J Med Biochem 2015; 34 (1) DOI: 10.2478/jomb-2014-0059

UDK 577. 1 : 61 **ISSN 1452-8258**

J Med Biochem 34: 3–12, 2015

Review article
Pregledni članak

PEDIATRIC NON-ALCOHOLIC FATTY LIVER DISEASE

OBOLJENJE NE-ALKOHOLNE MASNE JETRE U PEDIJATRIJI

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Summary: The rapidly increasing prevalence of childhood obesity and its associated co-morbidities such as hypertriglyceridemia, hyper-insulinemia, hypertension, early atherosclerosis, metabolic syndrome, and non-alcoholic fatty liver disease are major public health concerns in many countries. Therefore the trends in child and adolescent obesity should be closely monitored over time, as in the near future, we may anticipate a major increase of young adults with the stigmata of the metabolic syndrome, and of the related non-alcoholic fatty liver disease (NAFLD), that may lead to non-alcoholic steatohepatitis.

Keywords: paediatric obesity, non-alcoholic fatty liver disease, NAFLD, steatohepatitis

Kratak sadržaj: Naglo povećanje prevalence gojaznosti u detinjstvu i pratećih ko-morbiditeta kako što su hipertrigliceridemija, hiper-insulinemija, hipertenzija, rana ateroskleroza, metabolički sindrom, i ne-alkoholna masna jetra su glavni zdravstveni problemi u mnogim zemljama. To znači da trendove gojaznosti kod dece i omladine treba pratiti blagovremeno, s obzirom da se u bliskoj budućnosti očekuje povećana učestalost metaboličkog sindroma u ovoj populaciji, kao i pratećih oboljenja kao što je oboljenje ne-alkoholne masne jetre (NAFLD), koja može prouzrokovati ne-alkoholni steatohepatitis.

Ključne reči: pedijatrijska gojaznost, ne-alkoholna masna jetre, NAFLD, steatohepatitis

Impact of childhood overweight and obesity on health

The rapidly increasing prevalence of childhood obesity and its lot of associated co-morbidities, namely hypertriglyceridemia, hyper-insulinemia, hypertension, early atherosclerosis, metabolic syndrome, and non-alcoholic fatty liver disease (NAFLD) have become major public health concerns in developed and developing countries (1, 2). It follows that the trends in child and adolescent obesity should be closely monitored over time. However, this may prove to be more difficult than anticipated, as several definitions of child obesity are utilized, and as there are ethnic differences in body fat content (3). Body mass indices (BMI) (weight/height^2) of 30 kg/m^2 and of 25 kg/m^2 are widely accepted as the cut-off points for obesity and overweight respectively in adults (4). However no such definite values can be used in childhood and adolescence, as body mass index changes substantially from birth to adulthood (5, 6). To circumvent this issue, Cole et al. (7), using different national age- and sex-specific data sets, have developed age- and sex-specific BMI cut-off points to define overweight and obesity by using different national age- and sex-specific weight data sets. These are illustrated in *Figure 1*. Being less arbitrary, these data should permit a more precise evaluation of youth obesity and may change the future evaluation of paediatric obesity prevalence. Independently of the defi-

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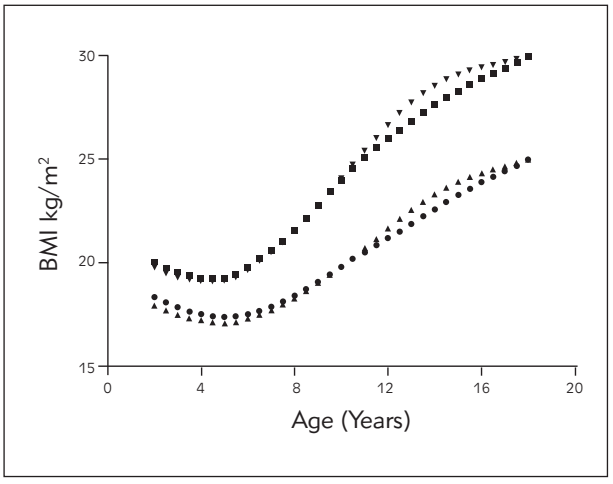

Figure 1 International age- and sex-specific cut-off points for BMI for overweight and obesity.

