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MINIREVIEWS

Multiomics biomarkers for the prediction of nonalcoholic fatty liver disease severity

Carlos J Pirola, Silvia Sookoian

Carlos J Pirola, Department of Genetics and Molecular Biology of Complex Diseases. University of Buenos Aires, Institute of Medical Research A Lanari, Buenos Aires, Argentina, National Scientific and Technical Research Council-University of Buenos Aires. Institute of Medical Research (IDIM), CABA 1427, Argentina

Silvia Sookoian, Clinical and Molecular Hepatology, University of Buenos Aires, Institute of Medical Research A Lanari, Buenos Aires, Argentina, National Scientific and Technical Research Council-University of Buenos Aires. Institute of Medical Research (IDIM), CABA 1427, Argentina

ORCID number: Carlos J Pirola (0000-0001-8234-4058); Silvia Sookoian (0000-0001-5929-5470).

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Correspondence to: Silvia Sookoian, MD, PhD, Senior Scientist, Clinical and Molecular Hepatology, University of Buenos Aires, Institute of Medical Research A Lanari, Buenos Aires, Argentina, National Scientific and Technical Research Council-University of Buenos Aires. Institute of Medical Research, Combatientes de Malvinas 3150, CABA 1427, Argentina. sookoian.silvia@lanari.fmed.uba.ar
Telephone: +54-11-52873905

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Abstract

This review intends to uncover how information from large-scale genetic profiling (whole genome sequencing, and whole exome sequencing) of nonalcoholic fatty liver disease (NAFLD), as well as information from circulating transcriptomics (cell-free miRNAs) and metabolomics, contributes to the understanding of NAFLD pathogenesis. A further aim is to address the question of whether OMICs information is ready to be implemented in the clinics. The available evidence suggests that any new knowledge pertaining to molecular signatures associated with NAFLD and nonalcoholic steatohepatitis should be promptly translated into the clinical setting. Nevertheless, rigorous steps that must include validation and replication are mandatory before utilizing OMICs biomarkers in diagnostics to identify patients at risk of advanced disease, including liver cancer.

Key words: Nonalcoholic steatohepatitis; Fibrosis; Liver biopsy; Genetics; *PNPLA3*; *TM6SF2*; Metabolomics; Proteomics; Transcriptomics; Nonalcoholic fatty liver disease; miR122

Core tip: It is expected that, in the near future, nonalcoholic fatty liver disease patients can be diagnosed and treated according to their own "molecular signature". Specific focus should be placed on prevention and early diagnosis through the application of biomarkers of disease risk. Selection of "personalized drugs" as well as tailored therapy according to the specific molecular signature should be further guaranteed.

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a chronic liver disease that affects adult and children populations around the world, with prevalence reaching alarming levels[1,2].

NAFLD may progress from a benign histological disease stage characterized by plain fat accumulation, usually referred to as simple steatosis or nonalcoholic fatty liver (NAFL), to a more severe histological form characterized by liver cell injury, a mixed inflammatory lobular infiltrate, and variable fibrosis named nonalcoholic steatohepatitis (NASH)[3,4].

Precise histological diagnosis, including disease stages (NAFL and NASH), is commonly based on liver biopsy[2]. Nevertheless, because this method imposes certain limitations, including potential complications such as bleeding and patients' abdominal discomfort, and needs to be performed in a special setting, noninvasive approaches are favored and have gained considerable attention. It is also noteworthy that the histological diagnosis of the severity of NAFLD might be potentially biased if a small portion of hepatic tissue is sampled.

Hence, significant clinical and research efforts are currently being directed toward the search for reliable biomarkers aimed at the prediction of the disease severity and prognosis.

Knowledge in the field of liver diseases, particularly NAFLD, has benefitted in the last ten years from the rapid development of high-throughput technologies, including genomics, transcriptomics, proteomics and metabolomics. This review intends to uncover how information from large-scale genetic profiling (whole genome sequencing and whole exome sequencing) of NAFLD, as well as information from transcriptomics and metabolomics, and the interplay of these personal characteristics with dietary factors may contribute to the diagnosis and risk prediction of NAFLD progression. In addition, the question of whether OMICs information is ready to be implemented in the clinics will be addressed.

A brief description of OMICs signatures, including their main applications as biomarkers in clinical practice, is provided in Figure 1. OMICs biomarkers may be considered either for screening purposes to assess the disease risk or exposure, or for the assessment of the disease severity and prognosis, and/or for monitoring treatment response (Figure 1).

ROLE OF GENETIC MARKERS IN THE PREDICTION OF NAFLD RISK AND DISEASE SEVERITY

Although the pathogenesis of NAFLD is not understood fully, a growing body of evidence indicates that the disease develops from a complex process involving many factors, including genetic susceptibility and environmental insults[5,6].

In fact, the results yielded by the first genome-wide association study on NAFLD[7] on the role of rs738409 C/G -a variant nonsynonymous single nucleotide polymorphism (SNP) of *PNPLA3* (patatin-like phospholipase domain containing 3, also known as adiponutrin or calcium-independent phospholipase A2-epsilon) have significantly contributed to the knowledge of the genetic component of NAFLD. This finding was subsequently widely replicated around the world, confirming that the G allele in the forward strand is significantly associated not only with an increased risk of fatty liver but the histological disease severity as well[8,9] (OR 1.88 per G allele). In fact, rs738409 explains about 5.3% of the total variance in NAFLD[9].

Furthermore, results of the first exome-wide association study of liver fat content indicate that rs58542926 (E167K), a nonsynonymous variant located in *TM6SF2* (Transmembrane 6 Superfamily Member 2), is significantly associated with increased liver fat content[10]. Nevertheless, in contrast to the effect of the variant located in *PNPLA3*, the rs58542926 exerts a moderate effect on the risk of NAFLD (odds ratio: 2.13)[11]. Subsequent studies have also revealed an association of rs58542926 with the disease severity[12-14], as well as dual and opposite role in cardiovascular disease prevention[11,12,15].

Thus, it is reasonable to speculate that genetic markers, particularly the 738409-G risk allele, may be used for individual risk assessment either alone or as a part of multi-score biomarkers (Figure 2). For example, Kotronen and coworkers evaluated the performance of rs738409 in predicting the risk of NAFLD by combining routine clinical and laboratory data and the rs738409 genotypes[16]. The authors observed a sensitivity of 86% and a specificity of 71% in the estimation of increased liver fat content[16]. Surprisingly, addition of the genetic information to the score improved the accuracy of NAFLD prediction by less than 1%.

