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Perspective

The role of hepatokines in NAFLD

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SUMMARY

Non-alcoholic fatty liver disease (NAFLD) is not only a consequence of insulin resistance, but it is also an important cause of insulin resistance and major non-communicable diseases (NCDs). The close relationship of NAFLD with visceral obesity obscures the role of fatty liver from visceral adiposity as the main pathomechanism of insulin resistance and NCDs. To overcome this limitation, in analogy to the concept of adipokines, in 2008 we introduced the term hepatokines to describe the role of fetuin-A in metabolism. Since then, several other hepatokines were tested for their effects on metabolism. Here we address the dysregulation of hepatokines in people with NAFLD. Then, we discuss pathophysiological mechanisms of cardiometabolic diseases specifically related to NAFLD by focusing on hepatokine-related organ crosstalk. Finally, we propose how the determination of major hepatokines and adipokines can be used for pathomechanism-based clustering of insulin resistance in NAFLD and visceral obesity to better implement precision medicine in clinical practice.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD), encompassing the pathology between non-alcoholic fatty liver (NAFL) and non-alcoholic steatohepatitis (NASH), is the main cause of chronic liver disease and hepatocellular carcinoma.[1–3] Furthermore, results from large meta-analyses reveal that NAFLD associates with increased risk of incident diabetes (2.2- to 2.7-fold),[4] cardiovascular events (1.5-fold),[5] new-onset heart failure (1.5-fold),[6] and extrahepatic cancers (1.5-fold).[7]

The relatively strong epidemiological relationships of NAFLD with cardiometabolic diseases point toward very close pathophysiological relationships between NAFLD and obesity-associated cardiometabolic diseases.[8–13] Globally, the prevalence of NAFLD in the general adult population is estimated at 25%,[14] while the prevalence of NAFLD is higher (~40%–60%) in overweight and obese subjects.[15,16] The highest global prevalence of NAFLD (~55%–70%) is found in patients with diabetes.[17,18] Thus, NAFLD has become an epidemic much like other non-communicable diseases (NCDs) including obesity, diabetes, cardiovascular disease, and obesity- and diabetes-associated cancer.[19,20] To what extent NAFLD is mainly a consequence or a cause of metabolic diseases is an important question. Both pathophysiological connections are of high clinical relevance. The identification of the most important mechanisms of metabolic diseases, which promote hepatic steatosis and progression of NAFLD to severe liver diseases—such as advanced fibrosis and cirrhosis—might reveal new strategies to prevent liver-related morbidity and mortality.[21] On the other hand, cardiometabolic mortality risk in patients with NAFLD is twice as high compared to liver-related mortality.[1,22] Thus, identification of NAFLD-associated disease mechanisms of cardiometabolic pathophysiology may reveal new strategies to reduce the risk of life-threatening cardiac events.

NAFLD AS A CONSEQUENCE OF METABOLIC DISEASES

An increased intake of glucose, fructose, and saturated fat induces hepatic *de novo* lipogenesis, subclinical inflammation in adipose tissue and liver, and insulin resistance in adipose tissue, liver, and skeletal muscle. These lifestyle parameters are also accompanied by an increased risk of type 2 diabetes in which insulin resistance causes hyperinsulinemia that can promote hepatic *de novo* lipogenesis while progressive β cell dysfunction aggravates hyperglycemia. Furthermore, increased free fatty acid (FFA), proinflammatory cytokines, ceramide, and dysregulated adipokine secretion from adipose tissue contributes to increased lipid storage in the liver. For example, excess FFAs promote hepatic mitochondrial dysfunction, increase oxidative stress, and uncouple oxidative phosphorylation. They also activate a fibrogenic response in hepatic stellate cells that can promote the progression to NASH and cirrhosis.[10,11,23–29] Adipose tissue dysfunction is largely attributed to age-related decline in sex hormones and sex-hormone receptor expression in men and women, and senescence of preadipocytes. Furthermore, these pathogenetic processes cause redistribution of adipose mass from the lower to the upper body and from subcutaneous to visceral depots, which lead to more ectopic storage of lipids in the liver.[30–36] Importantly, there is evidence from genetic studies that favorable adiposity alleles associate with a higher

subcutaneous fat mass and lower liver fat content, which may reduce the risk of type 2 diabetes, heart disease, and hypertension.[37]

Studies with lipodystrophy patients suggest that NAFLD may be a consequence of adipose tissue dysfunction. Genetically determined lipodystrophy is primarily characterized by dysfunction of subcutaneous adipose tissue, resulting in severe insulin resistance, visceral adiposity, and NAFLD.[38] Furthermore, patients with HIV and lipodystrophy associated with highly active antiretroviral therapy have peripheral lipoatrophy, visceral obesity, and severe forms of insulin resistance, dyslipidemia, and NAFLD.[39] In addition, acquired lipodystrophy may also play an important role in NAFLD. In this respect, we recently diagnosed and treated a 45-year-old woman with acquired lipodystrophy and severe hyperlipidemia and NAFLD, a condition that was based on immune checkpoint blockade-induced severe inflammation of lower-body subcutaneous adipose tissue.[40]

Besides dysregulated adipose tissue and reduced physical activity, impaired skeletal muscle function may strongly impact the pathophysiology of NAFLD. In this respect, variability in the metabolic response to exercise is being observed and the underlying mechanisms are being studied in the field of exerkine research.[41] In particular, the research about exercise-induced release and effects of myokines on liver, adipose tissue, brain, and the immune system is helpful to better understand the role of exercise and skeletal muscle function on metabolism and, more specifically, on the development of NAFLD.[42]

Dysregulated gut microbiota is also involved in the pathogenesis of NAFLD. The gut microbiota ferments indigestible carbohydrates, providing metabolites such as short-chain fatty acids to the liver. However, in the distal colon fermentation of peptides and proteins by microbiota also generates harmful products, such as branched-chain fatty acids, which might impair metabolic health.[43] In addition, most recently, the human gut microbiota was found to produce ethanol that might be clinically relevant for the pathogenesis of NAFLD.[44]

These findings highlight that there has been much progress during the last decade to identify the major mechanisms involved in the pathogenesis of NAFLD. However, there is still little knowledge about who will progress from simple steatosis to severe forms of NAFLD. For this purpose, large consortia focus on the identification of biomarkers identifying those patients at increased risk for progression to NASH and advanced fibrosis. For example, a non-invasive blood-based diagnostic test, called NIS4, comprising four independent NASH-associated biomarkers (microRNA 34A, alpha2 macroglobulin, HbA1c, and YKL-40) has been proposed.[45] Interestingly, microRNA 34A was recently identified as a new inhibitor of insulin signaling in adipocytes.[46] In addition, gut-microbiome-derived signatures were found to predict advanced fibrosis and cirrhosis.[47,48]

HETEROGENEITY OF THE NAFLD PATHOPHYSIOLOGY

While most patients with NAFLD are insulin resistant and have an elevated risk of cardiometabolic diseases, NAFLD induced by mutations in *GCKR* (glucokinase regulator), *PNPLA3* (patatin-like phospholipase domain-containing 3), and *TM6SF2* (transmembrane 6 superfamily member 2) unexpectedly do not display insulin resistance or an increased risk of cardiometabolic diseases. In fact, the fatty liver risk alleles of *PNPLA3* (rs738409) and *TM6SF2* (rs58542926) protect from cardiovascular disease. The precise mechanisms resulting in metabolically healthy fatty liver are mainly related to altered hepatic lipid droplet remodeling and very-low-density lipoprotein (VLDL) production and secretion.[49–51] In this respect, there is much evidence that hepatic triglyceride accumulation protects against fatty acid-induced lipotoxicity when saturated fatty acids are being channeled into triglyceride pools and metabolic precursors are being rerouted toward lipid synthesis, and these lipids are stored in triglycerides.[52–54]

