Research Article
NAFLD and Alcohol-Related Liver Diseases

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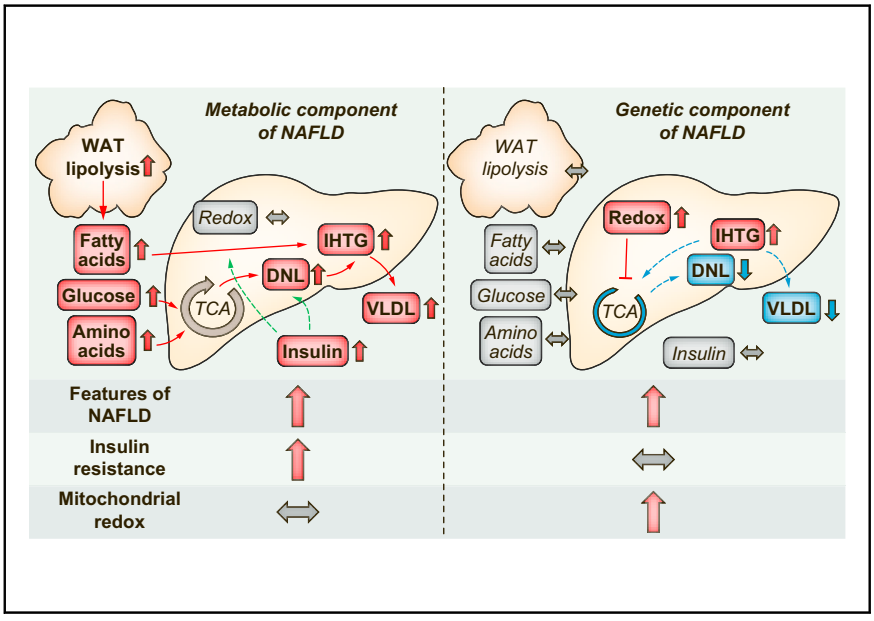
Distinct contributions of metabolic dysfunction and genetic risk factors in the pathogenesis of non-alcoholic fatty liver disease

Graphical abstract



Highlights

- The pathogenesis of NAFLD can be partly explained by a metabolic component and partly by a genetic component.
- The mechanisms underlying these components are fundamentally different.
- The metabolic component is characterized by a substrate surplus and increased rates of adipose tissue lipolysis and hepatic DNL.
- The genetic component is characterized by increased hepatic mitochondrial redox state and inhibition of pathways dependent on TCA cycle activity, such as DNL.
- These components additively increase the severity of NAFLD.

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Lay summary

The pathogenesis of non-alcoholic fatty liver disease can be explained in part by a metabolic component, including obesity, and in part by a genetic component. Herein, we demonstrate that the mechanisms underlying these components are fundamentally different: the metabolic component is characterized by hepatic oversupply of substrates, such as sugars, lipids and amino acids. In contrast, the genetic component is characterized by impaired hepatic mitochondrial function, making the liver less able to metabolize these substrates.

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Research Article
NAFLD and Alcohol-Related Liver Diseases

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Distinct contributions of metabolic dysfunction and genetic risk factors in the pathogenesis of non-alcoholic fatty liver disease

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Background & Aims: There is substantial inter-individual variability in the risk of non-alcoholic fatty liver disease (NAFLD). Part of which is explained by insulin resistance (IR) ('MetComp') and part by common modifiers of genetic risk ('GenComp'). We examined how IR on the one hand and genetic risk on the other contribute to the pathogenesis of NAFLD.

Methods: We studied 846 individuals: 492 were obese patients with liver histology and 354 were individuals who underwent intrahepatic triglyceride measurement by proton magnetic resonance spectroscopy. A genetic risk score was calculated using the number of risk alleles in *PNPLA3*, *TM6SF2*, *MBOAT7*, *HSD17B13* and *MARC1*. Substrate concentrations were assessed by serum NMR metabolomics. In subsets of participants, non-esterified fatty acids (NEFAs) and their flux were assessed by D_5-glycerol and hyperinsulinemic-euglycemic clamp (n = 41), and hepatic *de novo* lipogenesis (DNL) was measured by D_2O (n = 61).

Results: We found that substrate surplus (increased concentrations of 28 serum metabolites including glucose, glycolytic intermediates, and amino acids; increased NEFAs and their flux; increased DNL) characterized the 'MetComp'. In contrast, the 'GenComp' was not accompanied by any substrate excess but was characterized by an increased hepatic mitochondrial redox state, as determined by serum β-hydroxybutyrate/acetoacetate ratio, and inhibition of hepatic pathways dependent on tricarboxylic acid cycle activity, such as DNL. Serum β-hydroxybutyrate/acetoacetate ratio correlated strongly with all histological features of NAFLD. IR and hepatic mitochondrial redox state conferred additive increases in histological features of NAFLD.

Conclusions: These data show that the mechanisms underlying 'Metabolic' and 'Genetic' components of NAFLD are fundamentally different. These findings may have implications with respect to the diagnosis and treatment of NAFLD.

Lay summary: The pathogenesis of non-alcoholic fatty liver disease can be explained in part by a metabolic component, including obesity, and in part by a genetic component. Herein, we demonstrate that the mechanisms underlying these components are fundamentally different: the metabolic component is characterized by hepatic oversupply of substrates, such as sugars, lipids and amino acids. In contrast, the genetic component is characterized by impaired hepatic mitochondrial function, making the liver less able to metabolize these substrates.

Keywords: hepatic mitochondrial redox state; insulin resistance; metabolomics; *de novo* lipogenesis; adipose tissue lipolysis; PNPLA3; TM6SF2; MBOAT7; HSD17B13; MARC1.

