

## Non-invasive methods for diagnosis of non-alcoholic fatty liver disease in children and adolescents: literature review

### Métodos não invasivos para diagnóstico da doença hepática gordurosa não alcoólica em crianças e adolescentes: revisão da literatura

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#### **ABSTRACT**

With the significant increase in the prevalence of nonalcoholic fatty liver disease (NAFLD) in children and adolescents and knowing its association with other clinical conditions and its implications in adult life, noninvasive diagnostic methods may represent new paths for prevention, early diagnosis and follow-up of the evolution of the disease. Objective: To describe noninvasive methods for diagnosing NAFLD in children and adolescents. Methods: integrative review. The articles were searched in the PubMed, Lilacs, Medline, Embase and Google Scholar databases, from August to December 2021. The descriptors "non-alcoholic fatty liver disease", "non-invasive methods", "biomarkers", "child" and "adolescent" were used. Results: Several biomarkers have been associated with the diagnosis of NAFLD, and among the most recognized are the lipid and

glucose profile, liver enzymes, anti-inflammatory profile and assessment of body fat reserves. Antioxidant profile, uric acid, genome, lipidomics and proteomics evaluations are also predictors of steatosis; however, it remains to be determined whether these markers should be used together or in isolation for better results. Conclusions: Noninvasive markers for the diagnosis of NAFLD in children and adolescents are promising and, when used in association with clinical data, can be useful in guiding and managing this population for the prevention and control of NAFLD.

**Keywords:** nonalcoholic fatty liver disease, noninvasive methods, child, adolescent.

## RESUMO

Com o aumento significativo da prevalência da doença hepática gordurosa não alcoólica (DHGNA) em crianças e adolescentes e conhecendo sua associação com outras condições clínicas e suas implicações na vida adulta, os métodos diagnósticos não invasivos podem representar novos caminhos para prevenção, diagnóstico precoce e acompanhamento da evolução da doença. Objetivo: Descrever métodos não invasivos para o diagnóstico de DHGNA em crianças e adolescentes. Métodos: revisão integrativa. Os artigos foram pesquisados nas bases de dados PubMed, Lilacs, Medline, Embase e Google Acadêmico, no período de agosto a dezembro de 2021. Os descritores "doença hepática gordurosa não alcoólica", "métodos não invasivos", "biomarcadores", "criança" e "adolescente" foram usados. Resultados: Vários biomarcadores têm sido associados ao diagnóstico de DHGNA, e entre os mais reconhecidos estão o perfil lipídico e glicêmico, enzimas hepáticas, perfil anti-inflamatório e avaliação das reservas de gordura corporal. As avaliações do perfil antioxidante, ácido úrico, genoma, lipidômica e proteômica também são preditores de esteatose; no entanto, resta saber se esses marcadores devem ser usados juntos ou isolados para melhores resultados. Conclusões: Marcadores não invasivos para o diagnóstico de DHGNA em crianças e adolescentes são promissores e, quando utilizados em associação com dados clínicos, podem ser úteis na orientação e manejo dessa população para prevenção e controle da DHGNA.

**Palavras-chave:** doença hepática gordurosa não alcoólica, métodos não invasivos, criança, adolescente.

## 1 INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is characterized by hepatic alterations involving deposition of more than 5% of free fatty acids (FFAs) in hepatocytes in individuals without excessive ethanol consumption (>20 g/dial).<sup>1,2</sup>

NAFLD is the most common chronic liver disease in children and adolescents in the developed world, and it is considered to be a hepatic manifestation of metabolic syndrome (MS), in addition to being responsible for the increased prevalence of cardiovascular diseases in young individuals, who are mainly patients between 10 and 18 years old.<sup>3</sup>

The prevalence of NAFLD is diverse between regions, as it depends on aspects such as age and sex, genetic background, environmental conditions, diagnostic criteria and the nutritional status of the investigated individuals.<sup>3,4</sup> In Europe, the prevalence of NAFLD is estimated at 25%, and in the United States, it is estimated to be 34% in adults and 10-20% in children. In Asia, even though the average body mass index (BMI) is lower, rates as high as 30% have been reported.<sup>4,5</sup> In Brazil,

it is estimated that among children and adolescents, the prevalence of NAFLD is between 3% and 11% and that this number can vary

from 46% to 80% among obese youth. In Salvador-Ba, in a study carried out with obese children and adolescents, the prevalence was 62.2%, being more frequent in boys (58.9%).<sup>5</sup>