NAFLD AS A CAUSE OF METABOLIC DISEASES

Mendelian randomization (MR) is the use of genetic data to determine causal relationships between phenotypes and outcomes. In the case of NAFLD, the genetic variants are assumed to (1) associate with the risk factor of interest (hepatic steatosis), (2) be independent of confounders of the risk factor-outcome association, and (3) influence the outcome through their effect on the risk factor (the no-pleiotropy assumption). If the different pathways resulting in NAFLD do not involve adipose tissue dysfunction, insulin resistance, hyperlipidemia, or increased subclinical inflammation, then causal relationships of genetically determined NAFLD with cardiometabolic diseases may not emerge from MR analyses. In this case, horizontal pleiotropy (a genetic variant affects other traits that influence the outcome independently of the hypothesized exposure) may be operative. Thus, possibly owing to horizontal pleiotropy, genetically driven NAFLD was found to associate with the risk of type 2 diabetes and central obesity, but not with insulin resistance.[55]

In another MR analysis of data from the UK Biobank, a causal role of higher liver fat content in risk of type 2 diabetes was also observed; however, in that study, among the 10 variants associated with liver fat content, the liver fat-increasing alleles at *GPAM* (glycerol-3-phosphate acyltransferase, mitochondrial) and *C2orf16* (chromosome 2 open reading frame 16) were unexpectedly associated with lower risk of type 2 diabetes.[56] GPAM catalyzes the first step of triglyceride synthesis. Most recently a missense variant in GPAM was found to be associated with NAFLD and cirrhosis, but with lower serum triglycerides.[57] In a previous study, the same mutation was hypothesized to result in an increase in the hepatic triglyceride content with a compensatory increase in the triglyceride utilization to counteract the excess in lipid accumulation.[58] C2orf16 is a protein coding gene, and diseases associated with C2orf16 include hypobetalipoproteinemia.[59] These results may agree with the above-mentioned concept of metabolically healthy fatty liver, where altered hepatic triglyceride synthesis, lipid droplet remodeling, and VLDL production and secretion, when resulting in increased hepatic triglyceride levels, do not necessarily result in the impairment of glucose and lipid metabolism or increased subclinical inflammation.

Together, these results support the notion that different pathomechanisms of hepatic steatosis need to be very carefully considered when addressing the relationship of NAFLD with cardiometabolic risk. Besides these genetic approaches, data from pathophysiological-based research to integrate *in vitro* and

animal studies with observational studies in humans suggest that fatty liver is involved in the pathophysiology of hyperglycemia and cardiovascular risk. Mechanisms explaining this relationship include hepatic insulin resistance; atherogenic dyslipidemia; and synthesis of proatherogenic, procoagulant, and proinflammatory mediators that may promote cardiovascular disease and other cardiac/arrhythmic events.[12,27,60–62]

Recently, evidence for an important role of fatty liver in the pathophysiology of metabolic dysfunction was also provided by studying hepatocyte-derived extracellular vesicles (EVs). EVs containing miRNAs increase in response to hepatic lipid overload, which directly targets adipocytes. Thus, EVs containing the miRNA let-7e-5p stimulate lipogenesis and inhibit lipid oxidation through Pgc1α, which increases adipocyte lipid deposition.[63] Furthermore, hepatic fatty acids stimulate miRNA 122 in subjects with NAFLD, which regulates skeletal muscle lipid oxidation[64] and causes metabolic cardiomyopathy by dysregulating cardiac energy homeostasis.[65]

HEPATOKINES IN NAFLD

The search for other pathomechanisms by which fat accumulation in the liver may increase the risk of cardiometabolic diseases leads to the hypothesis that fatty liver has a different endocrine function compared to a healthy liver. Thus, by analogy to dysregulated adipokine secretion from inflamed and insulin-resistant adipose tissue, the fatty liver may differentially express and secrete proteins (hepatokines) into the circulation.[66] In 2006, we started investigating the hepatic expression and metabolic effects of the hepatokine fetuin-A in NAFLD subjects, revealing its detrimental signaling functions in heterologous tissues and organs.

More than twenty hepatokines have been identified and studied *in vitro* and with animals to establish their hepatic expression in fatty liver and their metabolic effects.[67–73] In this article, we focus on hepatokines associated strongly through clinical replication with cardiometabolic risk in patients with NAFLD, prospective studies for incident disease, or genetic analysis. Other hepatokines await detailed evaluation of their role in human cardiometabolic pathophysiology (Table 1). Furthermore, we depict in a broader picture how diet- and genetically induced fatty liver evolves and how the organ crosstalk involving nutrients, hormones, and organokines impacts the pathophysiology of fatty liver and cardiometabolic diseases (Figure 1).

CLINICALLY WELL-STUDIED DYSREGULATED HEPATOKINES IN HUMANS WITH NAFLD

Fetuin-A

Fetuin-A is the most widely studied hepatokine in humans. Fetuin-A is predominantly expressed in and secreted from the liver. Its hepatic expression is upregulated by FFAs via NF-κB signaling[95] and by glucose via ERK1/2 signaling.[96] Fetuin-A inhibits insulin receptor tyrosine kinase in liver and skeletal muscle.[97] In complex with fatty acids, fetuin-A activates Toll-like receptor 4, which can induce inflammatory signaling and insulin resistance in adipocytes and macrophages.[98,99] Furthermore, fetuin-A impairs glucose-induced insulin secretion via c-Jun N-terminal kinase and Ca^{2+}-dependent signaling,[100] which can disrupt functional maturation of pancreatic β cells.[101] Fetuin-A is downregulated by the E3 ubiquitin protein ligase FBXW7 (F box and WD repeat domain-containing 7), which might be an important mechanism to maintain glucose homeostasis.[102]

In humans, several studies from different research groups find that high circulating fetuin-A associates with fatty liver and insulin resistance.[67] Importantly, and in agreement with *in vitro* and animal data, fetuin-A plasma levels associate with insulin resistance, particularly when plasma FFAs are elevated.[103,104] This relationship is stronger in patients with NAFLD than in healthy controls.[105] Elevated fetuin-A plasma levels decline during lifestyle intervention-associated weight loss in parallel with a decrease of liver fat content[106,107] and with pharmacological therapy with thiazolidinediones, but not metformin.[108,109] In large clinical cohort studies, elevated plasma fetuin-A levels are associated with an increased risk of incident type 2 diabetes[74–77] and cardiovascular disease,[78] independently of established cardiometabolic risk markers. Some data from MR analyses support the causal relationship between fetuin-A levels and the risk of type 2 diabetes and cardiovascular disease,[77,80] whereas other MR analyses did not indicate causal relationships.[79,81] Thus, MR analyses need to be interpreted with caution because there is heterogeneity between populations—including strong genetic support in the Nurses' Health Study but no support in the CHARGE Consortium.[81] Regardless, these studies do not account for the interaction of circulating fetuin-A with circulating FFAs, which is an important mechanism of fetuin-A-induced subclinical inflammation. Moreover, as fetuin-A also inhibits vascular calcification,[110] this protective effect of fetuin-A may oppose its proinflammatory effect, particularly in patients with more advanced cardiovascular risk.