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Introduction

Non-alcoholic fatty liver disease (NAFLD) covers a spectrum of disease from steatosis, *i.e.*, excess accumulation of intrahepatic

triglyceride (IHTG), to steatohepatitis (NASH) and cirrhosis. The risk of NAFLD exhibits large inter-individual variation but the underlying mechanisms remain poorly understood.[1]

NAFLD is frequently associated with insulin resistance (IR) and features of the metabolic syndrome (metabolic component, 'MetComp').[2] IR of adipose tissue lipolysis increases the flux of non-esterified fatty acids (NEFAs), the main substrate for synthesis of IHTG, to the liver. In addition, IHTG synthesis from *de novo* lipogenesis (DNL) is accelerated.[2]

Genetic factors explain approximately half of the inter-individual variation in the risk of NAFLD (genetic component, 'GenComp').[3] Several common polymorphisms increase the risk of NAFLD, including those in patatin-like phospholipase domain containing 3 (*PNPLA3*, rs738409),[4] transmembrane 6 superfamily member 2 (*TM6SF2*, rs58542926)[5] and membrane bound O-acyltransferase domain containing 7 (*MBOAT7*, rs641738),[6] while variants in hydroxysteroid 17-beta dehydrogenase 13 (*HSD17B13*, rs72613567)[7] and mitochondrial amidoxime-reducing component 1 (*MARC1*, rs2642438)[8] are protective. Most individuals carry a combination of risk and protective alleles. In 2 large population-based cohorts, a genetic risk score (GRS), which was calculated based on all except the very recently described *MARC1* variant, conferred up to a 12-fold higher risk of cirrhosis.[9] A major strength of the use of a GRS compared to traditional cohort studies is the lack of reverse causation – the metabolic effects must be secondary to genetic variation, not *vice versa*.

In contrast to the 'MetComp', none of the risk-modifying polymorphisms affect insulin sensitivity.[4–8] Thus, consequences of IR such as increased lipolysis and DNL may not explain why the 'GenComp' modifies the risk of NAFLD. Lipolysis[10] and DNL[11] have hitherto only been directly measured in NAFLD associated with the *PNPLA3* I148M variant. While each variant has distinct effects on metabolic pathways,[12] it is unclear how these pathways converge to induce an identical histologic phenotype in NAFLD.

Several human studies have reported decreases in mitochondrial respiration in NASH[13–15] but the underlying mechanisms have remained unclear. We recently showed that a ketogenic diet inhibits mitochondrial respiration by increasing hepatic mitochondrial redox state, which was attributed to increased hepatic availability of fatty acids and their β-oxidation.[16] Hepatic mitochondrial redox state is known to play a key role in several liver diseases other than NAFLD, including alcohol-related liver disease,[17] and to predict mortality after acute liver failure,[18] alcoholic hepatitis[19] and liver transplantation.[20] Since the genetic risk variants increase IHTG and thereby potentially concentrations of intrahepatic fatty acids available for β-oxidation, we hypothesized that the 'GenComp' might increase hepatic mitochondrial redox state, leading to inhibition of tricarboxylic acid (TCA) cycle activity, and ultimately to mitochondrial dysfunction.

In the present study we examined how IR on one hand and genetic variants on the other, confer susceptibility to NAFLD. To this end, we tested the hypothesis that substrate surplus (NEFAs and *in vivo* lipolysis measured using D_5-glycerol, glucose, glycolytic intermediates, amino acids, and DNL measured by D_2O) characterizes individuals predisposed to NAFLD associated with IR ('MetComp'), while increased hepatic mitochondrial redox state inhibits pathways dependent on TCA cycle activity, such as DNL, in individuals predisposed to NAFLD due to a high genetic risk ('GenComp').

Patients and methods

Human participants

We recruited a total of 284 participants (*Discovery cohort*, Table S1) amongst those undergoing laparoscopic bariatric surgery at the Helsinki University Hospital, Finland. An additional 208 participants (*Validation cohort*, Table S1) were recruited amongst those undergoing laparoscopic bariatric surgery at the Kuopio University Hospital, Finland.[21] Another 252 participants who were not undergoing bariatric surgery were also recruited (*Non-bariatric cohort,* Table S1). Additional cohorts were assessed for DNL using D_2O (n = 61) and for adipose tissue lipolysis using hyperinsulinemic-euglycemic clamp and D_5-glycerol (n = 41) (Table S1). The following inclusion criteria were employed in all cohorts: i) age range from 18 to 75 years; ii) alcohol consumption <20 g/day for women and <30 g/day for men; iii) no known acute or chronic disease other than obesity, type 2 diabetes, NAFLD or hypertension on the basis of medical history, physical examination and blood count, plasma creatinine and electrolyte concentrations; iv) no clinical or biochemical evidence of liver disease other than NAFLD, or clinical signs or symptoms of inborn errors of metabolism; v) no history or current use of hepatotoxic compounds; vi) no pregnancy or breastfeeding in women.

Liver biopsies were obtained during surgery and assessed for histology, and in the *Non-bariatric cohort*, IHTGs were determined using ^{1}H magnetic resonance spectroscopy. Serum samples from all participants were analyzed using nuclear magnetic resonance (NMR) metabolomics. For details, please see the supplementary information.

Results

IR increases the features of NAFLD independently of genetic risk

To investigate the association between IR and features of NAFLD, we divided the participants of the *Discovery cohort* into 6 groups based on sex-specific quantiles of homeostatic model assessment of insulin resistance (HOMA-IR), an index of hepatic IR[22] (*H1–H6*, Table 1, Fig. 1A). The groups were similar with respect to age and sex. The groups with high IR had a higher prevalence of type 2 diabetes (Fig. 1B), BMI, waist circumference and circulating concentrations of glucose, insulin, hemoglobin A1c, triglycerides and liver enzymes compared to the groups with low IR (Table 1). The groups were similar with respect to the distribution of risk alleles in *PNPLA3*, *TM6SF2*, *MBOAT7*, *HSD17B13, MARC1* (Table 1) and the GRS (Fig. 1B).

The groups with high IR had more NAFLD and NASH than those with low IR (Table 1). Liver histology showed a marked increase in steatosis, features of NASH (ballooning, lobular inflammation and activity), fibrosis, and the total steatosis-activity-fibrosis (SAF) score as a function of HOMA-IR (Fig. 1D-I).

IR increases hepatic substrate supply

Next, we analyzed the serum metabolome of the participants using NMR (Table S2). To reduce dimensionality of the data, we first carried out principal component (PC) analysis, which revealed 12 distinct components (Table S3). To gain insights into the pathogenesis of IR-driven progression of NAFLD, we analyzed the associations between HOMA-IR and NMR metabolomics data. PCs 1, 5, 7 and 11 correlated positively and PC6 correlated negatively with HOMA-IR, adjusted for age, sex, BMI, diabetes,

Table 1. Clinical characteristics of the participants divided to groups based on sex-specific HOMA-IR (H1–H6).

