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The role of omics in the pathophysiology, diagnosis and treatment of non-alcoholic fatty liver disease

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ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) is a multifaceted metabolic disorder, whose spectrum covers clinical, histological and pathophysiological developments ranging from simple steatosis to non-alcoholic steatohepatitis (NASH) and liver fibrosis, potentially evolving into cirrhosis, hepatocellular carcinoma and liver failure. Liver biopsy remains the gold standard for diagnosing NAFLD, while there are no specific treatments. An ever-increasing number of high-throughput Omics investigations on the molecular pathobiology of NAFLD at the cellular, tissue and system levels produce comprehensive biochemical patient snapshots. In the clinical setting, these applications are considerably enhancing our efforts towards obtaining a holistic insight on NAFLD pathophysiology. Omics are also generating non-invasive diagnostic modalities for the distinct stages of NAFLD, that remain though to be validated in multiple, large, heterogenous and independent cohorts, both cross-sectionally as well as prospectively. Finally, they aid in developing novel therapies. By tracing the flow of information from genomics to epigenomics, transcriptomics, proteomics, metabolomics, lipidomics and glycomics, the chief contributions of these techniques in understanding, diagnosing and treating NAFLD are summarized herein.

1. Introduction – current status

NAFLD is recognized as the most common liver disease in developed countries with its incidence continuously rising in parallel to the increasing incidences of obesity and type 2 diabetes (T2DM) [1]. Significant efforts are currently underway to delve deeper into the pathophysiology of the disease, to create non-invasive tools for its diagnosis and staging, as well as to develop highly effective and specific treatments.

The disease is characterized initially by hepatic lipid accumulation (non-alcoholic fatty liver; NAFL), that can often progress to non-alcoholic steatohepatitis (NASH), liver fibrosis or cirrhosis, as outlined in detail elsewhere in this special issue [1]. Several triggers and series of events stimulating necroinflammatory processes in the liver have been recognized and described as "the multiple hit" pathogenetic mechanism leading to advanced NASH [2]. Additionally, multiple factors (such as lack of physical activity, unhealthy nutrition, concomitant alcohol consumption and presence of other metabolic diseases) have been

Abbreviations: 1H-MRS, magnetic resonance imaging and proton spectroscopy; AA, arachidonic acid; ACC, acetyl-coA carboxylase; ALT, alanine aminotransferase; ApoE, apolipoprotein E; APRI, aspartate aminotransferase to platelet ratio index; AST, aspartate transaminase; AUROC, area under the receiver operator characteristics; BMI, body mas index; CAP, controlled attenuation parameter; CHI3L1, chitinase 3 Like 1; ChREBP, carbohydrate-;responsive element-binding protein; CK-18, Cytokeratin-18; CRP, C-reactive protein; DAG, diacylglycerol; DHA, docosahexaenoic acid; DHEA, dehydroepiandrosterone; EPA, eicosapentanoic acid; F#, fibrosis (score); FADS, fatty acid desaturase; FFA, free fatty acids; FIB-4, fibrosis-4 score; FXR, farnesoid X receptor; GCKR, glucokinase regulatory protein; GGT, gamma-glutamyltransferase; GRS, genetic risk score; GWAS, genome - wide association studies; HAT, histone acetyltransferase; HCC, hepatocellular carcinoma; HDAC, histone deacetylase; HSD17B13, 17-b retinol dehydrogenase 13; HSI, Hepatic Steatosis Index; IGF, insulin growth factor; IGFBP, insulin growth factor binding protein; IL-10, Interleukin-10; DZNep, 3-deazaneplanocin A; PLA2, Phospholipase A2; IL-32, interleukin-32; IL-6, interleukin-6; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; DNMTis, DNA methylation inhibitors; HDACis, HDAC inhibitors; LSM, liver stiffness measurement; MBOAT7, membrane bound O-acyltransferase domain-containing 7; MetS, metabolic syndrome; miR, micro RNA; MRE, magnetic resonance elastography; MRI-PDFF, Magnetic resonance imaging derived proton density fat fraction; MS, mass spectrometry; MUFA, monounsaturated fatty acid; NAFL, non-alcoholic fatty liver; NAFLD, non-alcoholic fatty liver disease; NAS, NAFLD activity score; NASH, non-alcoholic steatohepatitis; NFS, NAFLD fibrosis score; OR, odds ratio; PC, phosphatidylcholine; PDGF, platelet-derived growth factor; PE, phosphatidylethanolamine; PNPLA3, patatin-like phospholipase domain-containing-3; PPAR, peroxisome proliferator-activated receptor; PUFA, polyunsaturated fatty acid; PTPRE, protein-tyrosine phosphatase epsilon; ROS, reactive oxygen species; SELDI, surface-enhanced laser desorption/ionization; SFA, saturated fatty acids; circRNA, circular RNA; lncRNA, long noncoding RNA; tRNA, transport RNA; SIRT, sirtuin; IgG, immunoglobulin G; HFD, high fat diet; SNP, single nucleotide polymorphism; SPM, specialized pro-resolving mediator; SWE, shear wave elastography; T2DM, Type 2 Diabetes Mellitus; TG, triglyceride; TGF, transforming growth factor; TM6SF2, transmembrane 6 superfamily member 2; TOF, time-of-flight; VCTE, vibration-controlled transient elastography; WC, waist circumference.

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Table 1
Most extensively studied scores and imaging modalities in NAFLD.

#	N	Comparisons	Prediction models	Sensitivity	Specificity	AUROC
Steatosis/any NAFLD						
	2815	C vs NAFLD	Ultrasonography	84.8%	93.6%	0.93
[4]	Meta-analysis					
[6]	Review	C vs S	a) Fatty liver index	a) 87%	a) 64%	a) 0.84
			b) HSI	b) 93%	b) 40%	b) 0.81
			c) SteatoTest	c) 90%	c) 54%	c) 0.80
			d) NAFLD liver fat score	d) 86%	d) 71%	d) 0.87
[7]	2735	a) S0 vs S1-S3	CAP	a) 69%	a) 82%	a) 0.82
	Meta-analysis	b) S0-S1 vs S2-S3		b) 77%	b) 81%	b) 0.87
		c) S0-S2 vs S3		c) 88%	c) 78%	c) 0.88
[8]	236	a) S0 vs S1-S3	CAP, M and XL	a) M: 75%	a) M: 75%	a) M: 0.82
		b) S0-S1 vs S2-S3		XL: 80%	XL: 74%	XL: 0.83
		c) S0-S2 vs S3		b) M: 80%	b) M: 81%	b) M: 0.89
				XL: 80%	XL: 83%	XL: 0.88
				c) M: 81%	c) M: 81%	c) M: 0.92
				XL: 86%	XL: 84%	XL: 0.93
[9]	635	a) S0 vs S1-S3	MRI-PDFF	a) 93%	a) 94%	a) 0.98
	Meta-analysis	b) S0-S1 vs S2-S3		b) 74%	b) 90%	b) 0.91
		c) S0-S2 vs S3		c) 74%	c) 87%	c) 0.90
NAFL vs NASH vs Fibrosis						
	3431	NAFL vs NASH	a) CK-18 M30	a) 75%	a) 77%	a) 0.82
[12]	Meta-analysis		b) CK-18 M65	b) 71%	b) 77%	b) 0.80
			c) FGF-21	c) 62%	c) 78%	d) 0.94
			d) Combined biomarker panel	d) 92%	d) 85%	
[226]	494	a) F0-F1 vs F2-F4	a) FibroTest	a) 8%	a) 100%	a) 0.85
	Meta-analysis	b) S0-S1 vs S2-S3	b) SteatoTest	b) 39%	b) 81%	b) 0.80
		c) NASH (NAS > 4)	c) ActiTest	c) 28%	c) 91%	c) 0.84
		d) NAS > 2	d) NashTest	d) 93%	d) 34%	
[227]	D: 150	a) F0-F2 vs F3-F4	ADAPT: age, diabetes,	a) D: 91%,	a) D: 73%	a) D: 0.86
	V: 281	b) F0-F2 vs F3-F4 in NAFL (Y vs N)	PLT, PRO-C3 (marker of collagen	b) 88%	b) 87%	V: 0.87
		c) F0-F2 vs F3-F4 in NASH (Y vs N)	type III formation)	c) 94%	c) 50%	b) 0.89
						c) 0.81
Fibrosis						
[11]	Review	a) F0-F1 vs F2-F4	VCTE	VCTE:	VCTE:	a) VCTE: 0.79
		b) F0-F3 vs F4	MRE	a) 66%	a) 80%	MRE: 0.92
				b) 89%	b) 86%	b) VCTE: 0.93
				MRE:	MRE:	MRE: 0.94
				a) 85%	a) 85%	
				b) 80%	b) 86%	
[228]	13,046	a) F0-F1 vs F2-F4	APRI	APRI:	APRI:	APRI:
	Meta-analysis	b) F0-F2 vs F3-F4	FIB-4	a) 59%	a) 77%	a) 0.70
		c) F0-F3 vs F4	BARD	b) 73%	b) 68%	b) 0.75
			NFS	c) 56%	c) 84%	c) 0.75
			CAP M	FIB-4:	FIB-4:	FIB-4:
			CAP XL	a) 64%	a) 70%	a) 0.75
			SWE	b) 78%	b) 71%	b) 0.80
			MRE	c) 76%	c) 82%	c) 0.85
				BARD:	BARD:	BARD:
				a) 44%	a) 70%	a) 0.64
				b) 83%	b) 59%	b) 0.73
				c) 52%	c) 84%	c) 0.70
				NFS:	NFS:	NFS:
				a) 66%	a) 83%	a) 0.72
				b) 81%	b) 70%	b) 0.78
				c) 80%	c) 81%	c) 0.83
				CAP M	CAP M	CAP M
				a) 92%	a) 57%	a) 0.83
				b) 89%	b) 77%	b) 0.87
				c) 97%	c) 78%	c) 0.92
				CAP XL	CAP XL	CAP XL
				a) 76%	a) 65%	a) 0.82
				b) 75%	b) 74%	b) 0.86
				c) 88%	c) 82%	c) 0.94
				SWE	SWE	SWE
				a) 85%	a) 94%	a) 0.89
				b) 90%	b) 92%	b) 0.91
				c) 100%	c) 86%	c) 0.97
				MRE	MRE	MRE
				a) 73%	a) 91%	a) 0.88
				b) 86%	b) 91%	b) 0.93
				c) 87%	c) 93%	c) 0.92
[229]	5366	a) F0-F2 vs F3-F4	ELF (HA, PIIINP, TIMP1)	a) 65%	a) 86%	a) 0.83
	Meta-analysis	b) F0-F1 vs F2-F4				b) 0.81

Table 1 (*continued*)

#	N	Comparisons	Prediction models	Sensitivity	Specificity	AUROC
Validation studies – studies in specific populations						
[5]	146	C vs NAFLD	a) Simple ultrasonography	a) 63%	a) 88%	a) 0.82
			b) 5- point ultrasonography score	b) 70%	b) 100%	b) 0.89
			c) ^{1}H-MRS			c) 0.96
[230]	383	a) S0 vs S1-S3	a, b, c) CAP	a) 80%	a) 83%	a) 0.87
		b) S0-S1 vs S2-S3	d, e, f) LSM	b) 70%	b) 76%	b) 0.77
		c) S0-S2 vs S3		c) 72%	c) 63%	c) 0.7
		d) F0-F1 vs F2-F4		d) 71%	d) 70%	d) 0.77
		e) F0-F2 vs F3-F4		e) 71%	e) 75%	e) 0.8
		f) F0-F3 vs F4		f) 85%	f) 79%	f) 0.89
[13]	424	a) NAFLD (Y vs N)	CK-18 M30	a) 63%	a) 83%	a) 0.77
		b) NASH (Y vs N)		b) 58%	b) 68%	b) 0.65
		c) Fibrosis (Y vs N)		c) 54%	c) 85%	c) 0.68
[224]	220	a) NAFLD (Y vs N)	a) SteatoTest	a) 73%	a) 72%	a) 0.73
	T2DM	b) NASH (Y vs N)	b) ActiTest	b) 74%	b) 62%	b) 0.70
		c) NASH (Y vs N)	c) NashTest	c) 71%	c) 60%	c) 0.69
		d) Fibrosis (Y vs N)	d) FibroTest	d) 64%	d) 73%	d) 0.72
[223]	213	a-d) NASH (Y vs N)	a) CK-18	a) 63%	a) 80%	a) 0.76
	T2DM	e-i) F0-F2 vs F3-F4	b) HAIR	b) 57%	b) 77%	b) 0.68
			c) NashTest	c) 71%	c) 58%	c) 0.66
			d) BARD	d) 98%	d) 5%	d) 0.61
			e) PRO-C3	e) 88%	e) 80%	e) 0.9
			f) APRI	f) 84%	f) 75%	f) 0.86
			g) FIB-4	g) 68%	g) 75%	g) 0.78
			h) FibroTest	h) 64%	h) 74%	h) 0.70
			i) NFS	i) 68%	i) 55%	i) 0.64
[231]	292	a) Fibrosis (Y vs N)	APRI	APRI:	APRI:	APRI:
		b) F0-F1 vs F2-F4	FIB-4	a) 63%	a) 76%	a) 0.75
		c) F1-F2 vs F3-F4	NFS	b) 65%	b) 71%	b) 0.73
			AST/ALT	c) 75%	c) 65%	c) 0.76
				FIB-4:	FIB-4:	FIB-4:
				a) 70%	a) 68%	a) 0.72
				b) 66%	b) 74%	b) 0.76
				c) 75%	c) 71%	c) 0.80
				NFS:	NFS:	NFS:
				a) 64%	a) 66%	a) 0.69
				b) 57%	b) 77%	b) 0.73
				c) 70%	c) 74%	c) 0.78
				AST/ALT:	AST/ALT:	AST/ALT:
				a) 38%	a) 76%	a) 0.59
				b) 54%	b) 68%	b) 0.65
				c) 54%	c) 73%	c) 0.68
[232]	3202	NFS	a) F0-F2 vs F3-F4	a) NFS: 89%	a) NFS: 37%	a) NFS: 0.74
		FIB-4	b) F0-F3 vs F4	FIB-4: 82%	FIB-4: 57%	FIB-4: 0.78
		ELF		ELF: 74%	ELF: 73%	ELF: 0.80
		VCTE		VCTE: 83%	VCTE: 61%	VCTE: 0.80
		Combinations		b) NFS: 94%	b) NFS: 24%	b) NFS: 0.73
				FIB-4: 88%	FIB-4: 41%	FIB-4: 0.75
				ELF: 83%	ELF: 55%	ELF: 0.76
				VCTE: 92%	VCTE: 38%	VCTE: 0.78

C, controls/healthy; D, Discovery; F, Fibrosis; N, No; S, Steatosis; V, Validation; Y, Yes. Sensitivities and specificities represent authors' chosen cutoff values; whenever optimal cutoffs are not specified, values with greater sensitivity are included. Whenever multiple scores are available for each comparison, the highest-performing scores are selected.

associated with increased risk of disease progression [3]. Still, we are not able to predict with great accuracy which patient and when will develop hepatic inflammation or fibrosis.

