Chapter 1

Non-alcoholic Fatty Liver Disease: What We Learn from Omics Studies

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1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is a serious hepatic disorder, affecting up to 30% of the general population of Western countries and approximately 15% in Asian population [1, 2]. The increased prevalence in developing countries is related with sedentary life style and lack of exercise. Moreover, the increase in NAFLD prevalence is also related with alterations in dietary intake caused by urban lifestyle which is represented by a 24% augmented energy...
intake because of the enhancements in the consumption of flour, cereal products, and added sugar and fats and/or in total fat and fruit intake [3]. In addition, the use of corn syrup or high fructose as sweeteners in beverages greatly contributed the prevalence of NAFLD [4]. Hence, various dietary models are being evaluated for prevention of NAFLD. The top studied dietary interventions include diets restricted in calories and carbohydrates with soy protein addition, low calorie diet rich in proteins, high protein diet, soft drinks with fructose compared to glucose sodas, and Mediterranean diet [5]. The fact that NAFLD is the second most common reason for liver transplantation emphasizes the burden of NAFLD to public health [6]. NAFLD comprises an entire pathological spectrum of diseases with successive stages of increasing severity, ranging from simple steatosis (SS), non-alcoholic steatohepatitis (NASH), and cirrhosis to hepatocellular carcinoma (HCC) [7]. The end result, HCC, is the fifth common cancer among all primary neoplastic diseases and affects one million individuals annually worldwide. Despite the common fact that hepatitis B or hepatitis C infection-associated liver cirrhosis or abusive alcohol consumption is the primary cause of HCC, recent studies reported that HCCs may also affect non-cirrhotic livers, most of them having no associated risk factors. Consequently, NASH is now evaluated as a significant risk factor due to the high prevalence of obesity and type 2 diabetes mellitus [8]. Recent reports stated that the risk to develop HCC in patients with metabolic syndrome is increased by 2.13 (odd ratio), while the increase rate is 4.4 in patients with NAFLD [9, 10].

NAFLD is closely associated with obesity, combined hyperlipidemia, type II diabetes mellitus, high blood pressure, and insulin resistance; it can be regarded as the hepatic manifestation of metabolic syndrome [11]. Insulin resistance, dyslipidemia, and cardiovascular risk factors are known to arise from abnormalities in fatty acid metabolism and systemic inflammation, but the exact link between metabolic syndrome and the onset and progression of liver injury is still unclear [12]. Steatosis, characterized by an accumulation of triglycerides in the liver parenchyma, may develop into NAFLD if the rates of hepatic uptake of circulating blood free fatty acids (FFA), which originates from excessive adipose tissue lipolysis, and de novo liver lipogenesis from glucose are greater than the rate of mitochondrial fatty acid oxidation or export as triglycerides within low-density lipoproteins. This phenomenon arises from abnormalities in glucose, fatty acid, and lipoprotein metabolism accompanied by the development of insulin resistance. On the other hand, upcoming evidence now suggest that triglyceride accumulation in the form of lipid droplets could instead be a parameter of excessive fatty acid trafficking, while non-triglyceride fatty acid metabolites would be the consequence of lipotoxicity of the NASH pathogenesis [13]. Insulin resistance results in an excessive flow of fatty acids from the adipose tissue and also hinders peripheral glucose removal. In the liver, fatty acid disposal causes excessive production of reactive oxygen species, followed by lipid peroxidation and augmented inflammatory response [14]. Still, the exact mechanism that explains progression of SS to NASH is not yet fully clear. Currently, liver biopsy is still the gold standard in diagnosis of NAFLD. The histological indication of NAFLD is determined as lipid accumulation in the hepatocytes in the absence of pathologies such as viral hepatitis or alcohol abuse [15]. However, liver biopsy has certain disadvantages. First and most important of all, it is an invasive procedure. Moreover, since NAFLD does not uniformly affect liver, this heterogeneity may cause some biases in biopsy results [16, 17]. Hence, there is an urgent need for non-invasive biomarkers to assess liver diseases such as NAFLD.
Currently, high-throughput Omics studies engage in to solve the complex mechanisms responsible for NAFLD and NASH development. The genomic studies focus on genome-wide association studies (GWAS) that identify biomarkers across whole genomes to determine genetic variations associated with a disease of interest. The technologies of high-throughput genotyping are now able to assay the common single nucleotide polymorphism (SNP) and find the association between SNPs and clinical conditions or measurable traits [18]. As in many other diseases, besides genetic factors epigenetics and transcriptomic alterations are involved in the development of NAFLD and NASH. Additionally, identification of specific proteins, either as novel biomarkers or as over-/under-expressed markers through proteomic studies, may have a massive effect by increasing the availability of biomarkers for early diagnosis and therapy [19].