	H1	H2	H3	H4	H5	H6
Group size, n	48	47	47	47	47	48
Age, years	49 (8)	50 (12)	48 (13)	49 (20)	48 (14)	49 (15)
Women, n (%)	35 (73)	34 (72)	34 (72)	35 (74)	34 (72)	35 (73)
Body mass index, kg/m^2	40.5 (6.9)	42.1 (7.6)	40.7 (7.6)	43.9 (8.0)	44.1 (7.2)	46.1 (8.1)***
Waist circumference, cm	118.5 (19.6)	122.5 (28.5)	122.3 (19.6)	130.0 (20.2)	125.0 (24.0)	135.0 (17.4)***
fP-Glucose, mmol/L	5.0 (1.0)	5.6 (1.0)	5.9 (0.9)	5.9 (1.0)	5.7 (0.8)	6.6 (1.5)***
fS-Insulin, mU/L	4.9 (2.6)	7.9 (2.3)	11.1 (2.9)	13.7 (3.6)	18.0 (4.4)	25.2 (12.4)***
Hemoglobin A_{1C}, %	5.6 (0.8)	5.7 (0.6)	5.7 (0.8)	5.8 (0.9)	5.7 (0.5)	6.1 (1.0)***
fP-Total cholesterol, mmol/L	4.1 (1.2)	4.2 (1.4)	4.2 (1.3)	4.0 (1.2)	4.2 (1.4)	4.1 (1.0)
fP-HDL cholesterol, mmol/L	1.32 (0.42)	1.19 (0.43)	1.12 (0.36)	1.18 (0.43)	1.14 (0.39)	1.07 (0.40)
fP-LDL cholesterol, mmol/L	2.6 (1.2)	2.5 (1.3)	2.5 (0.9)	2.4 (1.4)	2.5 (1.3)	2.4 (1.0)
fP-Triglycerides, mmol/L	1.01 (0.50)	1.09 (0.69)	1.32 (0.88)	1.22 (0.55)	1.31 (0.61)	1.37 (0.69)***
P-Alanine aminotransferase, U/L	31 (24)	24 (17)	34 (19)	30 (23)	34 (21)	42 (32)**
P-Aspartate aminotransferase, U/L	33 (15)	26 (8)	32 (13)	27 (11)	26 (13)	34 (23)**
P-Gamma-glutamyltransferase, U/L	27 (43)	24 (14)	37 (27)	25 (22)	34 (39)	37 (31)**
P-Albumin, g/L	38.2 (3.3)	38.0 (3.2)	38.1 (4.0)	38.2 (4.8)	38.8 (3.6)	38.0 (4.1)
B-Platelets, 10^9/L	238 (69)	241 (95)	244 (78)	258 (86)	264 (81)	263 (89)
NAFLD, n (%)	19 (40)	21 (45)	36 (77)	37 (79)	29 (62)	45 (94)***
NASH, n (%)	1 (2)	2 (4)	4 (9)	9 (19)	4 (9)	10 (21)***
PNPLA3 rs738409 (CC/CG/GG), n	30/12/6	27/20/0	25/18/4	26/20/1	26/19/2	28/17/3
TM6SF2 rs58542926 (CC/CT/TT), n	42/6/0	41/6/0	39/7/1	42/4/1	40/7/0	44/4/0
MBOAT7 rs641738 (CC/CT/TT), n	13/27/8	17/22/8	19/18/10	17/21/9	16/25/6	13/26/9
HSD17B13 rs72613567 (AA/A0/00), n	1/17/30	1/20/26	1/18/28	4/15/28	2/18/27	0/14/34
MARC1 rs2642438 (AA/AG/GG), n	5/19/24	4/17/26	0/16/31	3/16/28	2/13/32	4/24/20

B, blood; f, fasting; HOMA-IR, homeostatic model assessment of insulin resistance; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; P, plasma, S, serum.

Categorical data are presented as n (%) and continuous data as median (interquartile range). Significances were determined by Kruskal-Wallis analysis of variance test for continuous or Pearson's χ^2 test for trend was used for categorical variables. *p ≤0.05, **p ≤0.01, ***p ≤0.001. f, fasting; P, plasma; S, serum; B, blood.

use of lipid-lowering medications and the GRS (Fig. 1J). Correlations between individual metabolites in these PCs and HOMA-IR are shown in Fig. 1K.

IR was associated with a surplus of multiple metabolites including aromatic (phenylalanine and tyrosine) and branched-chain (leucine, isoleucine and valine) amino acids, glycolytic intermediates (lactate, alanine and pyruvate), fatty acids, and lipoprotein components (such as total TG, very low-density lipoprotein-TG, very low-density lipoprotein-cholesterol, diameter of very low-density lipoprotein, high-density lipoprotein-TG, and triglyceride-to-phosphoglyceride ratio) (Fig. 1K, Table S2). To rule out the possibility that these associations were secondary to diabetes, we performed the same analyses after excluding individuals with diabetes, yielding identical results (Fig. S1A).

To determine whether substrate surplus was associated with increased substrate flux, rates of *in vivo* adipose tissue lipolysis and hepatic DNL were determined using stable isotope techniques in subsets of individuals (Table S1). To this end, we divided 41 individuals with directly quantified adipose tissue insulin sensitivity into 2 groups with HOMA-IR either above ('High HOMA-IR', n = 20) or below ('Low HOMA-IR', n = 21) the sex-specific median (Fig. 1L). The groups were similar with respect to the GRS (Fig. 1M), while the 'High HOMA-IR' group had significantly higher IHTGs than the 'Low HOMA-IR' group (Fig. 1N). Insulin sensitivity of adipose tissue lipolysis, as determined by suppression of plasma NEFA concentrations and whole-body glycerol turnover during a hyperinsulinemic-euglycemic clamp, was significantly impaired in the 'High HOMA-IR' compared to the 'Low HOMA-IR' group (Fig. 1O-P).

Hepatic DNL was determined by D_2O in 61 individuals (Table S1), who were also divided into groups with HOMA-IR either above ('High HOMA-IR', n = 31) or below ('Low HOMA-IR', n = 30) the sex-specific median. The groups were similar with respect to the GRS (4.7±0.2 *vs.* 4.7±0.3, p = 0.95). The 'High HOMA-IR' group had significantly higher IHTGs (8.0±1.2 *vs.* 2.5±0.7 %, p <0.001) and hepatic DNL (9.9±1.2 *vs.* 5.0±0.7 %, p <0.001, Fig. 1Q) than the 'Low HOMA-IR' group.

To determine whether substrate surplus also characterizes the 'MetComp' in non-bariatric, non-diabetic individuals, we studied an additional *Non-bariatric cohort* (n = 252). These individuals were divided into 4 quartiles based on sex-specific HOMA-IR (*H1–H4*). The groups were similar with respect to age, sex and GRS but the groups with high HOMA-IR had higher BMI and IHTGs compared to groups with low HOMA-IR (Fig. S2A-F). The serum metabolomic profile that associated with HOMA-IR was virtually identical to that seen in the *Discovery cohort* (Fig. S2G, Fig. 1K).