The incorporation of genetic markers into noni-

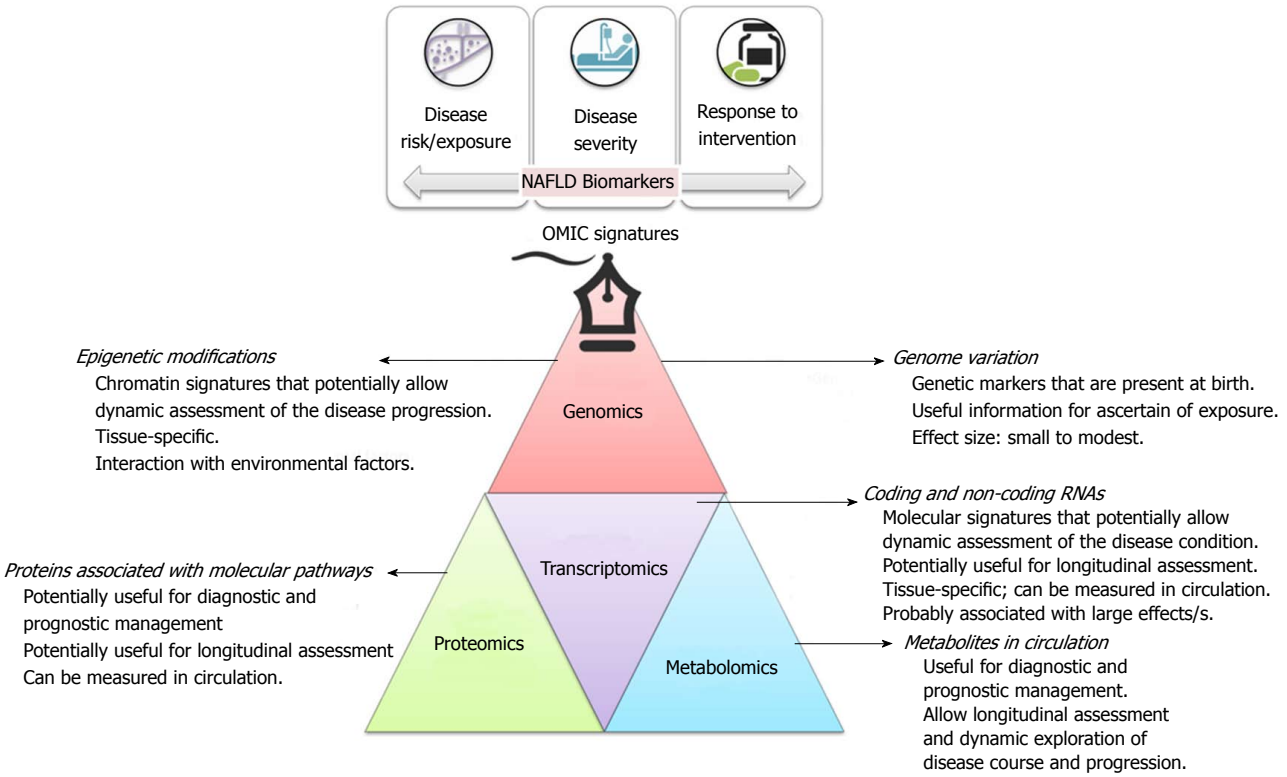


Figure 1 Brief description of OMICs signatures, including their main applications as biomarkers in clinical practice.

nvasive tests that discriminate between NAFL and NASH results in a more challenging strategy; despite these difficulties, there have been some interesting attempts. For instance, a risk score comprising of both clinical and genetic (*PNPLA3* rs738409 C>G, *SOD2* rs4880 C>T, *KLF6* rs3750861 G>A, and *LPIN1* rs13412852 C>T) risk factors resulted in an AUROC (Area Under the Receiver Operating Characteristic) of 0.80 to predict NASH in obese children with increased levels of liver enzymes[17], as shown in Figure 2.

Other examples include the *NASH Clin Score* that combines laboratory tests (AST, fasting insulin) and rs738409 genotypes, and the *NASH ClinLipMet Score* that combines laboratory test (AST, fasting insulin), circulating metabolites (glutamate, isoleucine, glycine, lyso PC 16:0; PE 40:6) and rs738409 genotypes[18], as depicted in Figure 2.

Furthermore, promising results have been reported on the use of genetic markers in predicting NAFLD-intervention response, as summarized in Figure 2. For example, it was observed that genetic variation in *PNPLA3* might confer sensitivity to liver fat content decrease in obese patients undergoing weight loss[19]. The findings yielded by this study, though based on a small number of subjects, suggested that weight loss was more effective in decreasing liver fat in subjects who were homozygous for the rs738409-G allele[19]. Likewise, rs738409 correlated with changes in metabolic profile and intrahepatic triglyceride content (IHTG) as measured by proton magnetic resonance spectroscopy in patients enrolled in a lifestyle modification program[20]. Concordant results were reported regarding greater improvement in hepatic steatosis after bariatric surgery in the risk-G-rs738409 allele carriers[21] (Figure 2).

A different approach to the use of genetic testing based on single base variations in the DNA sequence requires search for variants in mitochondrial DNA (mtDNA). Mitochondria contain their own genetic information in the mtDNA (16.5 Kb), which is maternally inherited; the 13 mtDNA-encoded proteins are all components of the *oxidative phosphorylation* (OXPHOS). A comprehensive exploration of the complete liver mtDNA-mutation spectrum in patients with NAFLD during different stages of the disease by next generation sequencing showed that the disease severity is associated with an increased liver mtDNA mutational burden, including point mutations in OXPHOS-genes that showed high degrees of heteroplasmy[22]. Given that the variability in the mt-genomes observed in NAFLD and NASH seems to originate from a common germline source, rather than from tissue-specific mutations, point mutations can also be assessed in samples of peripheral blood mononuclear cells[22].

ROLE OF EPIGENETIC MODIFICATIONS AS NONINVASIVE BIOMARKERS OF NAFLD AND NASH

The dynamic nature of epigenetic modifications is not only an ideal frame to explain the cross-talk between NAFLD and related phenotypes, including

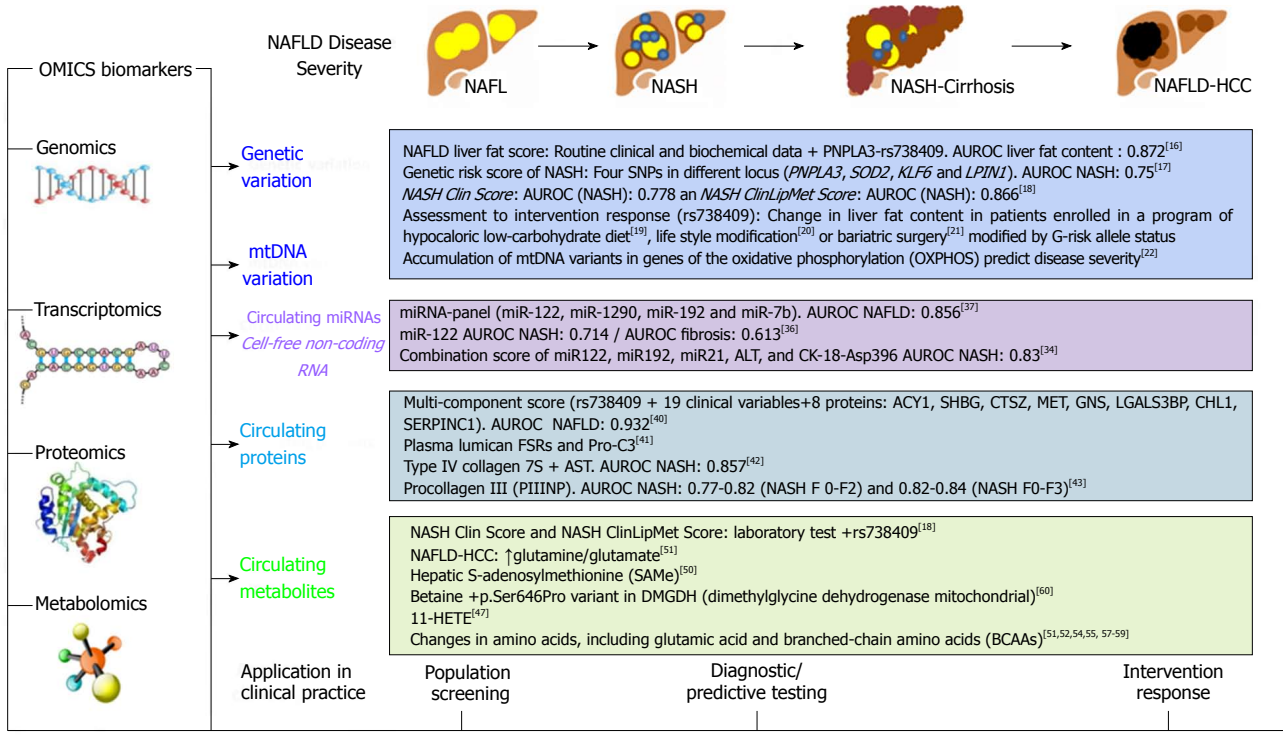


Figure 2 Summary of OMICs biomarkers in the prediction of nonalcoholic fatty liver disease severity.

insulin resistance[23], but is also an attractive target for therapeutic intervention[24]. Treatment-induced epigenetic remodeling of liver tissue was observed in a cohort of obese patients with NAFLD who underwent bariatric surgery[24]. In addition, changes in DNA methylation could be used as a target of a biomarker that allows monitoring, for instance, effectiveness of pharmacotherapy. Interesting results have been reported in the context of other non-cancer complex diseases, including rheumatoid arthritis[25], pediatric asthma[26] or anxiety disorders[27].

