Model I and Model II cut-off points.

Cut-off points	SN (%) (95% CI)	SP (%) (95% CI)	PPV (%) (95% CI)	NPV (%) (95% CI)
Model I				
Youden index (0.53): ≥ 41.5	69 (53-85)	84 (75-94)	71 (55-87)	83 (74-93)
≥ 15				
Whole sample (n=109)	94 (87-100)	44 (32-57)	49 (37-61)	93 (84-100)
Girls (n=61)	90 (74-100)	46 (30-63)	45 (28-62)	90 (75-100)
Boys (n=48)	100 (97-100)	41 (22-61)	53 (35-70)	100 (96-100)
≥ 25				
Whole sample (n=109)	82 (69-95)	63 (51-75)	55 (42-69)	86 (76-97)
Girls (n=61)	75 (54-96)	63 (47-79)	50 (30-70)	84 (69-98)
Boys (n=48)	89 (73-100)	62 (43-81)	61 (41-81)	90 (74-100)
≥ 60				
Whole sample (n=109)	49 (32-66)	94 (88-100)	83 (65-100)	77 (67-86)
Girls (n=61)	45 (21-69)	95 (87-100)	82 (54-100)	78 (66-90)
Boys (n=48)	53 (28-78)	93 (82-100)	83 (58-100)	75 (59-91)
Model II				
Youden index (0.02): ≥ 22	67 (46-88)	65 (51-79)	47 (29-65)	80 (67-94)
≥ 22				
Whole sample (n=75)	67 (46-88)	65 (51-79)	47 (29-65)	80 (67-94)
Girls (n=40)	50 (18-82)	57 (37-77)	33 (9-58)	73 (52-94)
Boys (n=35)	83 (58-100)	74 (54-94)	63 (36-89)	89 (73-100)
≥ 42				
Whole sample (n=75)	46 (24-68)	88 (75-98)	65 (39-90)	78 (66-89)
Girls (n=40)	33 (2-64)	93 (82-100)	67 (21-100)	76 (61-92)
Boys (n=35)	58 (26-90)	83 (65-100)	64 (31-97)	79 (61-98)
≥ 62				
Whole sample (n=75)	21 (3-39)	98 (93-100)	83 (45-100)	72 (61-84)
Girls (n=40)	17 (0-42)	100 (98-100)	100 (75-100)	74 (59-89)
Boys (n=35)	25 (0-54)	96 (85-100)	75 (20-100)	71 (53-89)
Model III				
Youden index (0.65): ≥ 63	65 (44-87)	100 (99-100)	100 (97-100)	86 (76-96)
≥ 24				
Whole sample (n=72)	82 (65-100)	69 (55-83)	56 (38-74)	89 (78-100)
Girls (n=40)	83 (58-100)	75 (57-93)	58 (32-85)	91 (78-100)
Boys (n=32)	82 (54-100)	62 (39-85)	53 (26-80)	87 (66-100)
≥ 44				
Whole sample (n=72)	62 (44-87)	88 (78-98)	71 (50-93)	84 (73-95)
Girls (n=40)	50 (18-82)	86 (71-100)	60 (25-95)	80 (64-96)
Boys (n=32)	82 (54-100)	90 (76-100)	82 (54-100)	90 (76-100)
≥ 54				
Whole sample (n=72)	65 (44-87)	92 (83-100)	79 (58-100)	85 (74-95)
Girls (n=40)	50 (71-100)	86 (71-100)	60 (25-95)	80 (64-96)
Boys (n=32)	82 (54-100)	100 (98-100)	100 (94-100)	91 (78-100)

SN: Sensitivity; SP: Specificity; PPV: Positive predictive value; NPV: Negative predictive value; CI: Confidence interval.

Table S5. Diagnostic performance of the HEPAKID screening protocol and its components in the exploratory sample and in the external validation sample.

	SN, % (95% CI)	**SP,** % (95% CI)	**PPV,** % (95% CI)	**NPV,** % (95% CI)
HEPAKID-algorithm (Step 1 ≥ 25 and Step 2 ≥ 25)				
Exploratory sample (n=95)	72 (55-89)	84 (74-94)	70 (53-87)	85 (76-95)
External validation sample (n=45)	70 (40-98)	84 (70-99)	64 (36-93)	87 (74-100)
HEPAKID index (step 1) (≥ 25 cut point)				
Exploratory sample (n=95)	82 (68–96)	62 (49-75)	53 (39-68)	86 (76-98)
External validation sample (n=45)	85 (61-100)	44 (25-63)	38 (19-57)	88 (68-100)
Biochemical screening (step 2) (≥ 25 cut point)				
Exploratory sample (n=95)	82 (69-95)	63 (51-75)	55 (42-69)	86 (76-97)
External validation sample (n=45)	85 (61-100)	69 (51-86)	52 (29-76)	92 (79-100)

SN: Sensitivity; SP: Specificity; PPV: Positive predictive value; NPV: Negative predictive value; CI: Confidence interval.

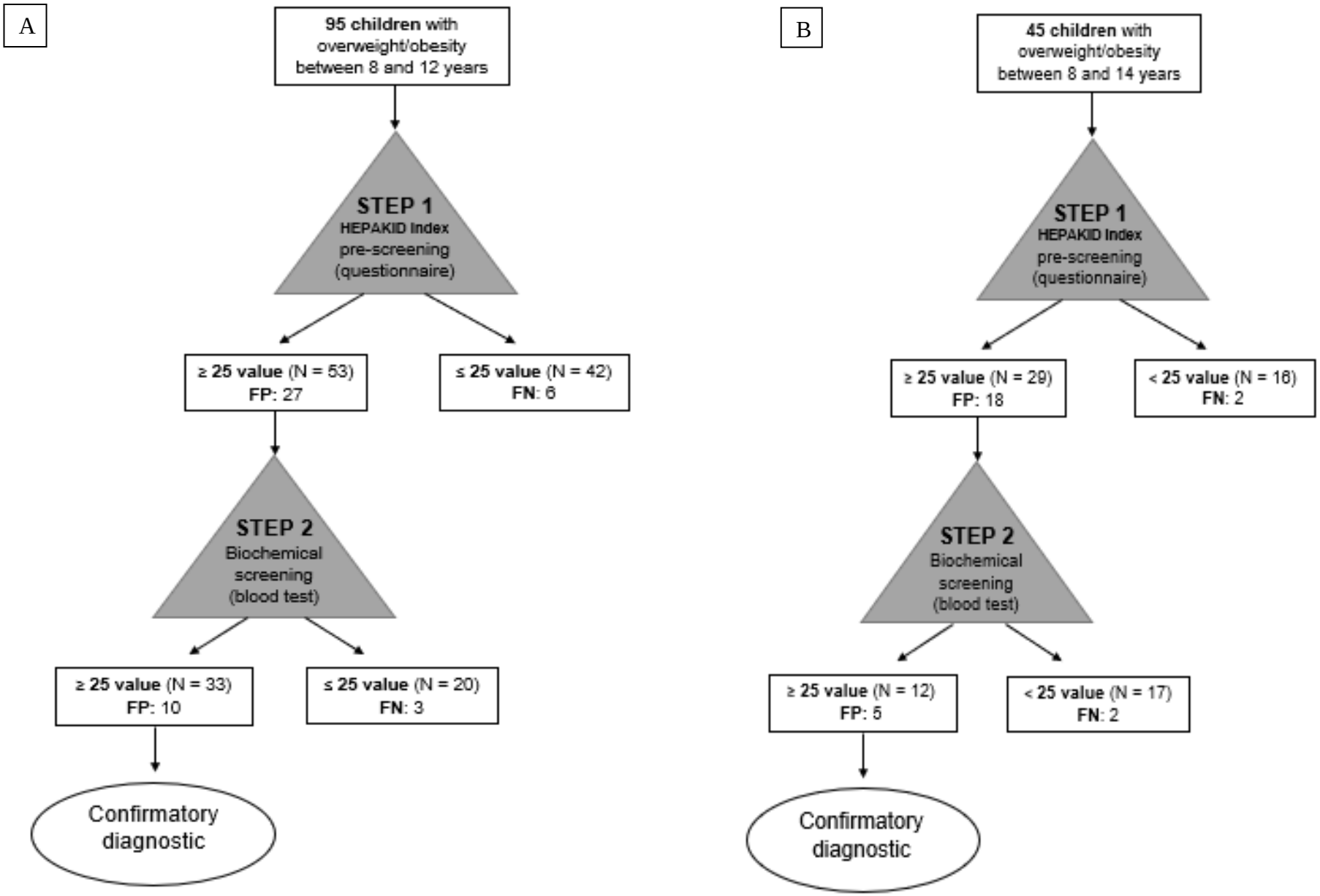

Figure S2: HEPAKID prediction protocol algorithm discriminatory capacity for screening pediatric MAFLD in the main sample (Panel A) and in the validation sample (Panel B). FP: False positive, FN: False negative.

Table S6: Guide for the application of HEPAKID prediction protocol algorithm.

<table>
<tr><td colspan="3">Guide for the application of HEPAKID prediction protocol algorithm:</td></tr>
<tr><td colspan="3">HEPAKID prediction protocol for the identification of children with overweight or obesity with elevated risk to suffer MAFLD.</td></tr>
<tr><td colspan="3">Population to perform the screening tool: Children aged between 8 and 14 years with overweight or obesity (primary care).</td></tr>
<tr><td rowspan="4">Step 1 (HEPAKID index)</td><td colspan="2">Information</td></tr>
<tr><td colspan="2">Complementary test: HEPAKID index questionnaire.

Information required for the questionnaire: Origin of the mother, gestational age (weeks), waist circumference, height, screen time behavior, sugar sweetened beverages consumption.

HEPAKID index questionnaire is available on https://bit.ly/2AQTUPa.</td></tr>
<tr><td colspan="2">Interpretation of the results</td></tr>
<tr><td>Children whose HEPAKID-index is ≥25
are derived to a blood test (Step 2)</td><td>Children whose HEPAKID-index is ≤25
Routine control of children with overweight/obesity and recalculation of equation every year.</td></tr>
<tr><td rowspan="4">Step 2 (Biochemical screening)</td><td colspan="2">Information</td></tr>
<tr><td colspan="2">Complementary test: Blood tests.
Parameters required for the screening: Glucose, insulin, TG, ALT, AST, GGT and ferritin.

Biochemical screening tool is available on https://acortar.link/1yeEyY.</td></tr>
<tr><td colspan="2">Interpretation of the results</td></tr>
<tr><td>Children whose biochemical screening score is ≥25
should be sent to a medical specialist to confirm the diagnosis.</td><td>Children whose biochemical screening score is ≤25
Routine control of children with overweight/obesity and blood test (recalculation of step 2) every year if overweight/obesity persist.</td></tr>
</table>

3.3 Study III

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MDPI

Communication

Circulating miRNAs as Biomarkers of Obesity and Obesity-Associated Comorbidities in Children and Adolescents: A Systematic Review

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ABSTRACT

Early detection of obesity and its associated co-morbidities in children needs priority for the development of effective therapeutic intervention. Circulating miRNAs have been proposed as biomarkers for obesity and its comorbidities. Therefore, we conducted a systematic review to summarize results of studies that have quantified the profile of miRNAs in children and adolescents with obesity and/or associated disorders. Nine studies aiming to examine differences in miRNA expression levels between children with normal weight and obesity or between obese children with or without cardiometabolic diseases were included in this review. We identified four miRNAs over expressed in obesity (miR-222, miR-142-3, miR-140-5p and miR-143) and two miRNAs (miR-122 and miR-34a) overexpressed in children with obesity and non-alcoholic fatty liver disease (NAFLD) and/or insulin resistance. In conclusion, circulating miRNAs are promising diagnostic biomarkers of obesity-associated diseases such as NAFLD and type 2 diabetes already in childhood. However, more studies in children using massive search technology and with larger sample sizes are required to draw any firm conclusion.

Keywords: miRNAs; childhood obesity; biomarkers

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; CVD, cardiovascular diseases; GGT, gamma-glutamyl transferase; IR, insulin resistance; MetS, metabolic syndrome; miRNA, microRNA; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; PBMC, peripheral blood mononuclear cells; T2D, type 2 diabetes mellitus; q-PCR, quantitative polymerase chain reaction.

INTRODUCTION

Childhood obesity is one of the most serious public health challenges of the 21st century (1). In 2016, it was estimated that 41 million children under the age of five and over 340 million children and adolescents aged 5-19 had overweight or obesity (2). Childhood obesity is associated with the development of type 2 diabetes mellitus (T2D), non-alcoholic fatty liver disease (NAFLD), metabolic syndrome (MetS), dyslipidemia and cardiovascular diseases (CVD) later in life and already in childhood (3). However, overweight and obesity, as well as their related diseases, are largely preventable; therefore, the prevention of childhood obesity and the early diagnosis of their associated diseases need high priority (1).

Biomarkers are measurable and quantifiable biological parameters which serve as indices for health- and physiology-related assessments, such as disease risk and diagnosis, psychological disorders, metabolic processes and abnormalities, etc. (4). Thus, biomarkers are useful to diagnose diseases or the susceptibility to suffer them. In this respect, the study and identification of biomarkers associated with obesity, T2D and CVD may be useful for early identification, proper treatment and good life assurance (5).

MicroRNAs (miRNA) are short, 21-23 nucleotides, single stranded, non-coding RNA molecules that are encoded in the genomes of complex organisms (6). MiRNAs are post-transcriptional gene expression regulators that have been implicated in a wide variety of cellular processes and disease conditions (7,8). Recently, miRNAs have been established as biomarkers for several disease states (9) and have been repeatedly studied in the context of metabolic diseases (8,10,11).

Several systematic reviews have gathered information about miRNAs´ role in adipose tissue (12) and in the development of CVD (13) or T2D (14). In recent years, a growing body of studies has determined the value of miRNAs as effective biomarkers to diagnose and assess the risk of obesity and its associated co-morbidities. However, most studies have been focused on and conducted in adulthood and, therefore, there is a lack of information regarding the role of miRNAs in childhood obesity. Considering the vast amount of information readily available on the regulatory roles of miRNAs together with the current pandemic of pediatric obesity, miRNAs might be foreseen as useful biomarkers for the future development of effective strategies for early diagnosis and therapeutic intervention of pediatric obesity and its associated diseases (12–14). Therefore, the purpose of the current systematic review is to hypothesize the biomarker role of circulating miRNAs in the early onset of obesity

and associated co-morbidities through the examination of available circulating miRNA profile data in children and adolescents with obesity, and in metabolic abnormalities related to obesity.

MATERIAL AND METHODS

The systematic review was conducted following the preferred reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) statement and was registered in the International Prospective Register of Systematic Reviews (PROSPERO reference number CRD42019135051).

Search strategy and eligibility criteria

We used the Population, Intervention, Comparison, Outcomes and Study design (PICOS) tool to formulate the question and facilitate the literature search (15). We conducted a literature search for all kind of studies providing data on differences in miRNAs expression between children and adolescents with obesity and its related cardiometabolic diseases and controls. The studies were considered eligible for their inclusion if: 1) the age of participants was between 3 and 19 years old, 2) they provided the quantified expression of miRNAs in the case and control groups, 3) the population of the studied group had overweight/obesity and/or associated metabolic diseases such as T2D, insulin resistance (IR), NAFLD and/or CVD risks factors, and 4) they were case and control studies or intervention studies. Studies that were not written in English or were grey literature, as well as reviews, editorials, opinions, letters, and meeting abstracts were excluded.