Adapted with permission from Cole TJ et al. (7). Data was obtained by averaging the national centiles. BMI: Body Mass Index. Filled circles: curve for overweight boys; filled square: curve for obese boys: filled upward triangles: curve for overweight girls; filled downward triangles: curve for obese girls.

nition of childhood overweight and obesity, secular trends that whilst their prevalence have plateaued in some countries it has continued to rise in others (8–11).

Hence, in the near future, we may anticipate a major increase of young adults with the stigmata of the metabolic syndrome (MetS), and of the related non-alcoholic fatty liver disease (NAFLD), that may lead to non-alcoholic steatohepatitis (NASH) (12, 13).

Epidemiology of the NAFLD and NASH

The term NAFLD refers to an array of conditions that range from hepatic steatosis alone to steatosis accompanied by inflammation and fibrosis (NASH) and cirrhosis (14). Due to the lack of specific non-invasive methods for the diagnosis of NAFLD, its prevalence in the paediatric population is ill defined. Schwimmer et al. (15), in an excellent retrospective autopsy-based study for all causes of death, conducted on 742 San Diego County children, aged between 2 and 19 years, reported a 9.6% prevalence of fatty liver disease (defined as 5% of hepatocytes containing macro-vesicular fat) after adjustment for age, gender, race, and ethnicity. Importantly, they pointed out that the prevalence rose to 38% in obese children. They further noted ethnic differences in the prevalence of fatty liver in obese children, Black children being less prone and Hispanic children being at higher risk. On the other hand, an early study in Japan conducted in 810 children aged between 4 and 12 years, estimated the prevalence at 2.6% based on ultrasonographic examination of the liver (16). The establishment of the prevalence of NAFLD in the adult population is inconsistent because subjects are mostly asymptomatic in the early phase of the disease, and because the most frequently used non-invasive biomarkers have variable sensitivity and sensitivity. As a point in case, ALT and AST have been shown to be both insensitive and nonspecific for diagnosing chronic liver disease (17, 18). This is even truer in youths. North- American, European and Asian studies have reported prevalence in obese children that spanned between 10% and 77% (19–22). The paucity of conclusive reports on the prevalence of NAFLD in youths and adolescents supports the development of better methodologies than the current biochemical marker alanine-aminotransferase (ALT) or aspartate-aminotransferase (AST), two surrogate routine work-up markers for hepatocyte apoptosis.

Natural history of NAFLD and NASH

Obesity is a major risk factor for developing NAFLD. Monteiro et al. (23) in a small group of overweight and obese youths, using the age- and sex-specific weight cut-off points derived by Cole et al. (7), have shown that intra-abdominal and total fat masses, evaluated by liver ultrasound, were important risk factors to develop NAFLD.

The development of NAFLD, clearly linked to the MetS, involves a multitude of extensively reviewed processes that are affected by the genetic background, the environment and the lifestyle (24–28). In brief, NAFLD refers to hepatic vesicular fat depots attributable to an impairment of free-fatty acid (FFA) mobilization and TG accumulation that likely result primarily from excessive carbohydrate consumption. These metabolic events inexorably lead to perturbations of insulin, insulin growth factors, leptin, adiponectin and other adipokines signalling pathways (28, 29). This first stage largely goes unnoticed and may be stable for years. In 5 to 10% of cases, patients evolve to the second stage in which hepatocellular ballooning (mostly in adults), inflammation and peri-sinusoidal fibrosis are observed (15). The inflammatory foci are most probably the result of a disrupted redox balance, linked to mitochondrial dysfunction, and activation of a number of cytokines that induce cell apoptosis (30, 31). This set of events is now being accepted as the »second hit« in the development of NASH (32). Finally, a fraction of patients (particularly adults) may progress to cirrhosis and liver failure that will require liver transplantation (33, 34). Brief overviews of the NAFLD/NASH histopathology characteristics as well as of the current imaging and biochemical markers used for their diagnosis follow.