The development of NAFLD is a complex multifactorial process that involves the disruption of multiple gene and protein mechanisms. Initially, Day and James suggested a “two-hit hypothesis” to define the development of NAFLD: The “first hit” corresponds to a primary hepatic lipid accumulation which is described as steatosis; the “second hit” is an oxidative stress leading to lipid peroxidation, followed by liver injury and inflammation [20]. Recently, this traditional “two-hit hypothesis” has been upgraded to “multiple parallel hits hypothesis.” It has been proposed that significant overlaps among insulin resistance, hepatic de novo lipogenesis, and subsequent hepatocyte injury also come into play in the progression from SS to NASH [21]. In addition, various candidate gene studies focusing on genetic factors of NAFLD development have further supported the “multiple parallel hits hypothesis” [22]. This review aims to sum up the current Omics studies such as genomics, transcriptomics, and proteomics to offer a better understanding of the pathogenesis of NAFLD.

2. Genomics in NAFLD

Accomplishment of Human Genome Project in 2003 greatly accelerates genome-wide association studies (GWAS) that enable researchers to identify biomarkers across genomes of population that are associated with a given disease. GWAS has a unique hypothesis-free approach that comes handy for examining genes that otherwise would have not been considered as candidates because of our limited knowledge of their function and for revealing as well non-protein coding regions of the genome that involve crucial regulatory alterations [14]. Thus, there are multiple GWAS conducted to identify genes that are associated with the development of NAFLD. According to genomic studies, the associated genes with the pathophysiology of NAFLD belong to hepatic lipid metabolism, ECM balance, cytokines, and insulin resistance [11] (Table 1).

The first GWA study that was performed by Romeo et al. notably increased the notion that genetic factors could affect the susceptibility of NAFLD [24]. In their study, Romeo et al. presented the association between a genome-wide survey of 9229 non-synonymous SNPs and hepatic fat detected by MR spectroscopy in 1032 African-American, 696 European-American, and 383 Hispanic adults residing in Dallas County and found that an allele in human patatin-like phospholipase domain containing 3 gene (PNPLA3) (rs738409, I148M)
was strongly contributing increased hepatic lipid levels, alanine aminotransferase levels, and hepatic inflammation. PNPLA3 gene that encodes adiponutrin is known to have lipase activity in vitro and has been shown to be involved in glucose and lipid metabolism [24]. Recent reports also supported the importance of this variant in NASH progression owing to its connection with fibrosis development [25]. The association of this variant in PNPLA3 gene (rs738409; I148M) with the susceptibility and histological severity of NAFLD was also confirmed by the study of Sookoian and Pirola which included 2937 subjects [26]. Chalasani et al. have examined 324,623 SNPs from the 22 autosomal chromosomes in 236 non-Hispanic white women with well-diagnosed NAFLD for their clinical and histological features [27]. They reported association of SNP rs2645424 on chromosome 8 in farnesyl-diphosphate farnesyltransferase 1 (FDFT1) with NAFLD activity score and SNP rs1227756 on chromosome 10 in a collagen XIII variant (COL13A1), with lobular inflammation. While they stated association of several variants with the degree of fibrosis or serum levels of alanine aminotransferase, they found no significant association between genotypes and steatosis, ballooning degeneration, portal inflammation, or other features of NAFLD. Transmembrane 6 Superfamily Member 2 (TM6SF2) gene is also found to be associated with NAFLD [30]. Minor allele frequency for the rs58542926 TM6SF2 polymorphism has been reported as 7% in Europeans, 4% in Hispanics, and 2% in African Americans [31], which are much lesser than MAF for PNPLA3 rs738409 (I148M) variant which has been reported as 49% in Hispanics, 23% in those of European ancestry, and 17% in African Americans [32]. Individuals with rs58542926 TM6SF2 polymorphism have shown to possess a greater risk of developing NAFLD (OR 2.13 (95% CI: 1.36–3.30)) [33]. Another GWAS conducted by Yuan et al. in three populations (total n = 7715) with replication in three additional cohorts (total n = 4704) analyzed genetic variations affecting plasma liver enzyme levels and reported six loci that have an effect on plasma levels of liver enzymes as well as confirming previously stated associations between the GGT1 locus and gamma glutamyl transpeptidase (GGT) levels and between the ABO locus and alkaline phosphatase levels [23].