It is worth noting that epigenetic modifications, *i.e.* DNA methylation, are not restricted to the nuclear genome, but can also be found in mt-genomes[28]. In fact, we found for the first time that hepatic methylation and transcriptional activity of the MT-ND6 (mt genome-encoded NADH deshydrogenase 6, a member of the OXPHOS complex 1) are associated with the histological severity of NAFLD[29]. This epigenetic change to mtDNA is potentially reversible by lifestyle interventional programs, as physical activity could modulate the methylation status of MT-ND6[29].

CELL-FREE DNA AND RNA AS NONINVASIVE BIOMARKERS OF NASH

Circulating molecular biomarkers, particularly cell-free DNA (cfDNA) and cell-free RNA (cfRNA) are focus of intensive research; however, the strategies employed in these studies are not necessarily novel. In fact, the first description of cell-free nucleic acids (cfNAs) was provided by Mandel and Métais in 1948[30]; indeed, these authors introduced the concept of liquid biopsy.

Basically, cfNAs refer to molecules of nucleic acids that circulate free of cells in the bloodstream and the source of which is primarily dying cells from distant tissues.

Considerable efforts have been dedicated to the use cfDNA for the prediction of liver fibrosis associated with NASH and alcoholic liver disease[31]; however, the preliminary results indicate substantial lack of specificity, as they can be completely unrelated to NASH-biology[32]. Furthermore, the fact that cfDNA circulates not only at very low concentrations but is also highly fragmented imposes analytical and technical challenges that are very difficult to overcome[33].

Conversely, detection of microRNAs (miRNAs), which are highly conserved noncoding small RNAs, has demonstrated quite robust performance, particularly in the circulating compartment. In addition, unlike cfDNA, cfmiRNAs are resistant to degradation as well as to several freeze–thaw cycles, making them ideal biomarkers for use in the clinical setting.

The circulating miRNA signature of NAFLD has been extensively explored in case-control studies, including patients with liver biopsy[34-37], Figure 2. Studies in which liver and circulating miRNA levels were compared demonstrated that cfmiRNAs are good predictors of NAFLD-disease stages[36]. Specifically, circulating miR122 and miR192 not only mirror histological and molecular events occurring in the liver, but have a reliable predictive power in differentiating simple steatosis from NASH[36]. Thus, it can be posited

that cfmiRNAs are reliable candidates for incorporation into multi-panel scores for the prediction of NAFLD and NASH (Figure 2).

For example, a miRNA panel, composed by the detection of miR122-5p, miR1290, miR27b-3p, and miR192-5p) showed a high diagnostic accuracy for NAFLD[37] (Figure 2). A combination score that included miR122, miR192, miR21, ALT, and CK-18-Asp396 exhibited an AUROC of 0.83 for the prediction of NASH[34] (Figure 2).

ROLE OF CIRCULATING PROTEINS IN THE PREDICTION OF NASH SEVERITY

The use of proteins that circulate in serum or plasma for predicting liver-related histological outcomes, specifically liver fibrosis, has been largely relegated probably because such approaches are technically challenging, while offering low performance and poor accuracy. The most remarkable example of this strategy is based on the use of plasma caspase-generated cytokeratin-18 fragments (CK-18) as a noninvasive alternative biomarker of NASH. Results from a large multicenter study showed that plasma CK-18 has relatively good specificity for NAFLD (AUROC: 0.77), NASH (0.65) and fibrosis (0.68). Nevertheless, the overall sensitivity for NAFLD (63%), NASH (58%) and fibrosis (54%) is limited, making this test inadequate for use as a single noninvasive screening test[38].

Interesting attempts to develop multi-component tests that integrate clinical and laboratory data, including circulating proteins, have also been made. For example, we have tested a diagnostic model based on a composite index using clinical and laboratory data, including circulating biomarkers such as soluble intercellular adhesion molecule-1 (sICAM-1), which was able to differentiate between patients with simple steatosis and NASH with a post-test probability for NASH of 99.5% when all positive tests were present[39].

There are similar proposals - though restricted to the prediction of NAFLD but not NASH - based on OMICs-derived data, including genetic information (rs738409), clinical variables, and measurement of different proteins (ACY1, SHBG, CTSZ, MET, GNS, LGALS3BP, CHL1, SERPINC1), which - if combined - seem to be quite reliable in disease risk identification (AUROC for steatosis 0.935)[40]. Nevertheless, it seems that this approach has limited cost-effectiveness for NAFLD-screening programs.

Latest advancements in this field focus directly on disease phenotypes, for example liver fibrosis, which target the detection of excess collagen synthesis rate both directly in liver tissue and noninvasively in blood[41].

The combination of type IV collagen 7S and aspartate aminotransferase (AST) in a multi-test for the prediction of NASH-fibrosis showed promising results[42]. Likewise, measurement of circulating procollagen III (PIIINP) has been quite accurate in the prediction of NASH (AUROC 0.77-0.82) and NASH-fibrosis (0.82-0.84)[43].

Unfortunately, proteomic analysis using state of the art technology is currently poorly developed in the field of NAFLD. In fact, robust attempts to refine, replicate and follow-up on putative discovered proteins have not been done, even though some promising studies have been carried out. For example, using MALDI TOF/TOF and western blot analysis of coupled tissue and serum samples allowed the identification of two interesting protein candidates, including the mitochondrial enzyme CPS1 (Carbamoyl-Phosphate Synthase 1) and GRP78, also known as heat shock protein family A (Hsp70) member 5, which could stratify the different phenotypes associated with the disease severity[44]. Results obtained by using similar approaches, including SELDI-TOF mass spectrometry[45] and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI TOF-MS)[46] have been published. Still, the identified peaks require validation, replication and large-scale testing.

CIRCULATING METABOLITES IN NASH PREDICTION

Initial case-control studies on plasma metabolomics of NAFLD have been performed years ago by Puri *et al*[47], who conducted a comprehensive analysis of plasma lipids and eicosanoid metabolites quantified by mass spectrometry. The authors reported a stepwise increase in lipoxygenase (LOX) metabolites, 5(S)-hydroxyeicosatetraenoic acid (5-HETE), 8-HETE and 15-HETE that characterized the progression from normal liver to NAFL to NASH[47]. Puri and colleagues found that the level of 11-HETE, a nonenzymatic oxidation product of arachidonic (20:4) acid, was significantly and specifically increased in NASH but not in NAFL patients[47]. Subsequent studies that included untargeted global metabolomic analysis revealed marked changes in bile salts and glutathione-related metabolites, as well as higher levels of branched-chain amino acids, phosphocholine, carbohydrates (glucose, mannose), lactate and pyruvate, in subjects with severe NAFLD[48]. Regarding bile salts, a recent study indicated that total conjugated primary bile acids were significantly higher in NASH[49].

A novel study in which the authors combined metabolomic data from experimental animals and human samples introduced the interesting concept that NASH might be sub-classified into two major subtypes according to the circulating pattern of triglycerides, diglycerides, fatty acids, ceramides and oxidized fatty acids[50].