ANGPTL3

Angiopoietin-like proteins (ANGPTL1–8) are secreted glycoproteins that regulate lipid metabolism. ANGPTL3 is exclusively expressed and secreted by the liver.[111] ANGPTL3 is upregulated by liver X receptor and downregulated by insulin, leptin, peroxisome proliferator-activated receptor-β, statins, and thyroid hormones.[71] ANGPTLs can inhibit lipoprotein lipase (LPL) activity. LPL activity promotes healthy storage of triglycerides in gluteofemoral compartment rather than the pathological visceral compartment, thereby improving lipid metabolism and insulin sensitivity.[112,113] Thus, elevated ANGPTL3 may contribute to cardiometabolic risk by inhibiting LPL.

ANGPTL3 levels are elevated in patients with NASH, but not in patients with simple steatosis.[114] In agreement, a systematic review and meta-analysis of 854 patients with fatty liver against 610 controls reveal elevated plasma ANGPTL8—but not ANGPTL3 or ANGPTL4—in patients with fatty liver.[115] Regardless, ANGPTL3 inhibition has a beneficial effect on insulin resistance and fatty liver in animals and dyslipidemia in humans.[82] Liver-targeted antisense inhibition of ANGPTL3 in patients with fasting hypertriglyceridemia, type 2 diabetes, and hepatic steatosis reduced triglycerides but did not reduce liver fat content or improve glycemic parameters.[83] As this study had a relatively small sample size (N = 105), it may have been underpowered to detect effects of inhibition of ANGPTL3 on hepatic steatosis and glucose metabolism.

Table 1. Hepatokines in NAFLD and their metabolic functions

Hepatokine	Expression in fatty liver	Effects on glucose and lipid metabolism *in vitro* and/or in animals	Site of action	Correlations in humans	Prediction of cardiometabolic diseases/ mechanisms in humans
Clinically well-studied dysregulated hepatokines in humans with NAFLD					
Fetuin-A	increased	obesity −/↑ insulin resistance ↑ subclinical inflammation ↑ insulin secretion ↓ β cell maturation ↓	liver, skeletal muscle, adipocytes, monocytes pancreatic islets	NAFLD ↑ insulin resistance ↑↑ subclinical inflammation ↑ type 2 diabetes ↑↑ CVD ↑ vascular calcification ↓	high levels predict type 2 diabetes[74–77] high levels predict CVD[78] MR results about causative effects: type 2 diabetes −/+[77,79] and CVD −/+[80,81]
ANGPTL3	increased (NASH)	insulin resistance ↑ inhibition of lipoprotein lipase and endothelial lipase	vascular endothelial cells, skeletal muscle, and adipose tissue	NAFL − NASH ↑	inhibition of ANGPTL3 improves insulin resistance −/+[82,83]
FGF21	increased	energy expenditure ↑ insulin resistance ↓ β cell survival ↑	adipose tissue, skeletal muscle, liver, and brain pancreatic islets	obesity ↑ NAFLD ↑ insulin resistance ↑ CVD ↑	genetic data indicate effects of FGF21 on food intake[84,85] FGF21 analogs decrease liver fat content and improve the blood lipid profile[86–89]
SHBG	decreased	sex hormone bioavailability ↑ sex hormone signalling ↑ hepatic lipogenesis ↓ endoplasmic reticulum stress ↓	reproductive organs liver	obesity ↓ NAFLD ↓↓ insulin resistance ↓↓ subclinical inflammation (↓) type 2 diabetes ↓↓ CVD ↓	low levels predict type 2 diabetes[90,91] low levels predict CVD[92] MR results about causative effects: type 2 diabetes +[90,91]
Selenoprotein P	increased	insulin resistance ↑ β cell function (↓)	liver, skeletal muscle pancreatic islets	insulin resistance ↑ subclinical inflammation (↑) CVD ↑	genetic data indicate effects of selenoprotein P on insulin resistance and β cell function[93]
Fetuin-B	increased	insulin resistance ↑	liver, skeletal muscle	NAFLD ↑ glucose effectiveness ↓	–
Follistatin	increased	hepatic glucose production ↑ adipose insulin resistance ↑	liver adipose tissue	NAFLD ↑ type 2 diabetes ↑ adipose tissue insulin resistance ↑	high levels predict type 2 diabetes and MR results about causative effects in type 2 diabetes (+)[94]
Hepatokines dysregulated in NAFLD that await precise clinical evaluation in humans					
LECT2	increased	insulin resistance ↑	skeletal muscle	visceral obesity ↑	–
Hepassocin	increased	insulin resistance ↑	liver, adipose tissue, and skeletal muscle	NAFLD ↑ hyperglycemia ↑	–
SMOC1	–	glucose production ↓	liver	hepatic insulin resistance ↓	–
TSC22D4/ LCN13	increased (TSC22D4) (NASH)	insulin resistance ↑	liver, skeletal muscle	NASH (TSC22D4 mRNA) ↑ insulin resistance (TSC22D4 mRNA) ↑ insulin resistance (LCN13 mRNA) ↓	–
Tsukushi	increased	possibly by regulation of thermogenesis	brown fat	type 2 diabetes ↑	–
Apolipoprotein J	(increased)	insulin resistance ↓	skeletal muscle	insulin resistance ↑ type 2 diabetes ↑	–

ANGPTL3, angiopoietin-related protein 3; CVD; cardiovascular disease; FGF-21, fibroblast growth factor 21; LCN13, lipocalin 13; LECT2, leukocyte cell-derived chemotaxin 2; MR, Mendelian randomization; NAFL, non-alcoholic fatty liver; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; SHBG, sex hormone-binding globulin; TSC22D4, transforming growth factor stimulated clone 22D4.

