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Controversies and Opportunities in the Use of Inflammatory Markers for Diagnosis or Risk Prediction in Fatty Liver Disease

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In the Western society, non-alcoholic fatty liver disease (NAFLD), characterized by the excessive accumulation of fat in the liver, represents the most common cause of chronic liver disease. If left untreated, approximately 15%–20% of patients with NAFLD will progress to non-alcoholic steatohepatitis (NASH), in which lobular inflammation, hepatocyte ballooning and fibrogenesis further contribute to a distorted liver architecture and function. NASH initiation has significant effects on liver-related mortality, as even the presence of early stage fibrosis increases the chances of adverse patient outcome. Therefore, adequate diagnostic tools for NASH are needed, to ensure that relevant therapeutic actions can be taken as soon as necessary. To date, the diagnostic gold standard remains the invasive liver biopsy, which is associated with several drawbacks such as high financial costs, procedural risks, and inter/intra-observer variability in histology analysis. As liver inflammation is a major hallmark of disease progression, inflammation-related circulating markers may represent an interesting source of non-invasive biomarkers for NAFLD/NASH. Examples for such markers include cytokines, chemokines or shed receptors from immune cells, circulating exosomes related to inflammation, and changing proportions of peripheral blood mononuclear cell (PBMC) subtypes. This review aims at documenting and critically discussing the utility of such novel inflammatory markers for NAFLD/NASH-diagnosis, patient stratification and risk prediction.

Keywords: chronic liver disease, NAFLD, NASH, inflammation, biomarker, liquid biopsy

INTRODUCTION

To date, non-alcoholic fatty liver disease (NAFLD) is the leading cause of chronic liver disease worldwide. It is a very heterogeneous disorder, that finds its aetiology in a complex and multifactorial interplay of different parameters, such as obesity, a sedentary life-style, the composition of the main nutrient-intake, insulin resistance, diabetes, alterations in the gut microbiome, and genetic predisposition (1). It mainly manifests as an excess fat disposition in the liver ($\geq$5% hepatic steatosis), independent of injury or inflammation, a condition termed isolated steatosis or non-alcoholic fatty liver (NAFL). When not intervened, NAFL may progress to non-

alcoholic steatohepatitis (NASH), which is characterised by the presence of injury, hepatocyte ballooning, inflammation, and a varying extent of fibrosis, and which enhances the risk of cirrhosis and hepatocellular carcinoma (HCC) development, thus strongly influencing liver-related mortality (2). Due to the current epidemic in excess body weight and the aging – and thus more diabetes-susceptible - world population, the NAFLD pathology will only increase further into modern society. Indeed, while in 2016, in the USA alone, the number of NAFLD patients clocked at 85.3 million, mathematical models estimate a prevalence of 100.9 million cases in 2030, a substantial increase of 18%. The numbers of patients suffering from the more severe NASH are thought to display an even more steep increase of 56%, going from 17.3 million patients in 2016 to an estimated 27 million in 2030. Subsequently, significant increases in the number of patients suffering from decompensated cirrhosis, HCC, and liver-related deaths will be observed (3).

Although the severity of NAFLD-prevalence and potential critical outcome, no efficient specific pharmacological interventions are currently available, besides the obligatory changes in lifestyle. Multiple clinical trials, however, are ongoing, evaluating the potential efficacy of novel pharmacotherapeutics in NAFLD patients (4). As the presence and severity of fibrosis has been identified as the major indicator of poor long-term outcome, including death and the need for a liver biopsy, in NASH patients (5, 6), it has become a critical determinant for the inclusion of NAFLD patients in clinical trials (7). Moreover, therapeutically obtained fibrosis regression is found to be closely correlated to the resolution of steatohepatitis and improvement of the NAFLD activity score (NAS) (8, 9), therefore highlighting the importance of evaluating the different NASH parameters for assessment of disease improvement. An accurate and efficient diagnostic tool for NAFLD evaluation is thus crucial to ensure a timely trial-inclusion of at-risk patients as well as subjecting these high-risk patients to more intense lifestyle interventions and surveillance for disease-related complications (10).

CURRENT CLINICAL DIAGNOSTIC MEANS FOR FATTY LIVER DISEASE

At present, the liver biopsy, either percutaneous or transjugular, remains the gold standard for disease evaluation in patients with NAFLD. This invasive procedure is however associated with multiple drawbacks, including the limited representation of the total liver mass, intra- and interobserver variability, pain and post-procedure complications, and an extensive financial cost causing a subsequent doubtable cost-effectiveness ratio (11). The poor inter-reader variability was recently stressed by a secondary analysis from a (negative) clinical trial, in which 678 paired biopsies from 339 patients were independently read by three hepatopathologists. Particularly, the scoring for histological NASH, as well as treatment responses (NASH resolution, fibrosis improvement) showed a low inter-reader reliability (12). In addition, the high financial cost and the potential

procedural risks, withhold the liver biopsy to be used as a tool for regular patient follow-up in medical practice, creating a severe hiatus in NAFLD-management. Multiple non-invasive techniques have therefore been suggested to be more suitable as manner of first-line investigation, including laboratory (blood) tests, and imaging-based tools (13).

Blood-based markers can be divided into direct markers, representing ECM production and degradation (hyaluronic acid, matrix metalloproteases, etc.), and indirect markers which indicate liver function and inflammation (aminotransferases, platelet counts, etc.) (11). Such circulating markers are often combined with risk characteristics (diabetes, age, etc.) into diagnostic scores to obtain maximum sensitivity and specificity. One popular example comprises the NAFLD fibrosis score (NFS), which includes age, aminotransferases, albumin, body mass index (BMI), platelet count, and glucose intolerance status, and which has a high negative predictive value for excluding NAFLD-patients with advanced fibrosis (14). Examples of other serological scoring tools are Fib-4, AST-to-platelet ratio index (APRI), Fibrotest, and enhanced liver fibrosis test (ELF). A recent meta-analysis, including 64 studies with a total of 13,046 NAFLD patients, compared the diagnostic performance of APRI, Fib-4, BARD score, and NFS for identification of advanced fibrosis, and reported the respective summary AUROCs of 0.77, 0.84, 0.76, and 0.84. This study concluded that Fib-4 and NFS have, of the analysed serological scoring tools, the highest accuracy and negative predictive value for ruling out advanced fibrosis (15). Moreover, as Fib-4 and NFS show the highest extent of validation in different NAFLD populations, with consistent accuracy in excluding advanced fibrosis (generating negative predictive values of over 90%), these scores are proposed as first-line screening method in clinical settings where more advanced/expensive tests are unavailable (16).