Data Sources and Search Strategies

We conducted a systematic literature search in PubMed and Web of Science database selecting the originals articles published until the 23rd of November 2018. The keywords used in the search strategy were related to the following topics: 1) Participants: children and adolescents, 2) Comparison: miRNA expression, 3) Outcome: obesity and/or cardiometabolic diseases. Different search strategies were used for PubMed and Web of Science. Thereby, the search strategy in PubMed database was: ("children" OR "adolescent" OR "youth" OR “teenager” OR “boy” OR “girl” OR “kids” OR “preschoolers”) AND (“obesity” OR “adiposity” OR “metabolic risk"

OR "cardiometabolic risk" OR "type 2 diabetes" OR "insulin resistance" OR "insulin sensitivity" OR "HOMA") AND (“microRNA” OR “miRNA” OR "micro RNA" OR “microRNAs” OR “miRNAs” OR “circulating MicroRNA” OR “MiR”). The search strategy in Web of science database was: ("child*" OR "adolesc*" OR "youth" OR teen* OR boy* OR girl* OR kids*) AND ("obesity" OR “adiposity” OR "metabolic risk" OR "cardiometabolic risk" OR "type 2 diabetes" OR "insulin resistance" OR "insulin sensitivity" OR "HOMA") AND (“microRNA” OR “miRNA” OR “circulating MicroRNA” OR “microRNAs” OR “miRNAs” OR “MiR”).

We found 102 scientific articles in the PubMed database and 172 in the Web of Science database. The 274 articles were imported into EndNote software (version X7, Thomson Reuters, USA) and duplicate files were removed, firstly automatically by the software and secondly by visual checking Figure 1.

Study Selection Process

Two independent reviewers (I.L./M.O.) checked the 216 articles after removing the duplicates. The title and abstract of these articles were examined to identify those that were likely to analyze the expression changes of miRNAs in children or young people with obesity, metabolic risk, CVD, T2D or IR.

Those articles in which it was not possible to know their content by reading only the title or the abstract were read full text to deliberate their final inclusion or exclusion in the systematic review. Disagreements about study selection were resolved by reaching consensus among reviewers.

Data Collection Process and Data items

One reviewer extracted the data from the included studies (M.O.) and its accuracy was checked by a second reviewer (I.L.). A specific database was created in Excel (Microsoft Corp., USA). The following fields were collected from each included study: 1) study (author identification and reference), 2) number of participants, age and sex, 3) weight and cardiometabolic status of the two groups (cases and controls), 4) biological sample from where the miRNAs were extracted, miRNA search technique (global search or specific miRNA search) and the used laboratory technique to quantify miRNAs expression, and 5) differences on the miRNA expression in each study between cases and controls. Supplementary material was reviewed in those cases where the full text did not provide all the relevant information needed for the data extraction.

Study Quality and Risk of Bias Assessment

Study quality was assessed by two independent reviewers (M.O. and I.L.) using the Appraisal systematical tool for Cross-Sectional studies (AXIS) (16) that is recommended to address issues that are often apparent in cross-sectional studies (17). The AXIS assesses study quality following different questions about introduction, methods, results, discussion and others. Additionally, study quality was also examined by the "Quality Assessment Tool for Quantitative Studies" developed by the Effective Public Health Practice Project (EPHPP) (18).

RESULTS

Study Selection and characteristics

We identified seven studies (78%) examining differences in miRNA expression between children with normal weight and obesity (19–25), one study investigating differences in miRNA expression between children with obesity and with or without NAFLD (26) and one study comparing miRNAs expression between children with obesity with or without IR (27).

The characteristics of the studies are shown in Table 1. Regarding age, two of the studies (19,20) included pre-school participants, three of them (21,23,27) studied participants between 6 and 12 years old, and the last four examined children and adolescents aged 6 to 18 years old together (22,24–26). The distribution of boys and girls into the cases and controls was similar in all the studies except in the study of Prats-Puig et al. (23), whose participants were only boys.

The participants were classified as cases or controls according to their BMI status, except in the study of Thompson et al. (26), in which they were classified according to their BMI and the presence or not of NAFLD, and in the study of Masotti et al. (27) in which all the participants had obesity and were assigned to the case or control group depending on the presence or not of IR.

In regard to the biological sample used, there were seven studies with circulating miRNAs obtained from plasma samples (19,21–23,25–27) and two studies that extracted miRNAs from peripheral blood mononuclear cells (PBMC) (20,24). Finally, two different methods/approaches were used to profile miRNA expression: massive parallel sequencing (Illumina's global miRNAs profiling workflow) or NanoString nCounter (microRNA panels) for global miRNA search, and TaqMan qPCR for the study of specific panel of miRNA seq.

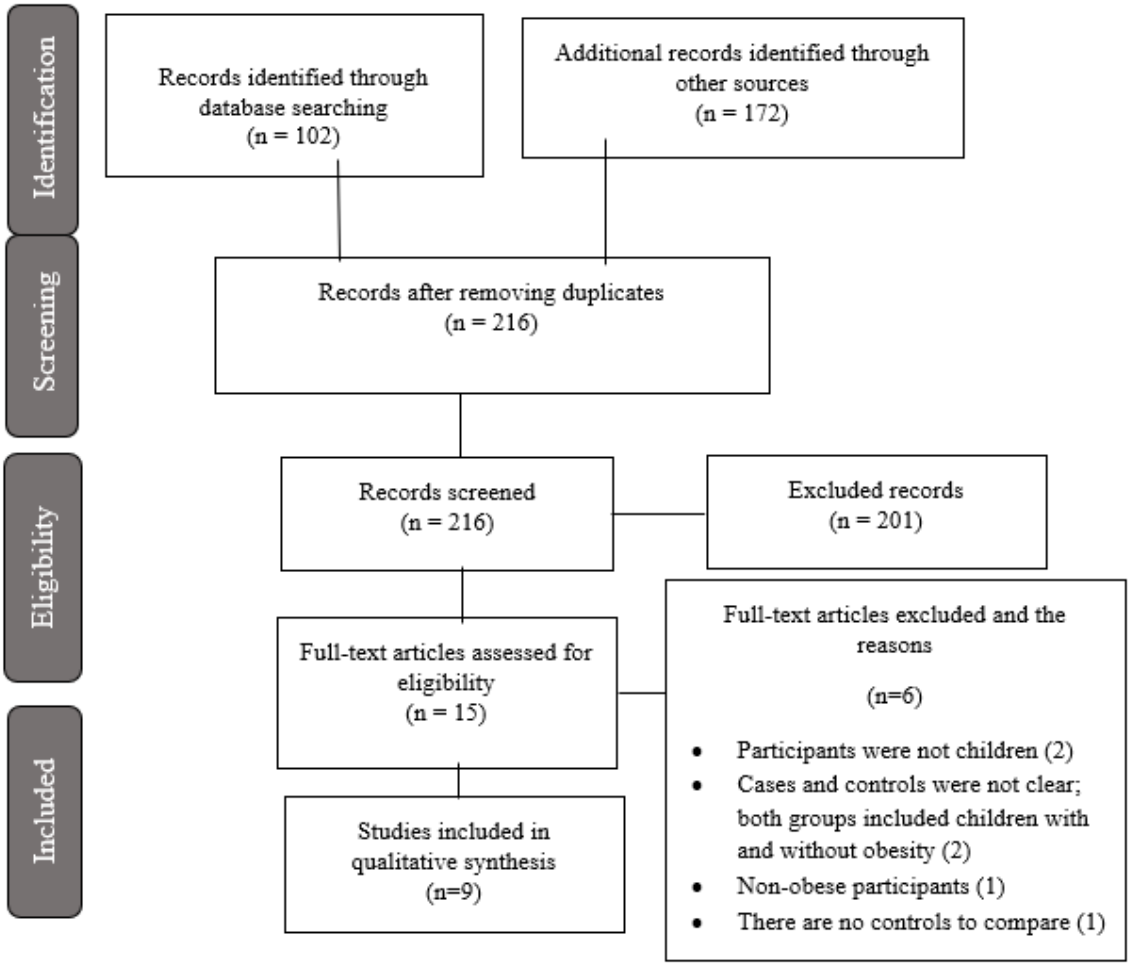

Figure 1. Presents the PRISMA consort diagram for the search strategy. The initial search retrieved 274 articles and a total of 9 studies were finally included after applying inclusion and exclusion criteria.

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Table 1: Characteristics of the studies examining differences in miRNA expression between obese and normal weight children and between obese children with and without cardiometabolic risk factors.

AUTHOR	Study aim	Biological sample	Search for miRNA and methodology		Participants	
			Type of search Number of miRNAs Sample size	**Technique/ method**	**Cases Sample size Sex (girls %) Age BMI**	**Control Sample size Sex (girls %) Age BMI**
Al-rawaf HA et al. 2018	To describe the circulating miRNA profile for adolescences and its association with circulating levels of leptin and adiponectin according to specific degree of obesity	Circulating miRNAs (Blood-plasma)	Specific search miRNAs N=10 N=150	qPCR	Obese 100 29 13.87±2.91 years BMI= 26.7±8.2 kg/m^2	Normal weight 50 44 13.8±2.88 years BMI= 17.4±4.3 kg/m^2
Cui et al. 2017	To screen candidate miRNAs as biomarkers for identifying obese children who are at risk of developing diabetes	Circulating miRNAs (Blood-plasma)	Massive - N=18 Validation miRNAs N=18 N=246	Global miRNAs profiling - Illumina qPCR	Obese N=100 51.5 61.0±10.4 months BMI=20.3±2.2 kg/m^2	Normal weight N=146 49.5 60.4±11.1 months BMI=15.1±1.06 kg/m^2
Ouyang et al. 2017	To characterize the miRNA profile in PBMC of obese children	Circulating miRNAs (Blood – PBMC)	Massive - N=12	Global miRNA Profiling - NanoString nCounter	Obese 50 39.7±2.2 months BMI=18.5±26kg/m^2	Normal weight 50 39.2±2.3 months BMI=13.5±15 kg/m^2
Thompson et al. 2017	To Evaluate whether circulating miRNAs that have been associated with NAFLD are altered in children with obesity, compared with healthy controls	Circulating miRNAs (Blood-plasma)	Specific search for miRNAs related to NAFLD miRNAs N=20 N=30	TaqMan RT-qPCR	Obese and NAFLD N=20 42.8 13.2±3.1 years BMI=34.7±10.4kg/m^2	Normal weight and non-NAFLD N=10 60 13.8±2.1 years BMI=20.1±2.5 kg/m^2

Iacomino et al. 2016	To identify circulating miRNAs potentially associated with early obesity in children	Circulating miRNAs (Blood-plasma)	Specific search miRNAs N=372 N=20	qPCR	Obese and overweight N=10 40 10.7±1.7 years BMI=31,7±4.3 kg/m^2	Normal weight N=10 50 10.5±2.67 years BMI=16,4 ± 1.7 kg/m^2
Masotti et al. 2016	To investigate the expression profile of circulating miRNA 1) fasting and 2)120min after OGTT in 6 IR obese preschoolers and 6 controls without IR.	Circulating miRNAs (Blood-plasma)	Specific search miRNAs N=179 N=12	qPCR	Obese + IR N=6 - 4.63 ±1.82 years BMI=20.9 ±2.9 kg/m^2	Obese without IR N=6 - 4.35± 0.85 years BMI=18.5 ± 1.2 kg/m^2
Can et al. 2015	To examine the relationship between 7 specific miRNAs and lipid metabolism in obese and non-obese children and adolescents.	Circulating miRNAs (Blood-plasma)	Specific search miRNAs N=7 N=86	qPCR	Obese N=45 57.7 14.71±1.76 years BMI=41.3±52.9 kg/m^2	Normal weight N=41 58.5 14.44±1.62 years BMI=18.9±2.1 kg/m^2
Prats-Puig et al 2013.	To examine the dysregulated circulating miRNAs in obese children.	Circulating miRNAs (Blood-plasma)	Massive - N=10	Global miRNA profiling – low-density TaqMan arrays (TLDAs)	Obese N=5 0 (only boys) 8.8±1.8 years z-BMI=3.36±0.43	Normal weight N=5 0 (only boys) 9.9±1.0 years z-BMI =-0.62±0.3
			Validation miRNAs N=15 N=125	qPCR	Obese N=40 55 9.2±1.4 years z BMI=2.69±0.59	Lean N=85 49 9.0±1.6 years z BMI=-0.32±0.71
Carolan et al. 2013	To investigate sCD163 levels, circulating iNKT frequency, cytokine profile and miR expression in obese and non-obese children.	Circulating miRNAs (Blood-PBMC)	Specific search miRNAs N=3 N=49	qPCR	Obese N=29 46.4 13.0±3.0 years z-BMI=3.4±0.5	Normal weight N=20 35 12.8±3.2 years z-BMI=0.2±1.1

PBMC: peripheral blood mononuclear cells, **NAFLD:** nonalcoholic fatty liver disease, **OGTT:** oral glucose tolerance test, **IR:** insulin resistance, **qPCR:** real-time polymerase chain reaction, **TLDA:** TaqMan low density arrays, **BMI:** body mass index**, z-BMI:** body mass index z-score.

Risk of bias within studies

The risk of bias assessment graph for the included studies is presented in Supplementary figures 1 and 2. Although some studies did not meet the overall objectives proposed in the introduction section and some issues described in the methodology were not very clear, in general terms, all the studies showed an adequate systematic methodology to include in the article.

Results of individual studies

All the studies showed statistically significant differences in the expression level of specific miRNAs between cases and controls. Differences in miRNA expression levels were expressed/quantified in terms of fold change or the ratio of mean expression level in cases to mean expression level in controls for each miRNA studied.

Seven studies (19–25) found significantly ($p<0.05$) dysregulated miRNAs in the sample of children with obesity compared with their normal weight peers (Supplementary Table 1). Three of them conducted a massive search of miRNAs (19,20,23). Cui et al. (19), after selecting 18 miRNAs candidates by massive search, observed that 8 of them were significantly dysregulated ($p<0.05$) in plasma of children with obesity (**Supplementary Table 1**). Ouyang et al. (20) found 8 miRNAs significantly ($p<0.05$) dysregulated in PBMC of children with obesity (**Supplementary Table 1**). In the study of Prats-Puig et al. (23), the massive search selected 16 candidate miRNAs and the authors found that 15 of them were significantly ($p<0.05$) dysregulated in plasma of children with obesity (**Supplementary Table 1**). Only the miR-222 was consistently up-regulated in two of these studies (Table 2) (19,23).

Four studies conducted a specific search for miRNAs by qPCR sets (21,22,24,25). Al-rawaf et al. (25) focused on a specific miRNA set of 10 miRNAs and observed that all of them were significantly ($p<0.05$) dysregulated in plasma of children with obesity (**Supplementary Table 1**). Three of them (miR-222, miR-142-3p and miR-140-5p) were also found overexpressed in the Prats-Puig et al. (23) study (**Table 2**), and miR-222 was found overexpressed in Cui et al. study (Table 2) (19). In contrast, miR-532-5p and miR-423-5p that were found down-expressed in the study of Al-rawaf et al. (25), were up expressed in the study of Prats-Puig et al. (23). Similarly, the miR-146a, that was found down-regulated in the study of Al-rawaf et al. was reported as up-regulated in the study of Cui et al. (19).