| Candidate genes | Cohort (n = Population size) | Reference |
|-----------------|-------------------------------|-----------|
| GGT1 and ABO    | n = 7715; replication in 4704 | [23]      |
| PNPLA3          | n = 11,340                    | [24–26]   |
| FDFT1 and COL13A1 | n = 236                     | [27]      |
| PNPLA3, NCAN, PPP1R3B, CCKR, and LYPLAL1 | n = 7126; replication in 592 cases and 1405 control | [28] |
| PNPLA3, TRIB1, CPN1, loci near HSD17B13, and MAPK10 | 61,089 | [29] |
| PNPLA3 and TM6SF2 | 2736                       | [30]      |

GGT1, gamma-glutamyltransferase 1; PNPLA3, patatin-like phospholipase domain containing 3; FDFT1, farnesyl-diphosphate farnesyltransferase 1; COL13A1, collagen type XIII alpha 1 chain; NCAN, neurocan; PPP1R3B, protein phosphatase 1 regulatory subunit 3B; CCKR, cholecystokinin receptor; LYPLAL1, lysophospholipase-like 1; TRIB1, tribbles pseudokinase 1; CPN1, carboxypeptidase N subunit 1; HSD17B13, hydroxysteroid 17-beta dehydrogenase 13; MAPK10, mitogen-activated protein kinase 10; TM6SF2, transmembrane 6 superfamily member 2.

Table 1. GWA studies of NAFLD.
Altogether, this numerous genomic studies propose the association of several genetic factors, especially those responsible for lipid metabolism, with NAFLD development. Still, further studies are required to deeply understand the effect of genetic variations on the pathogenesis of NAFLD to develop specific therapies that prevent the progression of the disease or specific treatments at each progressive step of the disease.

3. Transcriptomics in NALFD

The development of transcriptomic tools, predominantly real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) and microarrays, accompanied by supporting informatics and statistical methodologies enables researchers to investigate alterations in global mRNA levels in NAFLD. As in many other diseases, besides genetic factors, epigenetic and transcriptomic changes participate the progression of NAFLD. The term epigenetics is described as heritable alterations in gene expression patterns that are not encoded directly within DNA but are instead determined by related factors such as DNA methylation or histone modifications. While these epigenetic changes are heritable, they can also be modified in response to environmental effects [34]. The liver, being the key metabolic organ, is subjected to nutrition-derived factors that can alter its epigenetic signature. Two essential factors that play a role in epigenetic modifications of histones and DNA are acetyl-CoA and S-adenosylmethionine which are also directly involved in glucose or methionine metabolism, respectively [35]. Consequently, histone acetylation has been shown to participate to NAFLD development by triggering lipogenic and glycolytic genes, while abnormalities in S-adenosylmethionine levels have been demonstrated to result in lipogenesis, accumulation of hepatic triglycerides, and NAFLD [35].