Genetic risk increases the features of NAFLD independent of IR

To investigate the association between the GRS and features of NAFLD, we divided the 284 participants of the *Discovery cohort* into 6 groups based on the GRS (*G1-G6*, Fig. 2A-B, Table 2). The GRS groups had similar HOMA-IR, prevalence of type 2 diabetes, age, sex, BMI, waist circumference and circulating concentrations of glucose, insulin, HbA1c (glycated hemoglobin), and lipids (Fig. 2C-D, Table 2). By definition, groups with high GRS had a higher number of risk alleles in *PNPLA3*, *TM6SF2*, *MBOAT7*, *HSD17B13* and *MARC1* than groups with low GRS (Table 2).

The groups with high GRS had more NAFLD and NASH than those with low GRS (Table 2). Liver histology showed marked increases in steatosis, lobular inflammation and activity, fibrosis, and total SAF score as a function of the GRS (Fig. 2E-J).

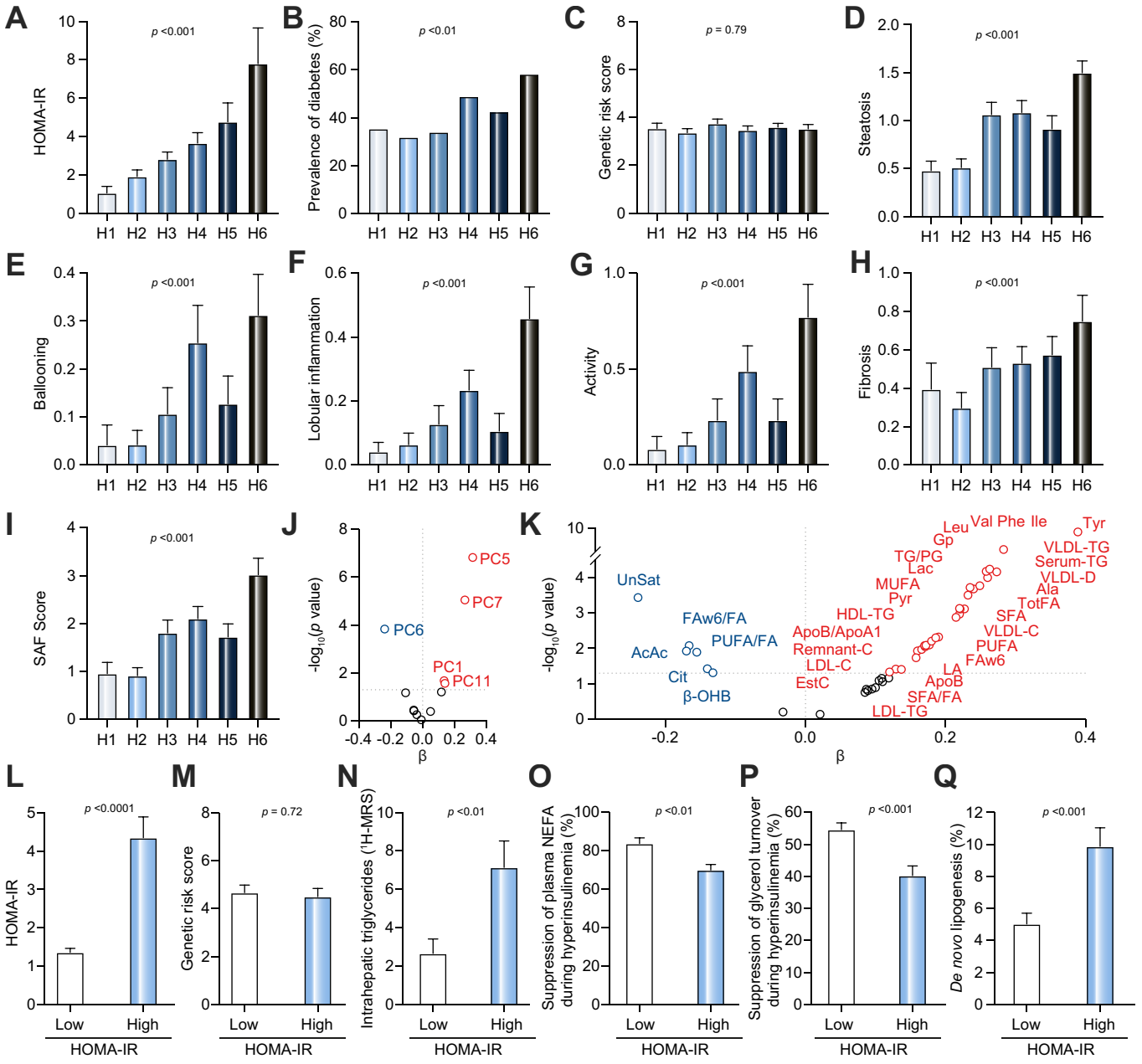

Fig. 1. IR increases the features of NAFLD and hepatic substrate supply independently of genetic risk. (A) HOMA-IR, (B) genetic risk score, (C) prevalence of type 2 diabetes, and histologically determined (D) steatosis, (E) ballooning, (F) lobular inflammation, (G) necroinflammatory activity, (H) fibrosis, and (I) total SAF score in quantiles based on sex-specific HOMA-IR (*H1–H6*). Significances were determined by the Jonckheere-Terpstra test for linear trend. (J) A volcano plot showing linear regression between HOMA-IR and the 12 PCs of metabolomics data, adjusted for age, sex, BMI, diabetes, lipid-lowering medication and GRS. Red denotes a positive and blue a negative correlation. (K) A volcano plot showing linear regression between HOMA-IR and metabolomics variables that were included in PC1, 5-7 and 11, adjusted for age, sex, BMI, diabetes, lipid-lowering medication and genetic risk score. Red denotes a positive and blue a negative correlation. Multiple testing was corrected by the Benjamini-Hochberg method. (L) HOMA-IR, (M) genetic risk score, (N) IHTGs as determined by ^{1}H-MRS, (O) suppression of plasma NEFA concentrations and (P) glycerol turnover during euglycemic hyperinsulinemia and (Q) hepatic *de novo* lipogenesis in independent cohorts. In L-P, 'Low HOMA-IR', n = 21 and 'High HOMA-IR', n = 20. In Q, 'Low HOMA-IR', n = 30 and 'High HOMA-IR', n = 31. Significances were determined by Student's *t* test. Data are in means±SEM. ^{1}H-MRS, proton magnetic resonance spectroscopy; AcAc, acetoacetate; Ala, alanine; Apo, apolipoprotein; β-OHB, β-hydroxybutyrate; C, cholesterol; Cit, citrate; D, diameter; EstC, cholesterol ester; FAw6, omega-6 fatty acid; Gp, glycoprotein acetyls; HDL, high-density lipoprotein; HOMA-IR, homeostatic model assessment of insulin resistance; IHTG, intrahepatic triglyceride; Ile, isoleucine; IR, insulin resistance; LA, linoleic acid; Lac, lactate; LDL, low-density lipoprotein; Leu, leucine; MUFA, monounsaturated fatty acid; NAFLD, non-alcoholic fatty liver disease; PC, principal component; PG, phosphoglyceride; Phe, phenylalanine; PUFA, polyunsaturated fatty acid; Pyr, pyruvate; SFA, saturated fatty acid; TG, triglyceride; TotFA, total fatty acids; Tyr, tyrosine; UnSat, unsaturation; Val, valine; VLDL, very low-density lipoprotein.