Iacomino et al. (21) followed a specific search strategy and selected a set of 372 miRNAs to be monitored. They found 8 miRNAs as significantly ($p<0.05$) dysregulated in plasma of children with obesity (**Supplementary Table 1**). Can et al. (22) after a specific search for seven miRNAs, observed that six of them were significantly ($p<0.05$) dysregulated in plasma of children with obesity (**Supplementary Table 1**). Carolan et al. (24) searched a specific set of three miRNAs and found that two of them were significantly ($p<0.05$) dysregulated in PBMC of children with obesity.

Table 2. Seven miRNAs expression was altered in more than one study. Differences in miRNA expression between children with obesity and normal-weight children.

miRNAs	Author	Effect size (cases vs controls)			p
		Relative expression level	Mean expression level	Fold change	
miR-222	Al-rawaf HA et al. 2018	14.5 *vs* 4.5	-	-	<0.001
	Cui et al. 2017	-	-	>6	<0.01
	Prats-Puig et al 2013.	-	41.08±30.59 *vs.* 25.43±17.87	-	0.001
miR-142-3p	Al-rawaf HA et al. 2018	12 *vs* 2.5	-	-	<0.001
	Prats-Puig et al 2013.	-	90.31±61.46 *vs.* 32.30±21.29	-	<0.0001
miR-140-5p	Al-rawaf HA et al. 2018	13.5 *vs* 4	-	-	<0.001
	Prats-Puig et al 2013.	-	32.66±18.13 *vs.* 23.15±17.50	-	0.001
miR-143	Al-rawaf HA et al. 2018	14 *vs* 3.5	-	-	<0.001
	Can et al. 2015	-	30.5 *vs* 115.35	-	0.001
miR-532-5p	Al-rawaf HA et al. 2018	8 *vs* 17	-	-	<0.001
	Prats-Puig et al 2013.	-	10.49±7.75 *vs.* 5.49±4.28	-	0.001
miR-423-5p	Al-rawaf HA et al. 2018	4 *vs* 14	-	-	<0.001
	Prats-Puig et al 2013.	-	2.16±1.35 *vs.* 1.13±0.77	-	<0.0001
miR-146a	Al-rawaf HA et al. 2018	4 *vs* 15	-	-	<0.001
	Cui et al. 2017	-	-	3.8	<0.01

The study of Thompson et al. (24) conducted a specific miRNA search for 20 miRNAs potentially involved in NAFLD and found 15 significantly ($p<0.05$) dysregulated in plasma of children with obesity and NAFLD compared with normal weight and non-NAFLD controls (**Table 3**). One of these miRNAs, mir191-5p, was also dysregulated in the study of Ouyang et al. (20). In the study of Masotti et al. (27), the expression profile of plasma circulating miRNAs at fasting and after an oral glucose test tolerance (OGTT) was investigated (**Supplementary Table 2**). They conducted a specific miRNA search for 179 miRNAs in both situations. They found that 14 miRNAs were significantly ($p<0.05$) dysregulated in fasting plasma of children with obesity and IR

compared to children with obesity and insulin sensitivity (**Table 4**). Two of these miRNAs, miR-122-5p and miR-34a-5p, were also found dysregulated in the Thompson et al.´s study (26), and another one, miR-320a, was also reported dysregulated in Iacomino et al.´s study (21).

Table 3. Differences in miRNA expression between children with obesity and non-alcoholic fatty liver disease (NAFLD) and normal-weight and non-NAFLD children.

miRNAs	Author	Effect size: fold change (cases *vs* controls)	p
miR-122-5p	Thompson et al. 2017	12.48	<0.0001
miR-34a-5p		5.09	<0.0001
miR-191-5p		7.21	<0.0001
miR-15b-5b		3.42	0.0004
miR-199a-5p		17.18	<0.0001
miR-222-3p		2.14	<0.0001
miR-223-3p		6.72	<0.0001
miR-181b-5p		3.29	0.0009
miR-23a-3p		5.3	<0.0001
miR-27b-3p		6.74	<0.0001
miR-21-5p		4.89	<0.0001
miR-451-5p		1.54	0.0404
miR-192-5p		3.78	<0.0001
miR-16-5p		1.56	0.0064
miR-29a-3p		2.81	<0.0001
miR-150-5p		1.79	0.0006
miR-214-5p		2.73	0.0213
miR-155-5p		2.63	0.0023
miR-103a-5p		3.38	<0.0001

Table 4. Differences in miRNA expression between children with obesity and insulin resistance and children with obesity and insulin sensitivity.

Differences in miRNA expression (fast).

miRNAs	Author	Effect size: fold change (Cases *vs* controls)	p
miR-122-5p	Masotti et al. 2016	2.82±0.49	0.037
miR-34a-5p		2.41±0.39	0.032
miR-320a		1.55±0.11	0.014
miR-505-3p		3.11±0,65	0.030
miR-26b-5b		1.63±0.17	0.020
miR-146a-5p		1.48±0.09	0.014
miR-148b-3p		1.47±0.18	0.032
miR-342-3p		1.46±0.25	0.050
miR-190a		-3.04±0.39	0.032
miR-200c-3p		-2.78±0.46	0.032
miR-205-5p		-2.60±0.44	0.032
miR-95		-1.72±0.26	0.032
miR-19a-3p		-1.55± 0.21	0.032
miR-660-5p		-1.50± 0.19	0.032

DISCUSSION

In this systematic review, we aimed to identify and unify circulating miRNAs dysregulated in excess adiposity and obesity-associated metabolic abnormalities in children and adolescents. Our findings show that: (i) there are still few studies focused on pediatric obesity with low number of participants and most of them use non-massive search methods for identifying dysregulated miRNAs; (ii) although there is a wide variability in the circulating miRNAs reported in the different studies, we can identify four circulating miRNAs, miR-222, miR-142-3, 140-5p and miR-143 that are overexpressed in children with obesity, and that (iii) miR-122 and miR-34a seem to be overexpressed in children and adolescents with NAFLD and/or IR.

The analysis of previous data carried out in this review also unveils four miRNAs (miR-222, miR-142-3, 140-5p and miR-143) as significantly overexpressed in children and adolescents with obesity in more than one report (Table 2). Of note it is that miR-222, miR-142-3 and 140-5p were identified after a massive search (19,23) and that the results obtained in studies in adults are in agreement with these findings (28). In this regard, elevated levels of these miRNAs were previously associated with higher BMI and were particularly up-regulated in the presence of severe obesity (28). In adults with morbid obesity, miR-142-3p, miR-140-5p and miR-222 were related to adiposity markers and, interestingly, their concentrations were substantially lowered after surgery-induced weight loss (28).

The identification of the miR-122 as potential biomarker of NAFLD in children with obesity is consistent with previous studies in adults and animal models (29). The miR-122 is mostly expressed in the liver, and it regulates cholesterol production and hepatic function (30). Indeed, in adults, miR-122 seems to be a key regulator of cholesterol and fatty acid metabolism in the liver (30), and it was associated with insulin resistance, obesity, metabolic syndrome, type 2 diabetes, and adverse lipid profile (31). Moreover, in adults, high levels of circulating miR-122 have also been associated with increased concentrations of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and gamma-glutamyl transferase (GGT), triglycerides, lower HDL-cholesterol levels, as well as with hepatic steatosis and the degree and progression of NAFLD (31–34). In agreement with these results, Brandt et al. observed that miR-122 circulating (plasma or serum) levels were higher in children with NAFLD than in non NAFLD overweight children and that miR-122 concentrations were associated with higher liver enzyme levels (i.e., ALT, AST and GGT) (38). Of note, in mice, the inhibition of miR-122 resulted in

lower plasma cholesterol levels, halted hepatic lipid synthesis, and enhanced hepatic fatty acid oxidation (30). Moreover, in adults, after bariatric surgery, hepatic function improvement significantly correlated with a decrease in circulating miR-122 levels (28). These findings strengthen the role of miR-122 as a sensitive and specific blood biomarker of liver function.

One study in young children (27) included in this review reported that the miR-122 was also associated with insulin resistance evaluated by means of an oral glucose tolerance test. In this line, some studies in adolescents, young and older adults observed that circulating miR-122 levels were correlated with insulin resistance; the miR-122 has been proposed suggesting that miR-122 could be involved in insulin resistance and might be used as a potential biomarker of diabetes risk (35–37) and progression (29). Moreover, several genes targeted by the miR-122 have been implicated in the pathogenesis of IR, including genes involved in muscle responses to insulin, such as PRKAB1, a subunit of AMPK, that is a critical regulator of metabolism in IR (36,38,39). However, it should be noted that the miR-122 is associated with high levels of triglycerides and cholesterol, and dyslipidemia is a common feature in patients with insulin resistance or diabetes (28,31,32,35). Thus, Ye et al. observed that this miRNA was up-regulated in patients who, in addition to type 2 diabetes, had NAFLD as compared with those who presented diabetes but not NAFLD (34).

In children and adolescents with obesity, the presence of NAFLD was associated with higher levels of miR-34a (26). This finding agrees with previous reports in adults in which this miRNA was proposed as a useful diagnostic biomarker of NAFLD (40,41) and non-alcoholic steatohepatitis (NASH) in patients with NAFLD (41). Also in animal models, hepatic miR-34a levels were elevated in dietary-obese mice and in ob/ob mice (42).

Previously reported data show that circulating miR-122 and miR-34a levels seem to be an extrahepatic biomarker of NAFLD and the progression of it, suggesting that both miRNAs might be able to serve as a non-invasive diagnostic marker against aggressive diagnostic methods such as liver biopsy.

In conclusion, circulating miRNAs are promising diagnostic biomarkers of obesity-associated diseases such as NAFLD and type 2 diabetes already in childhood. However, it was not possible to identify a concrete miRNA profile in children with obesity. Likewise, the limited number of studies, the low number of participants, the lack of homogeneity in participants according to their stage of puberty, and the use of different techniques for the identification and quantification of miRNAs (specific extraction methods for example) may have influenced the high variability found in the miRNA profile reported by the included studies. Nevertheless, findings presented

in the current review suggest that miR-122 and miR-34a may be overexpressed in children and adolescents with NAFLD and IR, and that circulating miR-222, miR-142-3, 140-5p and miR-143 are over-expressed in children with obesity. However, more studies in children using massive search technology and with larger sample sizes are required to draw any firm conclusion.

Conflict of interest: All authors declare no conflict of interest. The founding sponsors had no role in the design of study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

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Suplemmentary files:

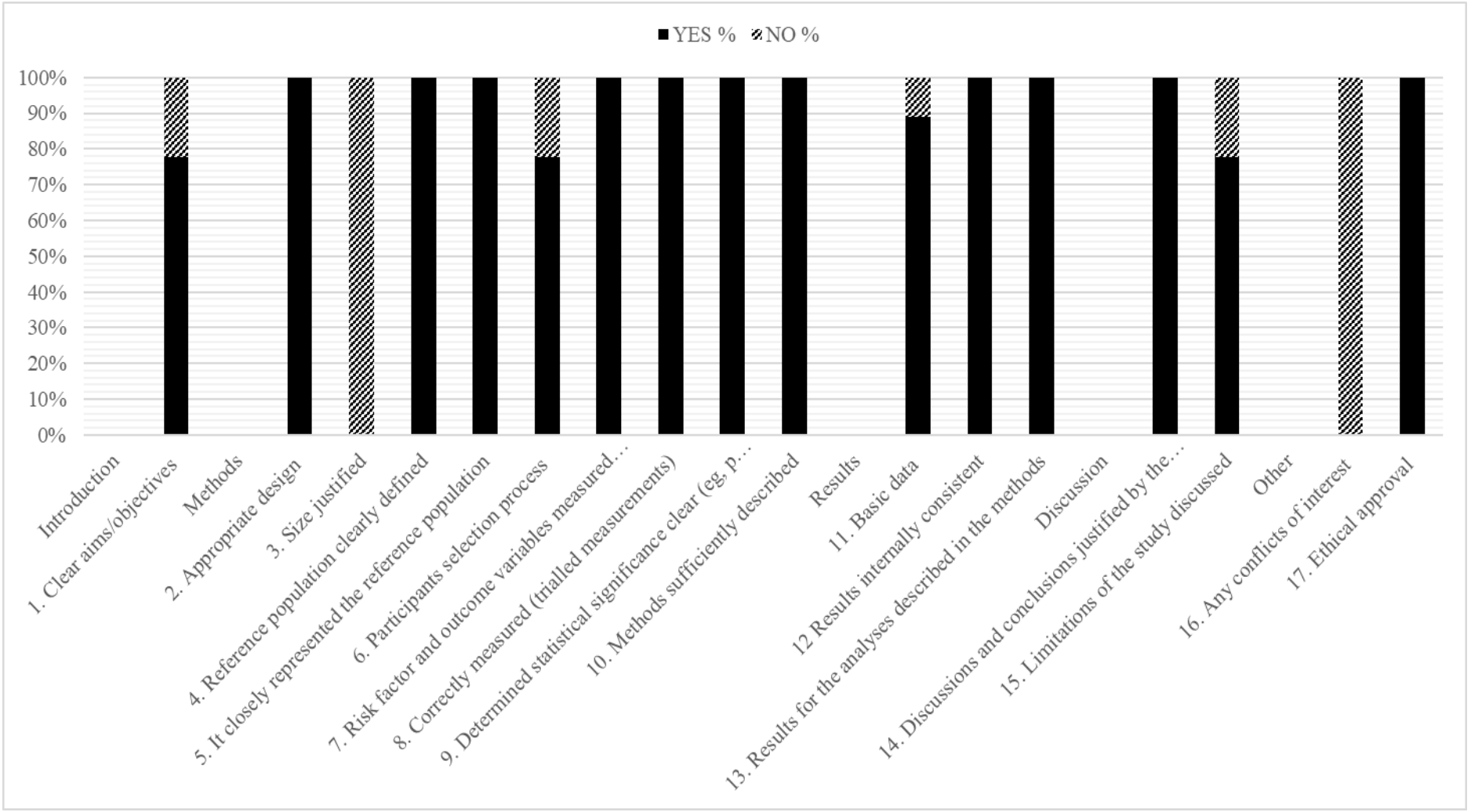

Figure S1: Risk of Bias Assessment and Study Quality (AXIS)