In the process of chronic liver damage, the liver acquires a stiffened character, due to the excessive deposition and accumulation of ECM by activated myofibroblasts. Such important change in elasticity may be visualized and quantified using different imaging-tools, having each their own accuracy, methodology, handling and interpretation (17). Especially the evaluation of liver stiffness through abdominal ultrasound has found its way into clinical practice, and comprises acoustic radiation force impulse (ARFI), (point) shear wave elastography, and (vibration-controlled) transient elastography (TE; Fibroscan), this latter being the most popular technique in current clinical practice (17). However, although specific adaptions (such as the development of an XL-probe) increase the utility of TE in patients with central obesity, excessive fat accumulation may still bear a risk of technical failure (18). One important advantage of TE is its potential to simultaneously evaluate the hepatic fat content through the additional feature of controlled attenuation parameter (CAP) (19). Furthermore, the combination of liver stiffness and CAP measures by TE with serological markers such as AST (FibroScan-AST/FAST score) can increase its accuracy in identifying patients at risk for progressive NASH (20).

Magnetic resonance imaging-based techniques, so called magnetic resonance elastography (MRE), represent another method of liver stiffness evaluation. MRE-results are less influenced by the presence of obesity, and may also simultaneously evaluate the extent of fibrosis and steatosis, the latter through use of the proton density fat fraction (PDFF), of which the accuracy has been suggested to be even higher than CAP (21). However, while MRI-based scoring tools have greater potential of correctly identifying the extent of steatosis and fibrosis in the affected liver, as compared to ultrasound-based techniques, due to their higher costs and low availability, they are more predominantly used in a research setting (22).

The above mentioned non-invasive diagnostic tools, are useful to narrow down the indication for liver biopsy, and help decide on the further clinical management of the patient. For example, in individuals suffering from obesity, who do not present any other feature of the metabolic syndrome, who have an APRI score ≤ 0.5, a FIB-4 score ≤ 1.1; and TE ≤ 6 kPa, the likelihood for the presence of significant liver fibrosis is very low, subsequently leading to a small liver-related mortality in the timeframe of 10 years (23, 24). In these cases, lifestyle changes combined with patient follow-up through use of stiffness measurement, are the best approach (2). However, in those patients with aberrant scoring values, the use of liver biopsy remains the reference standard for confirmation and evaluation of the presence of significant fibrosis (2).

Overall, the current non-invasive scoring tools lack sufficient sensitivity and specificity, especially for the early stages of liver disease, to make the liver biopsy completely aberrant. Additionally, almost no data is available on their usefulness in clinical follow-up. Therefore, novel diagnostic strategies are considered, focussing not only on the extent of fibrosis or damage of the affected liver, but also on liver inflammation, one other crucial aspect of liver pathogenesis.

INFLAMMATORY PROCESSES DURING NASH

The pathogenesis of NAFLD relies on multiple damaging "hits" (**Figure 1**), with the proliferation, dysfunction and inflammation of adipose tissue being one of them (25, 26). Indeed, in the visceral adipose tissue of NASH patients, an increased presence of, among other, CD11c+CD206 and CCR2+ macrophages can be found (27). Similar changes of intrahepatic accumulation of CCR2+ macrophages are present in the liver, particularly in patients with NASH-fibrosis (28). Such immune cell infiltration is accompanied by an increased release of chemokines and proinflammatory cytokines into the circulation, which have proven propagating effects on liver disease and insulin
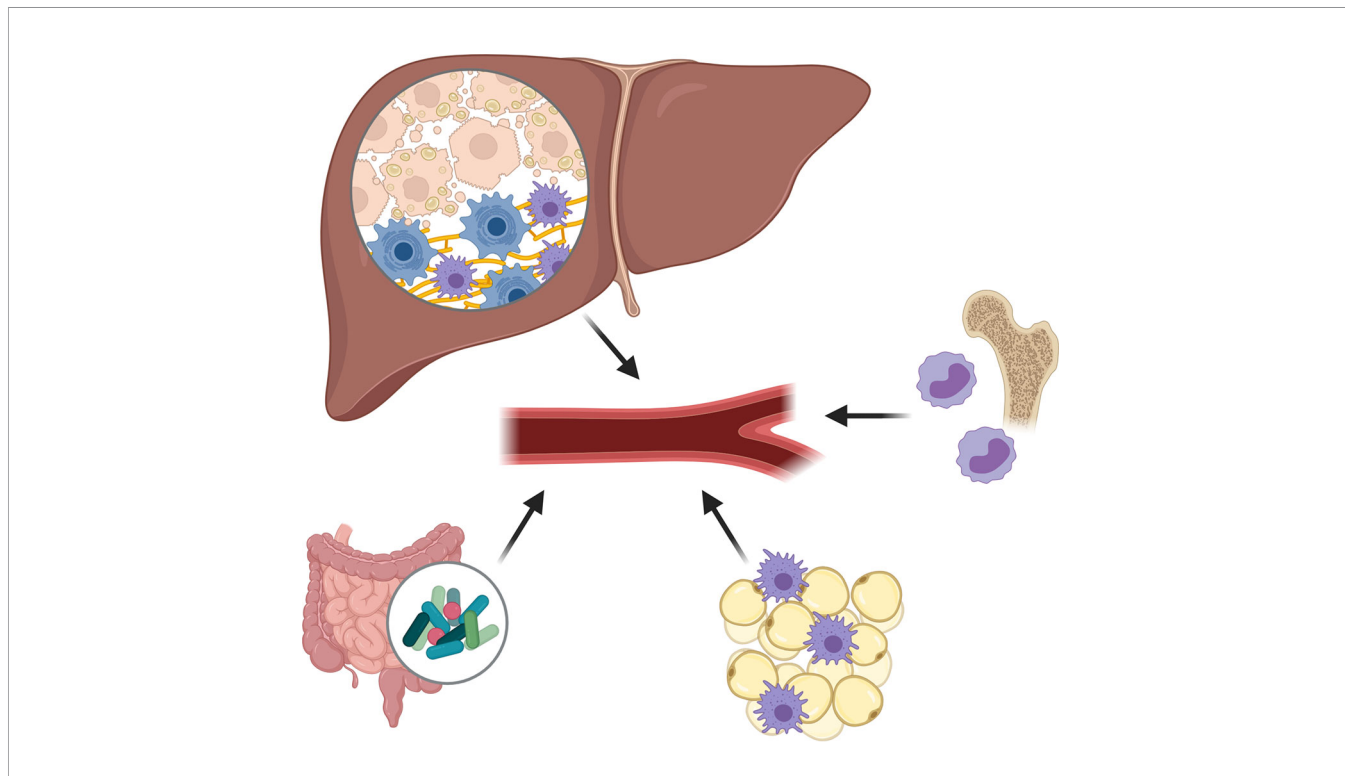
FIGURE 1 | Damaging hits during non-alcoholic fatty liver disease (NAFLD) pathogenesis. The pathogenesis of NAFLD relies on multiple damaging "hits", including the proliferation, dysfunction and inflammation of adipose tissue, the excessive presence of fatty acids and cholesterol in the liver and circulation, and important changes in gut permeability and microbiota composition causing an increased release of pathogen-associated molecular patterns. Subsequent to these damaging factors, an enhanced production and release of pro-inflammatory cytokines and chemokines is observed, creating a pro-inflammatory environment in which Kupffer cells become activated and monocyte-derived macrophages (MoMΦs) and other leukocytes are recruited to the liver.