In these groups, the disease is less common in children between 2 and 8 years of age, but it appears more frequently in those over 10 years of age, and the average age at diagnosis is between 11 and 13 years.<sup>6</sup> NAFLD is more frequent in male adolescents, and this finding has been attributed to changes in sex hormones, the fact that obesity is more prevalent in this age group and the longer exposure to risk factors for the disease.<sup>7</sup>

Young patients may have symptoms such as difficulty sleeping, fatigue, irritability, headache, nausea, and right upper quadrant discomfort, but a total absence of symptoms is not uncommon.<sup>8-10</sup> The lack of symptoms results in delayed diagnosis and subjects patients to longer exposure to the disease and harmful metabolic processes, as well as minimizing prevention since it is not routinely investigated.<sup>4</sup>

Overweight and obesity, metabolic syndrome, insulin resistance (IR), acanthosis nigricans, increased neck circumference (NC) and biochemical alterations are some of the clinical aspects associated with NAFLD.<sup>10</sup>

Recently, the discovery of genes whose polymorphisms are involved in the accumulation of fat in the liver points to the possibility that genetic factors also make up clinical aspects of NAFLD. Among the genes related to NAFLD, PNPLA3 predisposes to the production of an abnormal protein that promotes the accumulation of triglycerides in the liver, the loss of triglyceride hydrolase activity and a gain of lipogenic activity. Its variant I148 M, the main genetic risk factor for nonalcoholic steatohepatitis in adults, is associated with high levels of liver enzymes and a degree of steatosis in obese children. In addition, TM6SF2 E167K leads to the accumulation of liver fat through a reduction of very low-density lipoprotein secretion, and the GRP120 R270H variant reduces the anti-inflammatory action of the Kupffer cell receptor.<sup>7,11</sup>

The pathophysiology of this disease has not been completely elucidated; however, food intake and regulation of metabolism, transcription factors and lipid metabolic pathways are possible axes for its onset.<sup>12</sup>

Histological expression of NAFLD varies from steatosis to steatohepatitis and cirrhosis. Several factors contribute to disease progression, such as food intake, nutritional status, lack of physical activity and genetic predisposition.<sup>12,13</sup>

The test considered the gold standard for the diagnosis of NAFLD is a liver biopsy, an exam that is invasive and expensive. Ultrasonography (USG), a noninvasive and cheaper test than biopsy, can diagnose steatosis, but it cannot describe the liver histology.<sup>14</sup> Despite the availability of these

diagnostic procedures, not all cases can be evaluated from samples of the liver tissue or USG, which makes the search for new diagnostic methods important.

Considering the rapid increase in the prevalence of the disease and its various complications, greater knowledge about new aspects, such as noninvasive tests, is necessary to recognize new forms of prevention, early diagnosis and analysis of the disease's progression. Thus, this article aimed to describe noninvasive methods for diagnosing NAFLD in children and adolescents.

## 2 METHODOLOGY

This is an integrative review work and articles were searched in PubMed, Lilacs, Medline, Embase and Google Scholar databases, from August to December 2021. The descriptors "non-alcoholic fatty liver disease", "non-invasive methods", "biomarkers", "child" and "adolescent" and the Boolean operator "AND" were used in the searches. The searches were carried out independently by 3 evaluators and, after reading the titles and abstracts, articles were selected that were cross-sectional, case control or cohort, that the investigations were in children and/or adolescents and written in English, Spanish or Portuguese.

## 3 RESULTS AND DISCUSSION

A total of 79 articles were found that, after the analysis and selection made by the 03 evaluators, were summarized in 18 articles, read in full and used in the preparation of this review.

One of the most common investigations evaluated among these articles the association of elevated transaminases with NAFLD. Elevated liver enzymes, gamma glutamyl transferase ( $\gamma$ GT) and alanine aminotransferase (ALT), were identified as possible predictors of NAFLD; however, there is still no consensus on reference values for the assessment and diagnosis of this disease.<sup>3,15</sup> These increases seem to occur because in the presence of increased accumulation and decreased release of FFAs in the liver, beta oxidation and mitochondrial oxidation are induced, which generates oxidative stress and causes the destruction of hepatocytes. These changes can influence the biliary tract and increase serum levels of  $\gamma$ GT and ALT in young and obese patients during the initial stage of the disease.<sup>5,15,16</sup>

A high serum concentration of  $\gamma$ GT has been suggested to be an isolated predictor of NAFLD. For ALT, since NAFLD is diagnosed in children and adolescents with variable levels of this enzyme, it should be used in conjunction with other indicators and/or with imaging tests.<sup>16</sup> In a study carried out in Canada by Saad et al.<sup>17</sup>, an elevation of ALT, in the presence of MS in obese males, could be used as a noninvasive predictor of NAFLD.