As mentioned earlier, interesting strategies that combine clinical, genetic and lipidomic-derived variables into a multi-score have shown good predictive values in differentiating NAFL from NASH. Specifically, Zhou and coworkers reported on the performance of the *NASH*

Table 1 List of pathways involved in nonalcoholic fatty liver disease selected from significant *Q*-values that dependent on both genes and metabolites analyzed jointly

Pathway name	Q-joint
Solute carriers -mediated transmembrane transport	1.23E-12
Transmembrane transport of small molecules	9.66E-12
Transport of glucose and other sugars bile salts and organic acids metal ions and amine compounds	8.40E-10
Leukotriene biosynthesis	8.71E-10
Transport of glucose and other sugars bile salts and organic acids metal ions and amine compounds	1.91E-09
Transport of inorganic cations-anions and amino acids-oligopeptides	4.27E-09
Amino acid and oligopeptide SLC transporters	1.10E-08
Transport of inorganic cations/anions and amino acids/oligopeptides	2.40E-08
tRNA Aminoacylation	3.03E-08
Gamma-glutamyl cycle	3.61E-08
tRNA charging	5.96E-08
mRNA protein and metabolite induction pathway by cyclosporine A	8.47E-08
Class I MHC mediated antigen processing & presentation	1.73E-07
Na^+/Cl^- dependent neurotransmitter transporters	3.10E-07
Amino acid transport across the plasma membrane	3.72E-07
S-methyl-5-thio-alpha;-D-ribose 1-phosphate degradation	6.17E-07
Amine compound solute carrier transporters	6.17E-07
Protein digestion and absorption - homo sapiens (human)	2.13E-06
Amino acid interconversion	2.21E-06
Biochemical pathways part I	2.34E-06
Amino acid metabolism	3.96E-06
Aminoacyl-tRNA biosynthesis - homo sapiens (human)	6.88E-06
Metabolism of amino acids and derivatives	8.72E-06
Mineral absorption - homo sapiens (human)	1.47E-05
Cytosolic tRNA aminoacylation	2.86E-05
Mitochondrial tRNA aminoacylation	2.86E-05
tRNA Aminoacylation	2.86E-05
Histidine, lysine, phenylalanine, tyrosine, proline and tryptophan catabolism	0.000159
Gene expression	0.000181
Tryptophan catabolism	0.000275
Phase Ⅱ conjugation	0.000426
Phenylalanine and tyrosine catabolism	0.003
Glutamine and glutamate metabolism - homo sapiens (human)	0.00376
Glutaminolysis and cancer	0.00493
Glycine metabolism	0.0052
Glutamate glutamine metabolism	0.00665
Recycling of bile acids and salts	0.00669
Glycine serine alanine and threonine metabolism	0.0101
Branched-chain amino acid catabolism	0.0103

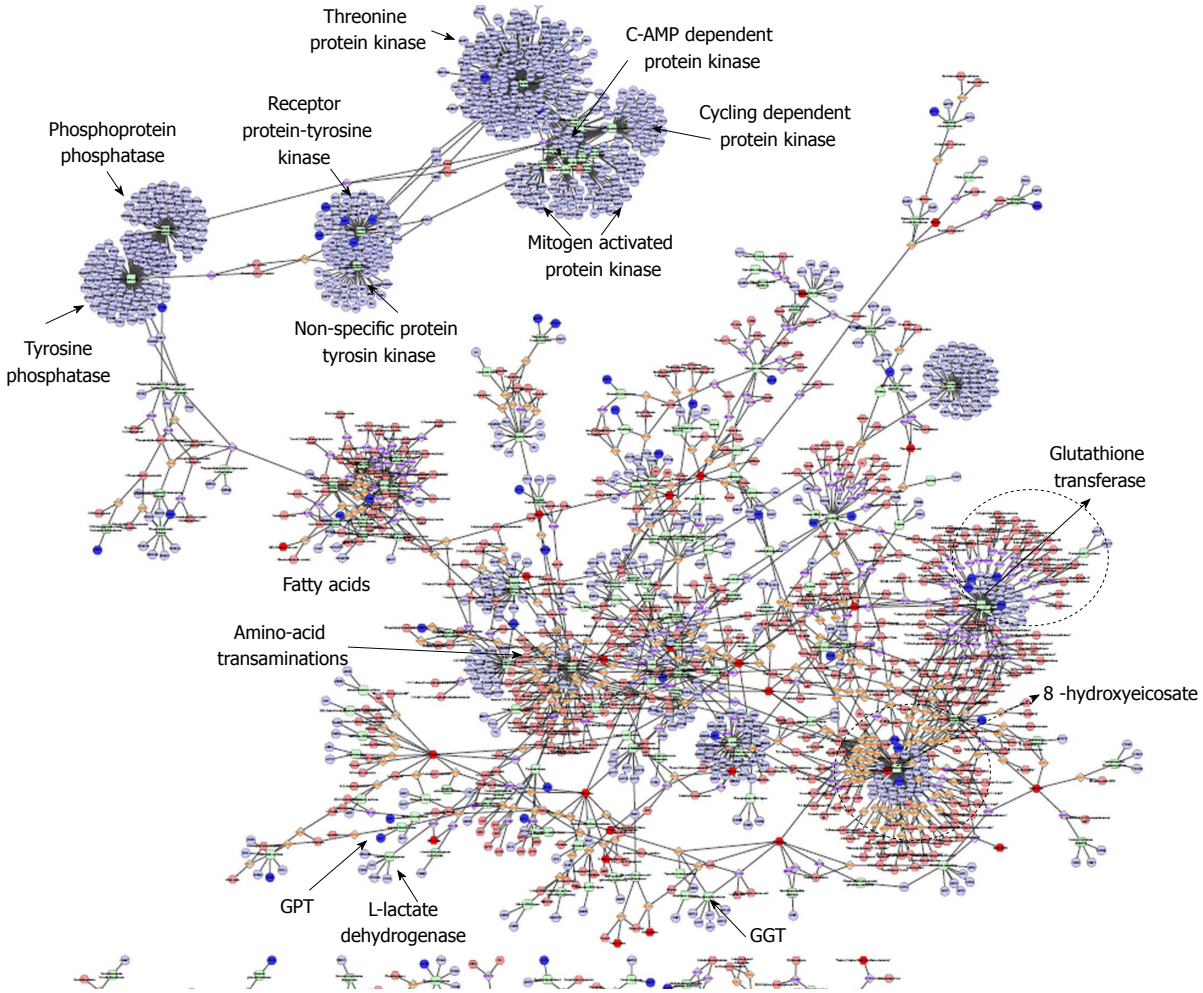
OMICs-integrative analysis was performed using the IMPaLA (integrated molecular pathway level analysis, http://impala.molgen.mpg.de)[67] platform. A joined adjusted *P*-value (*Q*-value) was calculated to control for multiple testing by false discovery rate.