resistance (the so called adipose tissue – liver axis), and which serve as fuel for local and systemic inflammation (25, 26, 29).

One other liver damaging "hit" concerns the excessive presence of fatty acids (FAs) and cholesterol in the liver and circulation (30). While during normal liver homeostasis, FAs are metabolized by the hepatocytes to form triglycerides, during NAFLD progression, an excessive amount of FAs, impaired beta-oxidation, and diminished export of triglycerides as very low-density lipoproteins can be observed, causing an accumulation of saturated FAs and oxidized cholesterol in the hepatocytes. The subsequent formation of lipotoxic species, and endoplasmic reticulum- and oxidative-stress, leads to profound hepatocyte damage, which may eventually cause the hepatocytes to undergo necrosis, apoptosis, or necroptosis. Such hepatocyte death is associated with the release of reactive oxygen species and their cellular content, including nuclear and mitochondrial DNA, purine nucleotides (ATP, UTP) and other damage-associated molecular patterns (DAMPs) (30, 31). These metabolic danger signals may activate a plethora of pattern recognition receptors (PRRs) expressed by various liver cells, and which will trigger the production and release of pro-inflammatory cytokines and chemokines, creating a pro-inflammatory environment in which Kupffer cells become activated and monocyte-derived macrophages (MoMΦs) and other leukocytes are recruited. This recruitment of pro-inflammatory cells, which significantly changes the immune cell composition of the liver during NAFLD, may occur *via* various chemoattractant-axes, including the CCL2/CCR2, CCL1/CCR8, CXCR6/CXCL16, and CCL25/CCR9-pathways, with the chemoattractants being secreted by activated Kupffer cells, liver sinusoidal endothelial cells, and hepatic stellate cells. Besides the enhanced recruitment, also an enhanced polarization of macrophages toward a pro-inflammatory ("M1-like") phenotype can be observed, potentially caused by stimulating cytokines such as TNF and IFN-γ (30, 32).

The major changes in gut permeability and microbiota composition, form an additional plausible harmful "hit". Multiple alterations can be observed that range from reduced microbial diversity, abundance of pathogenic microbiota, accumulation of bacterial metabolites, changes to the intestinal virome and reduced intestinal barrier (33, 34). With these changes, an increased release of pathogen-associated molecular patterns (PAMPs), e.g., lipopolysaccharide (LPS) can be observed in the portal vein and the systemic circulation, which stimulate the respective PRRs expressed by the various liver cells, and therefore further contribute to the pro-inflammatory liver environment (35).

Many experimental studies revealed that the "sterile" inflammation observed during NAFLD leads to a perpetuation of liver disease. Indeed, studies depleting certain types of immune cells (36, 37), or blocking the polarization and recruitment of inflammatory cells, have led to significant alleviation of fibrosis in various mouse models and early-stage clinical trials (32). However, it should not be forgotten that the inflammatory response is also crucial for healing and tissue repair, often observed during the early stages of liver injury

(38). While this dual function of the inflammatory system may have a more hampering effect on its use as therapeutic target, the overall presence of inflammation may represent an interesting tool for the diagnosis and follow-up of disease (**Table 1**). Therefore, next, we will describe and discuss potential new inflammatory markers for NAFLD-evaluation.

INFLAMMATORY MARKERS

C-Reactive Protein

During the acute phase of the inflammatory process associated with NAFLD development, an IL-6-dependent increased transcription and subsequent release of C-reactive protein (CRP) by the liver is observed. In the addition to the standard techniques for CRP evaluation (mainly through immunoassay), high-sensitivity CRP detection tests have been developed, which thus allow the detection of low-grade systemic inflammation. Various studies have identified elevated circulating high-sensitive (hs) CRP levels as indicators for histology- (39, 40) or ultrasound-based (41, 42) NASH. Elevated hsCRP levels were even found to be associated with the severity of NAFLD (43) and to distinguish patients with advanced fibrosis from those with mild fibrosis (40). This latter finding also correlated to the elevated CRP mRNA levels that were found in the liver tissue of NASH patients (40). Additionally, elevated hsCRP levels in NAFLD patients seem to have a predictive value for the development of cardiovascular complications (43). Such association between (hs)CRP levels and cardiovascular disease has already been proven outside the NAFLD pathology (44).

Although the previous mentioned studies claim the diagnostic utility of circulating hsCRP in NAFLD, results of other studies contradict this hypothesis, being unable to find any relationship between hsCRP and the extent of hepatic steatosis, necroinflammation, and fibrosis (45, 46), especially when hsCRP values are being corrected for visceral fat, BMI, gender or age of the patient (47, 48). Indeed, circulating hsCRP-levels are known to be associated with BMI, systolic blood-pressure, waist-to-hip ratio, insulin resistance, and concentrations of HDL cholesterol and triglycerides (49). Especially the amount of fat seems to be an important determinant of hsCRP levels, as it has been found that per 10% increase in BMI, circulating hsCRP levels increase by 19-20% (46).

Pentraxin 3

Pentraxin 3 (PTX3) is a member of the long chain pentraxin family, like CRP, and is a marker of the acute phase inflammatory response (50). It is predominantly produced by immune cells, although other cells and tissues may also contribute to its production, as a response to pro-inflammatory signals. Various studies have identified the elevated presence of circulating PTX3 in patients with NAFLD, as compared to controls (51–53), in NASH patients as compared to patients with simple steatosis (51, 54) and in correlation with the severity of hepatic fibrosis (51, 54, 55). Additionally, PTX3 levels were associated with triglyceride concentrations, LDL-cholesterol, and

TABLE 1 | Selected experimental inflammatory biomarkers in NAFLD.