Dyslipidemia is related to NAFLD and is established in the literature as a clinical aspect of the disease. In addition, low-density lipoprotein cholesterol (LDL-c) elevation is considered to be

an independent indicator in the work of El-Karakasy HM et al.<sup>18</sup>, and triglyceride (TGL) elevation is also a good indicator when it occurs in parallel with a high BMI and increased waist circumference (WC), which characterizes the existence of a phenotype of hypertriglyceridemic waist associated with the diagnosis of NAFLD.<sup>15</sup>

Based on the involvement of lipid metabolism alterations in NAFLD, a lipidomics assessment was performed, which showed a significant increase in molecular species of alkyldiacylglycerol (TG [O]) and phosphatidylethanolamine (PE) lipids and a significant decrease in alkyl/alkenyl-phosphatidylethanolamine (PE) [O]), alkyl/alkenyl-lysophosphatidylethanolamine (LPE [O]) and alkyl/alkenyl-phosphatidylcholine (PC [O]) in obese children with steatosis compared to obese children without steatosis.<sup>19</sup> The increase in TG [O] seems to be derived from the increased secretion of very low-density lipoprotein cholesterol (VLDL) or be due to de novo lipogenesis, which is increased in NAFLD.<sup>19</sup> The decrease in ether-phospholipids such as PC [O] and PE [O] may be associated with lower dietary intake of choline and ethanolamine, which are essential for the production of ether-phospholipids. Alternatively, the increase in saturated and monounsaturated fatty acids, associated with a diet rich in saturated fats and poor in polyunsaturated fatty acids, would lead to a shortage of docosahexaenoic acid and arachidonic acid, components that enrich the ether-phospholipids.<sup>19</sup> The exact role of LPE in NAFLD is unknown, but it is known that it is produced from the partial hydrolysis of PE and that it is a secondary component of the cell membrane.<sup>19</sup>

Additionally, considering the lower invasiveness and ready availability of plasma assessment, another analysis developed was proteomics, which identifies, describes and quantifies proteins involved in inflammatory processes and in the response and regulation to stress.<sup>20</sup> Some studies of this type were carried out in adults, and Pawel et al., in 2020, performed a similar analysis in children with and without NAFLD. The study found upregulation of afamin, retinol-binding protein-4, complement components and hemopexin and the downregulation of serum protease inhibitors, clusterin, immunoglobulin chains and vitamin D-binding protein. The authors considered these results to be important for describing a possible proteomic profile present in children with NAFLD; however, in this work, they did not justify such findings.<sup>20</sup>

Although the mechanism of uric acid involvement in NAFLD has not been fully explained, some studies in obese adults have described it as an independent marker of NAFLD in the early stages of the disease, given the fact that the adults and children evaluated are obese and are in the initial period of the disease.<sup>4</sup> One of the relevant issues involving uric acid is that, at concentrations considered normal, it exerts a neuroprotective effect, acting as a free radical scavenger. However, in the presence of oxidative stress, it promotes an increase in interleukin 6 (IL-6) and tumor necrosis factor (TNF- $\alpha$ ), becoming involved in the inflammatory process and losing the ability to distribute free radicals, as the latter are found in greater concentrations in these cases.<sup>4,21,22</sup> Another hypothesis

about the experience of measuring serum uric acid as a noninvasive marker is that it participates in the response to metabolic disorders, and it seems that in NAFLD, its expression is increased by hyperinsulinemia and its consequent decreased renal excretion.

Obesity is a risk factor for NAFLD widely cited in the literature. In obese individuals, there is greater FFA circulation and a greater chance of developing insulin resistance, which could explain its association with the disease under study.<sup>7,23</sup> However, these mechanisms cannot completely clarify the association of NAFLD with obesity, as not all obese children develop NAFLD. One of the explanations is that truncal adiposity is more decisive in the development of hepatic fat accumulation than peripheral adiposity.<sup>24,25</sup> Another issue is the possibility that visceral fat deposition is more influential in the accumulation of liver fat than subcutaneous fat, since it releases a higher concentration of FFAs directly to the portal vein, being more involved in the inflammatory process and consequently in the severity of liver injury.<sup>24,25</sup> On the other hand, subcutaneous suprailiac adipose tissue and intra-abdominal depth, measured ultrasonographically, were identified as independent predictors of hepatic steatosis in young people.<sup>25</sup>