An important reason for our limited ability to predict the course of the disease in a patient-specific manner is the lack of low-cost and easy-to use diagnostic tools, which would have enabled routine screenings and regular follow-ups of the population at high risk for the development of the disease. Liver biopsy is still considered the gold standard for diagnosing and staging NAFLD, but its high costs, labor-intensive character and non-negligible risks, in combination with recent advancement in non-invasive diagnostic procedures (Table 1), have significantly limited its use. For diagnosing hepatic steatosis, ultrasound is broadly used and is considered a well-tolerated and low-cost method. It demonstrates acceptable specificity but still suboptimal sensitivity, especially in earlier stages of the disease [4,5]. Additionally, it may not be available in primary care settings, whereas the diagnostic accuracy largely depends on the operator's experience. Thus non-invasive scores have been developed (Fatty liver index, Hepatic Steatosis Index, SteatoTest, NAFLD Liver Fat score) [6] (Table 1). These scores are following the "candidate risk factor" approach, are in most cases based on clinical and metabolic parameters (e.g. BMI, presence of T2DM, central obesity) selected on the basis of best clinical judgement, and they almost universally demonstrate suboptimal accuracy. Positive results have to be further confirmed with ultrasound and thus their use has been limited to date to epidemiological studies. Importantly, the currently available scores and ultrasound can detect steatosis up to a specific threshold but can poorly differentiate between the different levels of it. Efforts focusing on measuring the controlled attenuation parameter (CAP) with the M or XL-probes in ultrasound have reported different thresholds and higher but still suboptimal accuracies for steatosis level [7,8]. Magnetic resonance imaging derived proton density fat fraction (MRI-PDFF) is a very reliable method at predicting the level of steatosis, but its high costs and demands for relevant equipment and trained personnel limits its use to specific centers, thus rendering the method inappropriate for general screening [9].

Significant advancements have been also observed for detection of advanced liver fibrosis. Diagnostic scores such as FIB-4, NAFLD fibrosis score (NFS), BARD and Aspartate aminotransferase to platelet

ratio index (APRI) demonstrate acceptable but still suboptimal sensitivity and specificity [10] (Table 1). Vibration-controlled transient elastography and magnetic resonance elastography (MRE) have shown high diagnostic accuracy but are expensive and not widely available [11]. Finally, less tools are available for non-invasively differentiating NAFL from NASH. Cytokeratin-18 (CK-18) fragment has been mostly investigated, but its rather limited sensitivity at the individual level, in combination with the lack of a commercially available assay (certified for clinical purposes) and the large variability in the reported diagnostic cut-offs have significantly limited its clinical use [12,13]. Numerous, other algorithms have been also developed (Supplementary Table) based on selection of parameters according to their contribution in the pathogenesis of the disease in animal studies or their association with the risk for disease progression in epidemiological studies, but none of them has an established role in daily clinical routine. Thus, despite recent advances, there is still a great need for highly accurate low-cost and easy-to-use non-invasive models for differentiating NAFL from NASH and for assessing liver fibrosis stage.

In addition to the important limitations in the diagnosis of NAFLD, no specific and highly effective treatment exists. A large number of ongoing clinical trials are currently evaluating the efficacy of drugs that act through multiple mechanisms on liver fibrosis. However, most of the drugs that were aiming to improve liver fibrosis have failed so far. This suggests that either a treatment is required in earlier stages of the disease and before the establishment of advanced fibrosis or/and that the efforts to understand the pathophysiological mechanisms of the disease in order to develop more effective drugs and combination treatments should be intensified.

Omics technologies, the development of which has been significantly advanced in the last few years, can potentially be used to further investigate the pathophysiology of NAFLD, develop accurate diagnostic methods and identify therapeutic targets (Fig. 1). Their main advantage is that they can provide an enormous amount of data in a very short-period of time and with an unbiased approach. The rapid evolution of machine learning and artificial intelligence in the last years has also enabled the accurate analysis of large data sets produced by omics, leading to an avalanche of NAFLD-related information.

In this review, we are presenting the most important findings of omics studies and specifically of genomics, epigenomics, transcriptomics, metabolomics/lipidomics and glycomics in relation to the pathophysiology, diagnosis and treatment of NAFLD, including our own assessment of their future diagnostic and therapeutic potential.

2. Genomics

The emergence of high throughput sequencing and oligonucleotide arrays has kindled a series of genome - wide association studies (GWAS) and single nucleotide polymorphism (SNP) investigations that highlight the pathobiological role of previously obscure genes in the NAFLD spectrum (Fig. 2). For the purposes of this review, we will emphasize on three major genes that have been explored in large and diverse patient cohorts: *PNPLA3*, *TM6SF2* and *GCKR*.

2.1. PNPLA3

PNPLA3 (Patatin-like phospholipase domain-containing 3) is located in the 22q13 region of chromosome 22 and encodes adiponutrin, a protein with lipase and acyltransferase activity, expressed in liver and adipose tissue [14]. Adiponutrin variant p.I148M (rs738409) affects hepatic lipid composition by decreasing polyunsaturated fatty acid (PUFA) transfer from diacylglycerols (DAG) to phosphatidylcholine (PC), thus increasing PUFA content of triglycerides (TG) and DAGs while impairing PC synthesis and hindering lipid droplet hydrolysis [15]. *PNPLA3* was first introduced in NAFLD pathophysiology when a GWAS demonstrated a robust association of rs738409 C/G with intrahepatic TG levels independently of Body mass index (BMI), plasma lipid indices and insulin resistance. The variant was reported in Hispanics (45%), Caucasians (33%) and African Americans (24%) [16]. A recent GWAS confirmed its predominance in Hispanic patients [17]. Rs738409 is an established indicator of NAFLD risk with genome-wide significance owing to conventional genotyping, bioinformatics [18–21] and novel natural language processing algorithms [17,22]. Variants such as rs2896019, rs381062 [19] rs738408, and rs374720 [22] possess lesser significance.

Targeted genotyping delineates the effects of the rs738409 G-allele on hepatic fat accumulation, independent of age or sex [23,24], BMI [23–25], serum triglyceride and lipoprotein levels [23,25]. Positive associations with serum gamma-glutamyltransferase (GGT) [16] and aminotransferase activities are also reported [16,24,26–28], indicating a pro-inflammatory effect. A meta-analysis of 6071 NAFLD patients and 10,366 controls affirms rs738409 as a potent predictor of NASH risk (odds ratio (OR)=4.44), in an additive model of all polymorphisms, independent of ethnicity and age [18].

Rs738409 is an important indicator of histologically-confirmed steatosis [26]. G-allele carriage increases steatosis grade by 10% [24], and elevates the risks of steatosis score ≥ 2 (OR = 1.46), portal (additive OR = 1.57) and lobular (OR = 1.84) inflammation, Mallory-Denk bodies (OR = 1.55), NAFLD activity score (NAS) >3 (OR = 1.56) and fibrosis (OR = 1.50) [26]. Similar findings are replicated in subsequent GWAS [27] and SNP studies [28], with steatosis and fibrosis positively corresponding to risk allele frequency. Of note, the collective impact of p. I148M on steatosis, aminotransferases and fibrosis is enhanced by adiposity [29] and observed in pediatric cohorts [30]. Furthermore, rs738409 G-allele carriers are prone to hepatocellular carcinoma (HCC) (adjusted OR = 2.26, 5-fold risk for homozygotes compared to C-allele carriers) [31] and have increased risk of liver failure and liver-related death in fibrosis stage ≥3 [32].

2.2. TM6SF2

Located in 19p13, *TM6SF2* (transmembrane 6 superfamily member 2) encodes a regulatory protein of VLDL secretion, expressed in intestinal, renal and liver tissues [33,34]. *TM6SF2* variant p.E167K (rs58542926) affects PUFA biosynthesis and depletes PUFA from hepatic polyunsaturated PCs and TGs while enriching polyunsaturated Free Fatty acids (FFA), −yet reducing total FFA concentration-, thus impeding VLDL synthesis [34]. A GWAS of 86,704 patients first linked rs58542926 to elevated liver fat and NAFLD susceptibility, albeit decreased levels of plasma LDL and TG. Reported frequencies were 7.2% in Caucasians, 4.7% in Hispanics and 3.4% in African Americans [33]. Similar associations for SNPs near *TM6SF2* were previously reported [20].

Rs58542926-related NAFLD risk is significantly augmented by adiposity [29]. Rs58542926 is proinflammatory, being frequently correlated with elevated serum aminotransferase activity [28,33] but not GGT levels [28]. Histologically, a study of 1074 patients associates rs58542926 carriage with steatosis and fibrosis stage, attaining marginal significance for steatosis (OR = 1.38) [35]. Another SNP evaluation of 320 NAFLD patients indicates increased risks of steatosis grade ≥ 2 (OR = 1.90) and fibrosis grade ≥ 3 (OR = 2.35), adjusted for age, gender, BMI, T2DM and statin use [28]. Sookoian et al. illustrate positive correlations with NAFLD risk, disease severity and steatosis degree, but poor associations with inflammation, NAS, hepatocellular balooning and fibrosis [36]. Notwithstanding, the allele influences cirrhosis and predisposes to HCC in both unadjusted (OR = 1.92) [35] and adjusted models for age, sex, obesity and diabetes, or fibrosis (OR = 1.99/2.80) [37].

2.3. GCKR

GCKR (glucokinase regulatory protein), located in chromosome 2 [20], is expressed in liver tissue and inhibits glucokinase in hepatocyte nuclei. *GCKR* p.P446L (rs780094) variant blunts this inhibitory effect in

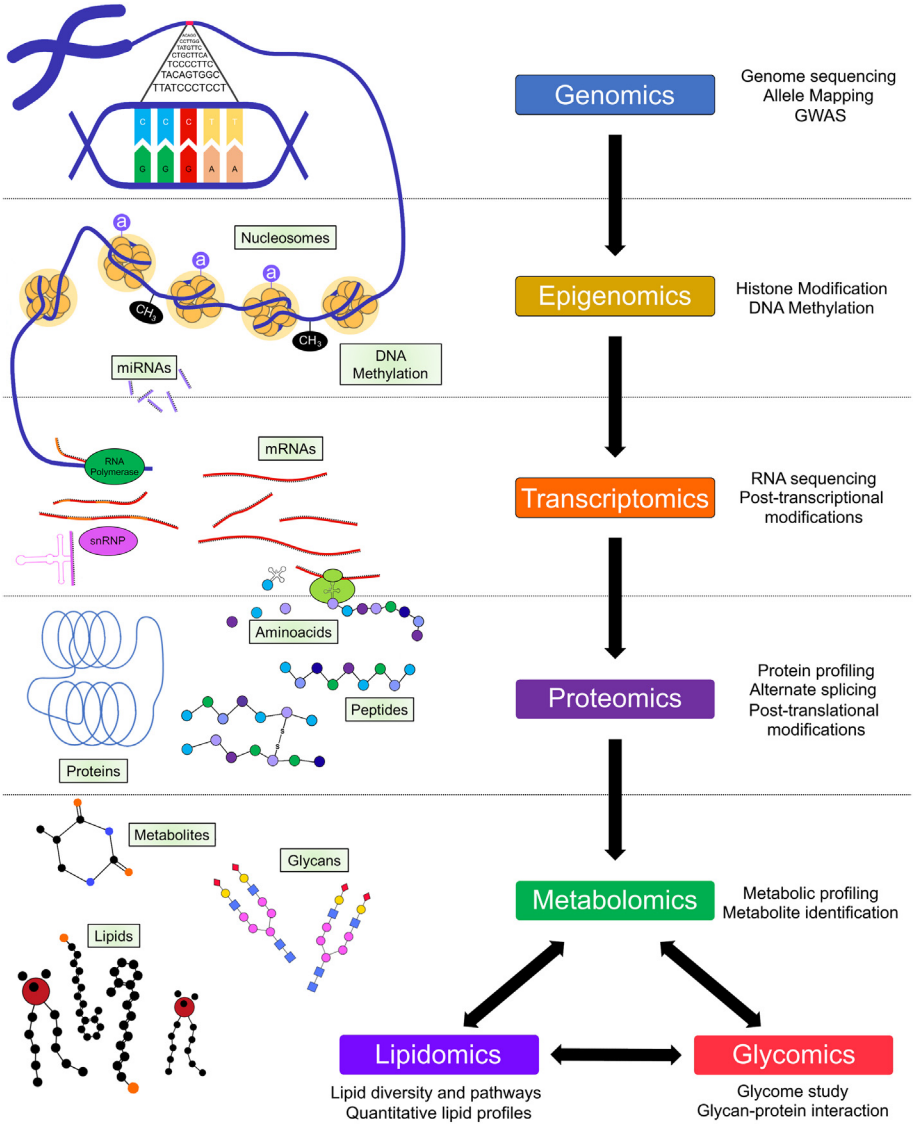

Fig. 1. Main omics procedures used currently in medicine and in NAFLD research.

response to fructose-6-phosphate, increasing glycolysis and glycogen production while concomitantly stimulating de novo lipogenesis [38]. The first loci near *GCKR* were linked to NAFLD in a large GWAS by Speliotes et al. [20]. Through multiple studies, rs780094 constitutes a significant index of NAFLD risk in homozygotes (OR 1.27) [39] with patient BMI further amplifying this effect [29]. The allele is mapped in high-risk pediatric and adolescent cohorts and, alongside rs1260326, increases fibrosis risk by 2.6-fold [30].

2.4. Other genes

Numerous additional genes are associated with NAFLD, including *MBOAT7*, *SERPINA1*, *APOB*, *IL28B*, *MERTK* and *HFE* [40]. *MBOAT* (Membrane bound O-acyltransferase domain-containing 7) rs641738 has been mildly associated with fibrosis but not liver function or steatosis [28] while it is also linked to inflammation [41] and NAFLD severity (OR = 2.6) [21]. Carriage of its T-allele doubles the risk of NAFLD-related HCC [37]. However, its alcoholic hepatitis-related effects are prioritized in available literature.

Hepatoprotective genes are also investigated. For instance, a large, replicated exome-wide analysis of >70,000 total individuals demonstrates that homozygous carriage of *HSD17B13* (17-b retinol dehydrogenase 13) minor variant rs72613567 T/A, diminishes the *PNPLA3*-related risk of liver injury and attenuates the risks of NAFLD NASH cirrhosis, by 30% and 49% respectively. [42].