Biomarker	Predominant Cellular source	Application	Conflicting results reported?	Strength of evidence
(high sensitivity) C-reactive protein	Hepatocytes, macrophages, lymphocytes	- NASH vs NAFL- NAFLD severity- prediction cardiovascular complications	Yes, by multiple studies. CRP seems strongly influenced by body fat.	+/-
Pentraxin 3	Neutrophils, macrophages, monocytes, dendritic cells	- NAFLD vs controls- NASH vs NAFL- NAFLD severity	Yes, 1 study reported the inability of pentraxin 3 to identify NASH	+
Interleukins				
IL-1β	Monocytes, macrophages	- NASH vs NAFL	No	++
IL-6	Monocytes, macrophages	- NAFLD vs controls- NASH vs NAFL- advanced fibrosis vs no or mild fibrosis	No	+++
IL-8	Monocytes, macrophages	- NAFLD vs controls- correlation to stage of fibrosis- correlation to NAFLD severity	No	+++
IL-12	Dendritic cells, monocytes, macrophages	- NAFLD severity	No	+ (only 1 study)
IL-32	NK cells, monocytes, T-cells	- NAFLD vs controls- NASH vs NAFL	No	+ (only 1 study)
Lipocalin-2	Neutrophils, hepatocytes	- NAFLD vs controls- NASH vs NAFL	No	+++
TNFα	Macrophages, NK cells, lymphocytes	- NAFLD vs controls	Yes, disputed correlation with severity of NAFLD	+
sTNFR1/2	Large variety of cell types	- NASH vs NAFL- advanced fibrosis vs no or mild fibrosis- Paediatric NAFLD vs controls- Paediatric NASH vs NAFL	No	+++
sCD14	Monocytes, macrophages	- NASH vs NAFL- correlation to NAS-score	Yes, disputed possibility to indicate NAFLD/NASH	+
sCD36	Macrophages, hepatocytes	- NAFLD vs controls- correlation to NAFLD severity	No	+++
sCD163	Monocytes, macrophages	- NAFLD vs controls- NASH vs NAFL- NAFLD severity- advanced fibrosis vs no or mild fibrosis- Treatment response- Paediatric NAFLD vs controls	No	++++
Extracellular vesicle-phenotypes				
CD14+	Monocytes, macrophages	- NAFLD vs controls- NAFLD severity	No	+ (only 1 study)
Vα21/Vβ11+	iNKT-cells	- NAFLD vs controls- NAFLD severity	No	+ (only 1 study)
CD14+/CD16+	Monocytes, macrophages	- advanced fibrosis vs no or mild fibrosis	No	+ (only 1 study)
Quantity immune cell subtypes & their phenotypes				
↑ CD14+CD16++ monocytes ↓ CD14++CD16- monocytes	N.A.	- NAFLD vs controls	No	++
CCR4 expression on monocytes	N.A.	- NAFLD vs controls	No	+ (only 1 study)
TLR6 expression on monocytes	N.A.	- NAFLD vs controls- NASH vs NAFL	No	+ (only 1 study)
CD25+CD45RA+CD4+ T-cells	N.A.	- NAFLD vs controls- Significant fibrosis vs no or mild fibrosis	No	+ (only 1 study)
CXCR3+CD4+ T-cells	N.A.	- NAFLD vs controls- Significant fibrosis vs no or mild fibrosis	No	+ (only 1 study)
PD1+CD25+CD45RA+CD4+ T-cells	N.A.	- NAFLD vs controls- Significant fibrosis vs no or mild fibrosis	No	+ (only 1 study)
Th17/rTreg	N.A.	- NASH vs NAFL	No	+ (only 1 study)
Th2/rTreg	N.A.	- NASH vs NAFL	No	+ (only 1 study)
Neutrophil-to-lymphocyte ratio	N.A.	- NASH vs NAFL- NAFLD severity- advanced fibrosis vs no or mild fibrosis- Liver disease related mortality	No	++++

References for the mentioned circulating markers can be found in the main text.

N.A., not applicable; NASH, non-alcoholic steatohepatitis; NAFLD, non-alcoholic fatty liver disease; NAFL, non-alcoholic fatty liver; IL, interleukin; NK, natural killer; TNFα, tumour necrosis factor alpha; sTNFR, soluble tumour necrosis factor receptor; CD, cluster of differentiation; iNKT, invariant natural killer; CCR, C-C chemokine receptor; TLR, toll-like receptor; CXCR, C-X-C chemokine receptor; PD1, programmed cell death protein 1; Th, T helper cell; rTreg, resting regulatory T cells.

waist circumference (53) and was found to be increased in obese children and adolescents with increasing severity of fatty liver (56). However, one study reports the inability to find any significant diagnostic value of PTX3 for the identification of NASH, nor any correlation with the severity of the disease (57), thus nuancing its diagnostic utility.

Interleukins

Interleukins (ILs), a heterogeneous group of cytokines which play essential roles on the activation and differentiation of immune cells, have been shown to play essential roles in NAFLD pathogenesis, what makes them interesting therapeutic targets (58–60). Their utility in diagnosis of NAFLD has been evaluated in various studies, and identified that several specific interleukins possess significant sensitivity and specificity as stand-alone marker, or in combination with other biomarkers. We describe some of the interleukins with diagnostic potential.

Circulating IL-6 levels show significant value for the discrimination of NAFLD patients from obese individuals (61). Moreover, a progressive increase during the NAFLD pathogenesis is observed, as proven by the potential of circulating IL-6 levels to identify patients with simple steatosis (NAFL) to healthy controls (62), to identify patients with NASH as compared to those with simple steatosis (63), and to discriminate NASH patients with advanced fibrosis, from patients with no or mild fibrosis (64). Interestingly, also the expression of its receptor (IL-6R) has been found to be elevated in the circulation of NASH patients (63). The elevated circulating IL-6 levels in NASH patients were found to be associated with the hepatic IL-6 protein levels and the overall degree of hepatic inflammation and fibrosis (65). It should be noted that on mRNA-level, one other study found no difference in hepatic IL-6 expression between individuals with NASH and those with simple steatosis (40).