Although there is a different contribution of tissues, measures of nutritional status such as BMI, WC, waist-to-height ratio and NC, used to measure total or central body fat, were identified as predictors of NAFLD when evaluated in conjunction with other indicators, such as biochemical or imaging.<sup>17,25,26</sup>

In an attempt to identify noninvasive NAFLD predictors, screening protocols for the disease in obese children and adolescents were tested. The results of these protocols were compared to imaging and biochemical tests; however, none of the tested protocols had an additional predictive value and are not indicated for the diagnosis of NAFLD, not even the one that tested new biomarkers.<sup>27,28</sup>

The inflammatory process of NAFLD has been established and appears to underlie obesity, MS, type II diabetes and/or IR. Thus, there is a strong justification for measuring the levels of TNF- $\alpha$ , interleukins and C-reactive protein (CRP) in individuals at risk or diagnosed with NAFLD.<sup>13</sup> TNF- $\alpha$  is responsible for the increase in lipolysis and the consequent increase in the flow of FFAs and their accumulation in the liver, by suppressing the activity of insulin receptor kinase, by suppressing the expression of genes involved in glucose uptake causing hyperinsulinemia, and by promoting the expression of IL-6, which reduces the expression of the insulin receptor substrate.<sup>29</sup> IL-6, in turn, stimulates the secretion of an acute phase protein of inflammation.<sup>5</sup>

Thus, another investigation was to test whether TNF- $\alpha$  and leptin had power in predicting the NAFLD Activity Score (NAS), which is used to predict the histological condition in patients with or without NAFLD. The results showed that there was an association between these two markers, alone or in combination, and  $NAS \geq 5$ . This seems to be a promising result because the determination

of NAS requires more biological resources and higher costs for its determination compared to the serum levels of TNF- $\alpha$  and leptin.<sup>29</sup> There was also, in a group of obese children and adolescents from Salvador-Bahia, an association of TNF- $\alpha$  and CRP with the degree of obesity and with IR and NAFLD when patients were severely obese.<sup>5</sup>

Thus, these results point to confirmation of the involvement of markers of the inflammatory process in NAFLD, and based on this understanding, it can be suggested that children and adolescents who present with risk factors for NAFLD or who are obese should be monitored with TNF- $\alpha$ , CRP and IL-6.

Another marker considered by many researchers to be an indicator of inflammation and fibrosis in NAFLD is cytokeratin-18 (CK-18). This protein is an intermediate fragment of hepatic epithelial cells and it reflects the degree of apoptosis of these cells and is considered by some researchers to be one of the possible mechanisms involved in the development of NAFLD. This process can cause activation of stellate cells associated with fibrosis.<sup>28,30</sup>

To investigate the involvement of CK-18 in the mechanism of NAFLD formation and progression in children and adolescents, a cross-sectional study was carried out in Ukraine in 2020. In this test, the highest level of CK-18, which may indicate the transformation of steatosis into steatohepatitis, was shown to be twice as high in adolescents with obesity and NAFLD in the presence of insulin resistance.<sup>30</sup> Additionally, in line with the analysis of the inflammatory process in NAFLD, a study was carried out that evaluated the dysmetabolism of branched-chain amino acids (BCAAs), as there is a metabolically unhealthy phenotype associated with obesity, steatosis, liver damage and inflammation.<sup>31</sup>

This study did not identify a link between inflammation and BCAA concentrations; however, in the studied sample, there was an increase in BCAA concentration, which was able to differentiate children with and without NAFLD. The association presented can be explained by the decrease in the expression of catabolic enzymes, which leads to an increase in BCAAs and, consequently, promotes an increase in oxidative stress, lipid accumulation and liver damage.<sup>31</sup>

In this same study, a linear regression model adjusted for age, sex and pubertal stage was tested, consisting of an analysis of BCAAs, ALT,  $\gamma$ GT, ferritin and insulin that predicted MRI-PDFF (magnetic resonance-derived proton density fat fraction) ( $R = 0.75$ ,  $p < 0.01$ ). The ROC analysis of this model revealed AUCs of 0.85, 0.85 and 0.92 for the detection of some degree of steatosis, for a moderate degree and for a severe degree, respectively.<sup>31</sup>

In NAFLD patients, the occurrence of small intestine bacterial overgrowth and increased intestinal permeability have already been shown, alterations that can be correlated with both the presence and severity of steatosis.<sup>32,33</sup> This is because the liver is the first line of defense against intestinal-derived antigens and one of the organs most exposed to toxic substances from the

intestine, such as bacteria and bacterial products, as it receives 70% of its blood from the intestine through the portal vein.<sup>32,33</sup>