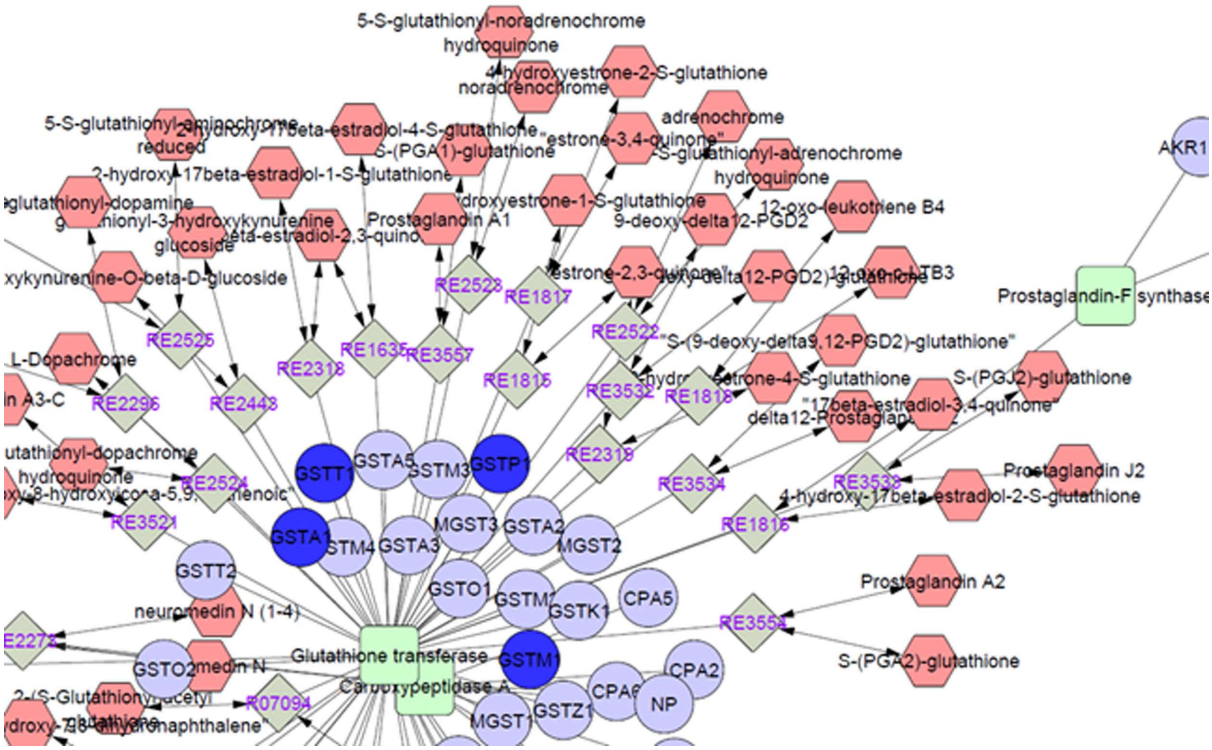
Clin Score, obtained through backward stepwise logistic regression analyses of biochemical variables (glutamate, isoleucine, glycine, lysophosphatidylcholine 16:0, phosphoethanolamine 40:6, AST, and fasting insulin), along with rs738409 genotypes[18]; this score identified patients with NASH with an AUROC of 0.866 (Figure 2).

Recent explorations on changes in liver metabolism during NASH development[51,52], along with the findings from high-throughput circulating profiling of patients with metabolic syndrome[53] suggest that elevated levels of alanine (ALT) and aspartate (AST) aminotransaminases in patients with NAFLD are the consequence of impaired liver metabolism of amino acids, including glutamate and aromatic amino acids, rather than a mere biomarker of liver injury[14,52,54]. This observation is consistent with the fact that NASH is associated with changes in the level of circulating amino acids[55], including L-glutamic acid, 2-hydroxyglutarate and alanine / pyruvate ratio, which are significantly associated with NAFLD-disease severity[52,56]. Changes in the level of branched-chain amino acids were described in pediatric population[57], and these findings were replicated in studies on adults as well[58].

Interestingly, alterations in multiple aminoacids, gamma-glutamyl dipeptides and lipids may be related to common genetic variations associated with NAFLD, as observed in earlier *in vitro* studies based on knocking down or over-expression of the pIle148Met (rs738409) isoforms[59].

Finally, a two-stage multicenter case-control study that combined results of NAFLD-histological variables, levels of circulating metabolites and genetic markers indicated that NASH is associated with decreased levels of betaine in circulation. Furthermore, the disease severity is associated with genotypes of the missense variant p.Ser646Pro (rs1805074) in *DMGDH* gene,


Threonine protein kinase
C-AMP dependent protein kinase
Receptor protein-tyrosine kinase
Phosphoprotein phosphatase
Cycling dependent protein kinase
Mitogen activated protein kinase
Non-specific protein tyrosin kinase
Tyrosine phosphatase
Glutathione transferase
Fatty acids
Amino-acid transaminations
8 -hydroxyeicosate
GPT
L-lactate dehydrogenase
GGT


5-S-glutathionyl-noradrenochrome hydroquinone
4-hydroxyestrone-2-S-glutathione
noradrenochrome
adrenochrome
2-hydroxy-17beta-estradiol-4-S-glutathione
S-(PGA1)-glutathione
2-hydroxy-17beta-estradiol-1-S-glutathione
S-glutathionyl-adrenochrome hydroquinone
Prostaglandin A1
9-deoxy-delta12-PGD2
12-oxo-leukotriene B4
glucoside
xykynurenine-O-beta-D-glucoside
RE2523
RE1817
RE2522
RE2525
RE1635
RE3557
RE2318
RE1815
RE3532
RE1818
RE2296
RE2443
RE2319
RE3534
RE2524
RE3521
RE1816
GSTT1
GSTA5
GSTM3
GSTP1
GSTA1
GSTM4
GSTA3
MGST3
GSTA2
MGST2
GSTT2
GSTO1
GSTM2
GSTK1
CPA5
neuromedin N (1-4)
RE3554
Glutathione transferase
GSTM1
GSTO2
medin N
CPA2
CPA6
NP
GSTZ1
MGST1
R07094
AKR1
Prostaglandin-F synthase
S-(9-deoxy-delta9,12-PGD2)-glutathione
S-(PGJ2)-glutathione
Prostaglandin J2
4-hydroxy-17beta-estradiol-2-S-glutathione
Prostaglandin A2
S-(PGA2)-glutathione