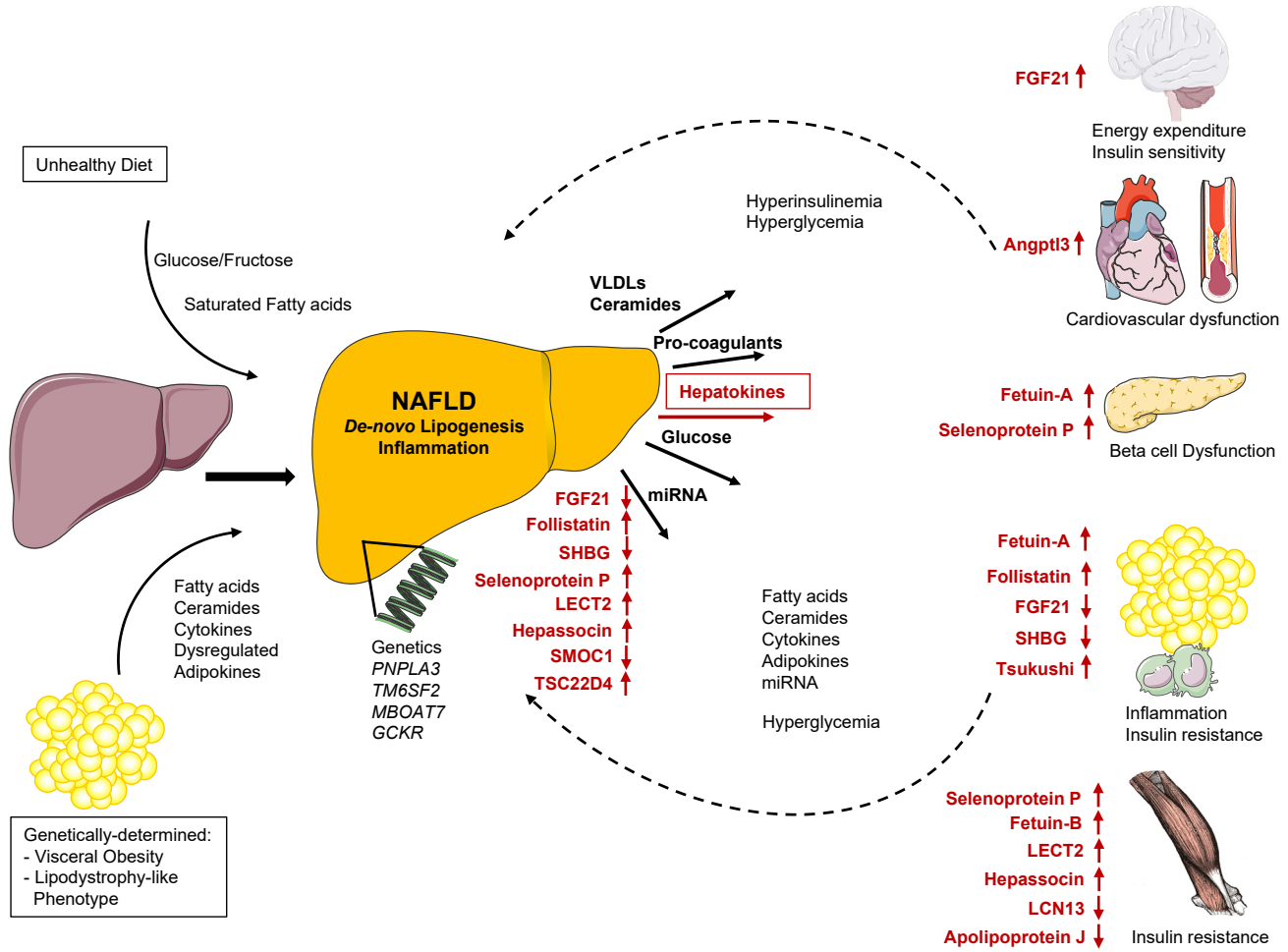


Figure 1. Causes and cardiometabolic consequences of NAFLD

An unhealthy diet involving a positive energy balance with increased caloric intake, particularly of glucose, fructose, and saturated fat, results in increased hepatic *de novo* lipogenesis and hepatic inflammation. This process is amplified by a lipodystrophy-like phenotype with expansion of visceral adipose tissue, resulting in increased release of fatty acids and ceramides and a dysregulated pattern of cytokines and adipokines. A genetic predisposition for hepatic lipid accumulation, inflammation, and fibrosis also contributes to the pathogenesis of NAFLD, albeit with wide range of metabolic effects. By increased/decreased secretion of very-low-density lipoproteins (VLDLs) with different amounts of ceramides, increased release of pro-coagulants, increased glucose output, and dysregulated secretion of microRNAs and hepatokines, the fatty liver impacts metabolically important organs and tissues and the cardiovascular system. The arrows shown with the hepatokines indicate increased/decreased organ function mediated by the respective hepatokines. The cardiometabolic pathologies brought about by the endocrine function of the fatty liver also impact the progress of NAFLD.

FGF21

Fibroblast growth factor 21 (FGF21) is one of the most widely studied hepatokines in animals. It has important metabolic regulatory properties in fasting and feeding.[116] Regarding its role in the protection from cardiometabolic diseases, administration of FGF21 to obese and diabetic mice and rats decreases plasma glucose and lipid levels; improves hepatic and peripheral insulin sensitivity, partially independent of less body weight, adiposity, or hepatic steatosis; and increases energy expenditure.[71,116] Regardless, it is difficult to identify these beneficial effects of FGF21 on human metabolism. There is ample evidence that FGF21 levels increase in individuals with obesity, insulin resistance, NAFLD, or type 2 diabetes. Increased production and release of FGF21 by the liver are hypothesized to occur under conditions that are associated with elevated hepatic lipid and carbohydrate signaling. By contrast, strong hepatic insulin resistance in mice lacking hepatic insulin receptor substrate (IRS) 1 and IRS2 reduces obesity and circulating FGF21.[117] Additionally, obesity and insulin resistance are thought to cause a state of FGF21 resistance in rodents and humans.[71,116] In clinical studies, FGF21 analogs reduce plasma triglyceride concentrations and increase HDL cholesterol levels but without a glucose-lowering or insulin-sensitizing effect.[116]

For example, a randomized, placebo-controlled, double-blind proof-of-concept trial in patients with obesity and type 2 diabetes treatment with the short-acting FGF21 analog LY2405319 resulted in improvements of dyslipidemia, reduction of body weight and fasting insulin levels, increase in adiponectin levels, and trend toward decrease in glycemia.[86] Another placebo-controlled, multiple ascending-dose study in overweight/obese subjects with type 2 diabetes treatment with the long-acting FGF21 analog PF-05231023 resulted in a decrease of body weight, improvement of plasma lipoprotein profile, and

increase of adiponectin levels, while glycemic control did not change.[87] Differences between mice and humans in the tissue expression and physiological functions of FGF21 may underlie the unexpected clinical findings[118]; however, a randomized, double-blind, placebo-controlled, parallel-group phase 2 study reveals less liver fat content and blood biomarkers of fibrosis in NASH patients treated with PEGylated FGF21.[88] In that study, which included patients with biopsy-confirmed NASH (fibrosis stage 1–3) and a hepatic fat fraction of at least 10%, a significant decrease in absolute hepatic fat fraction in the group receiving 10 mg pegbelfermin daily (−6.8% versus −1.3%; p = 0.0004) and in the group receiving 20 mg pegbelfermin weekly (−5.2% versus −1.3%; p = 0.008) compared with the placebo group was observed. Strong reduction of liver fat compared to placebo was also observed in a clinical study with LLF580, another Fc-hFGF21 fusion protein.[89] The beneficial effects of FGF21 on the improvement of hyperglycemia, dyslipidemia, NASH, and other comorbidities are attributed in part to its actions in hepatocytes and are also mediated in part by induction of adiponectin in adipose tissue or through a brain-liver axis.[119] Most recently, CNOT6L deadenylase was identified as an important inhibitor of the hepatic FGF21 and growth differentiation factor 15 (GDF15).[120] Thus, targeting CNOT6L may be a therapeutic approach to treat metabolic diseases.

SHBG

Sex hormone-binding globulin (SHBG) is predominantly expressed in and secreted from the liver. In 2007, glucose and fructose were found to reduce human SHBG production by hepatocytes via downregulation of hepatocyte nuclear factor-4alpha (HNF-4α).[121] Moreover, tumor necrosis factor alpha (TNFα) and IL1β downregulate the expression of SHBG by decreasing HNF-4α via MEK-1/2 and JNK.[122,123] SHBG expression is upregulated by thyroid hormones, adiponectin, and oleate, while the effects of insulin on SHBG expression are unknown.[124] SHBG regulates the level and the biological activity of circulating steroid hormones, including testosterone, dihydrotestosterone, and estradiol.[125] Among the sex hormones, androgens are generally the preferred SHBG ligands. In females, SHBG levels are mostly twice as high as in men. In the case of testosterone binding to SHBG, no significant differences for sex or obesity are observed; however, in morbid obese men the ability of SHBG to bind estradiol is increased.[126] Free SHBG binds to the G protein-coupled SHBG-R (SHBG receptor), which is activated at the cell surface by estradiol and 5α-androstan-17β-ol-3-one, which activate adenylate cyclases. In addition, SHBG may interact with megalin, an endocytic receptor found in reproductive tissues, and stimulate endocytosis of SHBG-bound androgens and estrogens.[127]