2.5. Diagnostic-prognostic perspective

12 years after identifying *PNPLA3* in NAFLD, research interest for genomics in metabolic liver disease continues to soar. Recently, large MRI-PDFF-assessed [43], and histologically-proven GWAS [44] of >10,000 total patients each, re-affirmed the robust clinical and histological genome-wide associations of *PNPLA3*, *TM6SF2*, *GCKR*, and *HSD17B13*. Novel fibroinflammatory loci in *SLC30A10* and *SLC39A8* were also studied [43]. However, a bench-to-bedside conversion of current results through tangible diagnostic and therapeutic applications has yet to take place.

PNPLA3 rs738409 constitutes the chief NAFLD predictor, adjusted for known risk factors (OR = 3.12) [21]. Yet its addition in an early predictive model of NAFLD, using routine variables, contributes trivially to score accuracy (area under the receiver operator characteristics (AUROC) = 0.866 and 0.872 respectively) [45]. *PNPLA3* is included in

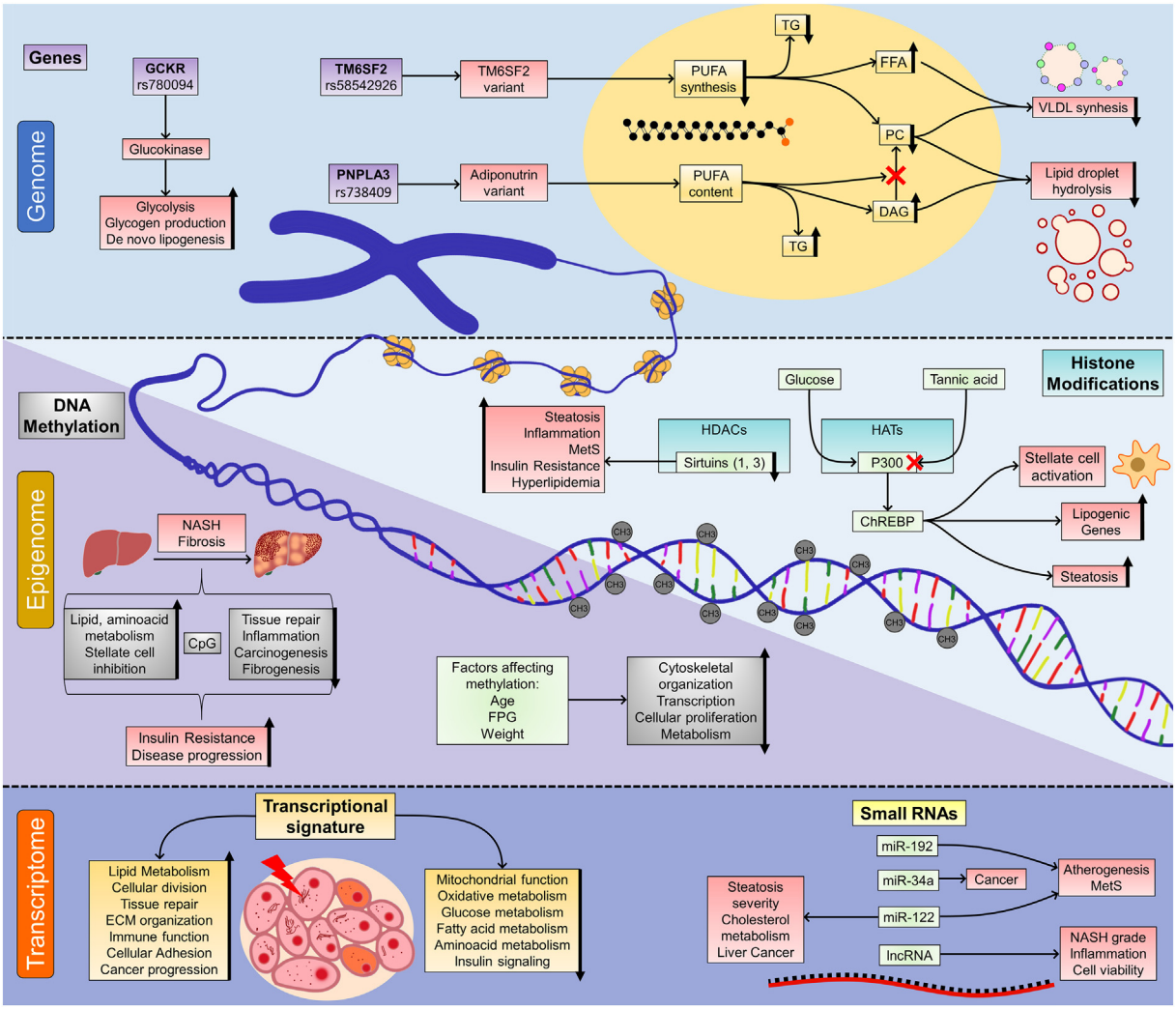

Fig. 2. Genomic, epigenomic and transcriptomic modifications in NAFLD pathophysiology. PNPLA3, TM6SF2 and GCKR are some of the most investigated genes in NAFLD. Adiponutrin (PNPLA3) variant I148M (rs738409) impairs PUFA transfer from DAGs to PCs, thus increasing PUFA in TG and DAG. TM6SF2 E167K (rs58542926) impairs PUFA synthesis, increases polyunsaturated FFAs and prevents PUFA incorporation into TGs and PCs. Both mechanisms lead to impaired VLDL synthesis and lipid droplet hydrolysis. GCKR P446L (rs780094) incites glycolysis, glycogen deposition and de novo lipogenesis by disinhibiting glucokinase. Epigenetic modifications characteristic of NAFLD progression include CpG site hypermethylation, thus reduced expression, of genes pertaining to lipid and aminoacid metabolism and stellate cell inhibition. Hypomethylation, thus increased expression, of genes pertaining to tissue repair, inflammation, carcinogenesis and fibrogenesis, increases insulin resistance and further propagates the disease. Methylation levels of cytoskeletal, transcriptional, proliferation-related and metabolic genes are affected by age, fasting glucose levels and body weight. At the histone level, depletion of sirtuins 1 and 3 and HDAC3 may propagate NASH and increase susceptibility to MetS, insulin resistance and hyperlipidemia. On the other hand, the glucose-activated HAT p300 activates ChREBP and thus precipitates stellate cell activation, elevates lipogenic gene expression and expedites steatosis, though these effects can be attenuated by tannic acid. Finally, the NAFLD transcriptome is characterized by overexpression of lipid metabolism, cellular stress, division and adhesion, extracellular matrix production and repair, cancer progression and immunomodulatory genes, whereas several pro-metabolic and insulin signaling genes are downregulated. miRNAs, especially miR-122, miR-192 and miR-34a, are linked to steatosis, cholesterol metabolism, liver cancer, atherogenesis and MetS, whereas other noncoding molecules, such as lncRNAs, are indicators of NASH grade and hepatocellular viability.

the NASH Score (Aspartate transaminase (AST, insulin, *PNPLA3* genotype) and the ClinLipMet Score (NASH Clin Score, metabolomic and lipidomic variables) with AUROCs of 0.778 and 0.866 respectively, yet its contribution to NASH prediction is not underlined [46]. In a more recent phenome-genome wide study of 27,744 patients, clinical data (age, sex, BMI, blood counts) comprise a fitter NAFLD predictor than rs738409 genotyping (AUROC = 0.785 and 0.574 respectively) and have 34% higher sensitivity compared to genotyping alone. Even though rs738409 carriage was associated with 3.1 years earlier NAFLD detection, its addition does not markedly amplify the forecasting model (AUROC = 0.788) [17].

Cumulative genetic risk scores (GRS) are frequently discussed (Table 2). A GRS of previously mentioned SNPs in *PNPLA3*, *TM6SF2*, *GCKR* and *MBOAT7* indicates a 3-fold increase in NAFLD risk starting from its second tertile [21]. When coupled with a multivariate insulin resistance score, a GRS of *PNPLA3* and *TM6SF2* SNPs detects NAS ≥ 3 (AUROC = 0.74) and fibrosis ≥3 when age included (AUROC = 0.82), yet GRS alone have AUROCs of ≤0.65 [41]. *PNPLA3* and *TM6SF2* polymorphisms are also included in a novel NASH score alongside diabetes, insulin resistance, AST and C-reactive protein levels, which predicts NASH more effectively compared to the older NASH Score (AUROCs of 0.787 and 0.729 respectively) and has enhanced accuracy in diabetic cohorts (AUROC = 0.835) [47]. Lastly, a recent investigation of 445,452 individuals formulates a GRS comprising *PNPLA3*, *TM6SF* AND *HSD17B13* SNPs. A maximum risk score of 6 designates a 26% increase in alanine aminotransferase (ALT) levels and a pronounced risk of cirrhosis and HCC (OR = 12 and 29 respectively) [48].

Genetic testing in NAFLD is currently not recommended by the American Association for the Study of Liver Diseases [49]. Thus far, genomics have not identified a consistent marker that reflects the accurate histologic features of NAFLD, partially owing to limited population sizes, inconsistent NAFLD validation methods and scarcity of

Table 2
Main genomics, epigenomics and transcriptomics-based diagnostic models of NAFLD.

#	N	Comparisons	Prediction models	Sensitivity	Specificity	AUROC
Steatosis/Any NAFLD						
[45]	D: 313 V: 157	C vs NAFLD	PNPLA3, MetS, T2DM, fasting insulin, AST, AST/ALT	D: 86% V: 84% Total: 85%	D: 71% V: 69% Total: 70%	D: 0.87 V: 0.86 Total: 0.87
[17]	8204	C vs NAFLD	PNPLA3, age, sex, 6 principal components			0.79
[112]	Transc: 40 D: 242 V: 183	C vs NAFLD	miR-122-5p, miR-1290, miR-27b-3p, miR-192-5p	D: 86% V: 90%	D: 73% V: 76%	D: 0.86 V: 0.89
[233]	446	a) C vs S < 34% b) C vs S 34–66% c) C vs S > 66%	11-SNP scoring model			a) 0.83 b) 0.94 c) 0.93
NAFL vs NASH						
[46]	D: 223 V: 95	Non-NASH vs NASH	NASH ClinLipMetScore: AST, insulin, PNPLA3 genotype, glutamate, isoleucine, glycine, LPC 16:0, PE 40:6	Total: 86%	Total: 72%	D: 0.88 V: 0.86 Total: 0.87
[108]	53	a) C vs NAFL b) NAFL vs NASH	a) miR-16 b) miR-34a			a) 0.96 b) 0.76
[113]	300	a) C vs NAFLD b) NAFL vs NASH	a) miRNA-122 b) miRNA-99a	a) 92% b) 94%	a) 85% b) 96%	a) 0.92 b) 0.91
[117]	198	a) NAFL vs NASH	miR-122, miR-192, miR-21, and CK-18 M30 fragment	91%	83%	0.83
[41]	177	NAS <3 vs NAS ≥ 3	*PNPLA3, TM6SF2,* age and enhanced lipoprotein insulin resistance index	48%	86%	0.82
[47]	D: 302 V: 151	NAFL vs NASH	PNPLA3, TM6SF2, diabetes, HOMA-IR, AST, CRP	D: 88% V: 97% Total: 91%	D: 68% V: 39% Total: 58%	D: 0.86 V: 0.79
[80]	35	NAFL vs NASH	DNA methylation of blood leukocytes in SIGIRR	71%	99%	0.88
NAFL vs NASH vs Fibrosis						
[88]	Transc: 125 D: 71 V: 160	a) C vs NAFLD b) C vs NAFLD (F ≥ 2)	IL-32 (transcriptomics-identified), ALT and AST	a) D: 94% V: 93% Total: 97% b) D: 21% V: 50% Total: 51%	a) D: 68% V: 86% Total: 74% b) D: 65% V: 82% Total: 85%	a) D: 0.85 V: 0.95 Total: 0.92 b) D: 0.88 V: 0.69 Total: 0.72
[103]	209	a) NAFL vs NASH b) NAFL vs Fibrosis	miR-122			a) 0.71 b) 0.61
[116]	687 D: 220 V: 467	NAS < 4 & F < 2 vs NAS ≥ 4 & F ≥ 2	NIS4: miR-34a, CHI3L1, HbA1c, a2-macroglobulin	D: 68% V: 74% Total: 76%	D: 77% V: 82% Total: 76%	D: 0.81 V: 0.81 Total: 0.82
Fibrosis						
[79]	26	F0-F2 vs F3-F4	DNA methylation of PPARγ	83%	93%	0.91
[91]	D: 72 V:17	F0-F1 vs F3-F4	a) 64-gene profile b) 20-gene subset	b) V: Accur = 94%		a) D: 0.98
[99]	Transc: 12 V1: 88 V2: 50	a) F0-F2 vs F3-F4 b) NAS ≤ 4 vs ≥ 5 c) NASH (Y vs N)	a) TGFB2/TGFB2-overlapping transcript 1 plus liver stiffness measurement b, c) RP11-128N14.5 lncRNA	a) 80% b) V1: 74% V2: 78% c) V1: 87% V2: 53%	a) 91% b) V1: 70% V2: 63% c) V1: 39% V2: 4%	a) 0.89 b) V1: 0.71 V2: 0.69 c) V1: 0.63 V2: 0.65
Meta-analysis						
[234]	4036	a) NAFLD (Y vs N) b) NASH (Y vs N) c) NASH vs NAFL	miR-122 miR-99a miR-34a Pooled miRNA panel	a) miR-122: 84% miR-99a: 82% miR-34a: 81% Pooled: a) 71% b) 74% c) 83%	a) miR-122: 72% miR-99a: 82% miR-34a: 83% Pooled: a) 76% b) 85% c) 85%	a) miR-122: 0.86 miR-99a: 0.87 miR-34a: 0.85 Pooled: a) 0.80 b) 0.86 c) 0.91

C, controls/healthy; D, Discovery; N, No; S, Steatosis; Transc, Transcriptomics; V, Validation; Y, Yes. Sensitivities and specificities represent authors' chosen cutoff values; whenever optimal cutoffs are not specified, values with greater sensitivity are included. Whenever multiple scores are available for each comparison, the highest-performing scores are selected.

biopsy-confirmed models. Most importantly, genomics are not dynamic processes, thus they cannot reflect the impact of environmental factors (high caloric intake, presence of obesity, T2DM etc.) in the pathogenesis and progression of NAFLD. Multi-gene models are promising tools of patient risk stratification but require additional validation.

2.6. Therapeutic perspective

PNPLA3 has also been investigated through early genetic interventions. Pre- and post-translational silencing of *PNPLA3* in p.I148M knock-in mice challenged with a sucrose-rich diet drastically improves intrahepatic fat profiles [50,51]. Additionally, *PNPLA3* silencing ameliorates steatosis and NAS independent of genotype, and improves inflammation, fibrosis and levels of acute phase, chemo-attractant and pro-fibrotic proteins, in p.I148M knock-in mice fed a NASH-inducing diet [51]. However, *PNPLA3* protects from hypercholesterolemia, gallstones, gout and acne in humans [52], therefore future pilot studies must be carefully considered. *TM6SF2* is another possible target, yet its substantial cardioprotective effects [53] indicate the complexity of such an approach, since the chief cause of death in NAFLD patients is cardiovascular disease [49].