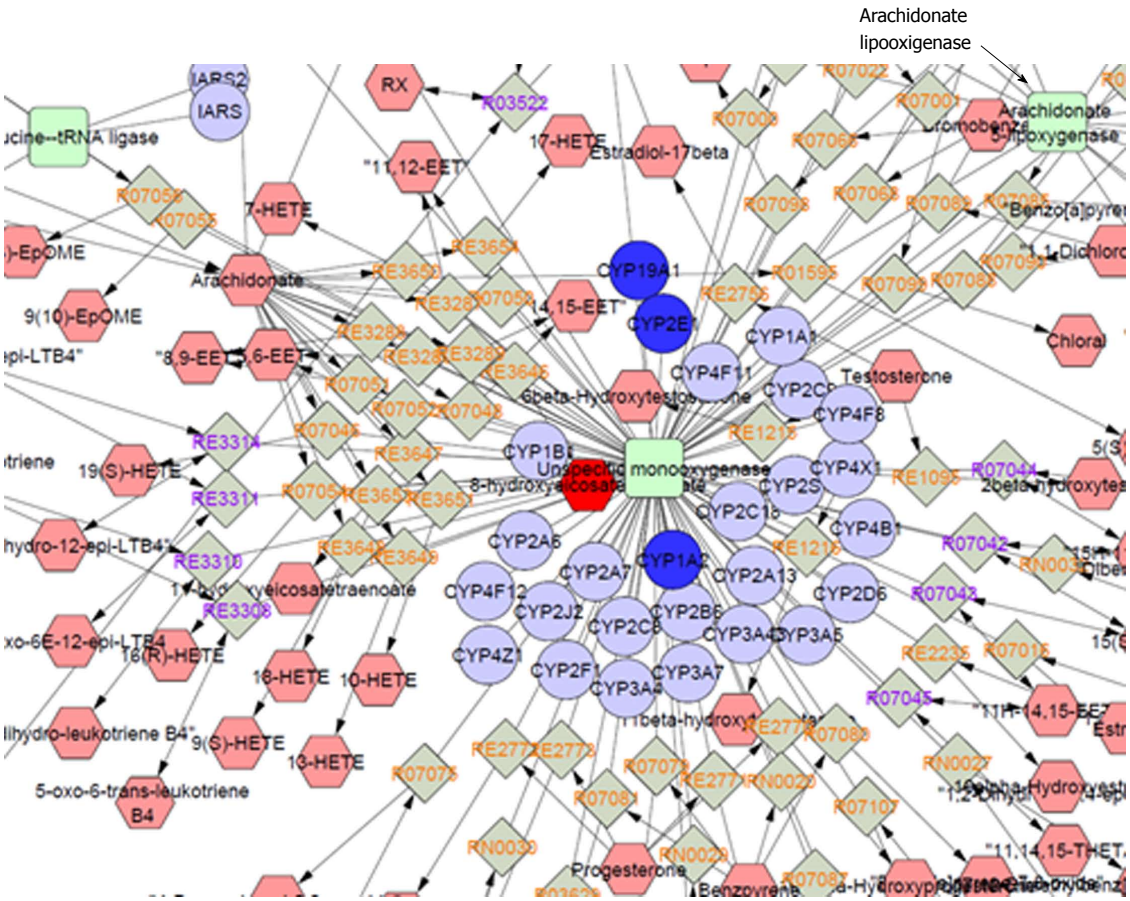


Figure 3 Whole interactome of compounds (hexagons), chemical reactions (diamonds), enzymes (squares) and genes (circles) associated with nonalcoholic fatty liver disease. Details on the set of genes and metabolites that were included in the analysis can be found in the main text; terms were filtered according to the ones already found in the databases. The interactome was built using Metscape[73], a plug-in for the widely used network analysis software Cytoscape[74] that supports calculation, analysis and visualization of gene-to-metabolite networks in the context of metabolism.

which encodes for the mitochondrial dimethylglycine dehydrogenase[60]. Betaine (N,N,N-trimethylglicine) performs a critical function in the pathway of methylogenesis by controlling the serum methionine levels; thus, the results of the aforementioned study[61] might be used to tailor therapeutic interventions based on metabolites that modulate the liver methylome.

INTEGRATION OF DATA DERIVED FROM GENOMICS/PROTEOMICS/ TRANSCRIPTOMICS AND METABOLOMICS TO PREDICT BIOMARKERS ASSOCIATED WITH NAFLD AND NASH

Integration of analyses carried out across multiple biological measurements or OMIC-platforms represents an emerging approach aimed at addressing the challenges imposed by the complex biochemical regulation processes[62].

For example, application of Systems Biology approaches, *i.e.* Gene Set Enrichment Analysis (GSEA)[63], to the field of genomic data has rendered novel knowledge of shared disease-pathways between alcoholic and nonalcoholic liver disease[64]. Likewise, integration of genomic data has highlighted the shared genetic basis of metabolic syndrome and NAFLD[5].

A similar approach can be employed in the field of metabolomics to analyze the enrichment of metabolites that are overrepresented (ORA) in a query-sample against the whole set of metabolites in metabolic pathways. In this context, metabolite set enrichment analysis (MSEA) is the metabolomic counterpart of gene set enrichment. Such analysis, which can be performed by using either commercial or freely available software[65], has been applied to demonstrate alterations in metabolic pathways associated with NAFLD[66].

As a proof of principle, as a part of this work, we performed OMICs-integrative analysis using the IMPaLA (Integrated Molecular Pathway Level Analysis, http://impala.molgen.mpg.de)[67] platform. Briefly, the analysis was conducted by integrating the information on metabolites, genes and proteins, allowing the joint adjusted P-value (Q-value) to be calculated.

Specifically, we selected a list of genes previously associated with NAFLD[5,64], and metabolites that are known to be altered in NAFLD/NASH[68]. Names on metabolites were curated using the compound ID conversion of the web-based MetaboAnalyst tool

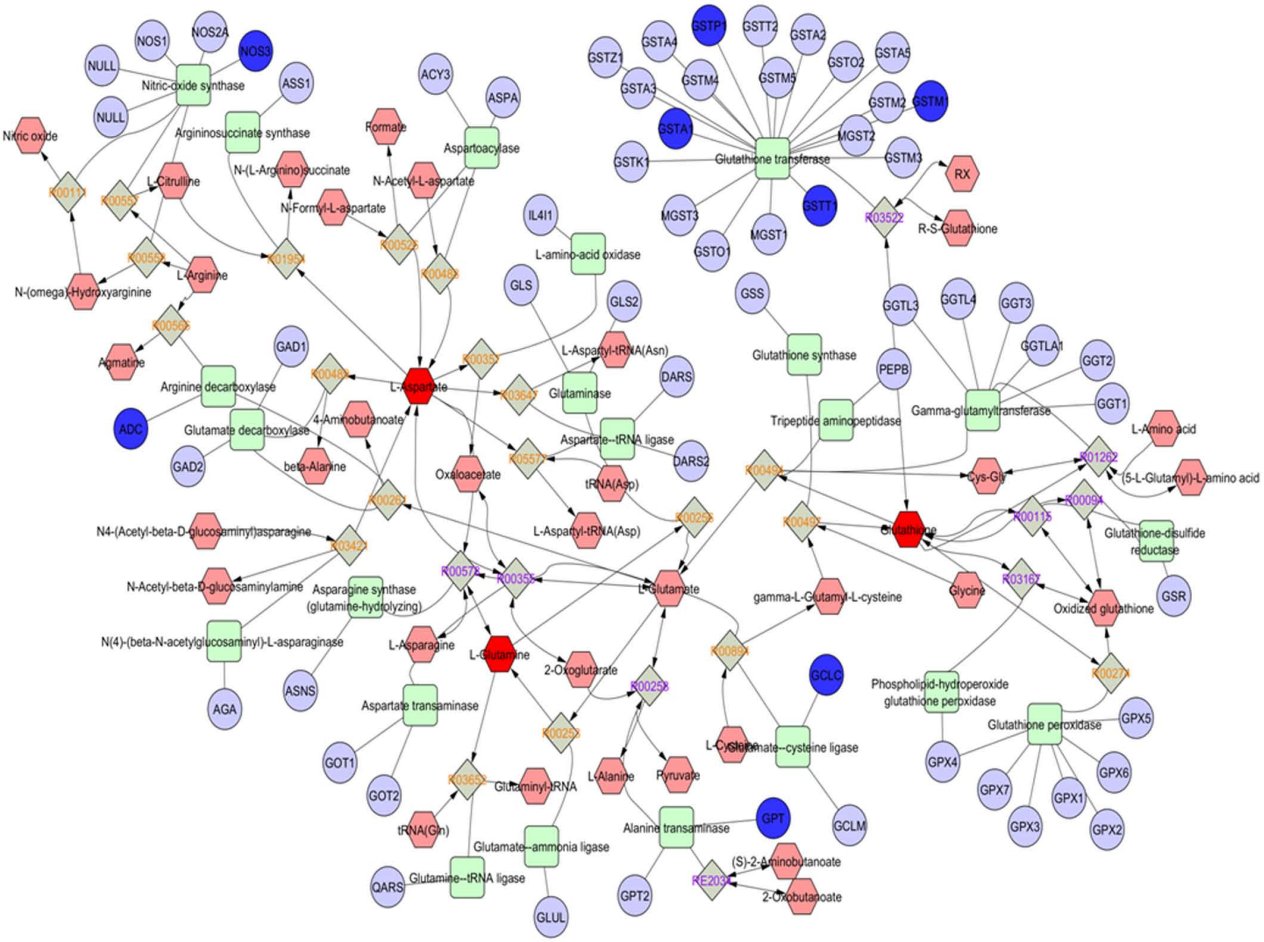


Figure 4 The urea-cycle, glutamate, and branched-chain amino acids in the biology of nonalcoholic fatty liver disease. Sub-network analysis showing the urea-cycle and metabolism of amino acids (L-arginine, L-proline, L-glutamate, L-aspartate and L-asparagine) that were extracted from the interactome shown in Figure 3. Compounds (common names in the Human Metabolome Database, http://www.hmdb.ca), chemical reactions, enzymes (KEGG database) and genes (HUGO symbols) are represented by hexagons, diamonds, squares and circles, respectively.