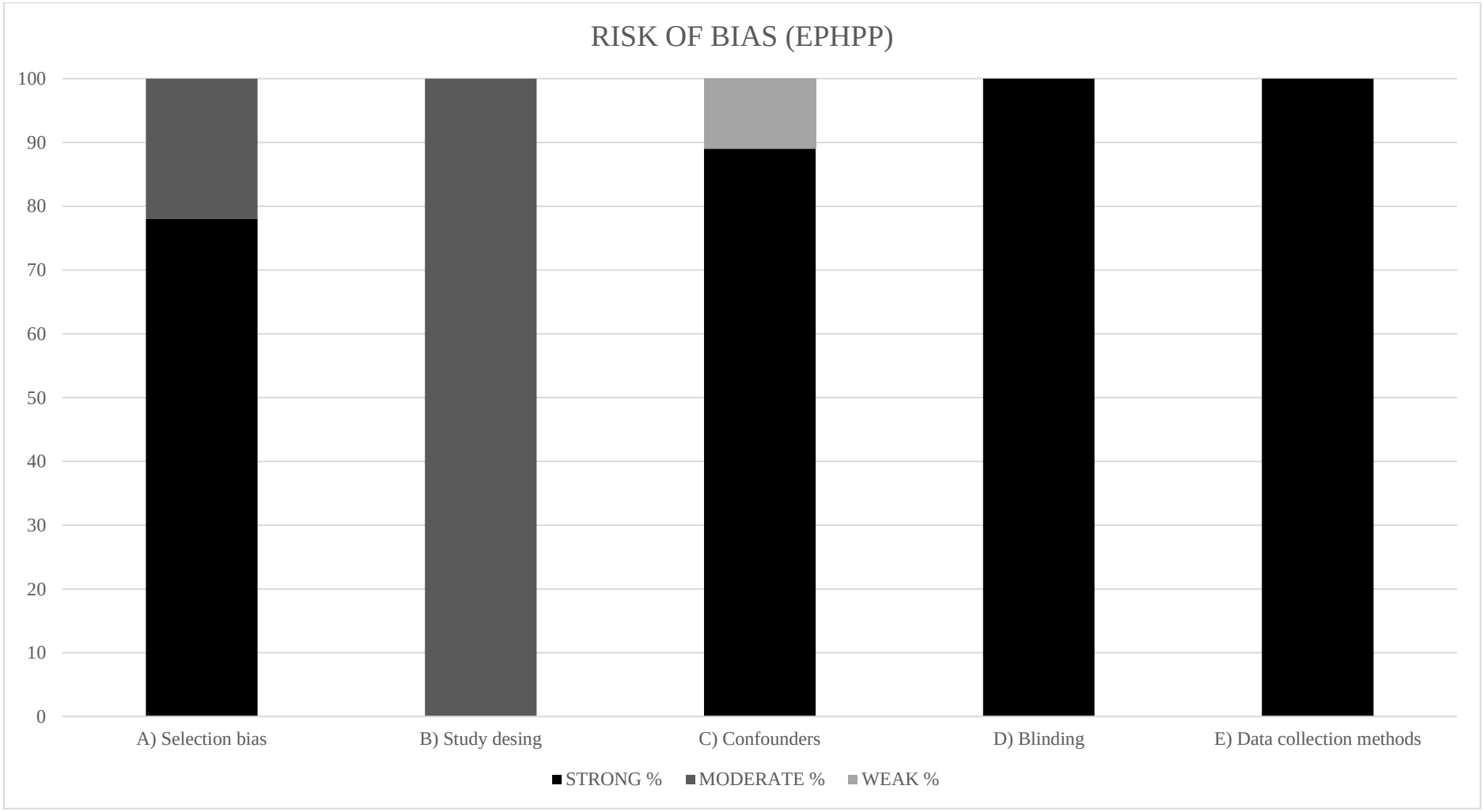

Figure S2: Risk of Bias Assessment and Study Quality (EPHPP; Effective Public Health Practice Project)

Table S1. Differences in miRNA expression between children with obesity and normal-weight children.

miRNAs	Author	Effect size (cases vs controls)			p
		Relative expression level	Mean expression level	Fold change	
miR-222	Al-rawaf HA et al. 2018	14.5 *vs* 4.5	-	-	<0.001
	Cui et al. 2017	-	-	>6	<0.01
	Prats-Puig et al 2013.	-	41.08±30.59 *vs*. 25.43±17.87	-	0.001
miR-142-3p	Al-rawaf HA et al. 2018	12 *vs* 2.5	-	-	<0.001
	Prats-Puig et al 2013.	-	90.31±61.46 *vs*. 32.30±21.29	-	<0.0001
miR-140-5p	Al-rawaf HA et al. 2018	13.5 *vs* 4	-	-	<0.001
	Prats-Puig et al 2013.	-	32.66±18.13 *vs*. 23.15±17.50	-	0.001
miR-143	Al-rawaf HA et al. 2018	14 *vs* 3.5	-	-	<0.001
	Can et al. 2015	-	30.5 *vs* 115.35	-	0.001
miR-532-5p	Al-rawaf HA et al. 2018	8 *vs* 17	-	-	<0.001
	Prats-Puig et al 2013.	-	10.49±7.75 *vs*. 5.49±4.28	-	0.001
miR-423-5p	Al-rawaf HA et al. 2018	4 *vs* 14	-	-	<0.001
	Prats-Puig et al 2013.	-	2.16±1.35 *vs*. 1.13±0.77	-	<0.0001
miR-146a	Al-rawaf HA et al. 2018	4 *vs* 15	-	-	<0.001
	Cui et al. 2017	-	-	3.8	<0.01
miR-26b	Cui et al. 2017	-	-	3.2	<0.01
miR-26b-5p	Iacomino et al. 2016	-	-	25.37	<0.05
miR-486	Cui et al. 2017	-	-	>6	<0.01
miR-486-5p	Prats-Puig et al 2013.	-	88.75±61.51 *vs*. 40.17±28.44	-	<0.0001
miR-486-3p	Prats-Puig et al 2013.	-	13.92±10.44 *vs*. 7.33±5.53	-	<0.0001
miR-130	Al-rawaf HA et al. 2018	16 *vs* 5	-		<0.001
miR-130a-3p	Ouyang et al. 2017	-	-	1.38	0.018
miR-130b	Prats-Puig et al 2013.	-	24.32±8.46 *vs*. 16.19±9.58	-	<0.0001
miR-520c-3p	Al-rawaf HA et al. 2018	6 *vs* 18	-	-	<0.001
miR-15a	Al-rawaf HA et al. 2018	9 *vs* 23	-	-	<0.001
miR-15b	Cui et al. 2017	-	-	>6	<0.01

miR-146b	Cui et al. 2017	-	-	>6	<0.01
miR-20a	Cui et al. 2017	-	-	3.2	<0.05
miR-197	Cui et al. 2017	-	-	- 4.2	<0.01
miR-301a-3p	Ouyang et al. 2017	-	-	1.33	0.011
miR-199a-3p/199b-3p	Ouyang et al. 2017	-	-	1.5	0.017
miR-191-5p	Ouyang et al. 2017	-	-	1.26	0.018
miR-361-5p	Ouyang et al. 2017	-	-	1.33	0.031
miR-126-3p	Ouyang et al. 2017	-	-	1.26	0.042
Let-7g-5p	Ouyang et al. 2017	-	-	-1.16	0.043
miR-4454	Ouyang et al. 2017	-	-	-2.12	0.043
miR-31-5p	Iacomino et al. 2016	-	-	4.9499	<0.05
miR-2355-5p	Iacomino et al. 2016	-	-	6.5216	<0.05
miR-1231	Iacomino et al. 2016	-	-	-8.7217	<0.05
miR-361-3p	Iacomino et al. 2016	-	-	-4.8918	<0.05
miR-136-5p	Iacomino et al. 2016	-	-	-4.8356	<0.05
miR-320a	Iacomino et al. 2016	-	-	-9.9692	<0.05
miR-206	Iacomino et al. 2016	-	-	-6.0515	<0.05
miR-335	Can et al. 2015	-	2.6 *vs* 11.6	-	<0.001
miR-27	Can et al. 2015	-	77.0 *vs* 124.00	-	0.032
miR-378	Can et al. 2015	-	6.0 *vs* 18.00	-	<0.001
miR-370	Can et al. 2015	-	501.0 *vs* 1687.0	-	0.045
miR-758	Can et al. 2015	-	175.45 *vs* 482.75	-	0.006
miR-221	Prats-Puig et al 2013.	-	8.49±7.01 *vs*. 50.36±45.32	-	<0.0001
miR-28-3p	Prats-Puig et al 2013.	-	5.21±2.80 *vs*. 8.84±4.06	-	<0.0001
miR-125b	Prats-Puig et al 2013.	-	0.48±0.38 *vs*. 0.92±0.88	-	0.001
miR-16-1	Prats-Puig et al 2013.	-	187.04±117.17 *vs*. 113.22±119.48	-	0.001
miR-328	Prats-Puig et al 2013.	-	7.06±4.02 *vs*. 11.44±9.97	-	0.001
miR-363	Prats-Puig et al 2013.	-	6.11±5.27 *vs*. 3.97±4.02	-	0.001
miR-122	Prats-Puig et al 2013.	-	37.44±35.74 *vs*. 23.46±24.83	-	0.001
miR-33a	Carolan et al. 2013	-	-	-	0.001
miR-33b	Carolan et al. 2013	-	-	-	0.017

Table S2. Differences in miRNA expression between children with obesity and insulin resistance and children with obesity and insulin sensitivity.

Differences in miRNA expression (fast).

miRNAs	Author	Effect size: fold change (cases *vs* controls)	p
miR-122-5p	Masotti et al. 2016	2.82±0.49	0.037
miR-34a-5p		2.41±0.39	0.032
miR-320a		1.55±0.11	0.014
miR-505-3p		3.11±0,65	0.03
miR-26b-5b		1.63±0.17	0.02
miR-146a-5p		1.48±0.09	0.014
miR-148b-3p		1.47±0.18	0.032
miR-342-3p		1.46±0.25	0.05
miR-190a		-3.04±0.39	0.032
miR-200c-3p		-2.78±0.46	0.032
miR-205-5p		-2.60±0.44	0.032
miR-95		-1.72±0.26	0.032
miR-19a-3p		-1.55± 0.21	0.032
miR-660-5p		-1.50± 0.19	0.032

Differences in miRNA expression after glucose oral tolerance test

miRNAs	Author	Effect size: fold change (cases *vs* controls)	p
miR-190a	Masotti et al. 2016	2.04±0.77	0.046
miR-200c-3p		3.88±0.82	0.015
miR-95		-3.31±1.35	0.044
miR-30b-5p		-1.82±0.54	0.027
miR-194-5p		2.05±0.53	0.028
miR-885-5p		4.42±1.42	0.044
miR-424-5p		-2.06±0.84	0.044
miR-301a-3p		-2.50±1.07	0.015
miR- 130b-3p		-2.32±0.92	0.046
miR-584-5p		2.45±1.08	0.046

3.4 Study IV

Peripheral blood mononuclear cells-expressed miRNA profiles derived from children with metabolic associated fatty liver disease and insulin resistance

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ABSTRACT

Background: miRNA have been proposed as potential biomarkers of metabolic diseases. Objectives: To identify potential miRNA biomarkers of early metabolic associated fatty liver disease (MAFLD) and/or insulin resistance (IR) in preadolescent children with overweight. Methods: A total of 70 children with overweight, aged 8.5-12 years old participated in the study. Hepatic fat was assessed by magnetic resonance imaging. Fasting blood biochemical parameters were measured and HOMA-IR calculated. Peripheral blood mononuclear cells (PBMC)-derived miRNA profiles associated with MAFLD (≥5.5% hepatic fat) and IR (HOMA-IR≥2.5) were identified using untargeted high-throughput miRNAs sequencing (RNA-seq). Results: A total of 2123 PBMC-derived miRNAs were identified in children with (21.4%) or without MAFLD. Among them, hsa-miR-143-3p, hsa-miR-142-5p and hsa-miR-660-5p were up-regulated, and p-hsa-miR-247, hsa-let-7a-5p and hsa-miR-6823-3p down-regulated. Importantly, children with MAFLD had consistently higher miR-660-5p expression levels than their peers without it ($p<0.01$), regardless of weight status. A total of 2124 PBMC-derived miRNA were identified in children with IR (28.6%) vs. children without IR, where thirteen of them were dysregulated ($p<0.05$) in children with IR. In addition, children with IR showed higher levels of miR-374a-5p and miR-190a-5p ($p<0.01$) and lower levels of miR-4284 and miR-4791 ($p<005$), than their peers without IR in both the whole sample and in those with overweight or obesity. Conclusions: Our study results suggest circulating miR-660-5p as a potential biomarker of the presence of MAFLD in preadolescent children, while circulating miR-320a, miR-142-3p, miR-190a-5p, miR-374a-5p and let-7 family miRNAs could serve as potential biomarkers of IR in children.

Key words:

MiRNA, Fatty liver, Insulin resistance, children, obesity, metabolic diseases

Abbreviations

ALT: Alanine aminotransferase, AST: Aspartate transaminase, BMI: Body mass index, GGT: Gamma-glutamyl-transferase, HbA1c: glycated hemoglobin, HDL: High-density lipoprotein, HOMA-IR: Homeostasis model assessment of insulin resistance, IR: Insulin resistance, MAFLD: Metabolic associated fatty liver disease, MRI: Magnetic resonance imaging, miRNA: microRNA, NW: normal weight, LDL: Low-density lipoprotein, OB: obesity, OW: overweight, SPSS: statistical package for social sciences, T2D: Type 2 diabetes mellitus, TG: Triglycerides.

INTRODUCTION

Metabolic associated fatty liver disease (MAFLD) is the most common liver disorder and the second common cause of liver transplantation (1). MAFLD has been considered the hepatic manifestation of metabolic syndrome and of systemic insulin resistance (IR) (2). The interaction between IR and MAFLD cause a vicious circle, where IR has determined such as one of the inductor of MAFLD, increasing hepatic de novo lipogenesis and impairing insulin-mediated suppression of adipose tissue lipolysis by inducing free fatty acids flux into the liver (3–5). In turn, MAFLD has been also directly associated with the aggravation of IR and, in consequence, with increased risk of developing type 2 diabetes (T2D), already in childhood (3,5,6).

The MAFLD term has been recently agreed among different expert groups in order to reflect more accurately the current knowledge of fatty liver disease associated with metabolic dysfunction (7,8). The definition of pediatric MAFLD is based on the evidence of intrahepatic fat accumulation in addition to one of the following three criteria: excess overall adiposity, presence of prediabetes or T2D, or as evidence of metabolic dysregulation defined as the presence of at least two cardiometabolic risks according to sex and age percentiles (8). It is estimated that MAFLD is present in nearly 10% of general pediatric population (9) and in 30% of children with overweight or obesity (10).

The development and progression of pediatric MAFLD is complex and multifactorial, and the underlying mechanisms have not been entirely elucidated (11). However, there is evidence that dietary habits, environmental and genetic factors can lead to the development of metabolic alterations directly associated with hepatic fat accumulation and inflammation (12,13). Although this disease is reversible and easily treatable in the early stages, its asymptomatic evolution, together with its high prevalence and costly (magnetic resonance imaging, MRI) and/or invasive (liver biopsy) diagnosis methods make early identification and treatment difficult (12). For that reason, the search for potential biomarkers has become a priority line in MAFLD research. Nowadays, there is evidence that excess adiposity and lifestyle factors such as sugar rich diets and sedentary behaviors are strong risk factors for the development and progression of hepatic steatosis through epigenetic mechanisms (11,14,15).

MicroRNAs (miRNAs), one of the major forms of epigenetic modulation, are short, noncoding RNA molecules (21-23 nucleotides) that have been proposed as potential biomarkers and therapeutic targets for

MAFLD (15,18) and type 2 diabetes in adults (19). In children, there are still few studies examining the miRNAs expression levels in relationship with IR or MAFLD (18–23). These studies, however, were performed through targeted analysis of several candidate miRNAs previously identified in adult studies. To date, as far as we are aware, there is no previous studies developed through a high-throughput untargeted search of miRNAs in pediatric population with MAFLD and/or IR. Therefore, the main objective of the present work was to identify potential miRNA biomarkers of early MAFLD and/or IR in preadolescent children, and, secondly, to analyze the associations of miRNA expression levels with cardiometabolic risk factors.

METHODS

Study design and participants

This cross-sectional formed part of the PREDIKID project (ClinicalTrials.gov ID: NCT03027726) whose overall aims were: (1) to evaluate the effect of a 22-week family based multidisciplinary intervention program including exercise on insulin resistance syndrome (IRS) risk in children with a high risk of developing T2D, and (2) to identify the profile of microRNA in peripheral blood mononuclear cells in children with a high risk of developing type 2 diabetes, and its response to a multidisciplinary intervention program including exercise. Details of sample calculation, randomization, the characteristics of the study participants, methodological procedures and measurements taken are available elsewhere (26).