In contrary to IL-6, the hepatic mRNA-expression levels of IL-8 are significantly increased in NASH patients with advanced fibrosis. IL-8 is an important chemoattractant for neutrophils and possibly other myeloid immune cells (66). Such changing IL-8 levels are reflected in the serum of these NASH patients, as elevated circulating IL-8 levels are found to correlate to the stage of fibrosis (67). Moreover, IL-8 levels can identify the presence of significant fibrosis in NASH patients (68), and are elevated in NAFLD patients when compared to both obese and non-obese controls (61). Additionally, a positive correlation between circulating IL-8 levels and the histological NASH parameters, lobular inflammation and hepatocellular ballooning, was observed in NASH patients suffering from (morbid) obesity (68, 69). The importance of IL-8 as diagnostic tool is further demonstrated by the possibility to, as only parameter in a panel of 24 analysed cytokines, significantly correlate with hepatic fibrosis after controlling for age, sex, BMI, hypertension, metabolic syndrome, and diabetes mellitus (67).

IL-12 is a pro-inflammatory cytokine, known for its aggravating effect on liver disease, through the induction of a T-helper (Th)-1 phenotype in Th cells and inhibition of the Th-2 phenotype (70). An elevation of circulating IL-12 levels is

associated with NAFLD-severity, as observed in an ultrasound-staged NAFLD population of 100 individuals (71).

Transcriptomics analysis on hepatic tissue identified up-regulated IL-32 levels in NAFLD patients, as compared to liver tissue obtained from obese individuals showing no signs of hepatic steatosis. Such elevated IL-32 mRNA levels were found to be associated with body mass index (BMI), aminotransferases, NAFLD activity score, and homeostasis model assessment of insulin resistance (HOMA-IR) index (72). Another transcriptomics-based study, including NAFLD patients with and without the PNPLA3 I148M genetic risk variant, identified a similar robust up-regulation of IL-32 in individuals with a severe phenotype (defined as NAFLD activity score ≥ 4, fibrosis stage ≥ 2, or presence of steatohepatitis), independently of the underlying genotype. The evaluation of circulating IL-32 levels identified its potential to diagnose NAFLD, and development of NASH. Surprisingly, these elevated levels were found to be independent of aminotransferases. No differences between PNPLA3 I148M carriers and non-carriers were observed (73).

The NAFLD pathology is strongly associated with inflammasome activation, with the nucleotide-binding oligomerization domain-like receptor pyrin domain containing 3 (NLRP3) inflammasome being the most extensively studied. In the presence of danger signals, the NLRP3 protein complex activates the protease caspase-1, which initiates the maturation of the pro-inflammatory cytokines IL-1β and IL-18. In the liver, the NLRP3-caspase-1 complex is predominantly expressed in KCs, but may also be present in parenchymal cells and other inflammatory cells (74). IL-1β plays a key role in liver disease, as it affects both steatosis, inflammation and fibrosis. Indeed, IL-1β stimulates triglyceride and cholesterol accumulation in hepatocytes, promotes the recruitment of immune cells through up-regulation of ICAM-1 on liver sinusoidal endothelial cells, and stimulates local inflammation through induction of IL-6 production (59). This strong association of IL-1β with disease severity is also reflected in the circulation, as elevated blood-levels of IL-1β are found in patients with NASH, compared to those with simple steatosis (75, 76). Interestingly, also elevated hepatic and circulating levels of IL-1 receptor antagonist (IL-1RA) are observed in NASH patients, and were even found to be correlated to the degree of lobular inflammation (77). While circulating IL-18 levels show a positive correlation with waist circumference, insulin resistance, the development of atherosclerosis, and triglyceride content in patients with metabolic syndrome (78), no changes in circulating expression levels were observed between NASH patients and patients with simple steatosis, or NAFLD patients and healthy individuals (79, 80). Remarkably, in mice receiving a "American lifestyle-induced obesity syndrome" diet, elevated levels of IL-18, but not IL-1β, were observed, in contrast to the above mentioned human data (81).

Neutrophil-to-Lymphocyte Ratio

Neutrophils, protagonists of the innate immunity response, differentiate from myeloid precursors in the bone marrow, and are recruited into the blood stream through various signalling

molecules such as granulocyte colony stimulating factor (G-CSF), CXC chemokine receptor (CXCR) 2 and 4 (82). Calculating the ratio of the number of neutrophils to lymphocytes (the neutrophil-to-lymphocyte ratio; NLR) harbors the potential to discriminate individuals with NASH from those with NAFL (83, 84). Indeed, using a cut-off value of 1.9, a sensitivity and specificity of respectively 72% and 70% is obtained for the identification of NASH-patients (85). Furthermore, NLR is able to discriminate NASH patients with advanced fibrosis, from those with mild-to-moderate fibrosis, indicating the significant association of NLR with the extent of histological features of NASH (83, 85). Additionally, due to the many effects of neutrophils on the immunological and metabolic features of NASH, NLR may also give information concerning hepatic steatosis, insulin resistance, hyperlipidaemia, imbalanced metabolic hormones, and abundance of pro-inflammatory signals (86). It is important to mention that NLR may also predict liver-disease related mortality. Indeed, analysis of the NLR in 570 patients with end-stage liver disease, of which 54 were NAFLD-patients, identified a significant correlation between increasing NLR and mortality within 3 months of listing for transplantation, and an overall association with high 3-month mortality when NLR ≥ 5 (87).

Lipocalin-2

Lipocalin-2 (LCN2), also known as neutrophil gelatinase-associated lipocalin (NGAL), is a secretory glycoprotein, which has a pro-inflammatory action, and which is thought to be strongly involved in the metabolic and cardiovascular complications associated with obesity (88). An overall increase in circulating LCN2 is observed in NAFLD patients, as compared to healthy controls, which additionally correlated to insulin resistance and inflammation (this latter was evaluated through CRP) (89). One other study, which included biopsy-staged NAFLD patients, identified the elevated circulating LCN2 levels in patients with NASH, as compared to NAFL patients or healthy controls. Moreover, in all NAFLD patients, LCN2 levels positively correlated to the injury-associated markers ALT, AST, and γGT. Co-localization of LCN2 with CD66b, a general neutrophil marker, in the liver biopsies of NASH patients further indicated the neutrophilic origin of the circulating LCN2 (90). Other recent studies renounce this neutrophil-exclusive origin of LCN2, claiming that also hepatocytes, adipocytes, and endothelia contribute to LCN2 production and secretion (91, 92), therefore questioning its exclusive representation of liver inflammation.