SHBG can exert a favorable effect on metabolism. In two mouse models of NAFLD, SHBG overexpression inhibits hepatic lipogenesis by reducing PPARγ through activation of the ERK-1/2 pathway.[128] Furthermore, SHBG protects against ER stress and its progression by reducing the expression levels of inositol-requiring enzyme 1 (IRE1α), which reduces the activity of transcription factor 6 (ATF6), DNA damage-inducible transcript 3 (CHOP), and immunoglobulin heavy chain-binding protein (BIP).[129] In agreement with findings from animal-based studies, a meta-analysis found that circulating SHBG levels are lower in patients with NAFLD, compared to healthy controls.[130] Using precise phenotyping methods, we found that the relationship between low circulating SHBG levels and fatty liver, and the increase of circulating SHBG levels during weight loss-associated reduction of hepatic steatosis, are independent of visceral obesity.[131] Furthermore, we found that mechanisms by which high circulating SHBG prevents the development of type 2 diabetes may involve protection from hepatic insulin resistance.[132] Most importantly, two large MR studies provide strong support that high circulating levels of SHBG protect men and women from developing type 2 diabetes.[90,91] By contrast, low SHBG plasma levels associate with more risk of incident cardiovascular disease.[92]

Selenoprotein P

In 2010, the glyco-hepatokine selenoprotein P was found to be dysregulated in hepatic steatosis.[133] Its hepatic expression is upregulated by glucose and palmitate, possibly involving the ER stress-activated JNK pathway. By contrast, selenoprotein P is downregulated by insulin. Metformin also reduces its expression through AMPK activation.[133,134] Selenoprotein P inhibits insulin signaling in hepatocytes and myocytes at the level of IRS1 and inhibits activation of AMPK.[133] Furthermore, selenoprotein P reduces glucose-induced insulin secretion in mice.[135] In humans, studies with relatively small sample sizes found plasma levels of selenoprotein P to be elevated in subjects with NAFLD[136–138] and in patients with type 2 diabetes.[133] Thus, selenoprotein P correlates with hyperglycemia, insulin resistance, and the carotid intima-media thickness[133,137,139]; however, in a meta-analysis, significant relationships of elevated selenoprotein P levels with type 2 diabetes, parameters of the metabolic syndrome, or NAFLD are weak or absent.[140] Genetic variants of the selenoprotein P plasma 1 gene (*SEPP1*) associate with acute insulin response and insulin sensitivity[93]; however, the relationships of selenoprotein P plasma levels with the incidence of type 2 diabetes or cardiovascular disease were not investigated by an MR analysis. Thus, based on its important effects on glucose metabolism in animal studies, more research is warranted to better understand the role of selenoprotein P in human metabolism.

Fetuin-B

Fetuin-B is primarily produced and secreted by the fatty liver and was found to induce insulin resistance in cultured hepatocytes and myotubes.[141] Fetuin-B causes glucose intolerance in mice, whereas liver-specific knockdown improves glucose tolerance without effects on insulin sensitivity.[141] Fetuin-B can aggravate hepatic steatosis in HepG2 cells and mice.[142] A meta-analysis and meta-regression in 3,800 patients with NAFLD and 3,614 controls revealed elevated fetuin-B plasma levels in subjects with increased liver fat content.[143] Animal and human data indicate that fetuin-B may regulate glucose effectiveness through an unknown mechanism.[141,144]

FST

The glycoprotein follistatin (FST) is expressed in many tissues throughout the body, but the liver is considered the primary organ responsible for circulating FST.[145] FST strongly regulates reproduction[146] and skeletal muscle growth[147] by inhibiting the TGF-β superfamily members myostatin and activins. In mice and humans, FST is secreted from the liver when its expression is upregulated by

high glucagon/insulin ratios during exercise and fasting—or by activated FoxO1 during hepatic insulin resistance.[148–151] Two isoforms are generated by alternative splicing, including membrane-bound Fst288 and circulating Fst315,[145,146,152] which interfere with activin and myostatin receptor binding and phosphorylation of Smad2/3 transcription factors.[153–155] Overexpression of FST has diverse effects in mice.[155] In muscle, Fst288 prevents β cell loss in obese *db/db* mice to maintain glucose tolerance without reducing adiposity[156]; however, non-specific overexpression of Fst315 can improve glucose tolerance while reducing obesity during a high-fat diet.[157,158] Regardless, overexpression of FST in liver (its natural site of expression) causes white adipose tissue insulin resistance and lipolysis, which delivers FFAs and glycerol to the liver to promote uncontrolled hepatic glucose production and glucose intolerance.[151] In hyperglycemic and high-fat-fed obese mice, disruption of FST restores glucose tolerance, white adipose tissue insulin signaling, and suppression of hepatic glucose production by insulin.[151] Furthermore, high circulating FST may promote NAFLD in humans by inducing insulin resistance in adipose tissue. Recently, we found FST to increase FFA release dose-dependently from human adipocytes.[94] Thus, hepatic FST might integrate white adipose tissue lipolysis with uncontrolled hepatic glucose production and NAFLD/NASH.[117,151,94]

Using variants of the glucokinase regulatory protein gene that are strongly associated with FST levels, we found that FST may be involved in the pathogenesis of type 2 diabetes. To validate the relationship between human FST and white adipose tissue insulin sensitivity, we investigated circulating FST in TUEF/TULIP (Tübingen Family Study/Tübingen Lifestyle Intervention Program) patients.[94,159] FST was higher in NAFLD subjects and it correlated significantly with BMI ($r = 0.27$, $p = 0.0001$), total body fat mass ($r = 0.28$, $p < 0.0001$), visceral fat mass ($r = 0.18$, $p = 0.008$), liver fat content ($r = 0.23$, $p = 0.0008$), and circulating FFAs during an oral glucose tolerance test at 0, 60, and 120 min ($r = 0.22–0.28$, $p \leq 0.001$).[94] By contrast, circulating FST correlated negatively ($r = -0.26$, $p = 0.0002$) with adipose insulin sensitivity. This correlation was sensitive to adjustment for leg fat mass, suggesting that FST might impair adipose insulin sensitivity predominantly in leg fat.[94] Since re-esterification of circulating FFAs is an important source of hepatic triacylglycerides in NAFLD patients,[23] FST-impaired adipose insulin sensitivity might stimulate NAFLD by shifting FFAs to the liver.

EMERGING HEPATOKINES DYSREGULATED IN NAFLD THAT NEED CLINICAL EVALUATION

LECT2

Leukocyte cell-derived chemotaxin 2 (LECT2) is dysregulated in fatty liver, but not yet carefully studied for relationships with clinical phenotypes or cardiometabolic diseases. LECT2 is primarily expressed and secreted from the liver, but also from adipose tissue, neurons, and white blood cells. *In vitro* and animal data suggest that LECT2 inhibits portal angiogenesis, promotes sinusoid capillarization, and worsens fibrosis.[160] It can also impair insulin signaling in skeletal muscle[161] and adipose tissue.[162] In humans, increased LECT2 plasma levels strongly associate with visceral obesity,[163] but not with NAFLD adjusted for waist circumference. In a relatively small study, circulating LECT2 is not associated with plasma glucose levels or early markers of atherosclerosis.[164] Larger studies need to investigate whether LECT2 can be used as a potential target for the treatment of NAFLD-related insulin resistance or marker of atherosclerosis.