Patient genotyping prior to therapeutic scheme initiation implies compelling curative applications. For instance, *PNPLA3* p.I148M carriers would not respond to statins and inhibitors of de novo lipogenesis, in contrast to *TM6SF2* and *GCKR* carriers [54]. Moreover, *HSD17B13* has already been implied as a therapeutic target [42], while it was recently established that carriage of *HSD17B13* rs6834314 diminishes the fibrotic effects of *PNPLA3* rs738409, thus indicating a novel objective for pathway-targeting therapies [55]. Further research could uncover powerful hierarchical and therapeutic tools in NAFLD genomics, jumpstarting a new era of clinicogenomics.

2.7. Conclusion – clinical perspective

Genomics are the first and one of the most extensively investigated omics so far. They have provided robust evidence validated by multiple studies about the causal relationship of certain common SNPs with development and rapid progression of NAFLD. Given the continuous evolvement of personalized medicine, we expect the information from genomics to be used in the near future for identifying the patients at higher risk for presenting a more aggressive course of disease and/or for potentially predicting the response to specific treatments. These may justify more intensive follow-ups or guide treatment decisions in high-risk populations. Sfince genomics are not dynamic processes and do not reflect the effects of environmental factors on NAFLD, we see less potentials for them as diagnostic markers and we expect for them to have only a small, if any, contribution in non-invasive diagnostic tests. Finally, due to the technical and clinical challenges related to gene-engineering as well as due to the complex pathophysiology of the disease, we recognize at the moment limited perspectives in the development of gene therapies for NAFLD.

3. Epigenomics

Epigenomics investigates the epigenetic modifications on cellular genetical material. Several studies have assessed the impact of epigenetic modifications in the development and progress of NAFLD (Fig. 2) as well as in the association of NAFLD with other metabolic diseases by focusing on DNA methylation, histone modifications and miRNA expression profiles that can significantly affect transcriptional activity.

3.1. Pathophysiology

Significant alterations in DNA methylation are observed when moving from NAFL to NASH and liver fibrosis [56–58]. These changes include alterations in DNA methylation that affect the expression of genes involved in glucose, lipid or acetyl-coenzyme A (CoA) metabolism (*PC*, *PLCG1*, *ACLY*) [57], insulin-like signaling (e.g. *IGF-1*, *IGFBP2*) and mitochondrial function (NADH dehydrogenase 6) [59]. Additionally, given that a specific DNA methylation signature develops with increasing age in humans, NASH seems to accelerate epigenetic age by promoting changes in methylation that are associated with hepatic collagen content [60]. An untargeted evaluation of DNA methylation in liver tissues of patients with NAFLD identified almost 70,000 CpG sites that were differentially methylated in patients with advanced liver fibrosis (F3-F4) compared to those with no or mild fibrosis (F0-F1). 76% of these sites were hypomethylated and 24% were hypermethylated in advanced liver fibrosis in NAFLD, whereas 7% of the reported methylations correlated with gene expression levels. The hypomethylation was associated with higher expression of genes related to tissue repair, liver inflammation, fibrosis and carcinogenesis (e.g. *FGFR2*, *COL1A1*, *CASP1*, *CCR7*, *CCL5*) whereas the hypermethylation with lower expression of genes involved in lipid and aminoacid metabolism [56,61]. These findings were also replicated in other studies [62]. DNA methylation seems to be particularly involved in the activation of hepatic stellate cells and their differentiation to myofibroblast that are crucial procedures for hepatic fibrogenesis [63,64]. Changes in methylation of specific genes have been linked with these processes. Genes promoting fibrogenesis such as *TGFβ1* and *PDGFα* are hypomethylated and thus highly expressed whereas genes inhibiting hepatic stellate cells activation such as the *PPARα*, *PPARδ* and *PPARγ* are hypermethylated and thus lower expressed in the liver of patients with advanced fibrosis compared to the ones with mild disease [65,66].

Apart from their involvement in NAFLD development and progress, hepatic alterations in DNA methylation may be associated with systemic metabolic outcomes. Specifically, a case-control study in NAFLD patients has shown that increased methylation in specific sites of the promoter of the PPARGC1A, which encodes the major regulator of mitochondrial biogenesis, is associated with lower mRNA expression of PPARGC1A in the liver, lower ratio of mitochondrial to nuclear DNA and increased insulin resistance, thus linking hepatic mitochondrial dysfunction of NAFLD with peripheral insulin resistance [67]. In agreement with the above finding, a study following untargeted procedures has identified 30 methylations that are affecting mRNA expression of hepatic genes involved in insulin signaling that are also highly correlated with fasting insulin independently of the presence of T2DM, thus providing further evidence for the relation of hepatic DNA methylation with insulin sensitivity [58]. Finally, a recent study focused on the identification of differentially methylated regions that form networks which are associated with the progression of NAFLD. They identified two important networks, one that included genes that affect cytoskeleton organization, transcriptional activity and cell proliferation and another that was associated with metabolic pathways. The CpG methylation levels in both networks were affected by age and fasting plasma glucose levels and for the second network the changes in methylation levels were partially corrected by controlling weight and blood glucose levels [68].

Histone modifications are also important epigenetic changes that affect transcriptional activity and refer to several posttranslational procedures such as acetylation, phosphorylation, methylation and ubiquitination. Among them, acetylation status has been most vigorously studied and is considered the net result of histone acetylation by histone acetyltransferases (HATs) and histone deacetylation by histone deacetylases (HDACs) [69]. Acetylation neutralizes lysin's positive charge, thus leading to a looser chromatin structure that facilitates transcription. The transcriptional coactivator p300 belongs to HAT proteins, whereas sirtuins belong to NAD-dependent class III HDACs.

Glucose-induced activation of p300 increases the transcription of carbohydrate-responsive element-binding protein (ChREBP) resulting in the stimulation of lipogenic genes through histone acetylation and thus promoting the development of NAFLD [69]. Tannic acid attenuated the effects of p300 leading to a decrease in the lipogenesis-related genes and to an improvement of NAFLD in mice [70]. Similarly, inhibition of cdk4 protein reduces the formation of C/EBPα – p300 complexes reducing liver steatosis and correcting age-associated liver changes [71]. P300 may also be involved in the activation of hepatic stellate cells and their transdifferentiation to myofibroblasts [72]. Among the Sirtuins, especially Sirtuin 1 (SIRT1) has been shown to participate in the regulation of hepatic metabolism and insulin sensitivity, with hepatic deletion of SIRT1 resulting in steatosis and inflammation, whereas overexpression of SIRT1 protecting from NAFLD [73–75]. Similarly, SIRT3 deficiency leads to insulin resistance, hyperlipidemia and steatohepatitis in mice [76] whereas a polymorphism of *SIRT3* is associated with metabolic syndrome and NAFLD in humans [77]. *HDAC3*, a member of human class I HDACs has been also implicated with circadian metabolic rhythm and its deletion leads to hepatic steatosis in mouse liver [78].

Altogether, there is increasing evidence that both DNA methylation as well as histone modifications play a crucial role in the development and progress of NAFLD.

3.2. Diagnostic perspective

An important question is whether the numerous epigenetic changes observed in NAFLD in combination with the highly dynamic nature of

epigenome can serve as diagnostic or prognostic markers of NAFLD or even as markers of treatment response. Very few studies have addressed this point so far. One of them that included a small number of patients with NAFLD has shown that alterations in hepatic DNA methylation may be sufficiently reflected in plasma. In this study, % of plasma DNA methylation of PPARγ was used to identify subjects with severe (Kleiner 3-4) vs mild fibrosis (Kleiner 1-2). The threshold of methylation was 81% and demonstrated 83% sensitivity and 93% specificity to differentiate between the two above categories [79]. Although validation of the findings in largest and more heterogenous cohorts is needed, it is one of the first studies to suggest that changes in DNA methylation can have beyond their pathophysiological significance also value as potential non-invasive biomarkers for the diagnosis and staging of NAFLD. A second study has also investigated differences in methylation of DNA from peripheral blood leukocytes that may be able to discriminate between NASH vs NAFL. The study identified six genes that their methylation correlates with lobular inflammation and can be potentially used as diagnostic biomarkers (with the best one showing 99% specificity and 71% sensitivity) for differentiating NASH from NAFLD [80]. Finally, in a large epigenome-wide association study consisting of 3400 participants of European ancenstry, 22 CpGs were found in peripheral blood to be associated with hepatic fat as assessed by computed tomography or ultrasound imaging and among them one was also associated with risk for new-onset T2DM [81]. Nevertheless, significantly more and larger studies are needed in order to decide whether epigenetic changes in the blood can have any diagnostic or prognostic value.

3.3. Therapeutic perspective

Targeting epigenomic changes for the treatment of diseases seems to be theoretically a very attractive option for a number of reasons. First, epigenomic alterations are involved in fundamental pathophysiological mechanisms of a disease through their impact on gene expression. Second, epigenetic changes are also highly dynamic and reversible, thus correction of them holds promise for reversal to "normal status". For example, after bariatric surgery, robust changes in methylation have been reported. Hypermethylation and consequently reduction of transcriptional activity of *PTPRE* (encoding protein-tyrosine phosphatase epsilon) may improve hepatic insulin sensitivity [57]. Exercise may also reduce methylation and improve mRNA levels of mitochondrial genes, thus improving mitochondrial function [59]. Third, it may be possible to combine treatments targeting epigenome with other drugs, having in that case a complementary role. Such treatments are currently being developed mainly against cancer diseases and consist of pharmacological DNA methylation inhibitors (DNMTis), HDAC inhibitors (HDACis) or activators. In NAFLD very few of them have been tested so far. Among them, the SIRT1 activator, resveratrol, improved dyslipidemia and steatohepatitis in a mouse model of atherogenic NAFLD [82]. Several on-going human clinical trials (two of them in Phase III) are currently investigating the efficacy of resveratrol in human NAFLD but results from the first studies have shown no significant effect on hepatic fat content [83–85]. Other medications are currently also being developed and have shown promising results in animal studies, such as the 3-deazaneplanocin A (DZNep) [86] or the HDAC inhibitor SAHA but none of them is in a more advanced stage of clinical development [87].

3.4. Conclusion – clinical perspective

Epigenomics are one of the least investigated "omics" in NAFLD. The few studies performed so far have shown that the extensive hepatic epigenetic modifications observed in NAFLD are causally related to the disease. It is still unclear though how accurately the hepatic epigenome is reflected in peripheral blood and thus it is questionable whether epigenetic changes studied at the level of peripheral blood cells may have any significant diagnostic value. Given also the limited amount of information from pathophysiological studies investigating the dynamic of epigenetic changes with NAFLD development and progression, it is probably premature to expect any drugs to be developed in order to target exclusively epigenome of NAFLD in the near future.

4. Transcriptomics

Transcriptomics refers to the quantitative assessment of all coding and non-coding RNA transcripts and reflects cellular transcriptional activity. The NAFLD transcriptome bridges genetic information to the steatotic, inflammatory and fibrogenic proteome profiles. Flexible modalities, such as RNA sequencing and microarrays, have outlined the roles of both regular and non-coding transcriptional components. Several ongoing efforts have been focusing on developing predictive models for NAFLD and NASH, albeit they have to overcome several challenges observed in all „omics" procedures (s. also Section 8. Conclusions – Challenges –Perspectives).

4.1. Gene expression and transcriptome profiling

Genetic predisposition determines NAFLD transcriptomic signatures, as shown by recent investigations on the carriage of *PNPLA3* I148M on transcriptome variability [88]. As described above, transcriptomics are also linked to epigenetic modifications in NAFLD [56,57].

Through global microarray transcriptional snapshots, NAFLD and especially NASH are characterized by overexpression of genes associated with lipid metabolism [89–92], acute phase regulators of insulin sensitivity [93], cellular division [89], DNA [89] and tissue repair [56], extracellular matrix organization [91,94–96], immune function [95] cellular adhesion and migration [91,92,96], signal transduction [91], P53 signaling [92] and cancer progression [96] among others (Fig. 2). Conversely, genes modulating mitochondrial function [93] oxidative, glucose, fatty acid and aminoacid or protein metabolism [56,91–93,96,97] as well as insulin signaling and transcription factors [91,94] are downregulated.

The implementation of large databases for differential gene expression links NAFLD with overexpression of *CD24*, *COL1A1*, *LUM*, *THBS2* and *EPHA3* and underexpression of *PZP* mRNAs [92]. Hepatic transcriptomes of NAFLD are distinct from those of normal and healthy obese subjects, yet a 132-gene signature, with extracellular matrix remodeling and immune system genes as its main components, distinguishes NASH from simple steatosis [95]. More specifically, the transcriptional snapshot of NASH is defined by upregulation of *PDGF*, *STAT*, *HNF-3* and *SMAD-4* pathway-related genes [97], as well as downregulation of *BNIP1, IGFBP1* [97], *SLC25A48, C4ORF48* [95] *SDC4*, *ATF3*, various inflammatory suppressors [94] and amino acid-metabolizing and reactive oxygen species (ROS) scavenger genes [97]. Interleukin-32 (IL-32) was recently ascertained as the most significantly upregulated transcript in advanced NAFLD and NASH (fold-change = 2.3 vs non-NASH), correlating with lipid accumulation and disease severity [88]. Additionally, several cancer-related genes, such as *AKR1B10, KRT23* and *GPC3*, are enhanced in NASH compared with NAFL or controls, by up to 155-fold and 460-fold respectively [96].

Concerning fibrosis, cross-sectional transcriptome analysis of hepatic cells defines 3820 and 2980 differentially expressed transcripts for the inflammatory and fibrotic states, respectively, and further underlines 16 fibrosis-defining genetic routes, pertaining to cytokine and extracellular matrix receptor interaction, focal adhesion and the PI3K-Akt signaling pathway [98]. Differentially expressed microarray signals, such as *UBE2V1*, *BNIP3L*, *RP11-128 N14.5*, linked to oxidative stress, inflammation, apoptosis and fibrogenesis, are upregulated in NAS ≥ 5 and fibrosis stages 3–4 [99]. Likewise, transcriptomics pinpoint dermatopontin, a propagator of extracellular matrix remodeling, which is robustly expressed (by 800-fold) in murine stellate cells, enhanced in human fibrotic livers and implicated in NAFLD fibrogenesis [100].