Histopathology

Figure 2 illustrates the histology of normal, NAFLD and NASH liver sections stained with haematoxylin and eosin. Panel A represent a normal liver section (×400); Panels B-D are examples at different magnifications of a paediatric pattern of non-alcoholic steatohepatitis. In the paediatric population, the prominent histological features are localized chronic inflammation and fibrosis, severe steatosis, perisinusoidal fibrosis but absence of ballooning degeneration (35). Evolution of NAFLD to cirrhosis has been shown in ≤ 1%–5% of cases in the paediatric population (36). Schwimmer et al. (37) have classified this pattern of injury respectively as types 2 and 1 NAFLD. However, in some cases, an overlap occurs, and a shift from a type 2 to a type 1 pattern has been described in older children (38). Histological principal features of adult and paediatric NAFLD are graded utilizing activity-scoring systems, (NAS) that are summarized in *Table I*. The degree of steatosis (0–3), lobular inflammation (0–2) and hepatocyte ballooning (0–2) and fibrosis (0–4) are combined into a score ranging from 0 to 11. A NAS score ≥ 5 denotes a diagnosis of NASH, whereas, 3–4 corresponds to a borderline lesion and ≤ 2 denotes non-NASH lesions. However, since the paediatric injury is characterized by the presence of chronic portal inflammation associated with portal fibrosis in the absence of hepatocyte ballooning, Aly et al. (40) have proposed to grade the fibrosis in paediatric NAFLD using the Index of Fibrosis, PNFI (0=none; 1=periportal or perisinusoidal fibrosis; 2=perisinusoidal and portal/periportal fibrosis; 3=bridging fibrosis; and 4=cirrhosis). In this 0–4 accessory grading, the histological spectrum ranges from steatosis, necro-inflammation, ballooning to cirrhosis.

Percutaneous liver biopsy remains the gold standard for the diagnosis of NAFLD and NASH. However this invasive procedure is not without risk, even when guided by imaging technologies. It also requires deep sedation, and risks of haemorrhage, infection or injury to the liver are not totally excluded (41). Other non-invasive methods have thus been explored as surrogate diagnostic tools.