For the current proposal, baseline data of 70 preadolescent children aged 8.5-12 years old and with complete and valid data on MRI-diagnosed hepatic steatosis (5.5% hepatic fat), IR and miRNA levels were analyzed. Having other hepatic pathology such as viral hepatitis, toxic hepatitis or autoimmune diseases were considered as exclusion criteria.

The study protocol, which complies with the ethical guidelines of the Declaration of Helsinki (2013 revision), was approved by The Euskadi Clinical Research Ethics Committee. Participants were recruited at the Pediatric Endocrinology Unit of the University Hospital of Araba, and at primary care clinics. The parents or legal guardians of each children provided written, informed consent.

Measurements

Hepatic fat and insulin resistance

Hepatic fat percentage was assessed by MRI using a Magnetom Avanto system (Siemens Healthcare, Erlangen, Germany) (26). The presence of MAFLD was determined as a hepatic fat percent ≥5.5% (27) in addition to one of the three following criteria: overweight or obesity, presence of prediabetes or T2D, or as evidence of metabolic dysregulation defined as the presence of at least two cardiometabolic risks according to sex and age percentiles (8). The homeostasis model assessment of insulin resistance [HOMA-IR=insulin (mU/L) × glucose (mmol/L)/22.5] was calculated by fasting serum concentrations of glucose and insulin (28). HOMA-IR ≥2.5 determined the presence of IR.

Anthropometric and biochemical parameters

Body mass (SECA 760), height (SECA 220), and waist circumference (SECA 201) were measured in duplicate following standard protocols. Thereafter, the body mass index (BMI) (kg/m2) and the waist to height ratio (WHtR) were calculated (29). Weight status was defined according to the body mass index (BMI) age and sex-specific cut-off values provided by Word Obesity Federation (30).

The plasma concentrations of cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), triglycerides (TG), glycated hemoglobin (HbA1c), glucose, insulin, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and gamma-glutamyl-transferase (GGT) were measured in fasting blood samples serum using standard protocols (26).

RNA purification and miRNA analysis

Total RNA from peripheral blood mononuclear cells was isolated using RNAeasy Kit (Quiagen). miRNAs profiles were analyzed using specific RNA-seq methodology. Briefly, gene libraries were prepared using TruSep Small RNA Sample preparation kit (Illumina, Inc) following manufacturer´s instructions. Libraries with 145 to 160 bp size were selected to undergo deep sequencing on Illumina's MiSeq Next Generation Sequencing system. Sequencing reactions were performed on Illumina's MiSeq Reagent Kit V3. Analysis of results were pre-

processed and analyzed using MiSeq Reporter, Bowtie, SAMtools and miRDeep software tools; as well as R/Bioconductor packages.

Bioinformatic analysis

Assignation of mapped sequencing reads to miRNA expression data using miRbase version 21 database was performed with featureCounts R function (31). Differential expression of miRNAs was tested using DESeq2 R package (32).

Statistical analysis

Differences in anthropometric and clinical characteristic between children with or without MRI-diagnosed MAFLD and between children with or without HOMA-IR determined IR were analyzed using the independent t-test or x2 test. T-test was performed to analyze differences in miRNAs expression between: (i) children with or without MAFLD, and (ii) children with or without IR. The miRNA expression levels were log2-trasformed for analysis. Partial correlations were performed to examine the association between miRNAs expression levels and biochemical parameter concentrations adjusting for sex, age and BMI. Statistical analyses were carried out with statistical software SPSS v.23.0 (IBM, Armonk, New York). Significance was set at α=0.05.

RESULTS

Clinical and anthropometric characteristic of participants according to the presence (21.4%) or absence of MAFLD, and to the presence (28.6%) or absence of IR are shown in **Table 1**. Children with MAFLD had significantly higher waist to height ratio, diastolic blood pressure and lower HDL, than their peers without MAFLD (**Table 1**). TG and ALT levels tended to be higher in children with MAFLD ($p<0.07$) when compared to those without MAFLD. Children with IR had significantly higher weight, BMI, TG, glucose and insulin levels and lower HDL levels, than their peers without IR (**Table 1**).

Table 1: Clinical characteristics among preadolescents with and without metabolic associated fatty liver disease (MAFLD) and with and without insulin resistance (IR) participating in the study.

	Non-MAFLD		MAFLD			Non-insulin resistance		Insulin resistance		
	N	**Mean (SD)**	**N**	**Mean (SD)**	***p***	**N**	**Mean (SD)**	**N**	**Mean (SD)**	***p***
Characteristics										
Age (years)	55	11.3 (1.2)	15	10.6 (1.0)	**0.025**	50	11.0 (1.2)	20	11.5 (1.1)	0.100
Girls (N. %)	55	31. 56	15	7. 47	0.352	50	24. 48	20	14. 70	0.116
Body composition										
Height (cm)	55	149.4 (7.3)	15	146.8 (9.0)	0.299	50	148.1 (7.5)	20	150.88 (7.9)	0.182
Weight (kg)	55	54.1 (9.4)	15	55.7 (15.4)	0.623	50	52.2 (8.4)	20	60.05 (14.1)	**0.006**
Body mass index (kg/m^2)	55	24.2 (3.2)	15	25.4 (4.5)	0.335	50	23.8 (2.9)	20	26.07 (4.5)	**0.013**
NW/OW/OB (N/%)	55	8,27,20.15,49,36	15	2,4,9.13,27,60		50	8, 23,19/16,46,38	20	2,8,10/10,40,50	
Waist to height ratio (x100)	55	50.18 (4.30)	15	53.60 (5.89)	**0.010**	50	50.0 (0.49)	20	52.0 (0.48)	0.246
Hepatic fat (%)	55	3.7 (0.9)	15	9.3 (3.7)	**<0.001**	50	4.7 (2.8)	20	5.3 (3.3)	0.474
Blood pressure										
Systolic (mmHg)	55	95 (10)	15	95 (8)	0.863	50	95 (9)	20	94 (11)	0.727
Diastolic (mmHg)	55	61 (7)	15	65 (6)	**0.028**	50	61 (7)	20	64 (6)	*0.052*
MAP (mmHg)	55	84 (8)	15	85 (6)	0.578	50	84 (8)	20	84 (7)	0.820
Biochemical parameters										
Cholesterol (mg/dL)	55	162.6 (25.5)	15	155.7 (34.6)	0.485	50	164.4 (29.3)	20	152.7 (21.6)	*0.071*
High-density lipoprotein (mg/dL)	55	51.1 (11.8)	15	43.7 (7.1)	**0.004**	50	52.1 (11.7)	20	43.2 (7.0)	**<0.001**
Low-density lipoprotein (mg/dL)	55	96.8 (21.1)	15	94.5 (30.8)	0.783	50	98.6 (24.9)	20	90.6 (17.9)	0.139
Triglycerides (mg/dL)	55	72.9 (31.6)	15	87.6 (24.6)	*0.065*	50	68.7 (26.2)	20	94.4 (34.1)	**0.005**
HbA1c_IFCC (mmol/mol)	37	35.6 (3.1)	12	36.0 (3.3)	0.696	34	35.3 (3.3)	15	36.5 (2.6)	0.206
Glucose (mg/dL)	55	84.7 (5.3)	15	84.7 (5.8)	0.989	50	83.1 (5.2)	20	88.6 (3.7)	**<0.001**
Insulin (µl/ml)	55	10.4 (4.9)	15	13.0 (5.5)	0.121	50	8.5 (2.3)	20	17.0 (5.1)	**<0.001**
HOMA-IR	55	2.20 (1.12)	15	2.74 (1.23)	0.114	50	1.76 (0.48)	20	3.72 (1.17)	**<0.001**
Aspartate aminotransferase (U/L)	55	23.1 (4.3)	15	24.5 (4.3)	0.287	50	23.9 (4.2)	20	22.1 (4.6)	0.131
Alanine aminotransferase (U/L)	55	18.6 (5.0)	15	25.2 (12.4)	*0.060*	50	20.4 (8.0)	20	19.0 (6.7)	0.452
Gamma-glutamyl-transferase (U/L)	55	14.3 (3.6)	15	16.7 (5.1)	0.100	50	14.6 (4.5)	20	15.5 (2.8)	0.295

HbA1c: Glycated hemoglobin; HOMA-IR: Homeostatic Model Assessment; IR: Insulin Resistance.; MAFLD: Metabolic Associated Fatty Liver Disease; MAP: Mean arterial pressure; NW: normal weight, OB: obesity; OW: overweight.

A total of 2123 circulating miRNAs were identified in our sample of children with or without MAFLD (**Supplemental Table 1**), where six of them were significantly dysregulated ($p<0.05$) in children with MAFLD – hsa-miR-143-3p, hsa-miR-142-5p and hsa-miR-660-5p were up-regulated, and p-hsa-miR-247, hsa-let-7a-5p and hsa-miR-6823-3p were down-regulated (**Table 2**). We observed that miR-660-5p expression levels were consistently higher in children with MAFLD than in their peers without it (**Table 3**). Thus, we observed similar results in the whole sample ($p<0.01$), and when we analyzed separately those children with overweight or obesity ($p<0.05$) and children with normal weight ($p<0.02$). In addition, MAFLD was significantly related to higher let-7a-5p and miR-142-5p and miR-142-5p expression levels only in non-overweight children.

Table 2: Mean fold change expression of circulating miRNA levels in children with metabolic associated fatty liver disease (MAFLD) compared to children without MAFLD and circulating miRNA levels in children with insulin resistance (IR) compared to children without IR.

miRNAs	**Fold change (log_2)**	**p**
Children with MAFLD *vs.* Children without MAFLD (N=70)		
p-hsa-miR-247	-1.00	**0.010**
hsa-let-7a-5p	-0.56	**0.019**
hsa-miR-143-3p	0.70	**0.027**
hsa-miR-142-5p	0.50	**0.046**
hsa-miR-6823-3p	-0.88	**0.047**
hsa-miR-660-5p	0.51	**0.049**
Children with IR *vs.* Children without IR (N=70)		
hsa-miR-320a	1.02	**0.002**
hsa-let-7d-5p	0.87	**0.002**
hsa-miR-4284	-1.03	**0.002**
hsa-let-7a-5p	0.61	**0.007**
hsa-miR-374a-5p	0.69	**0.009**
hsa-let-7g-5p	0.58	**0.012**
hsa-miR-185-5p	0.65	**0.014**
hsa-miR-142-3p	0.50	**0.021**
hsa-let-7b-5p	0.60	**0.029**
hsa-miR-15b-5p	0.61	**0.029**
hsa-miR-4791	-0.71	**0.033**
hsa-let-7f-5p	0.34	**0.037**
hsa-miR-190a-5p	0.54	**0.038**

IR: Insulin Resistance; MAFLD: Metabolic Associated Fatty Liver Disease.

Table 3: Mean expression difference of circulating miRNAs between children with and without MAFLD and children with and without IR.

	Children without MAFLD		Children with MAFLD			Non-MAFLD and normal weight		MAFLD and normal weight			Non-MAFLD and Overweight/Obesity		MAFLD and Overweight/Obesity		
	N	Mean (SD)	N	Mean (SD)	*p*	N	Mean (SD)	N	Mean (SD)	*p*	N	Mean (SD)	N	Mean (SD)	*p*
miR-247	55	3.12 (1.96)	15	2.22 (1.55)	*0.072*	8	2.24 (1.89)	2	2.60 (0.64)	0.676	47	3.27 (1.95)	13	2.17 (1.65)	*0.053*
let_7a_5p	55	8.75 (0.94)	15	8.49 (0.93)	0.358	8	8.54 (0.35)	2	8.11 (0.06)	**0.011**	47	8.78 (1.01)	13	8.55 (1.00)	0.465
miR-143-3p	55	3.00 (1.71)	15	3.79 (1.73)	0.131	8	2.75 (1.30)	2	5.35 (1.42)	0.188	47	3.05 (1.78)	13	3.55 (1.68)	0.357
miR-142-5p	55	6.52 (1.35)	15	7.13 (1.16)	*0.091*	8	6.67 (0.73)	2	8.21 (0.08)	**<0.001**	47	6.49 (1.43)	13	6.96 (1.15)	0.228
miR-6823-3p	55	1.38 (3.29)	15	0.13 (0.51)	0.149	8	2.10 (2.80)	2	0.00 (0.00)	0.340	47	1.26 (3.37)	13	0.15 (0.54)	0.247
miR-660-5p	55	1.69 (1.28)	15	2.69 (1.00)	**0.006**	8	1.30 (1.15)	2	3.12 (0.43)	**0.015**	47	1.75 (1.30)	13	2.62 (1.05)	**0.020**

	Children without IR		Children with IR			Non-IR and normal weight		IR and normal weight			Non-IR and Overweight/Obesity		IR and Overweight/Obesity		
	N	Mean (SD)	N	Mean (SD)	*p*	N	Mean (SD)	N	Mean (SD)	*p*	N	Mean (SD)	N	Mean (SD)	*p*
miR-320a	50	3.20 (1.78)	20	4.10 (1.64)	*0.054*	8	3.18 (1.59)	2	3.96 (0.45)	0.268	42	3.21 (1.83)	18	4.10 (1.73)	*0.079*
let-7d-5p	50	4.34 (1.54)	20	5.01 (1.55)	0.110	8	4.19 (0.87)	2	3.73 (0.85)	0.585	42	4.37 (1.64)	18	5.16 (1.56)	*0.088*
miR-4284	50	1.96 (2.93)	20	0.27 (2.56)	**0.003**	8	1.54 (2.66)	2	0.80 (4.50)	0.856	42	2.04 (3.00)	18	0.39 (2.44)	**0.002**
let-7a-5p	50	8.62 (0.91)	20	8.91 (1.12)	0.318	8	8.52 (0.37)	2	8.23 (0.19)	0.210	42	8.64 (0.98)	18	8.98 (1.16)	0.282
miR-374a-5p	50	2.04 (1.47)	20	3.25 (1.29)	**0.002**	8	2.36 (1.54)	2	3.21 (0.11)	0.165	42	1.98 (1.47)	18	3.26 (1.37)	**0.003**
let-7g-5p	50	7.15 (0.98)	20	7.46 (1.17)	0.308	8	7.17 (0.65)	2	7.12 (0.09)	0.872	42	7.15 (1.04)	18	7.50 (1.23)	0.305
miR-185-5p	50	2.04 (1.73)	20	2.76 (1.82)	0.138	8	1.86 (1.10)	2	2.36 (0.05)	0.235	42	2.07 (1.84)	18	2.81 (1.92)	0.181
miR-142-3p	50	3.29 (1.47)	20	3.93 (1.37)	*0.094*	8	3.47(0.63)	2	4.87 (0.51)	*0.088*	42	3.26 (1.58)	18	3.82 (1.40)	0.178
let-7b-5p	50	6.43 (1.46)	20	6.94 (1.41)	0.188	8	6.25 (0.64)	2	5.74 (0.04)	*0.062*	42	6.47 (1.58)	18	7.07 (1.43)	0.155
miR-15b-5p	50	4.90 (1.63)	20	5.25 (2.08)	0.504	8	5.39 (0.93)	2	4.49 (1.10)	0.440	42	4.81 (1.73)	18	5.34 (2.17)	0.367
miR-4791	50	3.37 (2.82)	20	1.37 (3.51)	**0.030**	8	2.74 (2.69)	2	2.21 (5.50)	0.915	42	3.50 (2.86)	18	1.28 (3.45)	**0.012**
let-7f-5p	50	8.68 (0.63)	20	8.77 (0.81)	0.644	8	8.76 (0.29)	2	8.34 (0.02)	**0.005**	42	8.66 (0.67)	18	8.82 (0.85)	0.491
miR-190a-5p	50	0.53 (0.90)	20	1.37 (1.11)	**0.005**	8	0.18 (0.58)	2	2.05 (0.49)	*0.052*	42	0.60 (0.93)	18	1.29 (1.14)	**0.031**

IR: Insulin resistance; MAFLD: Metabolic associated fatty liver disease; SD: standard deviation.