(http://www.metaboanalyst.ca/)[69,70]. We found 2,827 pathways; however, only 219 of 347 input gene-identifiers were mapped to 219 distinct physical entities found in these pathways (with a gene background size of 12655). Similarly, only 32 of 51 input metabolite-identifiers were mapped to 32 distinct physical entities found in the pathways (with a metabolite background size of 5340). Relevant findings, excluding data that was exclusively and heavily dependent on genes or metabolites, are shown in Table 1; pathways and the Q-values for gene and/or metabolite enrichment were jointly calculated.

It is interesting to highlight and discuss a few examples in more detail. For instance, in the pathway "SLC-mediated transmembrane transport" (Reactome database), the overlapping genes and metabolites are *CALM1* (Calmodulin 1), *G6PC* (Glucose-6-Phosphatase Catalytic Subunit), *FGF21* (Fibroblast Growth Factor 21), *GCK* (Glucokinase) and *GCKR* (Glucokinase Regulator), and taurocholic acid, D-mannose, creatinine, L-lactic acid, L-valine, L-isoleucine, L-phenylalanine, L-aspartic acid, L-tyrosine, carnitine, betaine, L-glutamine, linoleic acid, oleic acid, L-leucine and glycocholic acid, respectively.

Another interesting example is the pathway "Transmembrane transport of small molecules" (Reactome database), in which the overlapping genes and metabolites are *G6PC*, *CALM1*, *ATP1A1* (ATPase Na+/K+ Transporting Subunit Alpha 1), *TF* (Transferrin), *ABCC1* (ATP Binding Cassette Subfamily C Member 1), *FGF21*, *GCK*, *GCKR*, *HMOX1* (Heme Oxygenase 1), *ABCB1* (ATP Binding Cassette Subfamily B Member 1), *ABCC2* (ATP Binding Cassette Subfamily C Member 2), *ABCC3* (ATP Binding Cassette Subfamily C Member 3) and *ABCG2* ATP Binding Cassette Subfamily G Member 2), and L-glutamine, D-mannose, creatinine, L-lactic acid, L-valine, L-isoleucine, L-phenylalanine, taurocholic acid, L-aspartic acid, L-tyrosine, carnitine, betaine, linoleic acid, oleic acid, L-leucine and glycocholic acid, respectively.

Finally, in the pathway "Central carbon metabolism in cancer -Homo sapiens (human)" (KEGG database), the overlapping genes and metabolites are *PTEN* (Phosphatase and Tensin Homolog), *EGFR* (Epidermal Growth Factor Receptor), *MET* (MET Proto-Oncogene, Receptor Tyrosine Kinase), *PIK3CA* (Phosphatidylinositol-

Table 2 List of pathways involved in nonalcoholic fatty liver disease selected from significant *Q*-values independently on whether they represent the effect of gene/s or metabolite/s only

Pathway name	Pathway source	Q-joint
Adipogenesis	Wikipathways	2.00E-17
Non-alcoholic fatty liver disease (NAFLD) - homo sapiens (human)	KEGG	2.33E-17
Metabolism	Reactome	3.72E-17
AGE-RAGE pathway	Wikipathways	4.22E-17
Vitamin B12 Metabolism	Wikipathways	5.24E-17
Hepatitis B - homo sapiens (human)	KEGG	1.79E-16
Folate metabolism	Wikipathways	1.29E-15
Selenium micronutrient network	Wikipathways	3.87E-15
TNF signaling pathway - homo sapiens (human)	KEGG	5.77E-15
JAK-STAT-core	Signalink	1.99E-14
Adipocytokine signaling pathway - homo sapiens (human)	KEGG	7.07E-14
Nuclear receptors meta-pathway	Wikipathways	1.26E-13
IL1 and megakaryocytes in obesity	Wikipathways	2.73E-13
AGE-RAGE signaling pathway in diabetic complications - homo sapiens (human)	KEGG	3.79E-13
Spinal cord injury	Wikipathways	5.44E-13
Malaria - homo sapiens (human)	KEGG	7.09E-13
Metabolism of lipids and lipoproteins	Reactome	7.09E-13
SLC-mediated transmembrane transport	Reactome	1.23E-12
Pathways in cancer - homo sapiens (human)	KEGG	1.41E-12
Inflammatory bowel disease (IBD) - homo sapiens (human)	KEGG	2.25E-12
Lung fibrosis	Wikipathways	2.63E-12
Integrated pancreatic cancer pathway	Wikipathways	3.10E-12
PI3K-Akt signaling pathway - homo sapiens (human)	KEGG	3.28E-12
Chagas disease (American trypanosomiasis) - homo sapiens (human)	KEGG	4.67E-12
HIF-1 signaling pathway - homo sapiens (human)	KEGG	4.67E-12
AMPK signaling pathway - homo sapiens (human)	KEGG	9.56E-12
Transmembrane transport of small molecules	Reactome	9.66E-12
Central carbon metabolism in cancer - homo sapiens (human)	KEGG	1.41E-11
Jak-STAT signaling pathway - homo sapiens (human)	KEGG	5.75E-11
DNA damage response (only ATM dependent)	Wikipathways	7.27E-11
Cytokine-cytokine receptor interaction - homo sapiens (human)	KEGG	1.01E-10
Longevity regulating pathway - homo sapiens (human)	KEGG	1.02E-10
Toll-like receptor signaling pathway	Wikipathways	2.12E-10
Toll-like receptor signaling pathway - homo sapiens (human)	KEGG	3.94E-10
Toxoplasmosis - homo sapiens (human)	KEGG	4.73E-10
ABC transporters - homo sapiens (human)	KEGG	5.94E-10
Transport of glucose and other sugars bile salts and organic acids metal ions and amine compounds	Wikipathways	8.40E-10
Leukotriene biosynthesis	HumanCyc	8.71E-10
Insulin resistance - homo sapiens (human)	KEGG	1.14E-09
Transport of glucose and other sugars bile salts and organic acids metal ions and amine compounds	Reactome	1.91E-09
Sudden infant death syndrome (SIDS) susceptibility pathways	Wikipathways	2.12E-09
Cytokines and inflammatory response	Wikipathways	2.17E-09
AP-1 transcription factor network	PID	2.22E-09
FoxO signaling pathway - homo sapiens (human)	KEGG	3.05E-09
Leptin signaling pathway	Wikipathways	3.57E-09
Transport of inorganic cations-anions and amino acids-oligopeptides	Wikipathways	4.27E-09
Oncostatin M signaling pathway	Wikipathways	5.72E-09
Focal adhesion-PI3K-Akt-mTOR-signaling pathway	Wikipathways	6.53E-09
Amino acid and oligopeptide SLC transporters	Reactome	1.10E-08
Apoptosis	Wikipathways	1.41E-08
Apoptotic signaling pathway	Wikipathways	1.41E-08
Photodynamic therapy-induced NF-kB survival signaling	Wikipathways	1.84E-08
JAK STAT molecularvariation 1	INOH	2.04E-08
MAPK signaling pathway	Wikipathways	2.04E-08
Aryl hydrocarbon receptor	Wikipathways	2.35E-08
Transport of inorganic cations/anions and amino acids/oligopeptides	Reactome	2.40E-08
tRNA aminoacylation	Wikipathways	3.03E-08
gamma-glutamyl cycle	HumanCyc	3.61E-08
Glucose homeostasis	Wikipathways	4.08E-08
Validated transcriptional targets of AP1 family members Fra1 and Fra2	PID	4.13E-08
Hepatitis C and hepatocellular carcinoma	Wikipathways	4.26E-08
Calcineurin-regulated NFAT-dependent transcription in lymphocytes	PID	4.29E-08
Prostate cancer - homo sapiens (human)	KEGG	4.29E-08
Tuberculosis - homo sapiens (human)	KEGG	4.45E-08
Apoptosis - homo sapiens (human)	KEGG	4.54E-08