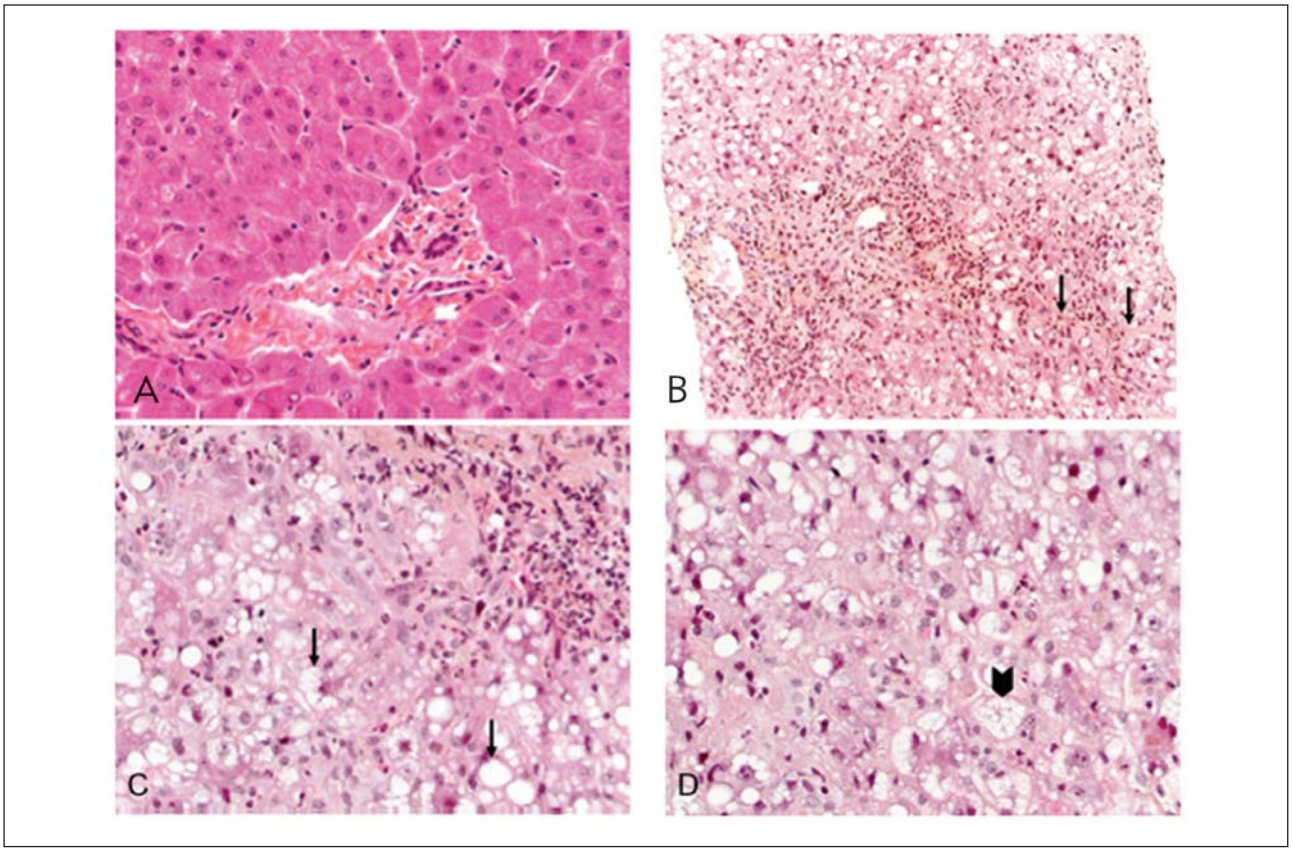

Figure 2 Histological patterns for normal liver, liver steatosis and liver steatohepatitis.

Hematoxylin and eosin stain A: normal liver (x400); B (X200), C (X400), D (X400): non-alcoholic steatohepatitis (pediatric pattern) an example of scoring following the NASH Clinical Research network Scoring System, portal-based fibrosis (arrows) score1C with portal inflammation score 3 and minimal lobular inflammation score 1, macro and micro-vesicular steatosis pan-acinar (black arrows) score 3 with contiguous patches, with few ballooning cells (arrow head) score 1. No granulomas, acidophil bodies, pigmented macrophages, mega mitochondria, glycogenated nuclei were observed.

Table I Histological NAFLD activity scoring systems.

Main scoring system (Ref. 39)	For adult and paediatric cases
Steatosis	0–3
Lobular inflammation	0–2
Hepatocyte ballooning	0–2
Fibrosis	4
Maximum score	11
Scores interpretation	
≥5	NASH
3–4	Borderline lesions
≤3	No NASH lesion
Accessory scoring for Fibrosis (Ref. 40)	Proposed for paediatric cases
No fibrosis	0
Periportal or perisinusoidal fibrosis	1
Perisinusoidal &portal/ eriportal fibrosis	2
Bridging fibrosis	3
Cirrhosis	4
Maximum score	4

Imaging techniques

In the last decade, a number of non-invasive tests based on ultrasonography, transient elastography, and magnetic resonance imaging (MRI) have been developed to assess the extent of liver fibrosis. Although widely available, traditional utrasonography has a poor sensitivity to detect mild steatosis and is inaccurate in quantifying liver fatty acid infiltration. Furthermore this technique is observer and operator-dependent, and its use in obese patients is subject of discussion (42, 43). Transient elastography, based on the assessment of liver stiffness, has also been shown to be useful in the evaluation of a significant fibrosis and cirrhosis (44–46). These approaches are however not universally accessible, and are mainly meant to detect fibrosis, an advanced stage of the disease.

Liver magnetic resonance imaging–estimated proton density fat fraction (PDFF) seems to be more sensitive and thus promising. It has been favourably compared to histopathology scores for hepatosteatosis. MR imaging-PDFF was significantly correlated to the histologic grades, and could discriminate between those patients with grade 0 and 1 or higher in one study (47) and between grade 1, 2 and 3 in another study (48). This technology, presently restricted to tertiary care institutions, is expensive and demands experienced staff. In summary these imaging techniques are useful in detecting steatosis, but are relatively inefficient in determining the level of liver damage, particularly at an early stage. Therefore there is a need to develop biomarkers that will be easily measured in central laboratories.