Hepassocin

Hepassocin is predominantly expressed in the liver and has mitogenic activity upon isolated hepatocytes.[165] It can protect against liver injury in rats.[166] Glucose increases hepassocin in HepG2 cells through the STAT3 and PP2A-HNF1 pathways.[167] Hepassocin induces insulin resistance in mice, possibly involving ERK1/2 and EGFR/JNK-mediated pathways in skeletal muscle.[168,169] It also promotes hepatic lipid accumulation through the ERK1/2-dependent pathway.[170] Serum hepassocin levels increase in patients with NAFLD[170] and correlate with fasting glycemia, but not fasting insulinemia.[168]

SMOC1

SPARC-related modular calcium-binding protein 1 (SMOC1) is a glycoprotein found in many tissues. SMOC1 is predominantly secreted from the liver and its gene expression and secretion are upregulated by glucose via a ChREBP-dependent pathway.[171] SMOC1 was found to inhibit CREB (cAMP-responsive element-binding protein 1) signaling in the liver, which reduces gluconeogenic gene expression and suppression of hepatic glucose output. Furthermore, SMOC1 was shown to increase glucose uptake into skeletal muscle of mice; however, the mechanisms of the respective action have not been identified. Results from euglycemic-hyperinsulinemic clamp studies with acute SMOC1 injection in lean, normoglycemic mice reveal that SMOC1 increased insulin-mediated suppression of hepatic glucose output but did not affect the rate of glucose disappearance. Thus, SMOC1 may improve insulin sensitivity via actions at the liver.[171] In humans, SMOC1 plasma levels are lower in insulin-resistant compared with insulin-sensitive individuals and correlate positively with hepatic insulin sensitivity; however, no correlation was observed between SMOC1 plasma levels and liver fat content.[171] Hepatic SMOC1 expression shows no significant differences across NAFLD stages. Circulating SMOC1 levels are not causally associated with type 2 diabetes or glycemic traits by MR analyses.[172]

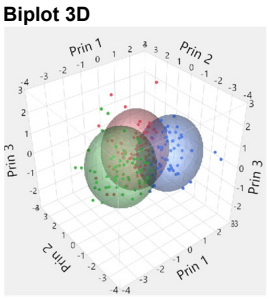
TSC22D4/LCN13

Transforming growth factor-stimulated clone (TSC)22D4 is a widely expressed protein that is thought to be involved in glucocorticoid and stress signaling, cell proliferation, and apoptosis. Hepatic inhibition of TSC22D4 reduces hyperglycemia, glucose intolerance, and insulin resistance in diabetes mouse models. Reduced TSC22D4 is accompanied by enhanced hepatic Akt T308/S473 phosphorylation in response to either exogenous insulin injection or a fasting-feeding regimen.[173] Furthermore, TSC22D4 inhibits systemic glucose homeostasis, at least in part through direct transcriptional inhibition of the small secretory protein lipocalin 13 (LCN13), which inhibits insulin sensitivity in skeletal muscle.[173] In humans, hepatic mRNA expression of LCN13 correlates positively with insulin sensitivity, while a negative correlation was found between hepatic mRNA expression of TSC22D4 and insulin sensitivity.[173] The regulation of hepatic TSC22D4 expression is poorly understood

Cluster variables:
Liver fat
Visceral fat
Adiponectin levels
Fetuin-A levels

Cluster method:
K-means cluster analyses

Number of clusters:
Cubic clustering criterion:
Ward's minimum variance method



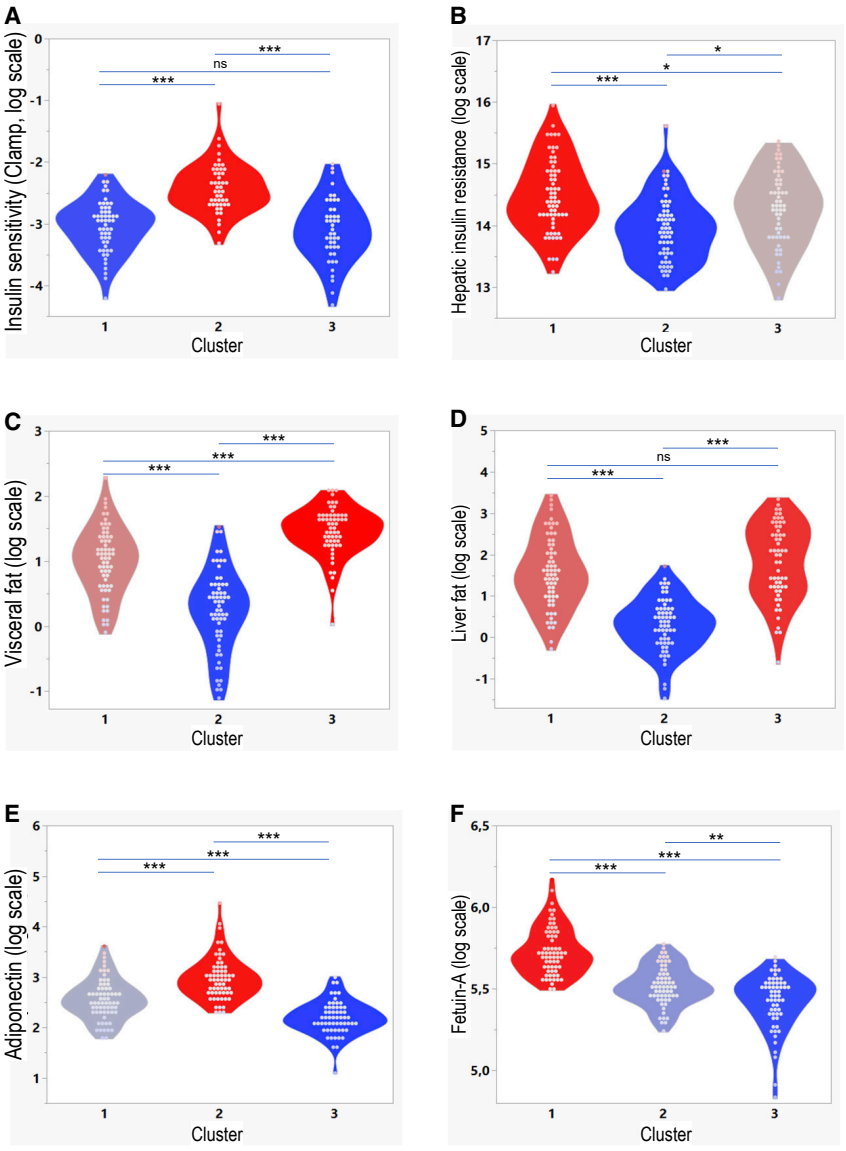


Figure 2. Relationships of liver fat/visceral fat/adiponectin/fetuin-A risk clusters

(A and B) Insulin sensitivity and (C–F) cluster parameters. In 185 subjects at increased risk of type 2 diabetes, K-means cluster analyses identified 3 clusters deriving from the parameters of (C) visceral fat content, (D) liver fat content, (E) the adipokine adiponectin, and (F) the hepatokine fetuin-A. The color coding indicates low values in blue, mean values in gray shading, and high values in red. p values for statistical differences between the clusters in Student's t tests (*$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$). The insert depicts the cluster analysis approach and the 3D biplot of the cluster results.

because TSC22D4 mRNA expression increases in obese patients without or with simple steatosis or with NASH.[174]

TSKU

Tsukushi, small leucine-rich proteoglycan (TSKU), is a hepatokine that increases expression and circulation during NAFLD and liver damage in mice and in humans.[175] The mechanism of its upregulation in mice may involve ER stress and inflammation. TSKU-deficient mice display enhanced adrenergic signaling and thermogenesis in brown adipose tissue, which reduces diet-induced obesity, insulin resistance, and hepatic steatosis.[176] In a small study, serum TSKU levels increase in patients with type 2 diabetes[177]; however, the role of TSKU in the pathophysiology of cardiometabolic diseases is unknown.