When comparing children with IR vs. children without IR, a total of 2124 circulating miRNAs were identified (**Supplemental Table 2**), where thirteen of them were significantly ($p<0.05$) dysregulated in children with IR – hsa-miR-320a, hsa-let-7d-5p, hsa-let-7a-5p, hsa-miR-374a-5p, hsa-let-7g-5p, hsa-miR-185-5p, hsa-miR-142-3p, hsa-let-7b-5p, hsa-miR-15b-5p, hsa-let-7f-5p and hsa-miR-190a-5p were up-regulated, whereas hsa-miR-4284 and hsa-miR-4791were down-regulated (**Table 2**). Children with IR showed significantly higher levels of miR-374a-5p and miR-190a-5p ($p<0.01$) and lower levels of miR-4284 and miR-4791 ($p<005$), than their peers without IR in both the whole sample and in those with overweight or obesity (**Table 3**). In addition, miR-let-7f levels were negatively associated with IR only in children with normal weight ($p <0.01$).

Association of miRNA expression levels with biochemical parameters

Figures 1 and 2 show the associations of MAFLD and IR, respectively, previously identified miRNA expression levels with cardiometabolic risk factors. Among MAFLD-associated miRNAs, it was observed that lower miR-247 ($p=0.017$) and higher miR-660-5p ($p=0.067$) expression levels were associated with higher percentage hepatic fat, and that higher expression levels of miR-142-5p were correlated with ALT plasma concentrations ($p=0.031$). Among IR-associated miRNAs, miR-374a-5p and miR-190a-5p were positively correlated ($p=0.004$ and $p=0.035$, respectively) and miR-4284 inversely ($p=0.034$) associated with HOMA-IR. In addition, miR-374a-5p and let-7b-5p miRNA expression showed significant correlations with TG plasma concentrations ($p=0.035$ and $p=0.031$, respectively).

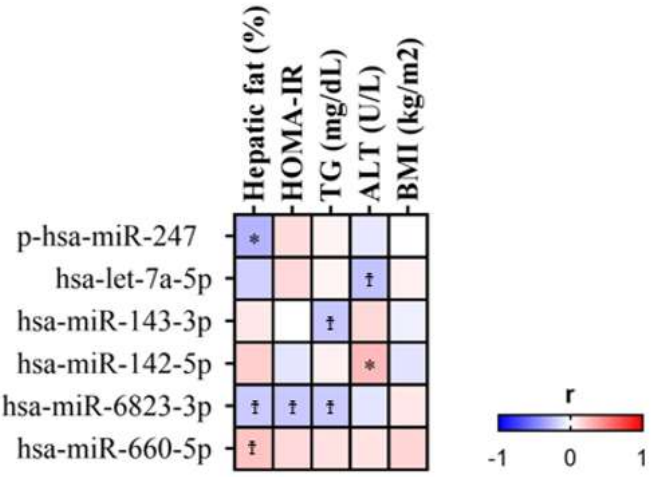

Figure 1: Correlation analyses of circulating miRNAs associated with MAFLD with cardiometabolic risk factors depicted by a heat map (N=70). Colors of the heat map represent the r values of the correlations analyses. Red color represents direct association, whereas blue color represents inverse associations. Intensity of color is proportional to the strength of the correlation. *p < 0.05. ϯ p< 0.07. Abbreviatures: ALT: Alanine aminotransferase; BMI: Body mass index; HOMA-IR: Homeostatic Model Assessment; IR: Insulin Resistance; MAFLD: Metabolic Associated Fatty Liver Disease; TG: Triglycerides. The analyses were adjusted with sex, age and BMI.

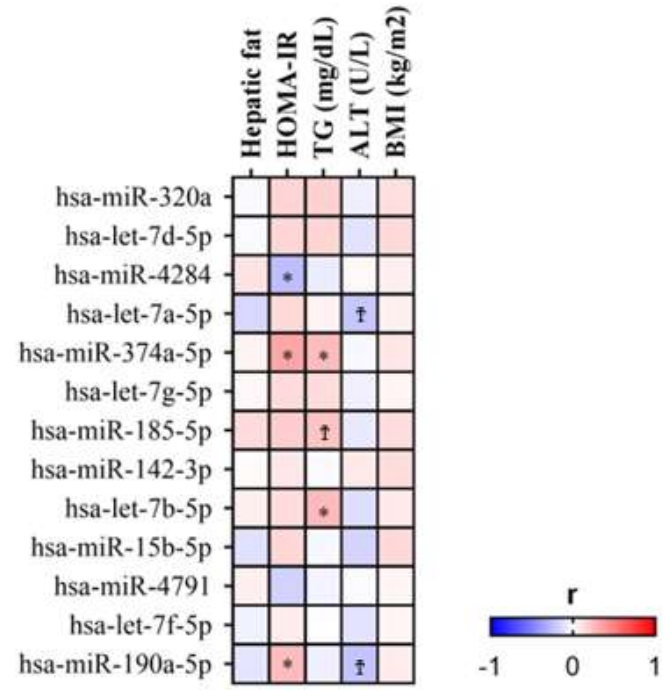

Figure 2. Correlation analyses of circulating miRNAs associated with IR with cardiometabolic risk factors depicted by a heat map (N=70). Colors of the heat map represent the r values of the correlations analyses. Red color represents direct association, whereas blue color represents inverse associations. Intensity of color is proportional to the strength of the correlation. *p < 0.05. ϯ p< 0.07. Abbreviatures: ALT: Alanine aminotransferase; BMI: Body mass index; HOMA-IR: Homeostatic Model Assessment; IR: Insulin Resistance; MAFLD: Metabolic Associated Fatty Liver Disease; TG: Triglycerides. The analyses were adjusted with sex, age and BMI.

DISCUSSION

In the present study, we conducted an untargeted high-throughput miRNAs sequencing and specific circulating miRNA profiles associated with MAFLD and IR in preadolescent children were detected.

To date, there is very limited data of the associations of circulating miRNAs with MAFLD. In adults, miR-122 is the most studied miRNA associated with the presence and severity of MAFLD (33). Other miRNAs such as miRNA-99a and miRNA-34a, have also been associated with MAFLD (30,31). In children, as far as we are aware, there are only three previous studies examining differences in miRNA expression levels between children with and without MAFLD. In contrast to our findings, these studies reported that the miRNA-122 was dysregulated in children with suspected MAFLD. Thus, two previous studies conducted in children and adolescents aged 8 to 18 years old (20,25), showed that miR-122 and miR-34a-5p expression levels were significantly elevated in those with obesity and ultrasound based (20) or MRI based (25) diagnosed-MAFLD compared with children with overweight or obesity without MAFLD. The association of the miR-122 levels with hepatic enzyme levels was also reported in three European cohorts of pre-pubertal children (21). However, previous studies were conducted following candidate miRNAs analysis of biomarkers of fatty liver in adults, and the untargeted approach for identifying novel biomarkers in children is lacking.

In our study approach of untargeted RNA sequencing, we did not detect significant differences in miR-122 or miR-34a levels between children with and without MRI-diagnosed MAFLD. Our results, however, show consistent associations of the miR-660 with MAFLD in preadolescent children. Indeed, we observed that (i) miR-660 was upregulated in children with MAFLD, (ii) children with MAFLD had higher mean expression levels than children without MAFLD, (iii) the results were consistent in children with overweight/obesity and in children with normal-weight, and (iv) mean expression levels of miR-660 were correlated with hepatic fat percent. Further studies conducted in vitro and in vivo animal models, have associated miR-660 (36) with the proliferation and activation of hepatic stellate cells and liver fibrosis which may explain our findings. These findings suggest that the miR-660-5p could be a potential specific biomarker of MAFLD, independently of the presence of overweight or obesity.

We also observed that the miR-142-5p was upregulated in children with MAFLD, and that non-overweight children with MAFLD had higher expression levels than their control peers. These results are in line

with studies in vitro and in vivo with animal models showing that the miR-142-5p was related to the accumulation of lipids in the hepatocytes and with increased hepatic steatosis (37). Nevertheless, these findings should be taken with caution. Indeed, we did not find any consistent and significant differences in miR-142-5p in the whole sample of children and mean expression levels of miR-142-5p were not significantly correlated with the percentage hepatic fat.

Nowadays, there are very few studies analyzing circulating levels of miRNAs in children with IR (22–25) and the results are controversial. Mohany et al. examined three circulating miRNAs (miR-486, miR-146b and miR-15b) in a sample of 120 children aged 6 to 14 years (24). The authors reported that the circulating levels of the three miRNAs were significantly higher in obese children with type 2 diabetes compared to either healthy controls or children with obesity but without type 2 diabetes. Lischka et al. analyzed the expression of 16 circulating miRNAs in children with severe obesity and observed that circulating levels of two of them, miR-34a and miR-122, were significantly higher in those children with prediabetes (25). In adults and animal models, many other miRNAs have been identified as potential biomarkers of insulin resistance or type 2 diabetes. Likewise, according to a meta-analysis of 39 case-control studies, miR-148b, miR-223, miR-130a, miR-19a, miR-26b and miR-27b could be proposed as biomarkers of diabetes (38).

In the current study, children with IR had elevated levels of miR-320a. This finding is in concordance with a previous study in children with obesity aged 2.0-5.8 years in which a specific search of 179 mRNAs was conducted (22),. In adults, circulating miR-320a has been previously associated with insulin resistance and with the progression of prediabetes to diabetes (39,40). In addition, this miRNA has been proposed as a predictor of the response to several pharmacological therapies for diabetes (39,40). In mice, it was observed that this miRNA could damage pancreatic b-cells, increase ROS levels and induce β-cell apoptosis (39,41).

We also found that the circulating miR-190a-5p levels were consistently higher in children with IR independently of their weight status, and that it was significantly correlated with HOMA-IR. In patients with type 2 diabetes, the miR-190a-5p was associated with the risk of developing diabetic retinopathy (42) In animal models, miR-190a-5p expression levels were higher in liver tissues of mice with liver fibrosis than in their respective controls (43).

We observed significant differences in mean expression levels of miR-142-3p between obese children with and without IR, in agreement with previous findings in adults (44,45) and children (21). In a sample of 250 school children, Al-rawaf et al. studied the association of specific miRNAs with different parameters associated with metabolic syndrome and reported higher levels of circulating miR-142 in those with higher HOMA-IR (23). The circulating miR-142-3p was also found up-regulated in adults with morbid obesity (46) and T2D (47) and was proposed as a potential biomarker for acute and chronic inflammation (48).

Likewise, we observed that miR-4791 and miR-4284 were down-regulated, and miR-374a-5p was up-regulated in preadolescent with IR and that there were significant differences in mean expression levels between in children with and without IR, either in the whole sample or in children with overweight or obesity, but not in normal weight children. These results suggest that the excess of overall adiposity might be influencing these miRNAs expression levels. There are very few studies examining these miRNAs and most of them have been explored in cancer disease (44- 46). Interestingly, in concordance with our results, one previous case-control study in non-obese Asian Indians patients with or without prediabetes or T2D patients, observed that the miR-347a-5p was correlated with HOMA-IR (49).

The use of the high-throughput untargeted analysis of circulating miRNAs methodology and the MRI-based diagnosis of MAFLD should be considered as important strengths of the current study. However, our relatively small sample size is recognized as a study limitation. More studies on bigger number of preadolescent children are to confirm or contradict our findings.

In conclusion, our study findings provide additional knowledge of the possible epigenetic regulation in MAFLD and IR. Disease specific miRNAs were detected among pediatric population, where miR-660-5p, miR-320a, miR-142-3p, miR-190a-5p, miR-374a-5p and let-7 family miRNAs of special interest. Our study results suggest circulating miR-660-5p as a potential biomarker of the presence of MAFLD in preadolescent children, while circulating miR-320a, miR-142-3p, miR-190a-5p, miR-374a-5p and let-7 family miRNAs could serve as potential biomarkers of IR in children.

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4. GENERAL DISCUSSION

4.1. Identification of anthropometric, sociodemographic and lifestyle factors associated with hepatic steatosis in children with overweight or obesity

In our Study I, we observed that WHtR, ethnic minority status, gestational age at birth, sugar-sweetened beverages (SSB) consumption, screen time and CRF (laps in 20mSRT test) were the most consistent anthropometric, sociodemographic and lifestyle factors associated with the presence of hepatic steatosis in children with overweight or obesity.

The most strongly associated factor with hepatic steatosis among the identified factors was belonging to an ethnic minority group. Previous studies have reported that ethnicity plays an important role in liver fat deposition due to genetic susceptibility and/or low socioeconomical status. In fact, in the United Sates, the prevalence of hepatic steatosis is higher in Hispanic, than in non-Hispanic children (22) and adults (66). Previous studies explained this finding as a genetic predisposition of the Hispanic population to suffer MAFLD. In fact, the prevalence of a SNP associated with increased risk of developing MAFLD, the *PNPLA3* rs738409, is more prevalent in Hispanic than in non-Hispanic individuals (43,67,68). In our study, ethnic minority was defined as belonging to a recognized ethnic minority for Spain or as having a foreign-born mother from a low- or middle-income country (130). Ethnic minority groups are different across countries; in our study, this group shares social disadvantages more than a genetic or biological background. Social disadvantages such as low income and parental education, occupation, minimal social network, non-traditional family structure, migrant status, ethnic or racial minority groups or unemployment have been associated with a higher prevalence of obesity and obesity-related comorbidities such as insulin resistance (70). Our results support these findings and extend to the presence of hepatic steatosis.

Our findings are in accordance with previously identified markers of paediatric hepatic steatosis. Thus, gestational age at birth, one of the most studied perinatal factor in the development of hepatic steatosis (75,77) and/or metabolic diseases in children was inversely associated with hepatic steatosis and pre-term children had higher risk to suffer hepatic steatosis. Regarding anthropometric measures, it should also be noted that the WHtR is one of the most frequently used anthropometric measures for identifying abdominal adiposity and cardiometabolic risk in children (131). In the current study, WtHR was associated with an increased risk of

MAFLD in children being a better predictor of the disease than BMI, which concurs with previous reports (92). In the same way, those lifestyle factors more strongly associated with suffering hepatic steatosis in our sample (screen time and SSB consumption) are also known to be strong determinants of paediatric hepatic steatosis (80,86,132,133).

4.1.1. Prediction accuracy of previously published paediatric screening tools.

In the Study I, we tested the accuracy of previously published paediatric screening tools for hepatic steatosis. All of them, the ped-NAFLD score (63) and the proposed different cut-off for plasma ALT levels (42,50,53) showed low accuracy and sensitivity in our sample of children, that limit their application as a routinary screening method.