Aminotransferases as biomarkers

Despite not being an exclusive hepatic enzyme, serum alanine aminotransferase (ALT) has been the most commonly used laboratory test for the diagnosis of hepatic ailment for the past 60 years. Yet we are still confronted with interpretation-related problems, particularly in paediatrics, as the there is no real consensus on the threshold value to adopt for detecting liver disease. The difficulties are due to a number of biological factors such as the circadian rhythm, anthropometric and demographic characteristics (49-52). The recent review of Pacifico et al. (53) covers the subject in details. Schwimmer et al. (54) have advocated the standardization of ALT reference intervals in children, and initiated the SAFETY study (Screening ALT For Elevation in Today's Youth) to develop them. To do so, they collected data from 43 acute care children's hospitals, from the 1999-2006 population-based National Health and Nutrition Examination Survey (NHANES) and from clinically characterized children with and without liver diseases. In addition they identified children with normal liver fat content using MRI. Using 982 NHANES healthy, metabolically normal and liver-disease-free participants, aged between 12 and 17 years, as reference group, they identified the upper limit of normal (95^{th} percentile) for ALT as 25.8 IU/L for boys and 22.1 IU/L for girls. Importantly, the prevalence of abnormal ALT values among the entire NHANES was 2.4% for boys and 1.3% for girls, if the hospitals' cut-off values were used. Disturbingly, the prevalence however rose to 15% and 8.6% respectively when the NHANES upper limit of normal were used, thereby demonstrating the importance of using well defined biology-based thresholds. The lack of uniformity in upper ALT thresholds is further illustrated by Wiegand et al (55) who reported a NAFLD prevalence of 11% in 16390 overweight, obese and extremely obese adolescents. This relatively low prevalence in such at-risk population can partially be explained by the higher than usual ALT and/or AST of >50 IU/L. Hilsted et al. (56) recently reported reference intervals for 21 common biochemical tests, among which ALT, in 1429 Danish school children and adolescents. Upon rejecting outliers according to the Dixon rule, they reported the ALT upper reference range as 47 IU/l with a 95% confidence interval ranging from 33 to 69 IU/L. These are different from the often referred upper threshold of 40 IU/L, and if applied would decrease the prevalence of NAFLD in a given population.