ApoJ

Apolipoprotein J (ApoJ, clusterin) is a sulfated secreted glycoprotein that is ubiquitously expressed and secreted by the liver.[178] Liver-derived ApoJ binds to its receptor LRP2 (LDL receptor-related protein 2) on the cell surface of skeletal muscle. ApoJ binding amplifies insulin action by specifically driving insulin receptor internalization.[178] In mice, ApoJ overexpression protects against Western diet-induced obesity and NAFLD.[179] Small human studies show that ApoJ plasma levels are upregulated in obesity, the metabolic syndrome, insulin resistance, and type 2 diabetes[180]; however, in humans, the relationship between ApoJ and insulin sensitivity or hepatic steatosis is unknown.

Adipo-hepatokines dysregulated in NAFLD

Several liver-secreted proteins with metabolic signaling properties that are dysregulated in NAFLD are also strongly expressed and secreted from adipose tissue, lung, and intestine. Among them are retinol-binding protein 4 (RBP4), pigment epithelium-derived factor (PEDF), growth differentiation factor 15 (GDF15), dipeptidyl peptidase-4 (DPP4), ANGPTL4 (angiopoietin-like 4), and ANGPTL8. These hepatoadipokines were found by *in vitro* and animal studies to have important effects on the regulation of glucose and lipid

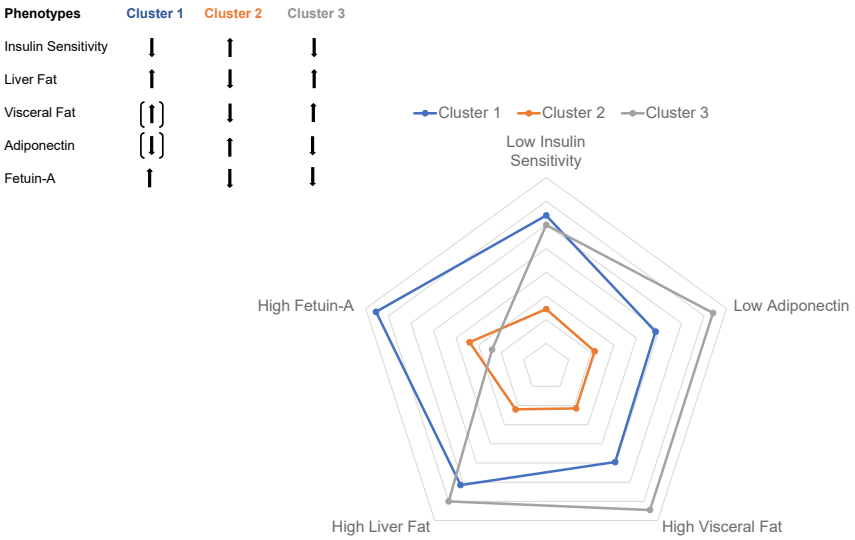


Figure 3. Radar charts of the liver fat/visceral fat/adiponectin/fetuin-A risk clusters with insulin sensitivity and the cluster parameters

Depicted are the median values of the *Z* scores of the parameters insulin sensitivity, measured from the frequently sampled oral glucose tolerance test; liver fat content; visceral fat mass; adiponectin levels; and fetuin-A levels. Insulin sensitivity and adiponectin levels were directionally flipped (−1 × *Z* score) to yield adverse variable effects. The insert depicts whether the parameters are increased or decreased in the 3 clusters.

metabolism[67–73,181]; however, in most cases, their impact on cardiometabolic diseases is incomplete. More recently, fibronectin type III domain-containing protein 4 (FNDC4), a secreted factor showing a high homology with the exercise-associated myokine irisin (FNDC5), was also proposed to modulate glucose and lipid metabolism as an adipokine and a hepatokine.[182,183]

HEPATOKINES AND ADIPOKINES FOR CLUSTERING INSULIN RESISTANCE

Studying the metabolic effects of dysregulated hepatokines in hepatic steatosis reveals how fatty liver can modulate glucose and lipid metabolism and subclinical inflammation. Now it is important to integrate this knowledge to predict progression of metabolic disease and apply precision medical strategies in humans. This research may help to better separate the role of fatty liver from visceral adiposity in the pathophysiology of insulin resistance and NCDs. In the Dallas Heart Study, visceral obesity—but not fatty liver measured precisely by magnetic resonance techniques—is associated with the incidence of prediabetes and diabetes.[184,185] These findings indicate that fatty liver alone may not be an important mediator of diabetes risk. Furthermore, only phenotypes with high visceral fat mass and either high or low liver fat content associate with increased risk of cardiovascular disease in the Dallas Heart Study. By contrast, low visceral fat mass/high liver fat content associated with increased risk of type 2 diabetes.[186] Previous work suggests that genetically determined fatty liver can be independent of insulin resistance and even protect against diabetes and cardiovascular disease.[18,49,51,56,187] Thus, separating metabolically healthy from metabolically unhealthy fatty liver is critical to advance our understanding of the role of fatty liver in cardiometabolic diseases.

Our previous work suggests that precisely measured fatty liver and visceral obesity may improve phenotype-based stratification of prediabetes subsets.[15] By analogy to the strategy that identified diabetes clusters,[188] we found that 899 individuals defined six clusters of individuals at increased risk of type 2 diabetes—two of which had a high prevalence of NAFLD (100% in cluster 5 and 75% in cluster 6). Clusters 5 and 6 also had high risk of diabetes, kidney disease, and all-cause mortality; however, subjects in both clusters also had very high visceral fat mass (5.73 L in cluster 5 and 5.64 L in cluster 6).[159] Furthermore, using K-means clustering of lipid content in the liver, pancreas, muscle, and visceral bed, we identified four fat distribution clusters. The cluster most strongly associated with the incidence of diabetes had the highest liver fat content and visceral fat mass.[189]

Using K-means clustering of liver fat content, visceral fat mass, hepatokines, and adipokines, we now propose to identify patients with insulin resistance that may differ in the contribution of fatty liver and visceral obesity to the pathophysiology. Reanalyzing previously published data from 185 subjects in the Tübingen Diabetes Family Study,[103,190] we clustered four metabolic parameters: circulating levels of fetuin-A and adiponectin, liver fat content, and visceral fat mass (supplemental information). We focused on circulating fetuin-A and adiponectin because these proteins independently associated with the incidence of type 2 diabetes in the EPIC (European Prospective Investigation into Cancer and Nutrition Potsdam) Study and the Nurses' Health Study.[84]

Three informative clusters are revealed by K-means analysis. Compared against cluster 2, clusters 1 and 3 had similar but low whole-body insulin sensitivity, and similar elevated liver and visceral fat content (Figures 2A, 2C, and 2D). Interestingly, relatively high adiponectin levels of cluster 2 are associated with the relatively low liver fat content and visceral fat mass (Figures 2C–2E). Of note, subjects in cluster 1 had the highest plasma fetuin-A levels, which associate with the highest hepatic insulin resistance (Figures 2B and 2F). The key differences of the phenotypes among the three clusters are depicted in a radar chart (Figure 3). Thus, the pathophysiology of metabolically unhealthy fatty liver-associated insulin resistance—characterized by high fetuin-A levels—may differ from the pathophysiology of visceral obesity-associated insulin resistance—characterized by low plasma adiponectin levels. In support of this hypothesis, circulating fetuin-A, but not adiponectin, correlated with hepatic insulin resistance (Table S1; Figure S1). Estimated adipose tissue insulin sensitivity (Figure S2B) and clamp-derived insulin sensitivity (Figure 2A) did not differ between clusters 1 and 3. As we used a relatively high insulin dose in our clamp, in our study insulin sensitivity measured by the clamp predominantly represents insulin sensitivity of glucose disposal. The hypothesis