4.2. MiRNAs and non-coding RNAs

Minor molecules such as micro-RNAs (miRNA) along with long noncoding RNAs (lncRNA) perform a variety of epigenetic and post-transcriptional functions, thus significantly affecting transcriptional activity in numerous diseases and among them also NAFLD [101]. For this reason, they are also considered important epigenetic modifiers. These processes are thoroughly delineated through large panels of differential miRNA expression [102–104] while the distinct NAFLD-related roles of miRNA subspecies were recently reviewed [101,105]. Of note, microRNA-122 (miR-122), deriving from chromosome 18, represents 70% of hepatic miRNA and has been implicated in cholesterol metabolism and liver cancer [106]. Cheung et al. first mapped a 63% underexpression of liver miR-122 in NASH patients compared with controls, through an evaluation of a 474-miRNA panel that identified 46 differentially expressed miRNA species and their corresponding targets [104]. MiR-122 was subsequently associated with steatosis severity [107]. The diminished liver expression of miR-122 in NASH is confirmed in a study of 84 miRNA species by Pirola et al., whereas serum miR-122, miR-192, miR-19 (a,b), miR-125b (associated with metabolic syndrome, atherogenesis and circulation) [103] as well as miR-34a, miR-16 (associated with cancer) [108], miR-21 and miR-451 [107] are upregulated in both NAFLD and NASH. Moreover, miR-301a, miR-34a and miR-375 alter lipid and carbohydrate metabolism, reflect NAFLD severity and are implicated in hepatocellular carcinomas [102].

Expression levels of lesser non-coding molecules have also been investigated [99,109,110]. Global hepatic RNA profiling has identified 535 lncRNA and 760 mRNA species overexpressed in NAFLD, whereas 1200 lncRNA and 725 mRNA species are underexpressed. These enriched RNAs chiefly belong to small molecule and organic acid metabolic pathways [109]. Functional transcriptome research of 4383 lncRNA species by Atanasovka et al. pinpoints elevated levels of hepatic lncRNA RP11-484N16.1 in NASH, and robustly associates it with NASH grade, lobular inflammation and NAS, while its knockdown in vitro aggravates hepatocellular growth and viability [110]. Identified and validated through whole serum transcriptome analysis, lncRNA RP11-128N14.5 has been linked to NASH diagnosis and is upregulated in NAS ≥ 5 [99]. Furthermore, transport RNA (tRNA) profiling pinpoints the differential expression levels of several anticodons for lysine, aspartate and glutamate in cirrhosis, in addition to various mitochondrial and amino acid tRNAs in cirrhosis versus NASH and NASH cirrhosis versus normal respectively [102]. The same study has also compared the abundance of 392 several small RNA molecules (ribosomal, small nuclear and nucleolar) between healthy, NASH and cirrhotic patients [102].

4.3. Diagnostic perspective

Various predictive modalities involving gene expression parameters, targeted measurements and miRNA panels with increased functionality are based on transcriptomics. In general, the reported accuracies for many of these tests have been high for diagnosing NAFLD or NAFL from healthy status but rather low for differentiating NAFL from NASH or for diagnosing advanced fibrosis. Additionally, even the most promising studies, have not been further tested or have performed poorly in other cohorts.

Among them, predictive models based on miRNAs have in theory higher chances to be robust and reproducible, since miRNAs can withstand numerous freeze-thaw cycles and long-term storage before degrading [111]. Several studies have identified miR-122 as a potential diagnostic biomarker. Most of them have shown that miR-122 alone or in combination with other miRNAs (e.g. miR-1290, miR-27, miR-192, miR-34, miR-99a) can accurately predict the presence of NAFLD or NAFL, but they all perform inadequately when trying to differentiate NAFL from NASH [103,108,112–114] (Table 2). Several diagnostic tests have been developed that include miRNAs together with other proteins in their algorithm. Among them, NIS4™, which includes miR-34a, alpha-2-macroglobulin, chitinase-3-like protein 1, has been recently tested in a population with type 2 diabetes ($n = 275$), showing an AUROC performance of 0.801 for diagnosing advanced NASH (NAS ≥ 4) and significant fibrosis (F ≥ 2), which was also higher than in other tests, such as FIB4 (0.704), ELF (0.704) or Fibrometer (0.678) but probably still suboptimal [115]. Additionally, the same test demonstrated 76% sensitivity and specificity in detecting NASH patients at risk of cirrhosis [116]. Another model integrating miR-122, miR-192, miR-21 and CK-18 fragments, can differentiate NASH from NAFL (AUROC = 0.83) [117] (Table 2).

New frontiers, such as circular RNA (circRNA) transcriptomics, are being investigated in animals. Researchers have already mapped and correlated circRNA species and their corresponding transcriptional pathways in mouse models of NASH, showcasing their associations with metabolism [118] and stellate cell activation [119]. Yet whether these results could yield meaningful conclusions that can be extrapolated in human cohorts remains unclear. Other prospective research questions in diagnostic NAFLD transcriptomics concern alternative RNA splicing [120] and the scrutiny of unexplored transcriptional components, such as CD24 [92].

4.4. Therapeutic implications

Curative and bedside implications of transcriptomics in NAFLD are considerably limited. From an interspecies aspect, the human and murine NAFLD transcriptomes are largely dissimilar, yet their likelihood increases in murine models following high-fat diets [90]. Hence the transcriptional signatures of NAFLD or NASH as well as the organic transcriptomic responses to relevant treatments for liver disease can be evaluated by numerous available pre-clinical in vitro prototypes and animal models [121]. For instance, mapping of the murine NASH transcriptome demonstrated an increase in the levels of Tsukushi, a novel adipose-related thermogenetic and metabolic hepatokine. Subsequent silencing of Tsukushi improved NASH and attenuated the hepatic transcriptional alterations associated with it [122]. Transcriptomics were also implemented in recent murine models assessing the NAFLD-related role of Poly rC binding protein 1, a cytosolic iron chaperone, whose deficiency exacerbates steatosis and inflammation, increases lipid biosynthesis and oxidative stress pathway expression levels, and is treated via reduced iron intake and vitamin E supplementation [123]. Lastly, in view of the COVID-19 pandemic, the expression levels of 4 SARS-COV-2 entry proteins were assessed by liver transcriptomics in human and murine NAFLD and NASH cohorts, with no significant changes being detected [124]. Future transcriptomic modalities should approach all different phases and components of transcription, kindle research for new remedies, and investigate the associations of liver disease with various pathologies precipitated by its metabolic milieu.

4.5. Conclusion – clinical perspective

Transcriptomics have been widely used and have contributed significantly to our understanding of the pathophysiology of NAFLD. Transcriptomics, with the exception of miRNAs, do not add significant value in non-invasive diagnostic scores, since their hepatic profile is not adequately reflected in peripheral blood. With the rapid improvement of available technologies and data analysis, transcriptomics are now routinely used in order to identify potential mechanistic pathways affected by drug candidates for the treatment of NAFLD.

5. Proteomics

Proteomics refers to the investigation of “proteome”, thus to all proteins expressed by a cell. The proteome, similar to transcriptome and epigenome, is highly dynamic. Cell activation, trafficking, differentiation or transformation as well as tissue type have a major impact on the

proteome. Additionally, posttranslational modifications (phosphorylation, acetylation, methylation, glycosylation etc.) as well as alternative splicing render proteome much more complex than transcriptome, with >120,000 proteins being expressed by a human cell. Finally, the proteome is downstream of genome and transcriptome, and thus it is stronger associated with the final phenotype [125].

Proteomic analyses have been performed for cell line - characterization or for protein investigation and identification in tissues under specific conditions or treatments, but their use has been in general rather limited, especially for biomarker or drug discovery [126]. There are several reasons for this. First, significant technical limitations exist that restrict the number of proteins that can be detected and can be identified with certainty. The first efforts involved two-dimensional gel electrophoresis and were able to detect some few dozens of spots that were subsequently matched to specific proteins with the use of MS. Later approaches managed to detect more protein peaks without though being able to identify the corresponding proteins. Advances in methods have enabled in the last years the identification of some thousands of proteins but still the largest part of proteome cannot be investigated with certainty with the available technologies. Second, reliable quantification of the levels of proteins, especially in blood has been very challenging. In plasma, three classes of proteins mainly exist: a) the highly abundant proteins, that include human serum albumin (approximately 50% of protein mass), apolipoproteins, coagulation markers and acute phase proteins of the immune system, that are measured in mg to ug/ml level, b) the organ-tissue secreted proteins without functional role in the circulation such as ALT, AST or troponins, measured in ug to ng/ml, c) the signaling molecules, such as cytokines or insulin, measured at ng to pg/ml level. This extreme range enables to identify no more than a few hundreds of the most abundant proteins. Consequently, several techniques with the use of monoclonal or polyclonal antibodies or plasma fractionation have been used to deplete most of the abundant proteins and focus on the organ-tissue secreted ones and on the signaling molecules. However, these methods can significantly impact the circulating levels of proteome (e.g. by non-specific binding of antibodies or due to differences in separation) thus leading to unreliable quantifications of final protein concentrations which reduces massively the reproducibility of the findings. Finally, proteomics measurements, in contrast to other procedures (e.g. transcriptomics, genomics or metabolomics) are labor intensive. Thus, most of the studies performed so far have been in very small study populations. These studies have led in some cases to the identification of potential biomarkers, which were though not further validated in independent cohorts.

While keeping in mind the above limitations, we next present the efforts to identify pathophysiological pathways and develop diagnostic models for NAFLD with the use of proteomics.

5.1. Pathophysiology

Several studies have investigated hepatic proteome alone or in combination with blood proteome, either in animal models or in humans with NAFLD, aiming to answer fundamental pathophysiological questions [127–129]. In one recent study, the levels and cellular distributions of 6000 liver proteins and 16,000 phosphopeptides have been assessed in the liver of mice developing hepatic steatosis due to high fat diet (HFD). This provided important fundamental information about the reorganization of organelles, lipid accumulation and cellular dysfunction that occurs with nutrient overload [129]. In another study, proteomics in liver biopsies identified almost 220 proteins that their levels significantly differ in patients with NAFLD compared to obese metabolically healthy individuals. The proteins that their levels were increased in NAFLD were involved in PPAR-signaling and extracellular matrix-receptor interactions whereas the ones that their levels were reduced, were mainly localized in mitochondria and participated in oxidative phosphorylation [128]. In a combined hepatic phosphoproteome and serum proteome analysis of 67 biopsy-proven NAFLD subjects, the ASK1-MAPK pathway that is activated by IL-10 was recognized as important for liver fibrosis, indicated by its strong association with higher % of hepatic collagen. In serum, alpha-2 macroglobulin and coagulation factor V were strongly associated with hepatic collagen [127]. It has thus been suggested that these pathways can potentially serve as therapeutic targets. In another approach, proteomic differences in liver tissues of obese patients with vs without T2DM were investigated. The analysis identified 850 proteins, from which 27 were significantly different between the two groups. Levels of proteins involved in methionine metabolism and especially of glutathione were reduced whereas levels of other proteins involved in oxidative stress were increased in the T2DM group [130]. Expanding on complications of the disease, the proteome of liver tissues with NAFLD from mice developing HCC has been assessed. Differences in tumor suppressor genes and oncogenes were observed, which were further investigated in human samples with NAFLD and HCC. Among them S100A11, which is secreted by cancer cells and stimulates cell proliferation and migration, was identified and associated with high-grade HCC and poor prognosis [128].

5.2. Diagnostic perspective

A small number of studies has aimed to develop diagnostic models with the use of proteomics (Table 3). When evaluating the results of these studies, we have to keep in mind the limitations and challenges related to such procedures, that are described below (8. Conclusions – Challenges –Perspectives). In one of the early efforts, combined genomic/proteomic analysis involving liver tissues for assessing gene expression and serum samples for assessing proteins was performed in patients with different stages of NAFLD. A SELDI-TOF mass spectrometry (MS) identified approximately 300 protein peaks with 16 of them being significantly different between groups. At that timepoint, it was not possible to identify in which proteins these peaks corresponded to but their masses were compared with the masses of 1440 serum proteins, which led to the identification of fibrinogen γ as one possible biomarker [131]. In another study including eighty morbidly obese patients, SELDI-TOF MS has identified in serum three protein peaks that their intensity significantly increased with the severity of NAFLD, i.e. when moving from steatosis to NASH. None of these peaks correlated with liver function tests or metabolic parameters and all of them returned to normal after bariatric surgery. With immunoSELDI assay these peaks were recognized as alpha- and beta-hemoglobin subunits [132]. One of the most comprehensive studies so far was able to identify 1700 serum proteins in a population with NAFLD. Among them 55 were significantly different between NALF and NASH with fibrosis stage 3 or 4 (F3/F4) and 15 between NASH vs NASH F3/F4 group. These proteins were involved in immune system regulation and inflammation, in coagulation cascades (including fibrinogen γ demonstrated by previous studies), in cell growth and proliferation (including IGFBP2, IGFBP3, also shown in targeted approaches to be relevant [133]) and many of them were apolipoproteins (demonstrated also in [134]) and blood carrier proteins. Six (fibrinogen β chain, retinol binding protein 4, serum amyloid P component, lumican, transgelin 2, and CD5 antigen-like) were selected to create a panel for the non-differential diagnosis of NAFLD and its stages, ranging from healthy liver status to NAFL, NASH or NASH with F3/F4 showing good to very good diagnostic accuracy (AUROCs 1.0, 0.83, 0.86, 0.91, respectively). This was though a study with small sample size and its findings have not been replicated so far [135]. In another approach, diagnostic models for differentiating between NAFLD vs healthy controls among the obese population undergoing bariatric surgery were developed. These models combined genomic information and especially the PNPLA3 genotype, phenomic data (i.e. selected biochemical measurements such as glucose, insulin, lipid profile, ALT) and proteomic data. Specifically, a proteomic analysis identified 1129 proteins with 30 of them being significantly different between the two groups. Among them was aminoacylase-1, sex

Table 3
Main proteomics and protein-based models for the diagnosis and staging of NAFLD.