Zonulin is a biomarker known to regulate intestinal permeability by modulating intracellular tight junctions and it participates in the development of innate intestinal immunity. Recognizing the role played by zonulin in the human body, a study carried out in Italy in 2014 tested the possible association of this mediator with the NAFLD stage. In this study, increased circulation of zonulin was correlated with steatosis and was associated with its severity in obese children and adolescents.<sup>34</sup>

According to the authors of this study, the results are clinically relevant; however, the study design and the fact that the results were not compared with patients with other intestinal diseases and without NAFLD limit understanding of the association between increased serum zonulin concentration and the disease investigated here. Keeping in mind the limitations, one can also consider the absence of control patients without compromising their nutritional status for comparison with the group tested by Pacifico et al.<sup>34</sup>

In adult patients, antibodies such as anti-mitochondrial antibody (AMA) and smooth muscle antibody (ASMA) are positive in patients without autoimmune liver disease but with chronic liver disease, which points to the possibility of the dosage of these antibodies being possible noninvasive markers for DHGNA.<sup>35</sup> In other studies, autoantibody positivity was present in apparently healthy children with no personal or family history of autoimmune disease, with rates of 3% for anti-nuclear antibody (ANA), 2.6% for ASMA and 1.1% for AMA.<sup>36</sup> Based on this prior knowledge, a cross-sectional study was carried out in California in 2008<sup>37</sup>, and its results did not show positivity for AMA, but the prevalence of positivity for ANA was 18% and positivity for ASMA was 32%. In their sample, positivity for ASMA was a significant predictor of histological severity in NAFLD. The real importance of autoantibody positivity in pediatric NAFLD is unknown, but this result is considered relevant in the search for noninvasive markers and deserves further investigation.<sup>35,36</sup>

In other cross-sectional studies, a series of biomarkers were evaluated and compared with liver biopsy.<sup>38,39</sup> Among the markers were some already tested in adults that had no association with NAFLD in children and adolescents. However, in those groups, patients with moderate to severe steatosis had higher levels of insulin-like growth factor (IGF-II), resistin, and soluble FAS ligand.

The role of IGF-II in NAFLD is not well understood; however, it is known to be involved in the development of diseases such as type 2 DM, cardiovascular diseases and cancer. Resistin, on the other hand, is a proinflammatory adipokine whose main properties associated with NAFLD are the ability to induce inflammation and insulin resistance.<sup>38,39</sup>

The Fas protein (CD95) is a membrane glycoprotein of the same family as TNF- $\alpha$ , and it may also be associated with chronic diseases with an expressive inflammatory profile, such as NAFLD,



in which case its participation is linked to its apoptotic mechanism when present in high doses.<sup>38,39</sup>

Another factor considered a possible noninvasive NAFLD marker is transforming growth factor beta (TGF- $\beta$ ). A study carried out with overweight adolescents, with or without NAFLD, showed an association of this marker with the disease. The explanation seems to lie in the fact that TGF- $\beta$  contributes to the inhibition of the growth of normal hepatocytes and stellate cells.<sup>40</sup>

Through the various attempts presented here to identify a noninvasive diagnostic method, a number of possibilities can be seen; however, there is still no consensus on which methods are better.

#### 4 CONCLUSION

Liver biopsy is still the gold standard for staging NAFLD, although it has many limitations, such as high cost, invasiveness and sampling variability and the fact that it is not indicated for screening, long-term monitoring of disease development or as a parameter for monitoring the response to treatment, especially among young people.

USG has been used as a parameter for the diagnosis of NAFLD. This is not an invasive test, but its limitations include the fact that it cannot evaluate the liver histology, and, therefore, it has to be complemented with other tests in clinical practice. Even with only moderate application, USG has become one of the means of early diagnosis of the disease and, in practice, serves as a trigger for further investigation into hepatic and metabolic health in asymptomatic youngsters.

The present review suggests that other methods considered noninvasive could assist in the diagnosis of NAFLD in children and adolescents when evaluated within a clinical context. However, it is still necessary to establish threshold values and markers that work better when isolated or associated with each other and to understand more securely how these markers work in eutrophic patients compared to obese patients.

These findings also reinforce the need to recognize the existence of the disease and its importance as a clinical problem that triggers relevant liver complications in adulthood, a fact that is often overlooked in clinical practice.

While these new tests are not fully established, it can be considered that the results are of great clinical importance because it is possible to know the biochemical profile of children and adolescents with NAFLD and use these markers, when available, as a screening tool for NAFLD in groups of young patients.

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