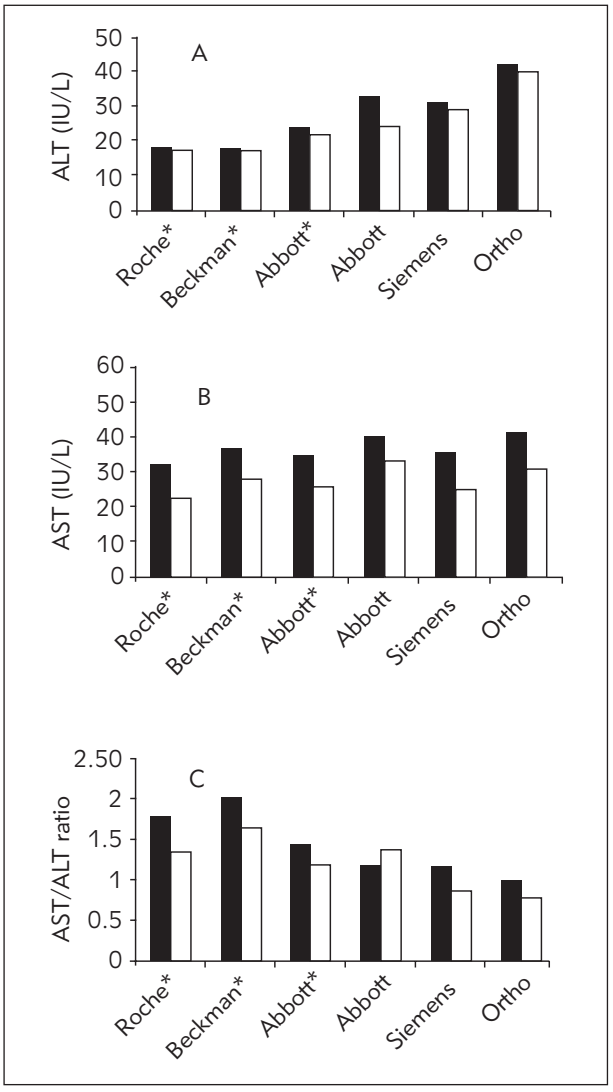

Figure 3A–C Transaminases Reference values according to the analytical platform.

Legend: *The transaminases were measured with exogenous pyridoxal phosphate added according to the Manufacturers instructions. Adapted from Estey et al. (54).

In the definition of reference values, methodological issues related to the analytical platforms, the buffer constitution, and the presence or absence of pyridoxal-phosphate as co-factor should not be underestimated. Neuschwander-Tetri et al. (57), using a CAP survey, have shown that although the intra-laboratory variations were small, there were non-negligible differences between analytical platforms. We have observed similar variations (*Figure 3A*) between 5 platforms tested in the CALIPER study (58). The short-term biological variability of serum ALT compounds the uncertainty of this test. Indeed, Lazo et al. (59) have reported that using the NHANES III cut-offs of normal levels, 31% of the adults with elevated ALT on the first measure would be classified normal upon a second investigation. This was linked to a high intra-individual variability for this enzyme that could be due to exercise and circadian rhythm. Aspartate aminotransferase (AST) is the second enzyme used in the context of defining liver cell damage. Likewise however will the reference values depend on the analytical platform (*Figure 3B*). The AST/ALT ratio is also commonly used to detect liver impairment. In a series of 70 adults with NASH, Sorbi et al. (60) have shown elevated AST and ALST with a median ratio 0.9 (range 0.3–2.8). Two groups reported independently abated AST/ALT ratios. The first by Iacobellis in 2006 (61), concerned 69 children aged between 4 and 19 years, with persistent elevation of ALT and AST associated with liver echography suggestive of fatty infiltration. The mean ± SD AST/ALT ratio in this series was 0.7±0.3 and 0.9±0.5 for patients without and with liver fibrosis respectively. The second study by Yang et al. (62), conducted in 77 obese children and adolescents, revealed a much lower AST/ALT ratios that were similar for the mild and significant fibrosis sub-groups. Interestingly, no mention was made of the methodology to measure the transaminases in either report. In view of the variability in the ALT and to a lesser extent AST paediatric reference ranges both according to age and to the analytical platform (51, 58) it is therefore inappropriate to use these values as thresholds for abnormality. The variable AST/ALT ratios obtained on the different platforms used for the CALIPER study (*Figure 3C*), clearly warrant a prospective reassessment of the validity of absolute values of ALT, AST and of the AST/ALT ratio in detecting NAFLD. Expressing the transaminases results as multiple of medians could possibly attenuate the methodological differences and allow a better comparison between studies.

Other markers

The natural history of NAFLD and NASH involves liver insulin resistance with a progressive intrahepatic fatty acid accumulation leading to mitochondrial dysfunction, oxidative stress, inflammation, and apoptosis followed by cell remodelling resulting in fibrosis. There are numerous non-invasive tests based on measuring extracellular turnover to detect and quantify liver fibrosis, with excellent performance late in the process (63–66). Hence it is logical to seek circulating biomarkers that that would not only precociously inform clinicians on the extent of liver damage, but also aid them monitoring the evolution of the disease and of the patient's response to treatment. Recent years have been fertile in the development of assays that allow for the measurement of such markers. However data showing their clinical validity are scarce. The following paragraphs give examples of proposed biomarkers related to paediatric NAFLD and NASH.