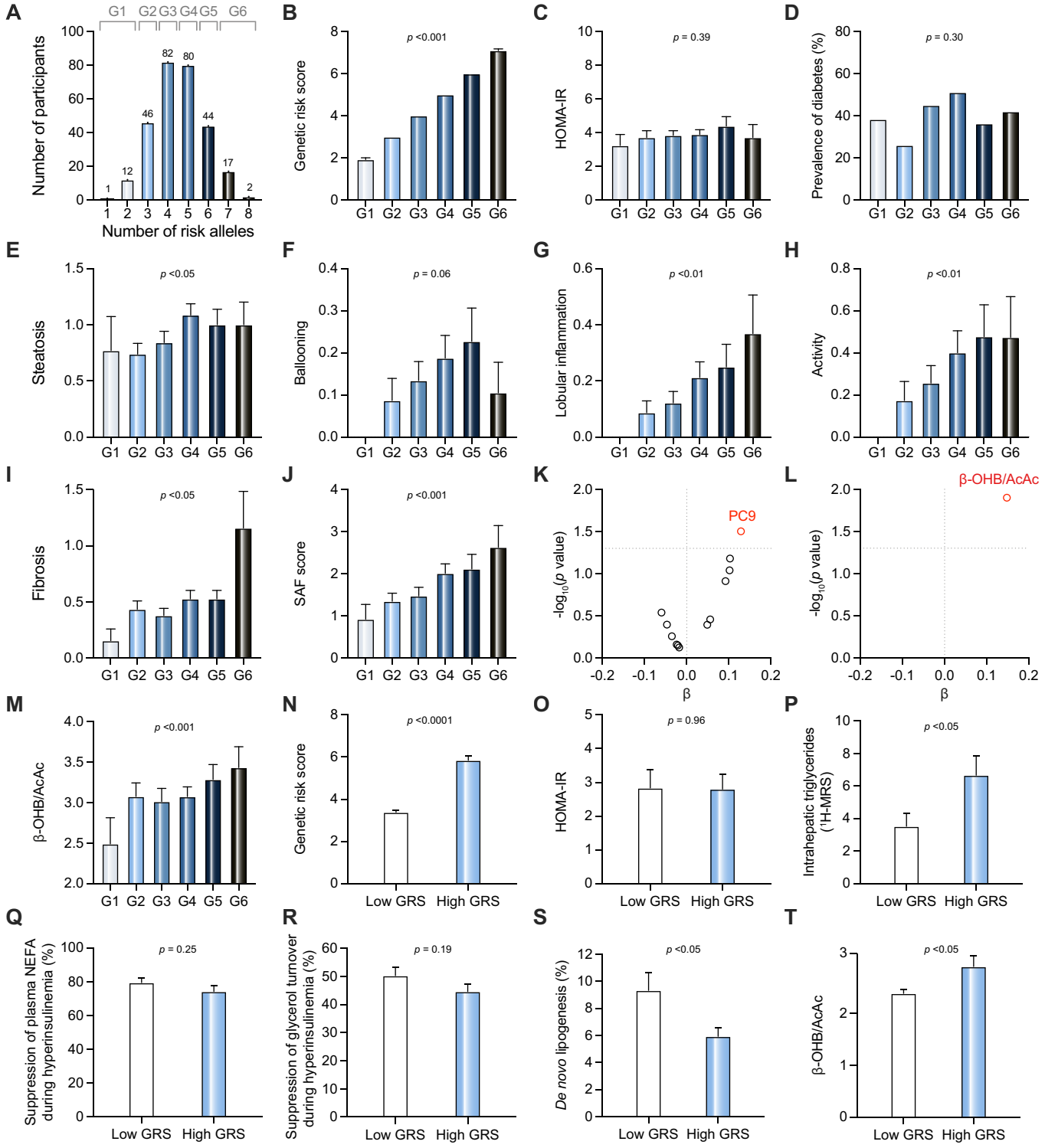

Fig. 2. Genetic risk increases the features of NAFLD and hepatic mitochondrial redox state independent of IR. (A) Number of participants with different number of risk alleles in *PNPLA3*, *TM6SF2*, *MBOAT7*, *HSD17B13* and *MARC1* and their allocation to genetic risk score groups (*G1-6*). *G1*: 1-2, *G2*: 3, *G3*:4, *G4*:5,*G5*:6, *G6:* 6-7 risk alleles. (B) GRS, (C) HOMA-IR, (D) type 2 diabetes, and histological (E) steatosis, (F) ballooning, (G) lobular inflammation, (H) necroinflammatory activity, (I) fibrosis, and (J) total SAF score in groups based on the genetic risk score (*G1-G6*). Significances were determined by the Jonckheere-Terpstra test for linear trend. (K) A volcano plot showing linear regression between GRS and the 12 PC factor scores of metabolomics data, adjusted for age, sex, BMI, diabetes, lipid-lowering medication and HOMA-IR. Red denotes a positive and blue a negative correlation. (L) A volcano plot showing linear regression between GRS and metabolomic variables that were included in PC9, adjusted for age, sex, BMI, diabetes, lipid-lowering medication and HOMA-IR. Red denotes a positive and blue a negative correlation. Multiple testing was corrected by the Benjamini-Hochberg method. (M) β-OHB/AcAc ratio in the GRS groups. Significance was determined by the Jonckheere-Terpstra test. (N) GRS, (O) HOMA-IR, (P) IHTGs, (Q) suppression of plasma NEFA and (R) glycerol turnover during euglycemic hyperinsulinemia, (S) hepatic *de novo* lipogenesis and (T) β-OHB/AcAc. In N-R, 'Low GRS', n = 21 and 'High GRS', n = 20. In S-T, 'Low GRS', n = 28 and 'High GRS', n = 33. Significances were determined by Student's *t* test. Data are means±SEM. AcAc, acetoacetate; β-OHB, β-hydroxybutyrate; GRS, genetic risk score; HOMA-IR, homeostatic model assessment of insulin resistance; IHTG, intrahepatic triglyceride; IR, insulin resistance; NAFLD, non-alcoholic fatty liver disease; NEFA, non-esterified fatty acid; PC, principal component.