Tumour Necrosis Factor Alpha

One of the major pathogenic drivers of the NAFLD pathology is tumour necrosis factor alpha (TNF-α), which mediates liver injury mainly *via* TNF-receptor-1 (TNFR1) signalling (93). Indeed, blocking this signalling pathway through use of anti-TNF-α (infliximab) (94, 95) or anti-TNFR1 (96) antibodies in rodent models of NAFLD, led to significant improvement of liver steatosis, inflammation, insulin resistance and extent of liver fibrosis. Furthermore, the presence of the rs1799964 single nucleotide polymorphism (SNP) in the TNF risk allele has

been found to be an independent risk factor for an enhanced rate of histological progression (97).

Besides its potential as a therapeutic target for treatment of NAFLD, its diagnostic utility has been widely discussed. In multiple studies, circulating levels of TNF-α have been found to identify the presence of NAFLD (98, 99), which would reflect up-regulated TNF-α mRNA expression in the affected liver (99). However, contradictory results concerning the sensitivity of circulating TNF-α levels for the identification of the different stages of the NAFLD pathology, have been reported. While some studies only report its potential to discriminate NASH-patients with cirrhosis from healthy subjects, being unable to show any correlation with the severity of the histopathology (100), others report its potential to distinguish NASH-patients with significant fibrosis from those with no or mild fibrosis (68).

Not only TNF-α itself, but also its soluble receptors (sTNFR1/2) have an increased presence in the blood stream of NASH patients, as compared to patients with simple steatosis (63, 99, 101). Furthermore, circulating levels of sTNFR-2 in NASH were even suggested to discriminate between advanced- and low stage-fibrosis. Interestingly, such sTNFR-2 were found to be higher in patients with diabetes mellitus (DM) compared to those without DM (102).

Lastly, circulating TNF-α and sTNFR1/2 levels might also have a diagnostic utility in paediatric NAFLD patients, as their levels were found to be increased in children with NAFLD (103) and NASH (104). However, these markers did not allow discrimination between advanced and no-mild liver steatosis (103).

CD14

CD14 is a multifunctional receptor, with a constitutive expression on the cell surface of various immune cells. In the liver, different macrophage populations express CD14 (105). Its main function is the recognition of LPS, or other components of the bacterial wall, causing the activation of a plethora of signalling cascades, eventually leading to cytokine production and shedding of its extracellular domain (sCD14) (106, 107). During NAFLD development, a strong correlation between the presence of CD14-positive immune cells, and the extent of necroinflammation and fibrosis has been observed (108). Additionally, the C/T (-159) polymorphism in the CD14 gene (rs2569190) has been closely linked to an increased risk in the development of NAFLD, however, without however influencing the degree of hepatic steatosis or fibrosis (109).

Circulating levels of soluble CD14 (sCD14), which has been claimed to be mainly derived from the liver (110), were found to be inversely correlated to insulin resistance and markers of liver injury (ALT and γGT) in both lean and obese individuals (111, 112). Additionally, changing levels of sCD14 accompanied the significant changes in hepatic necro-inflammation, and overall NAS-score, in obese individuals undergoing surgically induced weight loss (113). In a cohort of 113 NAFLD patients; sCD14 levels showed diagnostic value for the presence of NASH, and a strong correlation to liver inflammation (114), thus suggesting its diagnostic utility in this pathology. One other study, however, questions these results, as they only found such dynamic sCD14

levels in obese patients, as compared to healthy individuals, but not in relation to the development or severity of NAFLD/NASH (115).

CD36

One important example of a scavenger receptor, a pattern-recognition receptor, on phagocytic cells concerns CD36. Indeed, this receptor recognizes a plethora of both foreign material, such as lipids and lipoprotein components of bacterial cell walls, and endogenously derived ligands, including oxidized phospholipids, glycated proteins, and apoptotic cells. As activation of the CD36 causes the induction of pro-inflammatory signalling, its contribution to the development of inflammatory disease is evident (116). Besides macrophages, the presence of CD36 has also been identified on adipocytes, myocytes, enterocytes, and hepatocytes, where it regulates free fatty acid (FFA) transport and oxidation, VLDL secretion, and autophagy (117). Moreover, its stimulating effect on FFA uptake caused the CD36 receptor to act as driving force of the initiation and perpetuation of liver steatosis (118), thus displaying key functions in the different aspects of NAFLD pathogenesis.

The importance of CD36 in metabolic liver disease has been suggested by the close correlation between hepatic CD36 mRNA and protein expression and the liver fat content in morbidly obese patients (119, 120), and by its elevated presence in the livers of NAFLD patients compared to healthy controls (121, 122). This enhanced hepatic presence is represented in the blood-stream, as soluble CD36 (sCD36) was found to be increased in biopsy-staged NAFLD patients, compared to healthy controls, and as the circulating levels were even correlated with the histological grade of steatosis (123). sCD36 can also nicely distinguish patients with simple steatosis from healthy individuals (123, 124), and would correlate well with the extent of intrahepatic lipids (as measured by magnetic resonance spectroscopy) in NAFLD patients, therefore suggesting that this circulating marker especially represents the metabolic aspect of NAFLD pathology, and less of the immunological aspects. Lastly, it should be mentioned that sCD36 is tightly correlated to the presence of insulin resistance in obese patients with type 2 diabetes mellitus (125, 126), further suggesting its -especially-metabolic representation.

CD163

CD163 is a scavenger receptor, which has as main function the recognition of the tight complex of haptoglobin and haemoglobin, known to be formed after red blood cell haemolysis. While both monocytes and macrophages express CD163, its expression especially becomes elevated during macrophage-maturation (127, 128). The presence of a soluble form of CD163, sCD163, has been observed in the bloodstream, and is thought to be mainly derived from proteolytic shedding in response to various inflammatory responses such as LPS, oxidative stress and thrombin (128, 129). A significant portion of sCD163 is potentially derived from Kupffer cells, as sCD163-concentrations are found to be 23% higher in the hepatic vein, as compared to the portal vein, in patients with obesity or NAFLD

(130). Interestingly, no differences in sCD163 concentrations were observed between the portal and hepatic vein in healthy individuals (131).

In adult NAFLD patients, circulating levels of sCD163 are elevated, when compared to healthy controls. Furthermore, sCD163 has been described as able to discriminate NASH from simple steatosis, and to correlate with the extent of steatosis, inflammation, and hepatocellular ballooning (132). Indeed, other studies confirmed the close correlation of sCD163 with the histological extent of NAFLD (133), its utility for identification of NAFLD-induced advanced fibrosis (134), and its association with markers of liver necro-inflammation and glucose-homeostasis (135). sCD163 might also reflect treatment response, as decreasing circulating expression levels were observed in obese patients undergoing bariatric surgery (130), and NAFLD patients undergoing life-style intervention (135, 136)

Also in paediatric NAFLD cases, sCD163 may have diagnostic utility. Indeed, in children with biopsy-proven NAFLD or NASH, a significant increase in the amount of hepatic CD163-positive cells was observed in those with severe histological activity, and in close correlation with the presence and extent of fibrosis (137). Furthermore, in obese children, an elevated presence of sCD163 is observed in those with ultrasonographic-proven steatosis and elevated transaminase-levels (138).