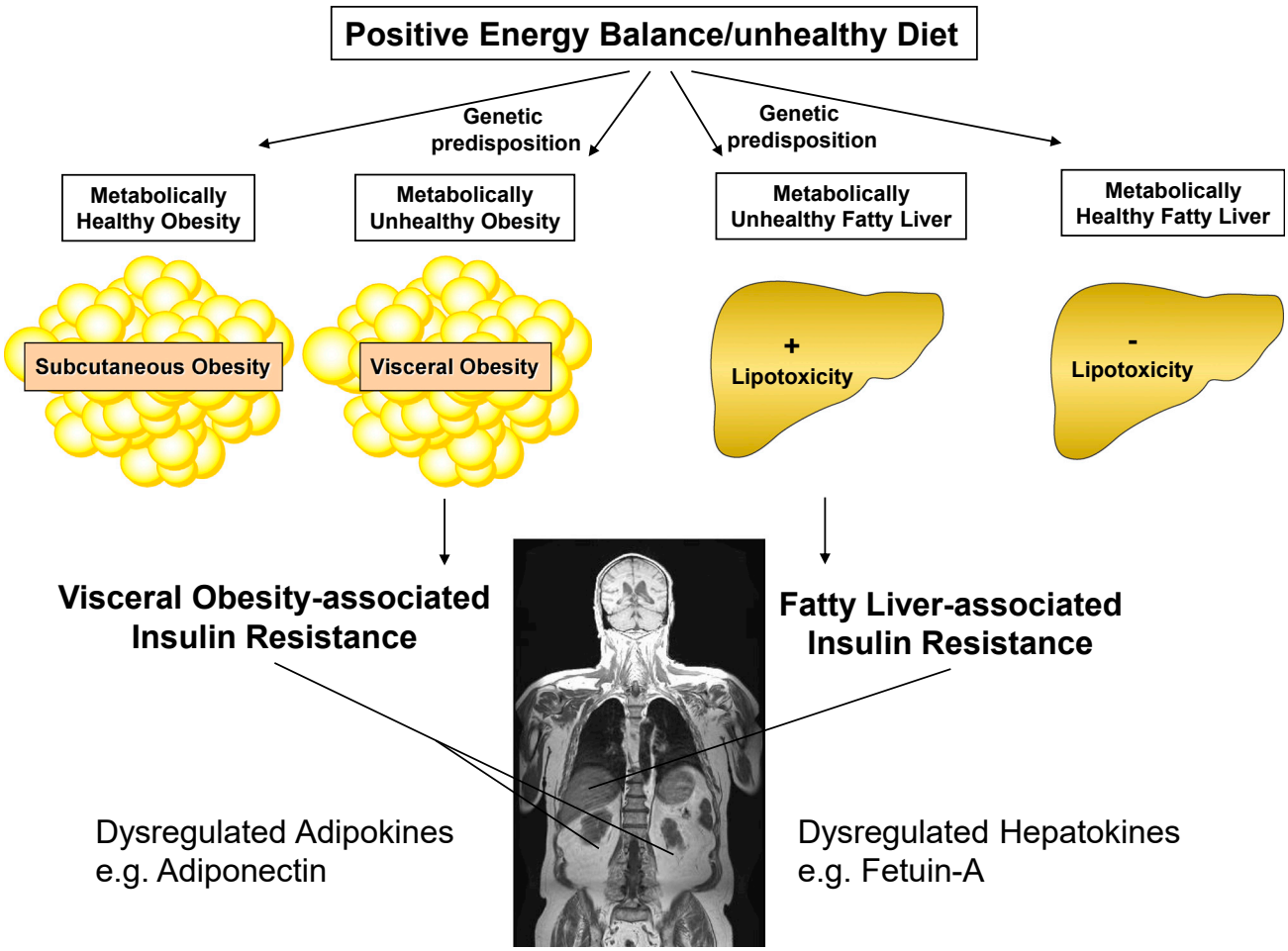


Figure 4. Impact of a positive energy balance and an unhealthy diet on insulin resistance and cardiometabolic diseases via effects on fat mass and distribution and hepatic steatosis

During conditions of a positive energy balance and unhealthy diets, subcutaneous and visceral adipose tissues expand in a manner that is predominantly genetically determined. Subcutaneous (metabolically healthy) obesity is not strongly associated with cardiometabolic diseases, whereas visceral obesity is a strong predictor of these diseases. Increased availability of fatty acids, increased subclinical inflammation, and dysregulation of adipokine production and release are thought to promote insulin resistance, atherosclerosis, and β cell dysfunction. This process is mainly characterized by dysregulated adipokine production and secretion. Accumulation of lipids in the liver is also genetically determined, and two distinct phenotypes have been identified. When hepatic detoxification processes are active, storage of lipids in the liver is not associated with metabolic diseases. By contrast, when lipotoxicity is present, hepatic glucose production increases and lipids are released, with an atherogenic profile. This process is mainly characterized by dysregulated hepatokine production and secretion.

that metabolically unhealthy fatty liver-associated insulin resistance may differ from the pathophysiology of visceral obesity-associated insulin resistance needs to be tested in animal studies and in human studies with precise measurement of tissue-specific insulin resistance using tracer methods.

Conclusion

There is ample evidence that NAFLD is an important consequence of insulin resistance and hyperglycemia, processes that are mainly induced by overnutrition and an unhealthy diet and a genetic predisposition for disproportionate body fat distribution. Research, which was predominantly conducted during the past 10 years, revealed that NAFLD may also have an important role in the pathogenesis of insulin resistance and cardiometabolic diseases. Support for this hypothesis has been mainly provided by *in vitro* and animal studies targeted to understand the role of dysregulated hepatokines in NAFLD; however, in most cases reliable clinical data in humans about the role of hepatokines in NAFLD are still missing, and it is important to collect such knowledge. As of today, examples of how to use the current knowledge about important organokines, including adipokines, myokines, and hepatokines, may help us better understand the role of visceral obesity, dysfunctional skeletal muscle, and fatty liver in the pathophysiology of insulin resistance (Figure 4). In this respect, for diagnostic purposes, the measurement of adipokines, myokines, and hepatokines could be used to better understand whether a patient's insulin resistance is predominantly driven by metabolic dysregulation of white adipose tissue, skeletal muscle, or the liver. Furthermore, if fatty liver is diagnosed, the measurement of hepatokines may help to separate a metabolically healthy from a metabolically unhealthy fatty liver.

The results of our cluster analyses support that metabolically unhealthy fatty liver-associated insulin resistance is characterized by high fetuin-A levels and that this phenotype may differ from the phenotype of visceral obesity-associated insulin resistance, which is predominantly characterized by low plasma adiponectin levels. Understanding whether and to what extent hepatokines are dysregulated may help to guide treatment. For example, insulin resistance and fatty liver associated with elevated circulating fetuin-A may be preferably treated with pioglitazone, particularly if adiposity is not the major issue. Also, insulin resistance accompanied by fatty liver, dyslipidemia, and low energy expenditure-associated hyperphagia may well respond to a treatment with FGF21 analogs, once approved for the treatment of impaired metabolic health. Future studies in the developing field of hepatokine research may reveal many other diagnostic and treatment strategy concepts that can contribute to a better implementation of precision medicine in clinical practice.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.cmet.2023.01.006.

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AUTHOR CONTRIBUTIONS

All authors contributed to the search of the published work and the interpretation of the data. N.S. analyzed the data and N.S. and M.F.W. wrote the paper.

DECLARATION OF INTERESTS

M.F.W. is an advisory board member of Housey Pharma (https://www.housey.com/).

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