Table 2. Clinical characteristics of the participants divided to groups based on genetic risk score (G1-G6).

	G1	G2	G3	G4	G5	G6
Group size, n	13	46	82	80	44	19
Age, years	49 (8)	49 (13)	51 (15)	49 (15)	49 (12)	47 (15)
Women, n (%)	9 (69)	40 (87)	64 (78)	56 (70)	26 (59)	12 (63)
Body mass index, kg/m^2	42.9 (11.3)	42.8 (10.3)	43.1 (10.3)	43.4 (7.7)	42.9 (6.1)	45.1 (10.0)
Waist circumference, cm	118.0 (33.5)	122.0 (72.0)	128.0 (18.8)	128.0 (21.5)	128.0 (20.6)	127.5 (29.3)
fP-Glucose, mmol/L	5.7 (1.4)	5.7 (0.8)	5.7 (1.2)	5.8 (1.5)	5.7 (0.9)	6.0 (1.1)
fS-Insulin, mU/L	7.9 (13.3)	12.4 (9.2)	12.0 (9.6)	13.3 (10.5)	14.7 (12.1)	11.0 (13.3)
Hemoglobin A_{1C}, %	5.8 (0.9)	5.7 (0.6)	5.7 (0.7)	5.7 (1.0)	5.7 (0.8)	5.7 (0.5)
fP-Total cholesterol, mmol/L	4.2 (1.3)	4.1 (1.6)	4.2 (0.9)	3.9 (1.3)	4.1 (1.4)	4.4 (1.3)
fP-HDL cholesterol, mmol/L	1.12 (0.54)	1.21 (0.32)	1.14 (0.47)	1.11 (0.39)	1.14 (0.42)	1.19 (0.39)
fP-LDL cholesterol, mmol/L	2.5 (1.1)	2.3 (1.4)	2.5 (0.9)	2.4 (1.4)	2.5 (1.2)	2.2 (1.3)
fP-Triglycerides, mmol/L	1.24 (1.65)	1.15 (0.74)	1.28 (0.65)	1.29 (0.78)	1.07 (0.55)	1.22 (0.46)
P-Alanine aminotransferase, U/L	32 (25)	27 (17)	34 (26)	30 (27)	35 (21)	34 (31)
P-Aspartate aminotransferase, U/L	26 (12)	25 (8)	30 (13)	30 (14)	31 (12)	36 (25)**
P-Gamma-glutamyltransferase, U/L	25 (28)	24 (20)	37 (35)	29 (28)	25 (26)	41 (53)
P-Albumin, g/L	38.1 (4.0)	37.4 (2.7)	38.9 (4.1)	38.3 (3.8)	38.6 (3.6)	37.2 (4.1)*
B-Platelets, 10^9/L	216 (76)	260 (60)	262 (87)	244 (83)	233 (59)	232 (97)*
NAFLD, n (%)	6 (46)	29 (63)	49 (60)	60 (75)	30 (68)	13 (68)*
NASH, n (%)	0 (0)	3 (7)	8 (10)	9 (11)	8 (18)	2 (11)*
PNPLA3 rs738409 (CC/CG/GG), n	13/0/0	37/9/0	54/28/0	40/38/2	17/22/5	1/9/9***
TM6SF2 rs58542926 (CC/CT/TT), n	13/0/0	46/0/0	77/5/0	70/10/0	31/11/2	11/8/0***
MBOAT7 rs641738 (CC/CT/TT), n	10/2/1	27/19/0	39/37/6	14/50/16	4/22/18	1/9/9***
HSD17B13 rs72613567 (AA/A0/00), n	3/5/5	4/26/16	2/34/46	0/25/55	0/11/33	0/1/18***
MARC1 rs2642438 (AA/AG/GG), n	8/4/1	4/32/10	5/34/43	1/27/52	0/6/38	0/2/17***

B, blood; f, fasting; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; P, plasma, S, serum.
Categorical data are presented as n (%) and continuous data as median (interquartile range). Significances were determined by Kruskal-Wallis analysis of variance test for continuous or Pearson's χ^2 test for trend was used for categorical variables. *p ≤0.05, **p ≤0.01, ***p ≤0.001.

Genetic risk increases hepatic mitochondrial redox state

To gain insights into the pathogenesis of NAFLD driven by genetic risk, we analyzed associations between the GRS and NMR metabolomics. Only PC9 correlated significantly with the GRS independently of age, sex, BMI, HOMA-IR, diabetes and use of lipid-lowering medication (Fig. 2K). This PC included only the β-hydroxybutyrate/acetoacetate ratio (β-OHB/AcAc), which is a *bona fide* marker of hepatic mitochondrial redox state (Fig. 2L, Table S3).[23–26] The β-OHB/AcAc ratio correlated significantly and dose-dependently with the GRS, independently of age, sex, BMI, HOMA-IR, diabetes and use of lipid-lowering medication (Fig. 2M, Table S2). β-OHB/AcAc did not associate with IR or type 2 diabetes (Fig. S3). To rule out the possibility that the association of the GRS with histological liver injury and β-OHB/AcAc was secondary to a single risk variant, we conducted sensitivity analyses using alternative GRSs, calculated by excluding a single risk variant at a time, yielding unchanged results (Table S4).

We also examined the impact of the GRS on rates of lipolysis and DNL. For this, the 41 individuals in whom lipolysis was determined were divided into groups of equal sizes with either 1-4 risk alleles ('Low GRS', n = 21) or 5-8 risk alleles ('High GRS', n = 20) (Fig. 2N). The groups were similar with respect to HOMA-IR but the 'High GRS' group had significantly higher IHTGs than the 'Low GRS' group (Fig. 2O-P). Insulin sensitivity of adipose tissue lipolysis, as determined by the suppression of plasma NEFA concentrations and whole-body glycerol turnover during euglycemic hyperinsulinemia, was similar between the GRS groups (Fig. 2Q-R).