In terms of ease in clinical applications, it is better for an epigenetic biomarker to be discovered in peripheral blood [36]. Hereof, a promising study that examined body mass index loss in obese adolescents reported significance of altered DNA methylation in Aquaporin-9 (AQP9), dual specificity protein phosphatase 22 (DUSP22), homeodomain-interacting protein kinase 3 (HIPK3), troponin T1 (TNNT1), and troponin I3 (TNNI3) genes [37].

MicroRNAs (miRNAs) which are short RNA molecules of about 22 nucleotides that control mRNA stability and thus transcription levels are crucial transcriptomic factors that can affect NAFLD development [14]. Studies have inconsistent results for their involvement in steatosis, possibly due to the variances of diagnostic methods, staging, and miRNA measurement [38]. The direct analysis of miRNA in the blood makes them ideal biomarkers for the distinct stages of steatosis. Recently, specific microarrays, such as muParaflo microRNA microarrays, greatly contributed to the examination of microRNAs in NAFLD. For instance, the expression miRNA-122, which constitutes 70% of the total liver miRNAs, has been shown to be augmented in the blood of NAFLD patients [39, 40]. The study of Cermelli et al. that compares miRNA levels of NALFD patients and healthy controls reported increased miRNA-34a and miRNA-16 levels in NAFLD patients. Also, they suggested that miRNA-122 and miRNA-34a might be a useful biomarker for the evaluation of NAFLD and NASH [39]. Besides miRNA-122, Pirola et al. reported association of miRNA-192 and miRNA-375 with the severity of the disease [40]. Nonetheless, since there is a dynamic and multifactorial relationship between
miRNAs and gene regulation, further studies and careful evaluations are required before miRNAs can be used as biomarkers in the diagnosis and staging of NAFLD.

In addition to epigenetic factors and miRNAs, alterations in gene expression profile also affect progression to NAFLD. Several cross-sectional studies performed on cohorts with various histological parameters (alcoholic steatohepatitis and NASH vs. no NASH, NASH vs. no NASH, control vs. steatosis vs. NASH, control vs. steatosis vs. NASH with steatosis >5% vs. NASH with steatosis <5%) reported the significant effect of Wnt pathway as a protagonist, besides genes participated in absorption, distribution, metabolism, and excretion (ADME); aldose reductase AKR1B10; and keratin family member KRT23 [41–45]. Studies also revealed that genes involved in cellular proliferation and ECM organization, such as dermatopontin (DPT), were differentially expressed in the liver transcriptome of NAFLD patients [46–48]. Unfortunately, subtle alterations in individual’s gene expression caused by interindividual heterogeneity of the disease and the adaptive nature of the pathological response limit clear-cut identification of patient categories and therefore complicate identification of transcriptomic biomarkers [41, 43, 45, 48].