Cell apoptosis markers

Hepatocyte programmed cell death has been shown to be an important factor in the initiation and progression of liver damage (67, 68). Three mechanistically related markers of cell apoptosis, Cytokeratin-18, N-terminal pro-collagen III and $CD14^+$ nK-cells have been reported. Upon activation of the apoptotic process, Caspase 3 (70) cleaves Cytokeratin18 (CK18), the major inter-filament protein of the hepatocyte cytoskeleton, yielding soluble fragments that are leaked into the circulation (69). Wieckowska et al. (70) were the first to demonstrate that CK-18 Caspase 3-generated fragments (C-terminal CK18Asp396 neo-epitope) were elevated in plasma of a small group of adult patients with suspected NAFLD. More recently, Feldstein et al. (71), in a multi-center validation study, derived form the non-alcoholic Steatohepatitis Clinical Research network (NASH CRN), demonstrated that the same circulating CK-18 neo-epitope predicted the severity of NASH. Shen et al. (72) using an identical technology with a cohort of 147 biopsy-proven adult patients with NAFLD, observed a higher serum concentration of CK18 fragments in NAFLD patients compared to controls with an excellent accuracy (AUROC >0.9). However this neo-epitope was less efficient in differentiating different stages of NASH. The authors, correctly warned that their study, and those of others, include a higher proportion of patients with risk factors and that the performance of biomarkers are likely to differ in a primary care setting, due to selection bias. Also, these markers are not specific to NAFLD or NASH as other diseases, such as hepatitis C will cause liver cell death and hence release of cytokeratin fragments into the circulation. Vos et al. (73) were the first to report the use of cytokeratin 18Asp396 neo-epitope as a diagnostic biomarker for NAFLD in children. They have shown that this biomarker was elevated in children with a clinical diagnosis of NAFLD (elevated ALT and hepatic steatosis determined by ultrasound or computed tomography). Two more recent reports confirm these data. The 1st, by Fitzpatrick et al. (74), compared the serum concentration of CK 18Asp396 neo-epitope in 3 small groups of children that had undergone a percutaneous needle liver biopsy for the purpose of classification (simple steatosis (NAS<2), borderline NASH (scores 2 & 3) and NASH (score 5). They included in their study the measurement of adiponectin, hyaluronic acid and high-sensitivity CRP, none of which showed to be effective in predicting steatohepatitis or fibrosis. The second report, by Lebensztejn et al. (75), also in children with biopsy-proven NAFLD, concluded that CK18Asp396 neo-epitope was suitable to predict liver fibrosis in children with NAFLD (AUC=0.672). As a note of warning, it must be realized that apoptosis is a fundamental process in almost all chronic liver conditions that include inflammation and fibrosis. Therefore although highly suggestive of NAFLD, other diseases have to be ruled out.

Cell microparticles

Circulating cell membrane fragments (microparticles) such as those from $CD14^+$ and Natural Killer T Cells (NKT) generated during the early phase of cell apoptosis can be quantitated by flow cytometry. Their interest lies in the fact that they can be measured by fluorescence–activated cell sorting (FACS). Their measurement has been described in several conditions (76–78). Kornek et al. (79) have recently shown, in a case-control study involving 67 adult patients with histology proven NAFL or NASH and 44 controls, that $CD14^+$ and iNTK generated micro-particles, known to mediate the development of NASH, were significantly augmented. They furthermore showed that the levels of these micro-particles correlated with levels of alanine aminotransferase and severity of NASH (histology proven) in patients with simple steatosis (NAFLD activity score [NAS] score <3) or NASH (NAS score >4). Along the same line of thought, De Vito et al. (80) reported in study involving 34 children with biopsy-proven NAFLD, that liver sections of patients with a NAFLD activity score (NAS) 5 contained a significantly greater number of immune cells with the common leukocyte antigen CD45 and the CD163 antigen, a member of the cysteine scavenger receptor family than those with a score < 5. Conversely there were fewer cells positive for the T cell lineage marker CD3 antigen. They further demonstrated an association between the number of $CD45^+$, $CD163^+$ and $CD3^+$ antigen-positive cells and the severity of histological features related to inflammation, fibrosis and ballooning. This technology holds many promises especially if coupled to the discovery and characterization of neo-epitopes of factors involved in inflammation processes and the early phase of apoptosis.