The Ped-NAFLD score is based on WtHR, ALT and HOMA-IR levels. The authors observed elevated sensitivity 89% and specificity 76% in their studied sample (63); However, in our study sample, this score achieved a high specificity (95%, CI: 87-100%), but very low sensitivity (33%, CI: 17-48%) and failed to detect 67% of children with hepatic steatosis. A previous study examined the performance of the Ped-NAFLD score in a sample of 119 children with severe obesity and observed a sensitivity of 75% and specificity of 68% (134). However, this study did not examine the accuracy of the score in overweight or mild obesity.

Similarly, the proposed ALT cut-offs test by the NASPGHAN (42), the ESPGHAN (50) or by other authors, showed very high specificity between (90% and 100%), but very low sensitivity (between 5% and 41%) in our sample. In fact, the NASPGHAN (42) and ESPGHAN (50) ALT cut-off points failed to identify 93% of children with an MRI-diagnosed fatty liver as having hepatic steatosis. Indeed, even the revised ALT cut-off of Schwimmer et al. (53) failed to detect hepatic steatosis in 59% of MRI-diagnosed children. These results are in line with previous studies in which the sensitivity varied between 24% and 48% (56,135). Although, the high specificity is a strength of ALT levels tests, the main limitation of these methods is their low sensitivity, that leaves most of the children with hepatic steatosis without early diagnosis and treatment.

4.1.2. Non-invasive screening tool based on anthropometric, sociodemographic and lifestyle information for the identification of children with high risk to suffer hepatic steatosis: The HEPAKID index

The main scientific contribution of the Study I was the development of a simple, non-invasive, sensitive, inexpensive and easy-to-perform screening method based on sociodemographic, lifestyle and anthropometric variables that can identify hepatic steatosis in pre-adolescent children with overweight or obesity (HEPAKID index). Indeed, a HEPAKID index score of ≥25.0 showed high sensitivity and reasonable accuracy identifying hepatic steatosis as detected by MRI. The HEPAKID index calculation is available on https://bit.ly/37WXV0j.

The HEPAKID index includes anthropometric data (WHtR), sociodemographic factors (ethnic minority status and gestational age at birth), lifestyle variables (SSB consumption, screen time) and CRF (laps in 20mSRT test), all of which are easily measured or collected in a brief questionnaire. The 20mSRT test is a routine test used to measure CRF in schools. However, as in clinical settings this information may not be available, a version of the HEPAKID index not taking into account CRF was also developed. https://bit.ly/2AQTUPa.)

In clinical practice, and particularly in primary care, the sensitivity of a screening tool is the main criterion for its selection because the objective is to identify patients who warrant further confirmatory diagnostic tests. The HEPAKID index identified 82% (79% in the model without CFR) of children with overweight/obese with hepatic steatosis (18% false negatives). However, it showed a specificity of 0.62 (0.59 in the model without CRF) and in consequence, 38% of children without the disease were identified as candidates for additional examination. Importantly, the HEPAKID-index was externally validated in a sample of 45 children with overweight or obesity, and the results were similar: high sensitivity (85%) and low specificity (44%).

The main strengths of the HEPAKID-index are that without any complementary test and without any invasive or costly method, i) it can be used in every child with overweight or obesity and, ii) it is able to identify the great majority of the children with hepatic steatosis. In contrast, its moderate specificity leads to the identification of children without the disease as a child with high risk of hepatic steatosis. This limitation suggested the need of a complementary screening method to improve the specificity before referring the children to a confirmatory diagnosis.

4.2. Identification of biochemical and genetic factors associated with hepatic steatosis in children with overweight or obesity

In our Study II, plasma TG, insulin, HOMA-IR, AST, ALT, GGT and ferritin levels were consistently associated with the presence of hepatic steatosis in children with overweight or obesity. In addition, we also observed that the distribution of carriers/non-carriers of the risk-alleles of four genetic variants, *PPARG* rs13081389, *PPARG* rs1801282, *HFE* rs1800562 and *PNLPLA3* rs4823173, was also significantly associated with the presence of the disease in our study sample.

These results are in concordance with previous studies in children with MAFLD, where plasma TG levels has been the most associated lipid dysregulation in obese children with MAFLD (96). In the same way, in adult and children studies, hepatic enzyme levels (AST, ALT and GGT) and HOMA-IR were previously identified as predictor biomarkers of fatty liver (93,134). Of note is that our results increase the knowledge about the implication of iron metabolism in the development of hepatic steatosis. In adults, ferritin was previously proposed as a predictor of liver injury (136); however, as far as we are aware, there is no previous studies in children analysing the association of ferritin with paediatric MAFLD.

The *PPARG* gene encodes the peroxisome proliferator-activated receptor (PPAR) gamma protein, which is a regulator of adipocyte differentiation and glucose homeostasis (137). This protein plays a key role in adipogenesis and adipocyte gene expression and has been associated obesity and T2D. The *PPARG* rs1801282 was identified to be associated with obesity, T2D, and insulin sensitivity in several studies (138) Similarly, the Patatin-like phospholipase domain containing 3 (*PNPLA3*) is the most studied gene in MAFLD. This gene encodes a transmembrane protein which is expressed predominantly in the liver, retina, skin and adipose tissue (139). In a previous study in adults with severe obesity, it was observed a statistically significant association between *PNLPA3* rs4823173 risk allele and the grade of hepatic steatosis (140).

4.3. Decision tree with high predictive potential for the identification of children with overweight or obesity candidates to confirmatory diagnosis: The HEPAKID prediction protocol

In the Study II, we developed three different models based on the biochemical and/or SNPs data in order to identify the most appropriate model to serve as a second-step screening tool for paediatric MAFLD. However, the exclusive application of these models, based on biochemical and/or SNPs data, showed moderate accuracy (sensitivity 67-82% and specificity 63-69%) to detect the disease.

The model I, based exclusively on biochemical parameters, showed limited applicability. Thus, the prioritization of high sensitivity (82%) with a cut point of ≥25, showed low specificity (63%); while the prioritization of high specificity (94%) with a cut-point of ≥60, showed very low sensitivity (49%). The model II, based on four SNPs associated with MAFLD (*PPARG* rs13081389, *PPARG* rs1801282, *HFE* rs1800562 and *PNLPLA3* rs4823173) also showed limited discriminatory capacity (67% sensitivity and 65% specificity). The model III, based on a combination of biochemical and genetic variables did not improve the accuracy enough (82% sensitivity and 69% specificity) in our study sample.

Indeed, although biochemical parameters such as HOMA-IR, TG, ALT, AST, GGT, or ferritin levels are increased in children with MAFLD (141,142), their prediction capacity is not enough for the screening of MAFLD. Similarly, considering the necessary technological resources for the analysis of the SNPs, the minimal specificity improvement of the prediction tool, and its high economic cost, models II and III becomes non eligible for the routine clinical practice. These results concur with previous reports in children and adults, where the addition of genetic information to clinical parameters in the calculations of the risk scores resulted in minimal improvements of sensitivity and specificity (109,143).

Genetic susceptibility seems to play a crucial role in the development and progression of MAFLD (144). Therefore, genetic variants have been proposed as potential biomarkers of MAFLD in adults (108) and children (105,110,145). However, MAFLD is a polygenetic disease where dynamic interactions between genes and environmental factors can modulate the development and progression of the disease (144). Therefore, we probably need more genetic information of MAFLD susceptible genes, as well as studies examining the genes-

environmental factors interactions, rather than just several SNPs, to establish accurate predictive models. In addition, to date, genetic variables are not easily available in routine clinical practice, which limits its application as a massive screening tool. Thus, the model I, based on easy to measure biochemical parameters, was proposed as the most appropriate model to serve as a second-step screening.

The most important contribution of Study II is the development of an easy to perform and minimally invasive prediction protocol for the identification of MAFLD among children with overweight or obesity, which encompasses elevated sensitivity, specificity and high accuracy. This protocol (HEPAKID prediction protocol), based on a short questionnaire and easy to measure biochemical parameters, may be useful in routine Primary Care clinical practice to identify early those children who should be referred to perform a confirmatory diagnosis.

The HEPAPKID prediction protocol is an accurate, sensitive (72%), specific (84%), simple and minimally invasive screening protocol for the identification of MRI-diagnosed MAFLD among children with overweight or obesity. In addition, the proposed protocol was validated in an external sample (N=45) showing similar results (sensitivity 70% and specificity 85%), which strengthens its prediction capacity.

This algorithm combines two consecutive steps without genetic information and/or difficult to measure biochemical parameters in routine clinical practice. In the first step, developed in Study I, children are classified as “at risk of having MAFLD” or “not” depending on the score achieved in the HEPAKID index pre-screening tool, available on https://bit.ly/2AQTUPa, which is derived from a questionnaire based on the recording of sociodemographic factors (ethnic minority status and gestational age at birth), anthropometric data (WHtR) and lifestyle variables (SSB consumption and screen time). In the second step, developed in Study II, those children identified in the previous step as “at risk” (HEPAKID index ≥25) have to be referred for a blood test to perform a second screening using common blood biochemical parameters (glucose and insulin to calculate HOMA-IR, TG, ALT, AST, GGT, and ferritin) available on https://acortar.link/1yeEyY. Those children with a score ≥25 in this second step should be sent to a medical specialist to confirm the diagnosis.

In adults, several prediction scores showed the elevated capacity of anthropometric and clinical parameters to predict the risk of suffering fatty liver disease (60–62). Nevertheless, in children, these scores had very limited accuracy (AUC-ROC between 0.68 and 0.75) (134). Previously proposed prediction scores or

algorithms for the screening of paediatric MAFLD (63,109,134) showed reasonable accuracy (between 0.81 and 0.88) and sensitivity (between 77% and 89%), but very limited application in external validations (specificity 68-95% and sensitivity 33-75%) (59,134). In addition, these models included non-easy to measure parameters such as blood leptin and adiponectin (63) or genetic information (109) that limit their routine applicability.

The combination of sociodemographic, anthropometric, lifestyle, and clinical information within the same algorithm seems to be the key to achieve high sensitivity (>70%), specificity (>80%) and elevated discriminatory capacity to identify children with MAFLD among those with overweight or obesity. Likewise, the high specificity achieved after performing the two steps, makes this tool useful for clinical practice avoiding unnecessary costly or invasive testing in patients without the disease, allowing its application in the entire child population with overweight or obesity. The proposed decision tree also contemplates the possibility of direct derivation to confirmatory diagnostic tests of children with moderate or severe obesity with MAFLD risk factors (such as family history of MAFLD, very high hepatic enzyme levels or hepatic symptomatology). Moreover, those children who maintain their overweight/obesity status, but who were not classified as children at risk of MAFLD in the first or in the second step, should be monitored and assessed yearly to avoid leaving any patient untreated in the future.

The study II complements our previous sensitive pre-screening tool, the HEPAKID index, developed in the Study I, adding the necessary specificity of a medical screening tool, but maintaining its simplicity, easiness and low economic cost. In any case, although the results were consistent in the validation sample, the proposed protocol should be externally validated in larger, multi-ethnic, and representative cohorts of children with overweight/obesity before its implementation in clinical settings.

4.4. Systematic review of the biomarker role of circulating miRNAs in the early onset of childhood obesity and associated co-morbidities

To the best of our knowledge, our systematic review (Study III) was the first systematic review examining the role of circulating miRNAs in childhood obesity and associated co-morbidities. This systematic review showed that: (i) there were still few studies focused in paediatric obesity, with low number of participants, and most of them using non-massive search methods for identifying dysregulated miRNAs, (ii) although there was a wide variability in the circulating miRNAs reported in the different studies, we identified four circulating miRNAs, miR-222, miR-142-3, 140-5p and miR-143, that were over-expressed in children with obesity, and that (iii) miR-122 and miR-34a seemed to be over-expressed in children and adolescents with MAFLD and/or IR.

The analysis of previous data carried out in this review, also unveil four miRNAs (miR-222, miR-142-3, 140-5p and miR-143) as significantly over-expressed in children and adolescents with obesity in more than one report. Of note that miR-222, miR-142-3 and 140-5p were identified after a massive search (146,147), and that these results were in agreement with previous studies in adults (148). In this regard, elevated levels of these miRNAs were previously associated with higher BMI and were particularly up regulated in the presence of severe obesity (148). In adults with morbid obesity, miR-142-3p, miR-140-5p and miR-222 were related to adiposity markers and, interestingly, their concentrations were substantially lowered after surgery-induced weight loss (148).

Regarding the miRNAs associated with paediatric MAFLD, only one study analysed miRNA potentially involved in this disease. Thompson et al. (121) conducted a specific miRNA search for 20 miRNAs potentially involved in MAFLD. Interestingly, miR-122 was the most up-regulated miRNA in children with obesity and MAFLD (N=20) compared with normal weight or overweight children without MAFLD (N=10). This result was consistent with previous studies in adults and animal models (120). The miR-122 is mostly expressed in the liver, and it regulates cholesterol production and hepatic function (149). Indeed, in adults, high levels of circulating miR-122 have also been associated with increased concentrations of ALT, AST, GGT, TG, lower HDL-cholesterol levels, as well as with hepatic steatosis and the degree and progression of MAFLD (150–153). In agreement with these results, Brandt et al. (122) observed that miR-122 circulating (plasma or serum) levels were higher in children with MAFLD than in non-MAFLD overweight children, and that miR-122 concentrations were associated with higher

liver enzyme levels (i.e., ALT, AST and GGT) (154). In the study included in this review, children and adolescents with obesity and MAFLD showed higher levels of miR-34a (121). This finding agrees with previous reports in adults in which this miRNA was proposed as a useful diagnostic biomarker of MAFLD (154,155), and non-alcoholic steatohepatitis (NASH) in patients with MAFLD (155). Also in animal models, hepatic miR-34a levels were elevated in dietary-obese mice and in ob/ob mice (156). However, the specific search of miRNAs, small sample sizes and the characteristics of control groups were important limitations of the studies included in the review.

Regarding the miRNAs associated with paediatric IR, only one study analysed those potentially involved in this disease. Masotti et al. (157), who conducted a specific search of 179 miRNAs, reported that the miR-122 was associated with insulin resistance evaluated by means of an oral glucose tolerance test. In this line, some studies in adolescents, young and older adults observed that circulating miR-122 levels were correlated with insulin resistance. In this line, the miR-122 has been proposed as a potential biomarker of the risk of developing diabetes (158,159) and its progression (120). Moreover, several genes targeted by the miR-122 have been implicated in the pathogenesis of IR, including genes involved in muscle responses to insulin, such as PRKAB1, a subunit of AMPK, a critical regulator of metabolism in IR (160–162). However, it should be noted that the miR-122 is associated with high levels of TG and cholesterol, and dyslipidaemia is a common feature in patients with insulin resistance or diabetes (150,151,158,163). Thus, Ye et al. observed that this miRNA was up regulated in patients who, in addition to type 2 diabetes, had MAFLD as compared to those who presented diabetes but not MAFLD (153).

Previously reported data showed that circulating levels of miR-122 and miR-34a may be extrahepatic biomarkers of MAFLD and its progression, suggesting that both miRNAs might be able to serve as a non-invasive diagnostic marker against more aggressive diagnostic methods such as liver biopsy. Nevertheless, the limited number of studies, the low number of participants, and the use of different techniques for the identification and quantification of miRNAs may have influenced the high variability found in the miRNA profile reported by the included studies. Therefore, more studies in children using massive search technology and with larger sample sizes are needed to confirm or not the results.