tRNA charging	HumanCyc	5.96E-08
Transcription factor regulation in adipogenesis	Wikipathways	6.27E-08
Sterol regulatory element-binding proteins (SREBP) signalling	Wikipathways	6.27E-08
Integrated lung cancer pathway	Wikipathways	6.43E-08
TNF related weak inducer of apoptosis (TWEAK) signaling pathway	Wikipathways	8.14E-08
mRNA protein and metabolite induction pathway by cyclosporin A	Wikipathways	8.47E-08
PPAR signaling pathway	Wikipathways	9.54E-08
Immune system	Reactome	9.57E-08
Regulation of lipid metabolism by peroxisome proliferator-activated receptor alpha (PPARalpha)	Wikipathways	1.13E-07
AMP-activated protein kinase (AMPK) signaling	Wikipathways	1.34E-07
Photodynamic therapy-induced NFE2L2 (NRF2) survival signaling	Wikipathways	1.52E-07
Leptin insulin overlap	Wikipathways	1.65E-07
Class I MHC mediated antigen processing and presentation	Wikipathways	1.73E-07
Caspase cascade in apoptosis	PID	1.99E-07
Overview of nanoparticle effects	Wikipathways	2.17E-07
Alpha6Beta4Integrin	NetPath	2.29E-07
VEGFA-VEGFR2 signaling pathway	Wikipathways	2.30E-07
HIV-1 Nef: Negative effector of Fas and TNF-alpha	PID	2.65E-07
Innate immune system	Reactome	2.69E-07
Na^+/Cl^- dependent neurotransmitter transporters	Reactome	3.10E-07
Colorectal cancer - homo sapiens (human)	KEGG	3.42E-07
Regulation of toll-like receptor signaling pathway	Wikipathways	3.64E-07
stress induction of hsp regulation	BioCarta	3.64E-07
Amino acid transport across the plasma membrane	Reactome	3.72E-07
Programmed cell death	Reactome	3.85E-07
Apoptosis modulation and signaling	Wikipathways	4.42E-07
SREBF and miR33 in cholesterol and lipid homeostasis	Wikipathways	4.84E-07
JAK STAT pathway and regulation	INOH	5.42E-07

OMICs-integrative analysis was performed using the IMPaLA (Integrated Molecular Pathway Level Analysis, http://impala.molgen.mpg.de)[67] platform. A joined adjusted *P*-value (*Q*-value) was calculated to control for multiple testing by false discovery rate.

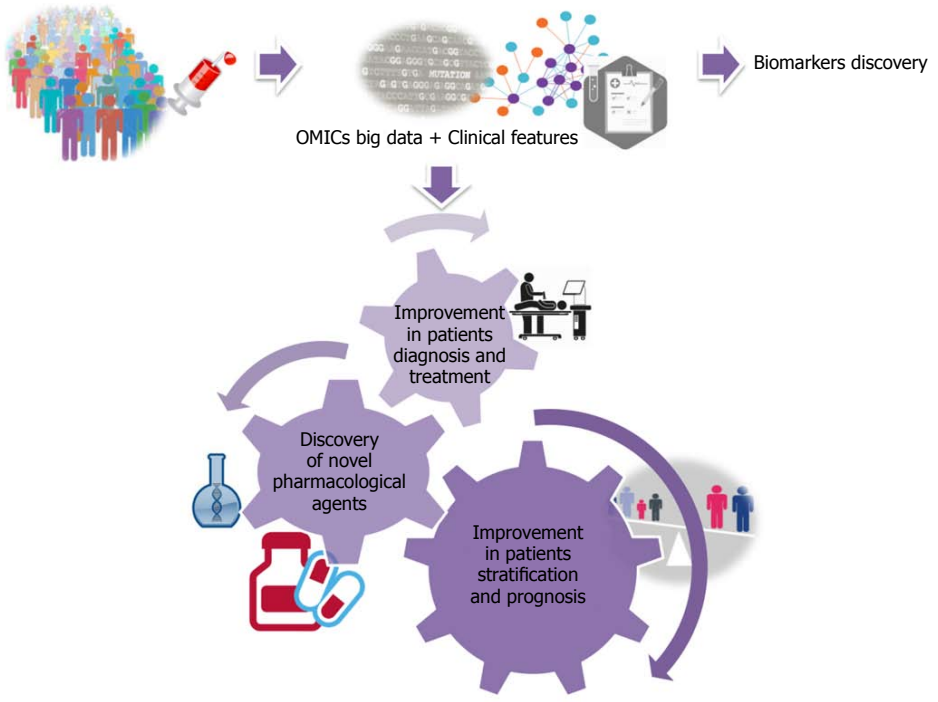


Figure 5 What to expect for the near future. A personalized nonalcoholic fatty liver disease approach by integrating OMICs big data with clinical information.

4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha), *MTOR* (Mechanistic Target Of Rapamycin Kinase), *AKT2* (AKT Serine/Threonine Kinase 2) and *GCK*, and L-glutamine, L-lactic acid, L-valine, L-isoleucine, L-phenylalanine, L-aspartic acid, L-tyrosine and L-leucine, respectively.

From these few examples, we may conclude that some pathways such as solute carrier (SLC) transporters should be further explored; in fact, available experimental data, while limited, support the participation of ABCC-family in NAFLD pathophysiology[71].

Nonetheless, the findings discussed above do not

necessarily indicate that no other important pathways are potentially involved in the biology of NAFLD. In fact, Table 2 illustrates the myriad of processes involved in the pathogenesis of a complex disease such as NAFLD. In addition, Figure 3 depicts the complexity of the interactome among the whole set of genes, enzymes, chemical reactions and metabolites associated with NAFLD. Figure 4 shows a sub-network emphasizing the importance of the urea-cycle and metabolism of L-arginine, L-proline, L-glutamate, L-aspartate and L-asparagine. Specifically, features in Figure 4 highlight the central role played by aminotransferases and gamma-glutamyl transferases in the frame of altered L-glutamine/L-glutamate, glutathione and BCAA levels, as already mentioned.

Finally, additional biomarkers that target immunity-related pathways, for example circulating levels of cytokines / chemokines, antibodies etc. might be useful in predicting NASH progression toward advanced phases[72].

CONCLUSION

Implementation of OMICs-derived biomarkers in the management and treatment of patients with NAFLD is still under extensive evaluation. Knowledge gained on genetic signatures associated with NAFLD and NASH, as well as the role of circulating cfmiRNAs and plasma metabolites, should be promptly translated into the clinical setting. Nevertheless, rigorous steps that must include validation and replication are mandatory before OMICs biomarkers are ready for use as diagnostic markers to identify patients at risk of advanced disease, including liver cancer.

What to expect for the near future: A personalized NAFLD approach by integration of OMICs - big data and clinical information (Figure 5): (1) It is expected that, in the near future, NAFLD patients can be diagnosed and treated according to their own "molecular signature"; (2) Specific focus should be placed on prevention and early diagnosis by the application of biomarkers of disease risk; (3) Selection of "personalized drugs" as well as tailored therapy should be made according to the specific molecular signature; and (4) Personalized lifestyle intervention is desirable but it is envisioned that the basic and general recommendations about alcohol restriction, healthy diet and exercise would remain the foundation of prevention and therapy.

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