Inflammatory factors

The activation of the innate immune system resulting from the low-intensity systemic inflammation, the second phase in the development of NAFLD (32), results in the up-regulation of the expression of pro-inflammatory cytokines such as Interleukin-6 (IL-6) and Tumour Necrosis Factor-alpha (TNFα) (81, 82), thereby making them logical candidates as markers for following the evolution from steatosis to NASH. This proved to be the case, as in a series of 47 adult patients with biopsy-confirmed NAFLD, Haukeland et al. (83) reported elevated plasma levels IL-6 levels even after correction for sex, BMI and age. Notably, the plasma levels of this cytokine were elevated in cases with simple steatosis, implying that it could be used as a sentinel for early events in the development of the disease. Wieckowska et al. (84) reported similar findings in 50 patients suspected with NAFLD. They further documented an increased IL-6 hepatic expression that correlated with the extent of inflammation and fibrosis. While several studies have described the influence of TNFα in diabetes and in

NAFLD (85-88), its case is not as straightforward. In the study of Haukeland et al. (83) although patients in the NASH group exhibited raised TNFα plasma levels, there was no difference between the NAFLD patients and controls, implying that it may not be useful in detecting early liver pathology. Serum levels of C-C motif chemokine ligand-2 (CCL2)/monocyte chemo-attractant protein-1 (MCP-1), responsible for hepatic monocyte/macrophage infiltration, inflammation and fibrogenesis, and CCL 19 were significantly elevated in patients with NAFLD compared to simple steatosis.

Future developments

Very few of above-cited reports were structured to test the diagnostic performance of the different factors associated with NAFLD. The advent of high throughput cutting-edge analytical tools has opened the Pandora box. They have allowed the development of genome-wide associations studies (GWAS) by different consortia that identified a plethora of metabolic pathways and networks associated with NAFLD. The enormous amount of information generated through these non-hypothesis driven biomarker discovery studies have now to be translated into effective clinical tools allowing precocious diagnosis of this condition. This will require large scale, well-structured controlled studies with common outcomes and design. Miller et al. (89) have published an excellent systematic review in which they cover the advantages and limitations of the biomarkers derived from non-targeted approach. A few examples of GWAS follow to give a glimpse of the wealth of information generated recently. In pilot GWAS involving 236 non-Hispanic Caucasian women, Chalasani et al. (90) analysing 324, 623 single nucleotide polymorphisms (SNPs) distributed on the 22 autosomal chromosomes, identified one SNP in the farnesyl diphosphate farnesyl transferase 1 gene (FDFT1), involved in cholesterol biosynthesis, associated with the NAFLD score. Following a similar approach, Kawaguchi et al. (91), demonstrated that the variant rs738409 of the patatin-like phospholipase domain-containing 3/adiponutrin (PNPLA3) gene encoding an isoleucine to methionine substitution at amino acid position 148 leading to an inactivation of its triglyceride hydrolytic property, was strongly associated with the severity of NAFLD in Japanese subjects. This was confirmed in other studies performed in different groups or populations (92–95).

Other approaches based on prior physiology knowledge have also been applied to define potential biomarkers. For instance, Bell et al. (96), using the proteomic approach, demonstrated differential circulating protein expression patterns between control obese subjects and patients with different levels of NAFLD. The biological processes in which there were significant changes in priority 1 protein concentrations included, as expected, those involved in the immune and inflammatory regulatory systems, in the cholesterol and triglyceride homeostasis, in the cell cycle control and in structural and extracellular integrity. Hence these data are confirmatory.

Conclusion

As can be perceived in this brief review, developing non-invasive biomarkers that are specific and sensitive particularly in the early phase of hepatic steatosis is difficult. The difficulty lies in that previous studies have used different techniques with different outcomes. The recent analytical tools coupled to powerful informatics have allowed non-hypothesis driven biomarker discovery. Their use will require that they go through the National Cancer Institute guidelines for the development of biomarkers (97), and that multi-center cohorts be used with standardized recruitment protocols, clinical end-points, analytical methods and reference intervals.

Conflict of interest statement

The authors stated that they have no conflicts of interest regarding the publication of this article.

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Received: May 14, 2014

Accepted: May 29, 2014