#	N	Comparisons	Prediction models	Sensitivity	Specificity	AUROC
Steatosis/any NAFLD						
[136]	D: 443 V:134	C vs NAFLD	Model including data from PNPLA3, 8 proteins and 19 phenotypic variables			D: 0.94 V: 0.91
[235]	70	C vs NAFLD	Model of 6 metabolites, including hemoglobin subunit α	89%	83%	
NAFL vs NASH vs Fibrosis						
[236]	99	a) C vs NASH b) C vs NAFLD	a) 15 protein peaks b) 51 protein peaks	a) 74% b) 86%	a) 89% b) 61%	
[132]	104	a, c) NASH (Y vs N) b, d) NAFL (Y vs N)	a) 50 protein peaks b) 67 protein peaks c) CM10-7558.4 (α-Hb subunit) d) CM10-7558.4 or CM10-7924.2 (β-Hb subunit)	a) 83% b) 78%	a) 67% b) 71%	c) 0.81 d) 0.73
[137]	135	a) NAFL vs HCC + NAFLD b) NASH vs HCC + NAFLD	Inter-alpha-trypsin inhibitor heavy chain 4	b) 86%	b) 75%	a) 0.93 b) 0.84
[135]	85	a) C vs NAFL vs NASH vs NASH F3-4 b) NAFL vs NASH F3-F4 D) C vs NAFLD	a) Panel of 6 proteins b) Panel of 3 proteins c) Panel of 2 proteins			a) 1.00, 0.83, 0.86, 0.91 b) 0.91 c) 1.00
[237]	110	a) C vs NAFLD b) NAFL vs NASH c) F0-F2 vs F3-F4	a) ApoE b,c) lymphocyte cytosolic protein1			a) 0.86 b) 0.81 c) 0.93
[238]	167 D: 117 V: 50	a) Non-NASH vs NASH b) F0-F1 vs F ≥ 2	a) 5 routine variables & 2 phosphoproteomic variables b) 6 routine variables & 3 phosphoproteomic variables	a) D: 81% Total: 77% b) D: 79% Total: 67%	a) D: 87% Total: 73% b) D: 73% Total: 75%	a) D: 0.86 V: 0.68 Total: 0.80 b) D: 0.80 Total: 0.75

C, controls/healthy; D, Discovery; F, Fibrosis; N, No; S, Steatosis; V, Validation; Y, Yes.

hormone-binding globulin, Galectin-3, antithrombin III and the hepatocyte growth factor. Logistic regression models for differentiating between NAFLD and healthy status were trained by using each type of data individually (i.e. genomic, phenomic or proteomic) as well as in different combinations in a large discovery cohort consisting of 443 subjects which were further tested in a validation cohort of 134 subjects. Highest AUROCs were achieved in validation cohorts when all three data sets were combined and was 0.914. However, the utility of such algorithms is questionable given that ultrasound or other simpler algorithms (e.g. fatty liver index, etc.) show similar or higher sensitivity and specificity. Additionally, diagnosing simply NAFLD does not initiate treatment decisions [136].

Other studies performed first a proteomic analysis in a relevant condition or in another species and then tested their findings in patients with NAFLD. For example, in one study, an HCC pig model was used and a proteomic analysis in serum was performed. The subsequent analysis identified the serum inter-alpha-trypsin inhibitor heavy chain 4 as the most important protein corresponding with NAFLD progression and HCC development. This was further tested in human serum samples and showed that the levels of the proteins were higher in NAFLD patients with HCC compared to those without [137]. Similarly, in another study a proteomic analysis in plasma of patients with liver cirrhosis vs healthy ones was performed. This identified 57 significantly different proteins which were involved in immune system regulation, inflammation, coagulation and fibrinolysis. Several of these proteins were subsequently tested in NAFLD patients and were significantly different in people with NAFLD and normal glucose tolerance compared to NAFLD with T2DM [138].

5.3. Therapeutic implications

Proteomic studies have rather limited contribution to drug development against NAFLD so far. Most of the treatment targets have been identified by animal studies supported by human physiology or epidemiological studies. Additionally, many of the medications that have shown efficacy in other metabolic diseases, such as obesity, T2DM and hyperlipidemia (e.g. GLP-1 receptor agonists, thiazolidinediones, statins) have been subsequently tested in NAFLD [139–142]. Moreover, many studies are focusing on a more targeted multilayer approach, thus selecting a group of proteins based on their structural and functional similarities and investigating them systemically in a spectrum of metabolic disorders. This approach is based on the tight pathophysiological connections between obesity, insulin resistance, T2DM and NAFLD and can lead to identification of proteins that have an important general role in metabolism or inflammation through multiple layers of evidence in different metabolic conditions. Such proteins may thus have higher chances to be relevant in the efforts for biomarker discovery and drug development in NAFLD. In this context, we have investigated four main hormonal systems consisting of approximately 25 hormones, i.e. activins-follistatins, gut hormones (proglucagon-peptides), IGF-axis and leptin-adiponectin in a series of studies, moving from conditions of acute or chronic energy deprivation to metabolically healthy energy status and to energy excess due to obesity, T2DM, cardiovascular disease and NAFLD [133,143–153]. We observed significant impairments in glicentin and major proglucagon fragment profiles in early stages of NAFLD (phase of augmenting insulin resistance), in follistatins and adiponectin during progression to NASH (phase of insulin resistance-inflammation) and in IGF-1/intact IGFBP3 in fibrosis in humans, thus showing great potential both as biomarkers as well as therapeutic targets [133,144,154].

Independently of the identification of novel therapeutic targets in the future, proteomics can also potentially help drug development through other approaches. For example, in one approach, targeted proteomics has succeeded in identifying changes (in most cases reduction) in concentrations of hepatic drug transporters in people with NAFLD compared to healthy ones. The reduction in hepatic drug transporters can have a major impact on pharmacokinetics and can drive clinical development decisions as well as dosing recommendations [155].

5.4. Conclusion – clinical perspective

Proteomics have not been widely used in NAFLD research. The proteins and signaling pathways involved in the pathophysiology of the disease have been almost exclusively identified by targeted

„hypothesis-driven" approaches based on data from mechanistic animal, observational human and in some cases large epidemiological studies. This is probably due to methodological challenges and technical limitations related to proteomics. We expect in the near future proteomics to be much more frequently applied in NAFLD studies. We expect that the main contribution of proteomics will not be in direct development of non-invasive diagnostic scores but rather in the identification of novel molecules and/or pathways with important role in the pathophysiology of the disease and with diagnostic and therapeutic potentials.

6. Metabolomics – lipidomics

Metabolomics refers to the investigation of small molecules and metabolic products, such as aminoacids, fatty acids and carbohydrates. Lipidomics are considered part of metabolomics and refer to the investigation of cellular lipids. Numerous studies have investigated the metabolome and lipidome in mouse models or patients with NASH. The evidence linking bile acid homeostasis as well as gut microbiome with the development and progress of NAFLD have been recently reviewed elsewhere [156,157]. In this section, we will focus particularly on the robust and extensive changes that are observed both in the hepatic (Fig. 3) as well as in the circulating lipidome, which have led to the development of numerous diagnostic models for NAFLD as well as to the identification of novel therapeutic targets.

6.1. Pathophysiology

The development of NAFLD is strongly associated with obesity and is characterized by increased lipid accumulation due to higher uptake of elevated FFA by unrestricted adipose tissue lipolysis and due to increased dietary TGs uptake. 60% of the hepatic triacylglycerol results from adipose tissue triglycerides after lipolysis, 25% from de novo hepatic lipogenesis and 15% from dietary TGs [158] (Fig. 3). The increased accumulation of triglycerides in the liver is associated with the formation of toxic intermediates that are mainly responsible for the initiation of inflammatory procedures and later of fibrotic changes in NAFLD (Fig. 3). Lipidomic studies have described specific changes in hepatic lipidome in patients with NAFLD [46,159–163]. The hepatic concentrations of saturated fatty acids (SFAs and specifically of palmitate acid, C16:0 and stearate acid, C18:0), free cholesterol, sphingolipids, glycerophospholipids and eicosanoids increase, whereas ω-3 PUFAs and specialized proresolving mediators (SPMs) of PUFAs decrease [164] (Fig. 3).

Specifically, SFA accumulation is positively associated with liver disease severity. In hepatocytes, SFA stimulate proinflammatory cytokine secretion by activation of the toll-like receptor-4 pathway, enhance endoplasmatic reticulum stress, increase ROS, decrease mitochondrial and peroxisome beta-oxidation by activation of JNK and induce apoptosis by activation of TRAIL-2 signaling pathway [164–166]. In non-parenchymal liver cells, SFA stimulate the production and secretion of

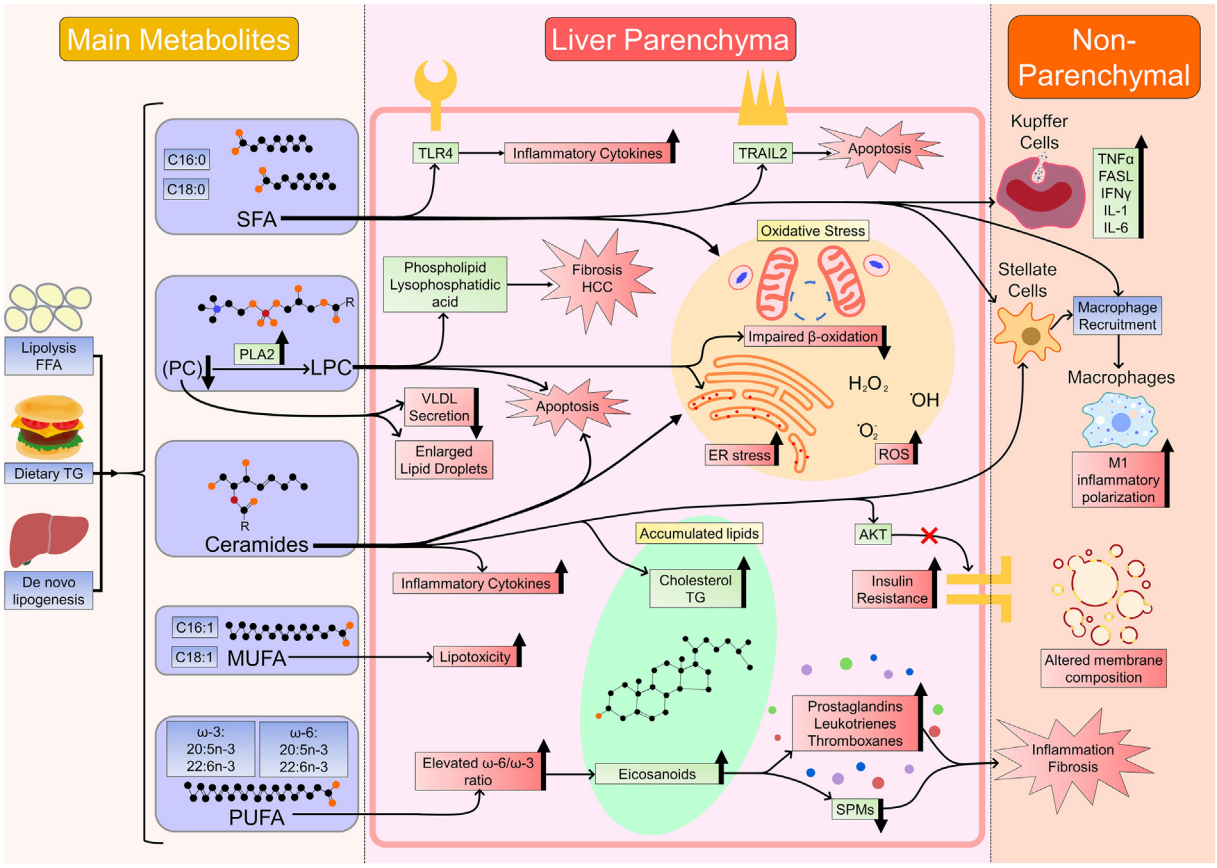

Fig. 3. Perturbations in lipidomic profile related to the pathophysiology of NAFLD. The uncontrolled lipolysis from adipose tissue, the increased dietary intake of TG and the upregulated de novo lipogenesis observed in NAFLD leads to elevated SFA, LPC, Ceramides and ω6/ω3 PUFA ratio. SFA stimulate the secretion of inflammatory cytokines via TLR4 and apoptosis via TRAIL2, whereas they increase oxidative stress, ER stress and impair β-oxidation in the mitochondria of hepatocytes. In stellate cells, they stimulate macrophage recruitment, whereas in Kupffer cells and macrophages SFA induce their polarization to the M1 proinflammatory state. Increased activation of PLA2 enzyme leads to formation of LPC and depletion of PC. PC are important for lipid droplet stability and their deficiency leads to large droplet formation and inadequate VLDL secretion. High LPC are also triggering mechanisms of impaired β-oxidation, apoptosis, fibrosis and HCC. High hepatic ceramide concentrations increase cholesterol synthesis and TG accumulation, promote insulin resistance by blocking Akt-mediated insulin signaling, induce the secretion of proinflammatory cytokines and stimulate apoptosis by increasing ROS generation, ER stress and β-oxidation impairment. In hepatic stellate cells, they increase extracellular matrix deposition and pro-angiogenic factors secretion promoting fibrogenesis. Finally, the high ω6/ω3 ratio leads to increased synthesis of proinflammatory molecules, such as prostaglandins, leukotrienes, thromboxanes in expense of the synthesis of anti-inflammatory SPMs, thus resulting in a pro-inflammatory and profibrotic net outcome.

proinflammatory and profibrotic cytokines from Kupffer cells and induce proinflammatory M1 polarization of macrophages [166,167]. Additionally, they stimulate the secretion of chemokines from hepatic stellate cells that recruit more macrophages in the liver [166,167].

Similar to SFA, lysophosphatidylcholines (LPC) have unfavorable hepatic effects. Specifically, they stimulate ER stress, cause mitochondrial dysfunction and increase apoptosis [168]. Additionally, they stimulate the release of hepatic extracellular vesicles from hepatocytes that trigger inflammatory procedures [169]. Moreover, the increased activity of the enzyme phospholipase A2 (PLA2) that catalyzes the formation of LPC from PC, leads to the rapid depletion of PC which affects hepatocyte membrane integrity and results in hepatocyte apoptosis, high release of lipotoxic lipids and increased inflammation [170]. Additionally, PC deficiency reduces VLDL secretion resulting in higher intrahepatic lipid degradation and formation of toxic intermediates. Furthermore, the low PC/PE ratio affects lipid droplet stability, leading to the formation of larger lipid droplets. Finally, LPC is metabolized by the enzyme autotaxin to phospholipid lysophosphatidic acid which stimulates liver fibrosis and development of HCC [171].

Ceramides are a type of sphingolipids whose hepatic levels are increased in NAFLD and correlate positively with disease severity [46,159–163]. In hepatocytes, ceramide promotes insulin resistance by inhibiting Akt-mediated insulin signaling, impairs beta-oxidation, induces ROS production, ER stress and proinflammatory cytokine secretion, enhances de novo lipogenesis, cholesterol synthesis and triglyceride accumulation and induces apoptosis [164,172–175]. Finally, ceramide stimulates fibrogenesis and angiogenesis by increasing extracellular matrix deposition and secretion of pro-angiogenic factors by hepatic stellate cells [176].