To examine the impact of genetic risk on hepatic DNL, the 61 individuals in whom DNL was measured were divided into groups with either 1-4 risk alleles ('Low GRS', n = 28) or 5-8 risk alleles ('High GRS', n = 33). The groups were similar with respect to HOMA-IR (2.6±0.4 *vs.* 2.8±0.6, p = 0.84), but the 'High GRS' group had a significantly higher IHTGs than the 'Low GRS' group (6.7±1.2 *vs.* 3.5±0.8 %, p <0.05). Hepatic DNL was significantly lower in the 'High GRS' group compared to the 'Low GRS' group (5.9±0.7 *vs.* 9.3±1.3 %, p <0.05, Fig. 2S). The 'High GRS' group also had a significantly higher β-OHB/AcAc ratio than the 'Low GRS' group (2.91±0.22 *vs.* 2.39±0.08, p <0.05, Fig. 2T).

The *Non-bariatric cohort* (n = 252) was divided into 4 groups based on the GRS (*G1-G4*). These groups were similar with respect to age, sex, BMI, and HOMA-IR, but the groups with high GRS had higher IHTGs compared to the groups with low GRS (Fig. S2H-M). GRS was not associated with serum metabolites in this cohort after adjusting for age, sex, BMI, lipid-lowering medication, HOMA-IR and multiple comparisons (Fig. S2N).

Hepatic mitochondrial redox state associates with features of NAFLD

We asked whether the β-OHB/AcAc ratio was related to histological features of NAFLD. The β-OHB/AcAc ratio was significantly associated with steatosis (14%), ballooning (23%), lobular inflammation (20%), activity (23%), fibrosis (17%) and SAF score (23% increase per 1 SD increase in the β-OHB/AcAc ratio) in the *Discovery cohort* – independent of age, sex, BMI and diabetes (n = 284, Table 3). The β-OHB/AcAc ratio was also significantly associated with steatosis, portal inflammation and fibrosis in the *Validation cohort* (n = 208, Table 3).

We examined whether histological features of NAFLD were proportional to the β-OHB/AcAc ratio in groups with different numbers of risk alleles and found this to be the case (R^2 up to 0.83, Fig. S4). Sensitivity analysis showed that the SAF score and the β-OHB/AcAc ratio were both related to the GRS and that these associations were not secondary to any particular genetic variant (Table S4). Although not included in the GRS due to its pleiotropic effects on IR, we also analyzed the rs1260326 variant in the glucokinase regulator, which has also been associated with

Table 3. Hepatic mitochondrial redox state associates with histological features of NAFLD.

	Beta	Lower 95%	Upper 95%	*p* value
Discovery cohort (n = 284)				
Steatosis	0.139	0.022	0.303	0.023
Ballooning	0.232	0.279	0.839	<0.001
Lobular inflammation	0.195	0.178	0.736	0.001
Activity	0.227	0.136	0.431	<0.001
Fibrosis	0.165	0.067	0.386	0.005
SAF score	0.230	0.060	0.186	<0.001
Validation cohort (n = 208)				
Steatosis	0.167	0.029	0.323	0.019
Ballooning	0.035	-0.221	0.369	0.621
Lobular inflammation	0.047	-0.153	0.305	0.514
Portal inflammation	0.153	0.087	1.396	0.027
Fibrosis	0.145	0.006	0.414	0.044

NAFLD, non-alcoholic fatty liver disease; SAF, steatosis-activity-fibrosis.
Betas between serum β-hydroxybutyrate/acetoacetate ratio and liver histology were calculated by linear regression, adjusted for age, sex, BMI and diabetes.

NAFLD,[27] and found it to be associated with the β-OHB/AcAc ratio (data not shown).

IR and hepatic mitochondrial redox state additively increase the features of NAFLD

To examine whether hepatic IR and hepatic mitochondrial redox state confer additive effects on the features of NAFLD, we divided the participants into 4 non-overlapping groups based on the sex-specific medians of HOMA-IR and β-OHB/AcAc ('Low HOMA-IR, Low β-OHB/AcAc', 'Low HOMA-IR, High β-OHB/AcAc', 'High HOMA-IR, Low β-OHB/AcAc' and 'High HOMA-IR, High β-OHB/AcAc') (Table S5).

The groups were similar with respect to size, age, and sex (Table S5). By definition, the 'High HOMA-IR' groups had higher HOMA-IR than the 'Low HOMA-IR' groups and the 'High β-OHB/AcAc' groups had higher β-OHB/AcAc than 'Low β-OHB/AcAc' groups (Fig. 3A-B). The 'High HOMA-IR' groups had higher BMI, waist circumference and circulating concentrations of glucose, insulin and triglycerides than the 'Low HOMA-IR' groups (Table S5).

The steatosis grade was higher in the 'High HOMA-IR' than the 'Low HOMA-IR' groups, irrespective of β-OHB/AcAc (Fig. 3C). Scores of ballooning, lobular inflammation and activity increased as a function of both β-OHB/AcAc and HOMA-IR (Fig. 3D-F). The prevalence of NASH was 3-4-fold higher in the groups with either isolated high HOMA-IR (11.4%) or isolated high β-OHB/AcAc (7.1%) than the group with none of these factors (2.8%), and 7-fold higher in the presence of both high HOMA-IR and high β-OHB/AcAc (20.8%, p <0.01) (Table S5). Thus, IR and hepatic mitochondrial redox state synergistically increase the features of NASH. Fibrosis was also markedly higher in the groups with either high HOMA-IR or high β-OHB/AcAc, or both, compared to the 'Low HOMA-IR, Low β-OHB/AcAc' group (Fig. 3G).

IR and hepatic mitochondrial redox state additively increase serum glycolytic intermediates and aromatic amino acids

Finally, we sought to identify a metabolic fingerprint underlying the synergistic effects of IR and hepatic mitochondrial redox state on NAFLD. Fig. S6 shows normalized z-scores of serum metabolite concentrations in the groups. The 'High HOMA-IR, High β-OHB/AcAc' group, characterized by a high prevalence of NASH (Fig. 3D-F, Table S5), had markedly increased serum concentrations of glycolytic intermediates (lactate, pyruvate and alanine) and aromatic amino acids (tyrosine and phenylalanine) (Fig. S6) compared to the 'Low HOMA-IR, Low β-OHB/AcAc' group, characterized by a low prevalence of NASH.