Extracellular Vesicles

Extracellular vesicles (EVs) are small, membrane-derived structures which can be divided into three subtypes (exosomes, microvesicles, and apoptotic bodies), and are released by the cells into their microenvironment. The EV-cargo may consist of messenger RNA (mRNA), micro-RNA (miRNA), long non-coding RNA (lncRNA), lipids, and proteins, and strongly reflects the cytosolic and membrane composition of its cell of origin, therefore suggesting the use of blood-circulating EVs to represent disease-associated cellular changes (11). Besides such changing cargo, also changes in the absolute numbers of circulating EVs may be observed during disease. For example, an elevated number of circulating EVs is observed in NASH patients, with the EV-numbers strongly correlating to NASH clinical characteristics and disease severity (139). These findings reflect the elevated number of circulating EVs identified in mice with diet-induced NASH (140). Moreover, while in these mouse models the circulating hepatocyte-derived (ASGR1$^+$ and CYP2E1$^+$) EVs were found to be already increased after 12 weeks of feeding, macrophage (Galectin 3$^+$)- and neutrophil (Ly-6G and Ly-6C)- derived EV were only elevated after 48 weeks of feeding, and were associated with the histological presence of inflammatory foci in the liver (140). These results suggest that circulating extracellular vesicles derived from specific subsets of inflammatory cells might have significant diagnostic utility. Such EV analysis in human patients identified the potential of CD14$^+$ EVs, derived from monocytes and macrophages, and Vα21/Vβ11$^+$ EVs, derived from invariant NK T (iNKT)-cells, to diagnose patients with NAFLD from healthy individuals. Furthermore, quantities of these specific EV-subsets were correlated to ALT levels and the overall

severity of NASH (141). Although CD4$^+$ and CD8$^+$ EVs were also up-regulated in NAFLD individuals, they displayed less diagnostic utility for the extent of steatosis (141). Another study identified changing numbers of CD14$^+$ and CD16$^+$ EVs in NAFLD patients with advanced fibrosis (F3-4) as compared to those with non-severe (F0-2) fibrosis, and the potential of these novel circulating markers to increase the diagnostic utility of the liver fibrosis score (LFS) (142). Although no NAFLD-patients were included, it is worth mentioning the study performed by Rautou et al, which identified significantly changing circulating levels of leuko-endothelial (CD31$^+$/41$^-$)-, pan-leukocyte (CD11a$^+$)- and lymphocyte (CD4$^+$)-derived EVs in patients with liver cirrhosis as compared to healthy controls. The number of leuko-endothelial (CD31$^+$/41$^-$)-derived EVs was found to be an indicator for systemic inflammation and the severity of cirrhosis (143). However, it is important to mention that the standardized preparation of EVs from plasma, their qualitative assessment (i.e., specific cargo content) as well as their accurate and reproducible quantification remain challenging and hamper the widespread use of EVs as NAFLD biomarkers at present (144).

Upon liver disease initiation and development, not only changing amounts of circulating EV subtypes and dynamic EV phenotypes can be observed, also significant changes in their miRNA cargo has been suggested as a potential diagnostic tool. While most studies focus on the total circulating miRNA content, consisting of protein (Ago2)-bound and EV-associated miRNAs, this diagnostic approach may lack sensitivity and specificity as delicate cell-type specific miRNA-dynamics may not be observed. Focusing on EV-associated miRNAs, representing the miRNA content of their cell of origin, may therefore be a more suitable diagnostic tool, as has been previously reported in a retrospective study using early-stage HBV/HCV patients (145). Indeed, various *in vitro* studies, mimicking the cellular changes observed during liver disease, identified a strong correlation between the dynamic cellular miRNA content, and the miRNA levels in their derived EVs (146–148). However, most of such studies have been executed using (primary) hepatocytes, and thus lack information concerning inflammatory cells and their EVs. Overall, upon the initiation and progression of liver disease, various circulating EV-associated miRNAs have dynamic expression levels (149). For example, EV-associated miRNA-122 has been found to increase in human NAFLD patients with significant liver fibrosis (150) and in dietary animal NASH models (151). Although most miRNAs are expressed by a variety of cell types, and show dynamics in various physiological or pathological conditions, some miRNAs may especially represent the increased inflammation associated with NAFLD-development. One such miRNA concerns miRNA-155, which is especially expressed in hepatocytes and macrophages, and which is known to control the innate and adaptive immune system during NAFLD (152). Indeed, miRNA-155 tightly contributes to TNFα production and LPS sensitization (152–154). Although its EV-associated expression levels have not yet been evaluated in human patients, increased levels are observed in LPS and CpG-administered mouse models of liver disease. Such enriched EV-associated miRNA-155 levels correlated well with the increased TNFα-levels and to the hepatic miRNA-155 content (155).

Dynamic Changes in PBMC-Subsets

During the initiation and perpetuation of NAFLD, the important dynamic changes in the phenotype and quantity of circulating immune cells have been proposed as a potential non-invasive diagnostic tool. While some studies have tried to characterize the complete circulating white blood cell content (156), others mainly concentrate their research on peripheral blood mononuclear cells (PBMCs), consisting of lymphocytes and monocytes, as they have been found to display the most important dynamics during the NAFLD pathology. Indeed, PBMC-analysis of the peripheral blood of NAFLD patients compared to healthy individuals, identified a significant elevation in the total monocyte fraction (157). Moreover, quantitation of the three different monocyte subtypes, being the classical CD14^{++}CD16$^-$, intermediate CD14^{++}CD16$^+$ and non-classical CD14$^+$CD16^{++} monocytes, identified an elevated number of non-classical monocytes, and a decreased number of classical monocytes in NAFLD patients. However, neither the quantitation of total monocyte levels, nor of monocyte subsets, was correlated to the severity of NASH (157–159). The different monocyte fractions showed strong association with age, triglyceride-content, and waist circumference (159). Interestingly, comparing monocyte-subsets between HCV- and NAFLD-patients, identified an increased proportion of non-classical monocytes only in the latter pathology (158).