Monounsaturated fatty acids (MUFAs) and PUFAs are also participating in the pathogenesis of NAFLD. The most well-studied and abundant MUFAs are palmitoleic acid (C16:1) and oleic acid (C18:1). MUFAs are lipotoxic but in a lesser degree compared to SFAs. Thus, a higher ratio of MUFA/SFA may be beneficial due to the lower ability of MUFAs to stimulate ER stress and apoptosis [164]. PUFAs consists of two classes, the ω-3 and ω-6 FA that are named after the position of the first double bond from the methyl end of the FA. Important ω-3 FA are considered the eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) and important ω-6 FA are the dihomo-γ-linolenic acid (20:3n-6) and arachidonic acid (AA, 20:4n-6). Most of the ω-3 and ω-6 FA are received through diet. 5–10% derive from 18-carbon fatty acids, whereas in the synthesis of highly unsaturated FA such as EPA, DHA and AA different enzymes are involved, such as elongase and desaturase enzymes (fatty acid desaturase-1 or 2; FADS1 or FADS2, and the Δ9 desaturase SCD-1) [177]. In NAFLD, a major dysregulation in the hepatic long-chain FA desaturation processes is observed, resulting in an elevated ω-6 to ω-3 ratio and increased flux in the ω-6 pathway [159,162,163]. The increased ω-6 concentrations lead to the synthesis of eicosanoids by their enzymatic oxidation (especially of AA but also of EPA and dihomo-γ-linolenic acid) with proinflammatory properties such as prostanglandines, thromboxanes and leukotrienes which induce hepatic inflammation. This occurs in expense of the synthesis of SPMs that mainly act to restore normal cell function and thus reduce chronic inflammation and fibrosis [178,179]. Finally, the high ω-6 to ω-3 ratio is associated with an impaired FADS1 activity that can affect cell membrane phospholipid composition resulting in membrane deficiency, cell necrosis and extracellular deposition of lipotoxic lipids that can aggravate hepatic injury [162,163].

6.2. Diagnostic perspective

Many studies have reported several diagnostic models based on metabolomics, lipidomics alone or combined with other biochemical and clinical parameters for the diagnosis and staging of NAFLD (Table 4). The best studies use populations of biopsy-proven NAFLD to create non-invasive algorithms based on blood measurements. The models aim to diagnose advanced fibrosis (≥3) (in most studies), differentiate NAFLD from healthy status, distinguish between NASH and NAFL or NASH vs non-NASH (in many studies), or detect the presence of fibrosis independently of its stage (in few studies). Challenges related to the development of these models are described later (s. 8. Conclusions – Challenges – Future Perspectives). Additionally, important parameters that affect study quality include: a) validity of the methods and tools used for data generation, i.e. the use of targeted vs untargeted approaches, level of confidence for the identity of detected lipids and quality of samples. Such information is rarely provided in the published studies and is in general difficult to assess or objectify. Advances in mass-spectrometric methods and efforts to standardize the way to report identified lipids has helped to improve methodology in the latest years, b) type of statistical analysis performed in the study. Here, most of the early approaches were focusing on identification of the most important parameters, which were subsequently hand-picked according to univariate methods for creating an algorithm based on logistic regression. This approach inserts though important bias in the analysis, which leads to overfitting, thus the algorithm is tailor-made for the investigated population and will often demonstrate low reproducibility in other populations. Novel methods involving machine learning techniques (such as supervised or unsupervised learning with neural networks) have revolutionized data analysis in the last years and are providing a robust framework for future studies, c) use of validation cohorts. Formation of several consortia worldwide have enabled the evaluation of the created algorithms in different cohorts in the last years. Below we are presenting the most promising studies that managed to create diagnostic algorithms for the diagnosis and staging of NAFLD.

In a large study involving 467 subjects with biopsy-proven NAFLD two diagnostic algorithms based on multiple logistic regression were developed. These algorithms were subsequently validated in a second independent cohort of 192 patients. The first diagnostic algorithm consisted of 11 triglyceride species and focused on differentiating NAFLD from healthy status. It demonstrated a sensitivity of 94% and specificity of 57% in the validation cohort. The second algorithm consisted of 20 triglyceride species and differentiated with 70% sensitivity and 81% specificity the presence of NASH from NAFL [180]. Based on the above findings and methodology, the OWLiver® test was created, which includes 25 triglycerides and aims to discriminate NASH from NAFL patients. The test, which was initially trained in white patients without T2DM, performed poorly in a multiethnic cohort of patients with T2DM (AUROC = 0.69). This shows that patient heterogeneity is an important factor and that tests developed in non-diabetic populations may not be as accurate in patients with T2DM [181]. In another study, information from metabolomics and lipidomics were combined with biochemical and genotypic measurements to create an algorithm for differentiating NASH from non-NASH (i.e. combined healthy and NAFL). 223 subjects were used to train the algorithm and 95 to validate it. The final model consisted of AST, insulin, PNPLA3 genotype, glutamate, two aminoacids (glycine and isoleucine) and two lipids (LysoPC 16:0 and PE 40:6) and was able to differentiate NASH from non-NASH with 85% sensitivity and 72% specificity [46].However, the non-NASH population consisted both of healthy subjects (53%) and of NAFL (47%). It is though important to be able to distinguish accurately NAFL from healthy status. Additionally, sensitivity and specificity in most tests is high when healthy subjects are included, but the same tests may not perform as well when NAFL is directly compared with NASH.

In a smaller proof-of-concept study a different approach was followed. A lipidomics, glycomics and targeted hormonal analysis was performed. Results were analyzed with different machine learning methods and 19 different diagnostic models were reported for classifying the subjects simultaneously to healthy or NAFL or NASH group. The models consisted of varying number of variables (from 10 up to 29) and demonstrated high sensitivities and specificities [144]. The variables included in the models were mainly lipids and often adiponectin, which

Table 4
Selected metabolomics and lipidomics panels for the diagnosis and staging of NAFLD.

#	N	Comparisons	Prediction models	Sensitivity	Specificity	AUROC
Steatosis/any NAFLD						
[239]	Metab: 30 V: 230	a) S ≤ 5% vs S > 6% b) S ≤ 5%vs S 6–30% c) S ≤ 5%vs S 31–60%	α-ketoglutarate	a) 80%	a) 63%	a) 0.74 b) 0.68 c) 0.78
[240]	D: 287 V: 392	a) C vs NAFLD b) Prediction of liver fat %	a) TG 16:0/18:0/18:1, PC 18:1/22:6, PC(O-24:1/20:4) b) TG(48:0), PC(18:1/22:6), PC(O-24:1/20:4)	a) D: 70% V: 65% b) D: 70% V: 69% Total: 69%	a) D: 79% V: 73% b) D: 76% V: 74% Total: 75%	a) D: 0.80 V: 0.77 b) D: 0.79 V: 0.78 Total: 0.79
[184]	559 Pediatric/adolescent D: 2/3 V: 1/3	C vs NAFLD	11 metabolites and 3 clinical variables (WC, whole-body insulin sensitivity index, TG)	73%	97%	0.94
NAFL vs NASH						
[46]	D: 223 V: 95	Non-NASH vs NASH	NASH ClinLipMetScore: AST, insulin, PNPLA3 genotype, glutamate, isoleucine, glycine, LPC 16:0, PE 40:6	Total: 86%	Total: 72%	D: 0.88 V: 0.86 Total: 0.87
[241]	D:374 V: 93	NAFL vs NASH	BMI-dependent metabolic profile of 292 metabolites and 51 unidentified variables	D: 71% V: 56% Total: 62%	D: 92% V: 89% Total: 97%	D: 0.87 V: 0.85 Total: 0.84
[242]	D: 73 V: 49	C vs NAFL vs NASH	OxNASH: 13-HODE/linoleic acid, age, BMI, AST	Different cutoff points D: 81% V: 84%	 D: 97% V: 63%	D: 0.83 V: 0.74
[243]	108	a) C vs NAFL b) C vs NASH c) NAFL vs NASH	a) Urinary Indoxylsulfuric acid b) Urinary Indoleacetic acid c) Urinary Pyroglutamic acid			a) 0.79 b) 0.82 c) 0.65
[180]	D: 467 V: 192	a) C vs NAFLD b) NAFL vs NASH	a) 11 triglyceride species and BMI b) 20 triglyceride species and BMI	a) D: 98% V: 94% b) D: 83% V: 70%	a) D: 78% V: 57% b) D: 94% V: 81%	a)D: 0.90 V: 0.88 b) D: 0.95 V: 0.79
NAFL vs NASH vs Fibrosis						
[244]	78	a) NAFL vs NASH b) NAFL vs NASH+F ≤ 2	C16:1n7/C16:0 FA ratio			a) 0.71 b) 0.69
[144]	80 D: 2/3 V: 1/3 repeated 100×	a) C vs NAFL vs NASH b) F0 vs F1-F4	a) 19 models with 10–29 variables (lipids, glycans, hormones) b) 3 models with 10 lipids, 5 glycans, 5 fatty acids	a) 91% b) 97%	a) 95% b) 99%	a) 0.97 b) 1.00
[183]	D: 156 V1: 142 V2: 59	a) F0-F2 vs F3-F4 b) Non-NASH vs NASH	a) 10 metabolites b) 31 metabolites	a) D: 90%	a) 79%	a) D: 0.94 V1: 0.94 V2: 0.84 b) D: 0.89 V2: 0.82 (10/31 metabolites)
Fibrosis						
[245]	106	a) NAS 1–4 vs NAS ≥5 b) F0-F1 vs F2-F4	a) C15:0, C18:1n7c, AST, ferritin b) C15:0, C16:1n7t, C18:1n7c, C22:5n3, age, ferritin, APRI	a) 73% b) 82%	a) 90% b) 90%	a) 0.82 b) 0.92
[246]	227	a) F0-F2 vs F3-F4 b) C vs F3-F4 c) C vs F4	Top 10 urinary steroid metabolites, BMI and age			a) 0.92 b) 0.99 c) 1.00
[247]	D: 44 V: 105	F < 3 vs ≥ F3	a) 16-OH-DHEA-S/etiocholanolone-S b) 16-OH-DHEA-S/DHEA-S	V: a) 76% b) 81%	V: a) 85% b) 80%	V: a) 0.85 b) 0.84
Hepatocellular balooning						
[248]	132	Balooning (Y vs N)	Collagen IV 7 s, plasma choline, LPE(e-18:2)	89%	71%	0.846
Validation studies						
[181]	220	a) C vs NAFLD b) Non-NASH vs NASH c) C vs NAFLD (selected subset) d) Non-NASH vs NASH (selected subset)	a, c) Panel of 11 triglyceride species and BMI b, d) Panel of 20 triglyceride species and BMI (as in [180])			a) 0.64 b) 0.69 c) 0.79 d) 0.87
[223]	213	Non-NASH vs NASH	a) 11 triglyceride species and BMI b) 20 triglyceride species and BMI (as in [180])	66%	69%	0.68

C, controls; D, Discovery; F, Fibrosis; N, No; S, Steatosis; V, Validation; Y, Yes. Sensitivities and specificities represent authors' chosen cutoff values; whenever optimal cutoffs are not specified, values with greater sensitivity are included. Whenever multiple scores are available for each comparison, the highest-performing scores are selected.

its protective role against steatosis, inflammation and liver fibrosis is well established [152,153,182]. A validation cohort did not exist, but 2/3 of the study population was used to develop the models and 1/3 to validate it, with this procedure being repeated 100 times with several slices of data. This approach helps to control against random results but it still does not address population heterogeneity. Nevertheless, the ability to diagnose between three and not two conditions (healthy, NAFL, NASH) can be attractive for large screenings of high-risk

population in primary care setting and without the need for any additional imaging [144] and should be further evaluated in future studies.

Several studies have attempted to develop non-invasive algorithms for differentiating between advanced (stage 3–4) vs not advanced liver fibrosis (stage 0–2). In one of the most comprehensive studies so far, a cohort of 156 subjects was initially used to develop an algorithm after a metabolomic and lipidomic analysis, which finally consisted of 10 metabolites/lipids. Among the 10 parameters, six were cholesterol-derived precursors of steroid hormones which were significantly reduced in advanced fibrosis, one was the primary conjugated bile acid, glycopholate, that was increased in advanced fibrosis and one the aminoacid taurine that is associated with bile acid conjugation and its levels were decreased in fibrosis. Finally, the other two parameters were palmitoleate acid and fucose, which were both higher in advanced fibrosis. Increased fucosylation, as described below (s. glycomics) is one of the main findings of studies that investigated alterations in circulating glycome in NAFLD. The derived algorithm was further validated in a twin and family cohort consisting of subjects that have undergone an MRI for calculation of hepatic steatosis and a MRE for assessing advanced fibrosis as well as in another cohort of biopsy-proven NAFLD. The model demonstrated 90% sensitivity and 79% specificity for detecting advanced fibrosis, which was higher compared to FIB-4 index (39% specificity) and NAFLD fibrosis score (59% specificity) [183].

Finally, very few studies have tried to develop such models in pediatric population. The most comprehensive one so far included 222 children and adolescent with biopsy- or MRI-proven NAFLD and 337 without the disease. Metabolomics identified 11 metabolites of interest which together with waist circumference, whole-body insulin sensitivity index and triglycerides could detect the presence of NAFLD with 97% specificity and 73% sensitivity [184].

6.3. Therapeutic implications

NAFLD is considered an unmet clinical need. Several treatments that are currently in the pipeline restore many of the abnormalities observed in the lipidome. Specifically, agonists of farnesoid X receptor (FXR) reduce lipotoxicity by promoting mitochondrial beta-oxidation, decreasing de novo lipogenesis and stimulating cholesterol excretion [142]. In Phase 2 clinical trials and in an interim analysis of an ongoing phase 3 clinical trial, obeticholic acid reduced hepatic steatosis, inflammation and fibrosis compared to placebo [142,185–187].

Dual PPARα and PPARδ agonists (e.g. elafibranor) also stimulate mitochondrial and peroxisome β-oxidation as well as omega-oxidation [188–191]. PPARγ agonists reduce circulating FFA and consequently the flow of SFA to the liver, they improve insulin sensitivity and guide macrophage and Kupffer cell polarization from the proinflammatory M1 phenotype towards to the anti-inflammatory M2 one [142,167]. Various selective PPARγ modulators, dual PPARα/γ agonists (e.g. Saroglitazar) or Pan-PPAR agonists (e.g. lanifibranor) have shown promising results in human studies and are currently under evaluation in phase 2 or phase 3 clinical trials [142,192–194]. In this context, it was recently announced, that lanifibranor met the primary endpoint of a reduction in steatosis activity fibrosis score, including NASH resolution without worsening of fibrosis in a Phase II clinical trial, thus being the first study that met both FDA and EMA regulatory endpoints for accelerated approval [195].

ACC inhibitors (e.g. firsocostat) attenuate the function of acetyl-coA carboxylase (ACC) that catalyzes the conversion of acetyl-CoA to malonyl-CoA. Consequently, malonyl-CoA is reduced and hepatic de novo lipogenesis as well as mitochondrial FA beta oxidation are downregulated [142,196]. In humans, ACC inhibitors reduce hepatic steatosis and on-going clinical trials will evaluate their effect also on inflammation and liver fibrosis [142,196].

Regarding LPC accumulation, animal studies have shown that interruption of the generation of LPC from PC with inhibition of PLA2 results in normalization of PC levels, prevention of NAFLD and reversal of NASH and fibrosis [158,197].