4. Proteomics in NAFLD

The improvement of novel proteomic tools accelerated researches in NAFLD diagnosis and discovery of biomarkers. The first study that examined serum protein profiles in NAFLD by surface-enhanced laser desorption ionization time of flight mass spectrometry (SELDI-TOF MS) on 98 obese patients, with 91 NAFLD patients (12 steatosis alone, 52 steatosis with non-specific inflammation, 27 NASH) and 7 patients without NAFLD as obese control, reported 12 significant protein peaks. However, because of the inherent limitation of low mass accuracy in SELDI-TOF MS, researchers could only identify fibrinogen γ and proposed a possible association with fibrosis [49]. The study of Bell et al. identified significant alterations in 55 proteins between NAFLD and NASH with advanced fibrosis by performing an ion-intensity-based, label-free quantitative proteomic approach (LFQP) [50]. They also reported significant changes of 15 proteins between early NASH and NASH with progressed fibrosis. From their data, a 6-protein diagnostic method that includes fibrinogen β chain, retinol-binding protein 4, serum amyloid P component, lumican, transgelin 2, and CD5 antigen-like and a 3-protein diagnostic method consisting of component C7, insulin-like growth factor acid labile subunit, and transgelin 2 were developed to diagnose the progressive stages of NAFLD (AUROC ranging from 0.83 to 0.91). Moreover, they also presented that alanine aminotransferase (ALT) was a low-grade diagnostic protein for the evaluation of different stages of NAFLD (AUROC = 0.53) [50]. Several other studies were also consistent with the fact that ALT is not a suitable NAFLD diagnosis biomarker, and no optimal ALT levels are present to evaluate advanced fibrosis [51]. Even with the inability to discover unique biomarkers that could distinguish between NAFLD and NASH, the study of Bell et al. greatly contributed into the understanding of the pathogenesis of NAFLD and NASH [50]. Generally, most of the proteins identified by several proteomic studies suggest
the association of immune system regulation, inflammation, hepatic ECM structure, and protein carriers in the blood with NAFLD (Table 2). Nonetheless, even proteomics is a great tool for gaining deep insight on the pathogenesis and progression of the disease; unfortunately, these tools cannot yet offer specific biomarkers with major clinical value to diagnose NASH or discriminate NASH and steatosis.

5. Conclusions

In conclusion, the Omics studies explained throughout the review supported the fact that NAFLD is a complex disease caused by several phenomena such as accumulation and traffic of various lipids in the liver and triggered inflammation responses. Altogether genomic, transcriptomic, and proteomic studies are in accordance with the basic detectable pathogenic mechanisms of NAFLD which are mitochondrial energetic and structural abnormalities, triggered inflammatory response via multiple targets, and lipotoxicity.

| Protein categories            | Protein markers                                      | Reference |
|------------------------------|------------------------------------------------------|-----------|
| Protein carrier              | Apolipoproteins                                      | [52]      |
|                              | CD5 molecule-like (CD5L)                             | [53]      |
| Metabolic pathways           | Carbamoyl phosphate synthetase I (CPS1)              | [54]      |
|                              | Glucose-regulated protein 78 (GRP78)                 | [54]      |
|                              | Uric acid                                            | [55]      |
| Acute phase protein          | High sensitive C-reactive protein (Hs-CRP)           | [56]      |
|                              | Hemoglobin                                           | [57]      |
|                              | Serum fucosylated haptoglobin (Fuc-Hpt)              | [58]      |
|                              | Pentraxin 3 (PTX-3)                                  | [59]      |
| Anti-inflammatory and antioxidant | Bilirubin                                            | [60]      |
| Extracellular matrix         | Hyaluronic acid                                      | [61]      |
|                              | Type IV collagen 7S                                  | [62]      |
|                              | Laminin                                              | [63]      |
|                              | Lumican                                              | [64]      |
|                              | Matrix metalloprotease 9 (MMP-9)                     | [65, 66]  |
| Immune cells and cytokines   | C–C motif chemokine ligand 2 (CCL2) and Monocyte chemotactic protein 1(MCP1) | [67]      |
|                              | Retinol-binding protein 4 (RBP4)                     | [50, 68–70]|

Table 2. Proteomic studies of NAFLD.
Advances in genomic and transcriptomic tools allow researchers to inspect significant genetic polymorphisms and epigenetic alterations, along with miRNA levels in different stages of the NAFLD progression. Noticeably, individuals with unfavorable genetic polymorphisms coupled with disadvantageous biological environment carry a high risk of developing NAFLD. Moreover, development of novel proteomic methodologies also supported the biomarker studies in NAFLD which aim to discover key protein molecules that carry significant clinical importance in the concourse of the disease. Even though a few candidate serum protein markers achieve to distinguish NAFLD and NASH, further validation studies of these biomarkers in larger cohorts are still required before they can be clinically used in the diagnosis and evaluation of the disease progression. Overall, further advancement of Omics studies is still required to deeply understand the pathophysiology of NAFLD and discover specific biomarkers for clinical use.

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