Besides the important changes in monocyte subsets, a change in receptor/ligand expression on their cell membrane may be observed. For example, the expression of CCR4, which is known to recognise the chemoattractants CCL2, CCL4, and CCL5, is significantly enriched on monocytes, especially intermediate monocytes, from NAFLD patients as compared to healthy controls. Also sialic acid binding Ig-like lectin (SIGLEC)-1, also known as CD169, which is involved in monocyte recruitment, knows enrichment on intermediate and classical monocytes of NAFLD patients (158). Monocytes isolated from patients with NAFLD demonstrated increased expression of the inflammatory cytokines IL-6, TNF-a, and IL-1b, particularly in patients with fibrosis (160). Last, TLR6 expression is up-regulated on monocytes, and is useful as marker to differentiate NAFLD patients form obese individuals, and even to distinguish patients with NASH from those with simple steatosis (161).

Further characterisation of the circulating immune cell landscape led to the identification of decreased levels of total CD3$^+$ cells, CD8$^+$ T cells, CD56^{dim} NK cells, NKG2D$^+$ NK cells, NKG2D$^+$ CD56^{dim} NK cells, NKG2D$^+$ iNKT cells, PD1$^+$CD4$^+$ T cells, CXCR3$^+$CD4$^+$ T cells, PD1$^+$CD25$^+$CD45RA$^+$CD4$^+$ T-cells, and mucosal-associated invariant T (MAIT) cells, and elevated numbers of total CD4$^+$ T cells, CD25$^+$CD45RA$^+$CD4$^+$ T-cells and Th2 cells, when NAFLD patients were compared to healthy controls (162, 163). Changing levels of PD1$^+$CD4$^+$, PD1$^+$CD25$^+$CD45RA$^+$CD4$^+$, and CXCR3$^+$CD4$^+$, CD25$^+$CD45RA$^+$CD4$^+$ T-cells were even able to discriminate patients with significant fibrosis (F2-4) from those

with no or mild fibrosis (163). Lastly, based on the observation of a diminished amount of resting Tregs (rTregs; CD4$^+$ CD45RA$^+$CD25^{++}) in patients with NASH, compared to those with NAFL, the Th17/rTreg and Th2/rTreg ratios have showed significant diagnostic value in NASH patients (164).

Overall, NAFLD affects the composition and functional properties of circulating immune cells, which likely reflects the hepatic pathology as well as extrahepatic metabolic disorders (e.g., in adipose tissue). While this is interesting from a conceptual perspective for understanding NASH, this has as of now not yet translated into a more accurate disease phenotyping or risk stratification. Part of this gap might be explained by technical challenges (e.g., requirement of fresh cells, laborious work-flow for multi-panel characterization), but part might be related to the complex biology, in which systemic immune responses are not solely driven by changes in the diseased liver.

CONCLUDING REMARKS

One major hiatus in the clinical management of NAFLD patients remains the inability of non-invasive scoring tools to identify those patients with risk of NAFLD progression, and their lack in sensitivity and specificity to detect small changes in disease perpetuation or progression. An overwhelming number of studies have identified novel circulating markers, suggesting their utility for routine clinical practice. Indeed, in the search for such novel diagnostic tools, preference has been given to serological markers, as they harbor multiple features of the ideal biomarker, being their ease of sampling, wide availability, small sampling error, good cost-effectiveness, the possibility to execute repeated measures, possibility of automatization, and limited observer-related variability (11). Due to the important contribution of the inflammatory system in the NAFLD pathogenesis, circulating markers reflecting such inflammatory actions may represent innovative diagnostic tools.

One of the major drawbacks for the implementation of novel discovered biomarkers in clinical practice is their discovery in, often, small cross-sectional studies, lacking external validation. Very few studies investigated dynamic changes of inflammatory biomarkers in response to treatment, but the high number of ongoing clinical trials in NAFLD/NASH is expected to close this gap (165). Indeed, while the ideal patient cohort is heterogeneous in various parameters such as age, gender, and ethnicity, most of the reported studies rely on highly selected and specific patient populations, such as morbidly obese individuals. For example, while most studies have been performed in ethnicity-homogeneous populations, often with an Asian or Caucasian background, important ethnicity-dependent differences in the diagnostic utility of biomarkers have been reported (166). It is also important to mention that, due to the important perpetuating effects of type 2 diabetes mellitus (T2DM) on the risk of NAFLD and advanced fibrosis, the diagnostic utility of biomarkers may be altered due to presence of T2DM. Indeed, several studies reported that diagnostic tools, which were developed and validated in non-diabetic populations, may underperform when applied to NAFLD patients suffering from

T2DM (167, 168). When comparing the results obtained from different studies, it is often difficult to compare the diagnostic utility of the proposed markers, due their validation against different standards, often liver biopsy or ultrasonography. Additionally, without the use of liver biopsy as reference, the credibility of the proposed marker is often lower, especially due to the inability to report the correlation of the marker with the various stages of NAFLD propagation and its histological-associated changes. When comparing studies concerning the same diagnostic marker, comparison of the negative and positive predictive value is often complicated due to the use of different cut-off values.

Due to the complex interplay of the various damaging "hits" in the pathogenesis of NASH, including the dysfunction/inflammation of adipose tissue and an overall increased systemic inflammation (169), circulating inflammatory markers are unable to provide information solely on hepatic inflammation, but instead represent the overall (hepatic and extrahepatic) inflammatory status. Significant differences in the level of extrahepatic inflammation between NASH-patients, e.g., lower levels of adipose tissue inflammation observed in NAFLD patients with the PNPLA3 I148M genetic variant as compared to weight-matched NAFLD patients homozygous for the wild type allele (170), may therefore cause the observed discrepancies between various published studies.

To gain popularity in the clinical community, a proposed novel biomarker should provide significant additional and useful information, impossible to obtain through use of the clinical routine parameters or imaging systems. While most of the clinical serological scoring tools are unable to differentiate patients with NAFLD from those with simple steatosis, some of the described inflammatory markers claim this possibility (**Table 1**). However, almost all studies lack extensive validation to ensure credibility of these obtained results. It should also be mentioned that inflammatory markers are not liver-specific, and therefore demand critical interpretation, as they can be strongly influenced by comorbidities (171). As each individual biomarker has his own strengths and weaknesses, the combination of several of such circulating markers, or their combination with demographic characteristics or imaging-based results in the creation of mathematical models, probably holds the highest potential in the search for the ideal non-invasive diagnostic tool.

AUTHOR CONTRIBUTIONS

Concept and design, drafting of the manuscript, and critical revision by JL and FT. All authors contributed to the article and approved the submitted version.

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