4.5. Potential miRNA biomarkers of early MAFLD and/or IR in preadolescent children and its association with metabolic risk factors

In the study IV, we conducted an untargeted high-throughput miRNAs sequencing and specific circulating miRNA profiles associated with MAFLD and IR in preadolescent children were detected.

To date, there is very limited data on the associations of circulating miRNAs with MAFLD. In adults, miR-122 is the most studied miRNA associated with the presence and severity of MAFLD (151). Other miRNAs such as miRNA-99a and miRNA-34a, have also been associated with MAFLD (151,164). In children, as far as we are aware, there are only three previous studies examining differences in miRNA expression levels between children with and without MAFLD. In contrast to our findings, these studies reported that the miRNA-122 was dysregulated in children with suspected MAFLD. Thus, two previous studies, one of them included in our systematic review (Study III), conducted in children and adolescents aged 8 to 18 years old (121,123), showed that miR-122 and miR-34a-5p expression levels were significantly elevated in those with obesity and ultrasound based (121) or MRI-based (123) diagnosed-MAFLD compared with children with overweight or obesity without MAFLD. In our study IV conducted with an untargeted RNA sequencing approach, we did not detect significant differences in miR-122 or miR-34a levels between children with and without MRI-diagnosed MAFLD. Our results, however, show consistent associations of the miR-660 with MAFLD in preadolescent children. Indeed, we observed that (i) miR-660 was upregulated in children with MAFLD, (ii) children with MAFLD had higher mean expression levels than children without MAFLD, (iii) the results were consistent in children with overweight/obesity and in children with normal-weight, and (iv) mean expression levels of miR-660 were correlated with hepatic fat percent. These findings suggest that the miR-660-5p could be a potential specific biomarker of MAFLD, independently of the presence of overweight or obesity. Further studies conducted in vitro and in vivo animal models, have associated miR-660 (165) with the proliferation and activation of hepatic stellate cells and liver fibrosis which may explain our findings.

Nowadays, there are very few studies analysing circulating levels of miRNAs in children with IR (123,157,166,167) and the results are controversial. Mohany et al. examined three circulating miRNAs (miR-486, miR-146b and miR-15b) in a sample of 120 children aged 6 to 14 years (167). The authors reported that

the circulating levels of the three miRNAs were significantly higher in obese children with T2D compared to either healthy controls or children with obesity but without T2D. Lischka et al. analysed the expression of 16 circulating miRNAs in children with severe obesity and observed that circulating levels of two of them, miR-34a and miR-122, were significantly higher in those children with prediabetes (123). In adults and animal models, many other miRNAs have been identified as potential biomarkers of insulin resistance or T2D. Likewise, according to a meta-analysis of 39 case-control studies, miR-148b, miR-223, miR-130a, miR-19a, miR-26b and miR-27b could be proposed as biomarkers of diabetes (168).

In our study, children with IR had elevated levels of miR-320a. This finding is in concordance with a previous study in children with obesity aged 2.0-5.8 years, included in our systematic review (Study III), in which a specific search of 179 mRNAs was conducted (157). In adults, circulating miR-320a has been previously associated with insulin resistance and with the progression of prediabetes to diabetes (169,170). In addition, this miRNA has been proposed as a predictor of the response to several pharmacological therapies for diabetes (169,171).

We also found that the circulating miR-190a-5p levels were consistently higher in children with IR independently of their weight status, and that it was significantly correlated with HOMA-IR. In patients with T2D, the miR-190a-5p was associated with the risk of developing diabetic retinopathy (172). In animal models, miR-190a-5p expression levels were higher in liver tissues of mice with liver fibrosis than in their respective controls (173).

We observed significant differences in mean expression levels of miR-142-3p between obese children with and without IR, in agreement with previous findings in adults (114,174) and children (166). In a sample of 250 school children, Al-rawaf et al. studied the association of specific miRNAs with different parameters associated with metabolic syndrome and reported higher levels of circulating miR-142 in those with higher HOMA-IR (166). The circulating miR-142-3p was also found up-regulated in adults with morbid obesity (163) and T2D (175) and was proposed as a potential biomarker for acute and chronic inflammation (176).

Likewise, we observed that miR-4791 and miR-4284 were down-regulated, and miR-374a-5p was up-regulated in preadolescent with IR, and that there were significant differences in mean expression levels between children with and without IR, either in the whole sample or in children with overweight or obesity, but not in normal weight children. These results suggest that the excess of overall adiposity might be influencing these miRNAs expression levels. There are very few studies examining these miRNAs and most of them have been explored

in cancer disease (177–179) . Interestingly, in concordance with our results, one previous case-control study in non-obese Asian patients with or without prediabetes or T2D patients observed that the miR-347a-5p was correlated with HOMA-IR (180).

The use of the high-throughput untargeted analysis of circulating miRNAs methodology and the MRI-based diagnosis of MAFLD should be considered as important strengths of the current study. However, our relatively small sample size is recognized as a study limitation. More studies on bigger number of preadolescent children are to confirm or contradict our findings.

In conclusion, the Study IV findings provide additional knowledge of the possible epigenetic regulation in MAFLD and IR. Disease specific miRNAs were detected among paediatric population, where miR-660-5p, miR-320a, miR-142-3p, miR-190a-5p, miR-374a-5p and let-7 family miRNAs of special interest. Our study results suggest circulating miR-660-5p as a potential biomarker of the presence of MAFLD in preadolescent children, while circulating miR-320a, miR-142-3p, miR-190a-5p, miR-374a-5p and let-7 family miRNAs could serve as potential biomarkers of IR in children.

5. CONCLUSIONS AND CLINICAL HEALTH IMPLICATIONS / CONCLUSIONES E IMPLICACIONES CLÍNICAS

5.1. Conclusions

The conclusions of the present International Doctoral Thesis are:

I. Sociodemographic and lifestyle factors such as ethnic minority, prematurity at birth, elevated WtHR, SSB consumption, screen time and low cardiorespiratory fitness are consistently associated with the presence of hepatic steatosis in children with overweight or obesity.

II. At present, available screening methods for MAFLD identification in children show two main disadvantages: 1) ALT levels- based cut-points and screening tools showed elevated specificity, but very low sensitivity, and 2) the need of biochemical analysis and/or genetic information in every child with overweight or obesity, which is certainly a large amount of blood testing in children that very often are apparently healthy.

III. The HEPAKID index is the first sociodemographic, lifestyle and anthropometric data-based screening tool for identifying children with overweight or obesity whit elevated risk to suffer hepatic steatosis. The HEPAKID index is a non-invasive, sensitive, inexpensive and easy to perform pre-screening method ideal to use in paediatric primary care setting. However, its limited specificity, suggests the need to additional screening methods for the proper identification of the children with hepatic steatosis.

IV. Biochemical parameters such as, plasma TG, insulin, HOMA-IR, AST, ALT, GGT and ferritin levels, as well as the presence of risk-alleles of *PPARG*rs13081389, *PPARG*rs1801282, *HFE*rs1800562 and *PNLPLA3*rs4823173 polymorphisms, are consistently associated with the presence of hepatic steatosis in children with overweight or obesity. However, their prediction capacity is not enough for the screening of MAFLD. In addition, to date, genetic variables are not easily available in routine clinical practice, which limits their application as a massive screening tool.

V. The HEPAKID prediction protocol shows high sensitivity, specificity and discriminatory capacity to identify paediatric MAFLD in children with overweight or obesity. The combination of sociodemographic, anthropometric, lifestyle, and clinical information within the same algorithm identifies with high sensitivity, specificity and accuracy, as wells as low time-consuming and economic cost children with overweight or obesity who likely suffer from MAFLD, and who should be referred for confirmatory diagnosis.

VI. Circulating miRNAs could be promising diagnostic biomarkers of obesity-associated diseases, such as MAFLD and T2D, already in childhood. However, it was not possible to identify a concrete miRNA profile in children with obesity in the literature.

VII. Circulating miR-660-5p seems to be a biomarker of the presence of MAFLD in preadolescent children, regardless of weight status.

VIII. Circulating miR-320a, miR-142-3p, miR-190a-5p, miR-374a-5p and let-7 family miRNAs could serve as potential biomarkers of IR in children.

5.2. Clinical applications

The main clinical application of the current International Doctoral Thesis is the development of an accurate screening protocol for the identification of overweight or obese children with high risk of MAFLD in primary care. This tool may help to improve the diagnosis and treatment of the disease in paediatric population.

The HEPAKID prediction protocol complies with all of the criteria to be an interesting and useful screening tool in primary care: i) the target disease, MAFLD, is highly prevalent among children with overweight, and it is the most prevalent hepatic disease in developed countries and the second indication for liver transplantation, ii) non-treated MAFLD increases the risk of other chronic diseases such as T2D and CVD, iii) MAFLD is reversible in its early stages, but it can be progress causing an irreversible disease with severe complications and high economic costs, such as NASH, cirrhosis, and hepatic failure, iv) lifestyle-based interventions are effective treatments for MAFLD, v) this protocol based on two easy to perform steps can be applied to all the children diagnosed with overweight or obesity in primary care. The first step of the protocol identifies with elevated sensitivity the children with elevated risk of hepatic steatosis and it does not require any complementary test; only the fulfilment of a simple questionnaire based on anthropometric, sociodemographic and lifestyle data. Those children with elevated risk have to be referred for blood testing to perform the second step. The step, based on easy to measure biochemical parameters, identifies with elevated specificity those children who should be referred for confirmatory diagnosis, vi) its simplicity and low economic cost permits its application to all of the children diagnosed with overweight or obesity. However, it should be validated in larger paediatric multi-ethnic cohorts of children in order to test its reliability.

5.1. Conclusiones

Las conclusiones de esta Tesis Doctoral Internacional son:

I. Factores sociodemográficos y de estilos de vida como la pertenencia a una etnia minoritaria, la prematuridad, un elevado índice cintura-talla, el consumo de bebidas azucaradas, el tiempo de visualización de pantallas, y la baja capacidad cardiorrespiratoria están consistentemente asociados con la presencia de esteatosis hepática en niños/as con sobrepeso u obesidad.

II. Los métodos de cribado de MAFLD pediátrica existentes muestran dos desventajas importantes: 1) los métodos de cribado basados en los niveles de ALT plasmática muestran una especificidad elevada, pero muy baja sensibilidad, y 2) la necesidad de realizar una extracción y analítica sanguínea en un elevadísimo número de niños/as que presentan sobrepeso u obesidad, y que en muchos casos están aparentemente sanos, supone un enorme coste sanitario.

III. El índice HEPAKID es la primera herramienta de cribado basada en datos antropométricos, sociodemográficos y de estilos de vida capaz de identificar a niños/as con sobrepeso u obesidad con elevado riesgo de padecer esteatosis hepática. Este cuestionario no invasivo, sin coste adicional y fácil de cumplimentar es idóneo para realizar el cribado de la esteatosis hepática pediátrica en atención primaria. Sin embargo, su especificidad es limitada lo que sugiere la necesidad de complementarlo con pruebas adicionales para una óptima identificación de la enfermedad.

IV. Los niveles elevados de TG, HOMA-IR, ALT, AST, GGT y ferritina en plasma, así como la presencia de alelos de riesgo de las variantes genéticas *PPARG*rs13081389, *PPARG*rs1801282, *HFE*rs1800562 y *PNLPLA3*rs4823173 se asocian consistentemente con la presencia de esteatosis hepática en niños/as con sobrepeso u obesidad. Sin embargo, estos marcadores presentan dos limitaciones importantes: insuficiente capacidad predictiva de la MAFLD y, actualmente, insuficiente acceso a la información genética en la práctica clínica diaria. Estas características limitan su aplicabilidad como método de cribado masivo en la práctica clínica.

V. El protocolo de predicción HEPAKID muestra una alta sensibilidad, especificidad y capacidad discriminatoria para identificar la MAFLD en niños/as con sobrepeso u obesidad. La combinación de datos sociodemográficos, antropométricos, clínico y de estilos de vida en el mismo algoritmo logra la identificación de

los niños/as con sobrepeso u obesidad susceptibles de padecer MAFLD y que deben ser derivados a unidades especializadas para la confirmación del diagnóstico, con suficiente sensibilidad, especificidad y precisión, así como con una mínima inversión de tiempo y recursos económicos.

VI. Los miRNAs circulantes son biomarcadores prometedores de enfermedades asociadas a la obesidad como la MAFLD y la diabetes mellitus de tipo 2. Sin embargo, no ha sido posible identificar un perfil de miRNAs concreto asociado con las mencionadas comorbilidades en niños/as con obesidad en la literatura científica actual.

VII. El miRNA circulante miR-660-5p parece ser un biomarcador predictivo de la presencia de MAFLD en niños/as preadolescentes, independientemente de su peso corporal.

VIII. Los miRNAs circulantes miR-320a, miR-142-3p, miR-190a-5p, miR-374a-5p y los de la familia let-7 podrían servir como potenciales biomarcadores de la resistencia a la insulina en población pediátrica.

5.2. Aplicaciones clínicas

La aplicación clínica más relevante de esta Tesis Doctoral es el desarrollo de una herramienta para la identificación de MAFLD en niños/as con sobrepeso u obesidad. Este protocolo puede ser de gran ayuda en atención primaria para identificar a los niños/as con elevado riesgo de padecer MAFLD y, así mejorar su diagnóstico temprano y, en consecuencia, su tratamiento en la edad pediátrica.

Este protocolo cumple con todos los criterios para ser una herramienta de cribado útil e interesante para su implementación en atención primaria; i) la enfermedad que identifica es un problema de salud pública alarmante, siendo la enfermedad hepática más prevalente de los países desarrollados y la segunda causa de trasplante hepático; ii) la MAFLD no tratada tempranamente incrementa, además, el riesgo de desarrollo de otras enfermedades crónicas como la diabetes mellitus tipo 2 y las enfermedades cardiovasculares, iii) existe un tratamiento eficaz basado en la mejora de los etilos de vida; iv) en los primeros estadios, la enfermedad es reversible si se detecta y trata precozmente, pero puede derivar en una enfermedad irreversible con graves complicaciones y gran coste económico como la esteatohepatitis y la cirrosis, y en el peor de los casos fallo hepático; iv) el método de cribado propuesto está basado en dos sencillos pasos, mínimamente invasivos:. el primer paso, no necesita ninguna prueba complementaria y con cumplimentar un sencillo cuestionario basado

en información antropométrica, sociodemográfica y de estilos de vida se puede identificar con gran sensibilidad a aquellos niños/as con un elevado riesgo de padecer esteatosis hepática. Estos niños/as, identificados como de alto riesgo, serán derivados a extracción sanguínea y análisis de marcadores bioquímicos de rutina. El segundo paso, permitirá identificar con elevada especificidad a aquellos niños/as que deben de ser derivados a unidades especializadas para confirmar el diagnóstico; v) Su sencillez y bajo coste permite que se aplique en todos los niños/as diagnosticados con sobrepeso u obesidad en atención primaria; vi) la búsqueda de niños/as con la enfermedad es un proceso continuo, contemplando en el mismo protocolo el seguimiento de niños/as con sobrepeso u obesidad, pero sin riesgo actual identificado.

La validación y mejora de esta herramienta de cribado puede ser de gran ayuda en la detección precoz de MAFLD pediátrica. Esta herramienta permite identificar a los niños/as susceptibles de padecer MAFLD con elevada sensibilidad, especificidad y precisión, así como con una mínima inversión de tiempo y recursos económicos.

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