Several treatments are targeting also ceramide levels by aiming to reduce ceramide synthesis (de novo or through hydrolysis from sphingomyelin) or increase ceramide degradation. The reduction of ceramide synthesis can be achieved by inhibiting relevant enzymes (i.e. palmitoyltransferase) but it should be limited only to hepatic ceramides, since global reduction of ceramides can negatively affect nervous system [198]. Alternatively, lower ceramide synthesis can be achieved by blocking intestinal FXR activation [199]. However, in that case, FXR blocking should be limited to the intestine and not affect FXR activation in the liver which acts beneficially. In other approaches, reduction of the accumulation of specific ceramides (i.e. C16:0 ceramide) is targeted with the use of several inhibitors (e.g. DES-1 70) or degradation of ceramides is stimulated by enzymes (e.g. acid ceraminidase). These have till now been tested only in animal studies and not in humans.

Many treatments have targeted to restore the increased ω-6/ω-3 ratio by ω-3 supplementation. Although the findings from animal studies had been very promising, and the first results from human studies had shown an improvement in liver steatosis, later studies could not demonstrate any benefit in steatosis, necro-inflammation or fibrosis in patients with NASH [142,200–202]. Newer experimental strategies are focusing not on the supplementation of ω3 but on restoration of the ω-6/ω-3 ratio with the use of ω-3 desaturase that leads to the production of ω-3 from ω-6 PUFAs, showing beneficial effects in steatosis and necro-inflammation in animal studies [203].

Finally, different experimental approaches are aiming to decrease proinflammatory eicosanoids and increase the concentrations of SPMs. Specifically, some of them are targeting the inhibition of PLA2α enzyme that catalyzes the cascade leading to AA and lysophospholipids generation, showing promising results in animal studies [197,204]. Others are targeting the production of proinflammatory lipids by inhibiting the enzyme 5-LOX [205,206] which catalyzes the lipooxygenation of AA to leukotriene lipids or they aim to block the binding of leukotrienes to their receptors [207]. The 5-LOX inhibitor tipelukast has been approved by FDA for a phase IIa clinical trial. Finally, other approaches are aiming to increase the SPMs RvD1, RvE1, protectin DX and maresin-1 leading to robust improvement of fibrosis in animal models of NAFLD [164,208,209]. Given that SPMs are rapidly inactivated by oxidoreductases and that SPM receptors are downregulated in obesity, recent efforts are also focusing on improving the pharmacologic properties of them by creating either resistant to deactivation analogues [210] or by enhancing their delivery on their site of function.

Altogether, most drugs in on-going clinical trials are targeting, among other mechanisms, hepatic lipidome in order to improve NAFLD. Additionally, there is a significant number of medications that have shown beneficial effects on animal models of NAFLD by improving hepatic metabolome/lipidome and remain to be further clinically tested.

6.4. Conclusions – clinical perspective

Metabolomics/Lipidomics are one of the most investigated omics in NAFLD. They have provided important information about the pathophysiology and course of the disease and changes in metabolome and lipidome have a major role in mechanisms of NAFLD development and progression. Thus, most drug candidates aim to restore the abnormalities in metabolic and lipid profile observed in NAFLD. These abnormalities are not only observed in the liver but often also in peripheral blood, which have led to the extensive use of metabolomics/lipidomics for the development of non-invasive tests for the diagnosis and staging of NAFLD. Many of these tests have reported excellent performance in original studies, which were though not reproducible in other cohorts. This is due to several issues and challenges that are observed in all omic procedures and have to be addressed (s. below 8. Conclusions- Challenges - Perspectives) for the field to move forward. Nevertheless, among the “omics”, we still consider that metabolomics/lipidomics have the best potentials for leading to the development of important tools for diagnosis and staging of the disease.

7. Glycomics

7.1. Physiology- pathophysiology

Glycomics refers to the comprehensive investigation of glycome, i.e. of glycan structures that circulate either as free glycans or they are bound to proteins (glycoproteins), lipids (glycolipids) or phospholipids (glycophospholipids). Glycome is highly dynamic as it is affected by transcriptome, proteome, environmental factors (nutrition) and cellular secretory machinery [125,211]. Glycans can affect protein morphology and interaction with other proteins as well as regulate nutrient storage and sequestration [212]. Additionally, they can protect cell stability and facilitate cell to cell interactions [212]. Glycosylation is the process of the formation of the glycoconjugates (glycoproteins or glycolipids) [213] which can happen both intra- and extracellularly. In the extracellular glycosylation, enzymes secreted mainly from liver hepatocytes and platelets (i.e. glycosyltransferases) are involved [213]. Changes in glycan profile have been observed in numerous inflammatory diseases and in different types of cancer and have been often linked causally with the pathogenesis and progression of these diseases [213–215].

In NAFLD, few glycomic studies have been performed so far (Table 5). These have either aimed to assess the circulating glycome in untargeted approaches or they have focused on the identification of glycans related to specific proteins (i.e. haptoglobin, transferrin, IgG2, IgA1, alpha-1 antitrypsin, ceruloplasmin). The findings of these studies suggest that in NAFLD and during its progression from NAFL to NASH and liver fibrosis higher concentrations in fucosylated, sialylated and agalactosylated glycans are observed. Sialic acids in glycolipids or glycoproteins have diverse functions, including formation of a protective cell surface barrier, involvement in interactions of white blood cells with endothelial lining of blood vessels, recognition by pathogens and toxins and facilitation of cell migration by some cancers [216]. Circulating sialic acid levels have been positively associated with metabolic syndrome and with NAFLD [217,218]. Fucosylated glycans are also involved in a variety of physiological and pathological procedures, including cell adhesion and migration, angiogenesis, malignancy, tumor metastasis as well as immune cell development and regulation [219]. Consequently changes in fucosylation have been reported in numerous inflammatory conditions, such as in rheumatoid arthritis, chronic pancreatitis, sickle cell disease and Crohn's disease [219]. In the liver, fucosylation serves as a signal for the secretion of fucosylated glycoproteins from normal hepatocytes into bile [219]. In ballooning hepatocytes, which are observed in NASH, the fucosylation-related sorting machinery is dysfunctional which may result in the secretion of the fucosylated glycoproteins in the sera instead of bile [219]. Similarly, hypogalactosylation (lack of galactose in the formed glycoprotein or glycolipid) especially of IgG has been associated with inflammatory response and with a number of autoimmune diseases [213].

7.2. Diagnostic perspective

Most of the glycomic studies in NAFLD have tried to identify glycans or glycoproteins that can serve as blood biomarkers for differentiating between NAFL and NASH or for detection of the presence of liver fibrosis and its stage (Table 5). The diagnostic accuracy of most of these tests has been limited, with some reporting higher accuracy for diagnosing NASH and others for diagnosing advanced fibrosis. This suggests that although changes in circulating glycome/glycoproteins are observed in NAFLD, these are not sufficient in order to be used alone for the development of diagnostic models of the disease and thus combination with other clinical or biochemical parameters are needed. In this context, fucosylated-haptoglobin showed below 70% accuracy at differentiating NAFL from NASH, but 76–81% when combined with Mac2bp [220]. Similarly, in our study, which detected with the use of mass-spectrometry the highest number of glycans so far, the concentrations of glycans in serum were able to differentiate between the presence of liver fibrosis or not with 76% sensitivity and 74% specificity [144]. Glycans alone could poorly differentiate between NAFL and NASH but when combined with lipid species in models of 20 variables (18 lipid species and 2 glycans) the sensitivity and specificity for discriminating between NASH, NAFL or healthy liver status increases significantly.

7.3. Therapeutic perspective

Regarding potential treatments, no study thus far has investigated whether targeting glycome can be a therapeutic option in NAFLD. Treatments aiming to decrease fucosylation are currently under evaluation in autoimmune diseases and cancer. In this context, inhibition of

Table 5
Glycomics models for the diagnosis and staging of NAFLD.

#	N	Comparisons	Prediction models	Sensitivity	Specificity	AUROC
[249]	57	NAFL vs NASH	a) Glycan *m/z* 1955 b) Glycan *m/z* 2032 c) Glycan m/z 2584			a) 0.833 b) 0.863 c) 0.866
[250]	D: 51 V: 224	NAS < 3 vs NAS 3–4 vs NAS ≥ 5	Log(NGA2F/ NA2)	D: 79% V: 86%	D: 50% V: 43%	D: 0.75 V: 0.66
[220]	D: 124 V: 382 US: 803	a) Hepatocellular ballooning (yes vs no) b) F0-F1 vs F ≥ 2 c) NASH vs NAFL d) F3 detection	a) Fucosylated Hp b) Mac-2 binding protein c, d) Fucosylated Hp and Mac-2 binding protein	a) D: 72% b) D: 81% c) D: 81% V: 71% d) US: 52%	a) D: 72% b) D: 78% c) D: 79% V: 82% D) US: 89%	a) D: 0.82 b) D: 0.77 c) D: 0.85 V: 0.84 d) 0.77
[144]	80 D: 2/3 V: 1/3 repeated 100×	a) C vs NAFL vs NASH b) F0 vs F1-F4	a) 19 models with 10–29 variables (lipids, glycans, hormones) b) 3 models with 10 lipids, 5 glycans, 5 FA	a) 91% b) 97%	a) 95% b) 99%	a) 0.97 b) 1.00
[251]	60	a) NAFL vs NASH b) F0-F2 vs F3-F4	GlycoNashTest (log(NGA2F/NA2)) and its components glycans NGA2F, NA2	a) 90% b) 90%	a) 45% b) 71%	a) 0.74 b) 0.87
Validation studies						
[252]	510	a) NASH vs non-NASH b) NAFL vs NASH c) Fibrosis (Y vs N)d) F0-F2 vs F3-F4	Mac-2 binding protein	a) 70% b) 68% c) 66% d) 87%	a) 82% b) 73% c) 76% d) 60%	a) 0.82 b) 0.76 c) 0.75 d) 0.74

C, controls; D, Discovery; N, No; Se, sensitivity; Sp, specificity; V, Validation; Y, Yes.

fucosylation with a 2-deoxy-d-galactose, a fucosylation inhibitor, reduces inflammation in rheumatoid arthritis by decreasing inflammatory macrophages and Th17 cells in the lymph nodes as well as by reducing the levels of TNFα, interleukin-6 (IL-6), and antibodies to type II collagen in the serum [221]. Similarly, inhibition of fucosylation by 2-fluorofucose suppresses the proliferation, migration and tumor formation of HepG2 liver cells [222]. Whether similar interventions can be beneficial in NAFLD by reducing inflammation, fibrosis or HCC formation should be addressed in future studies.

7.4. Conclusion – clinical perspective

Glycomics are one of the least investigated "omics" in NAFLD. Given the strong relation between glycoprotein-glycolipid formation and liver function, more studies assessing the potentials of glycans in biomarker discovery and drug development are needed.

8. Conclusion – challenges - perspectives

Significant advances in omics technology and in data analysis have provided us with important information about the pathophysiology of NAFLD, have contributed to the creation of models for the diagnosis and staging of the disease and have identified potential therapeutic targets. Certain omics have been widely used so far either for identifying high risk patients (genomics), for evaluating the effect of treatments in key pathophysiological mechanisms (transcriptomics) or for developing non-invasive diagnostic tools (metabolomics/lipidomics).

Major challenges exist that have to be overcome in order to increase the benefits and the reproducibility of the results deriving from the use of these technologies in NAFLD research:

a) *Technical aspects:* The analysis in most omics procedures provide relative and not absolute concentrations for the measured parameters. The relative concentrations derive often after normalization to total MS signal, assessed by some researchers from the peaks and by others from the area below the peaks. Background noise, sample quality, variability between different MS runs, differences in capabilities between MS machines and in level of confidence in the identification of the investigated species (lipids, proteins etc.) can have an important impact in the final relative concentrations and jeopardize reproducibility of the results, especially when training or validating non-invasive, omics-based diagnostic tools.
b) *Study design - Clinical aspects:* Several methodological aspects related to study design can have a significant impact in the interpretation of omics results and especially in the development of non-invasive tests. For example, a diagnostic model trained and validated in a patient population of a liver clinic, where a high prevalence of advanced fibrosis is observed, may not perform as well when used in the general population and in primary care setting, where advanced fibrosis is less common. Additionally, most of the omics studies have been performed so far in small populations or in cohorts with specific characteristics. No adjustments for confounding factors (medications, co-existence of metabolic diseases, BMI, age etc.) are being performed. Thus, these models often do not account for population heterogeneity, which is crucial for the accuracy all non-invasive diagnostic panels. This has been repeatedly shown in studies that focused on patients with overweight, obesity and especially with T2DM. Several and diverse biomarker panels (including SteatoTest, ActiTest, NashTest-2, cytokeratin-18, FibroTest, OWLiver tests) were evaluated but none of them demonstrated optimal performance for diagnosing NASH or liver fibrosis especially in patients with T2DM [5,13,181,223,224]. Third, very few tests have been evaluated prospectively. This is a very important step, which even other simpler algorithms or imaging modalities have failed to pass [225]. Additionally, depending on their mechanism of function, medications may have different levels of impact on the metabolic and lipid profile in the liver which may affect circulating metabolome/lipidome and its ability to reflect the stage of the disease through a diagnostic algorithm originally designed in untreated patients.

Altogether, diagnostic models based on omics have yet to reach exceptional levels of performance, while satisfactory study outcomes need to be cautiously scrutinized and repeatedly validated into large and diverse patient cohorts both cross-sectionally as well as prospectively. To this direction, FDA has recently approved the parallel evaluation of diagnostic biomarker panels in NASH drug development - biopsy-proven clinical trials. Specific criteria should be fulfilled in these evaluations which include: a) the selection of the biomarkers before study initiation in order to protect the prospective character of the evaluations, b) submission of guidelines for the use and interpretation of the measurements from the proposed diagnostic panels, c) detailed description of the assay performance specifications in order to ensure independent reproducibility, d) detailed presentation of statistical analysis to ensure replications of the results, e) validation of the final results from a second independent to the trial data set. Aim of this approach is first to develop non-invasive models that will be able to identify subjects who are most likely to fail or pass in the liver biopsy screening of the clinical trial, thus accelerating study recruitment. The ultimate goal though still remains to be able to replace liver biopsy with highly accurate and specific non-invasive diagnostic tests both in clinical trials as well as, in the near future, in daily clinical routine.

In conclusion, omics are contributing to the major evolution observed in health research in the last years, that is characterized by the use of big and diverse data analyzed by advanced mathematical and computational tools in order to identify new ways of understanding human biology and health. The power of these technologies creates significant opportunities for addressing unmet clinical needs such as the non-invasive diagnosis and treatment of NAFLD. In this context, it seems reasonable to consider the first proof-of concept studies in this area of translation research as contributions towards the understanding and establishment of the necessary methodology and tools in order to achieve major leaps in the treatment of our suffering fellow human beings in the near future.

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Declaration of competing interest

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