Discussion

Herein, we show that both IR and high genetic risk, defined by the number of risk alleles in *PNPLA3*, *TM6SF2*, *MBOAT7*, *HSD17B13* and *MARC1*, increase the features of NAFLD independently of each other and other known risk factors. The 'MetComp' was characterized by a substrate surplus and flux (adipose tissue lipolysis and hepatic DNL), while the 'GenComp' was not. In contrast, the 'GenComp' was associated with an increased hepatic mitochondrial redox state, as determined by the serum β-OHB/AcAc ratio, and inhibition of hepatic pathways dependent on TCA cycle activity, such as DNL. Individuals with both 'MetComp' and 'GenComp' had both abnormalities and more severe NAFLD than those with only one or none of these factors.

The associations of NAFLD with IR and redox metabolism[28,29] and specific metabolite profiles of distinct NAFLD-related variants have been characterized previously.[12] However, to the best of our knowledge, there are no previous studies investigating the mechanisms underlying the distinct components of NAFLD, *i.e.* IR and cumulative genetic risk. We extend previous studies by showing that the mechanisms underlying 'MetComp' and 'GenComp' of NAFLD are fundamentally different.

'MetComp' was characterized by increased circulating concentrations of NEFA and substrates of the DNL pathway, including glucose, glycolytic intermediates and branched-chain and aromatic amino acids (Fig. 1K). These changes reflected a higher flux of these substrates as shown by direct measurement of lipolysis and DNL, the 2 major pathways contributing to IHTGs (Fig. 1O-Q). The finding of accelerated adipose tissue lipolysis, which in absolute terms is the major pathway contributing to IHTG in NAFLD, is consistent with previous studies involving patients with NAFLD and NASH. Although the absolute contribution of DNL to IHTG is lower than that of lipolysis, it is in relative terms the most abnormal pathway in NAFLD.[30,31] The present data show that DNL is specifically increased in 'MetComp' associated with IR and obesity (Fig. 1Q). Several factors could contribute to this phenomenon. These include stimulation of DNL by insulin and substrate surplus, reflecting at least in part increased intake of simple sugars and amino acids. Obesity is

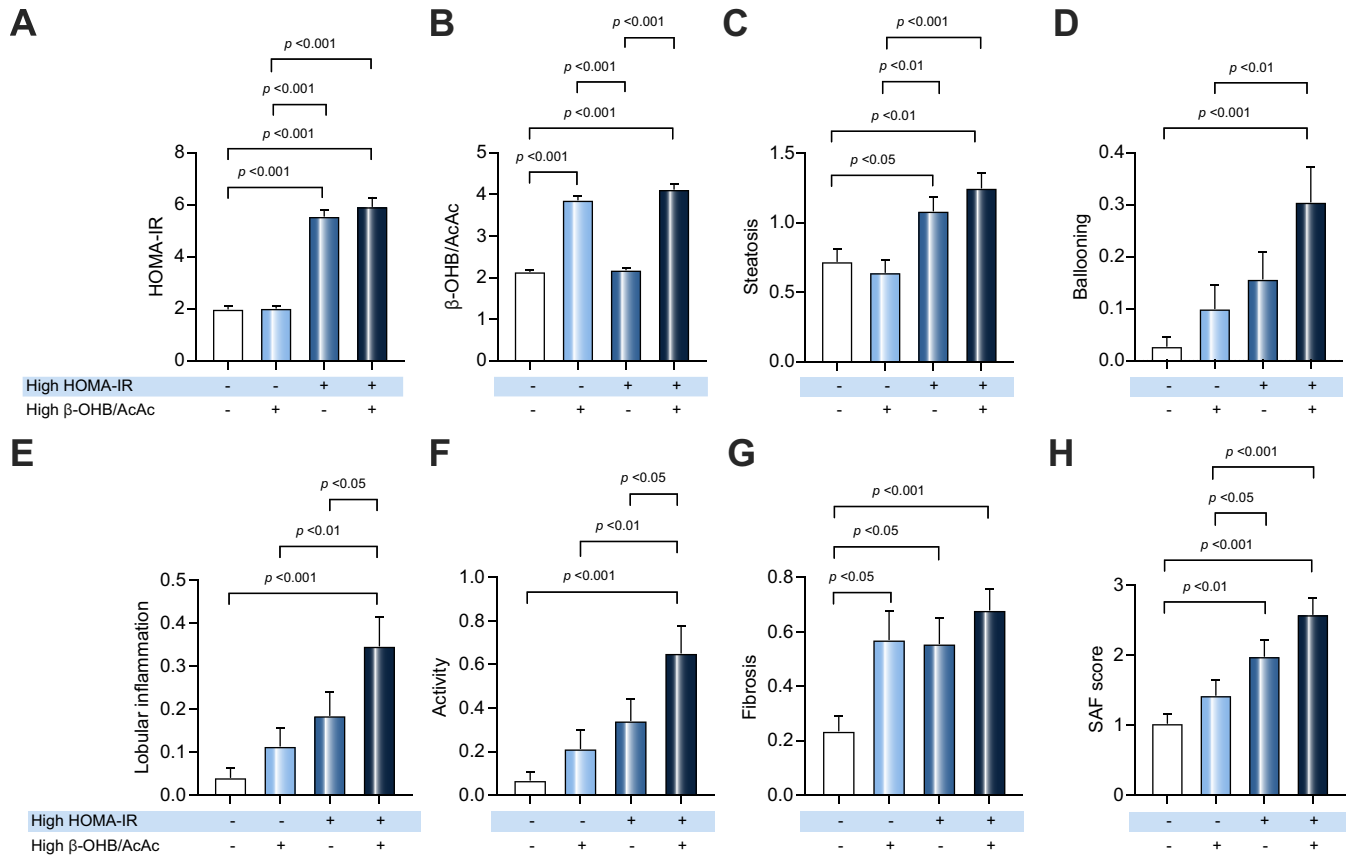

Fig. 3. IR and hepatic mitochondrial redox state additively increase the features of NAFLD. (A) HOMA-IR, (B) β-hydroxybutyrate/acetoacetate ratio (β-OHB/AcAc), and histologically determined (C) steatosis, (D) ballooning, (E) lobular inflammation, (F) necroinflammatory activity, (G) fibrosis, and (H) total SAF score in quantiles based on sex-specific medians of β-OHB/AcAc and HOMA-IR. 'Low HOMA-IR, Low β-OHB/AcAc', n = 72, 'Low HOMA-IR, High β-OHB/AcAc', n = 70, 'High HOMA-IR, Low β-OHB/AcAc', n = 70, 'High HOMA-IR, High β-OHB/AcAc', n = 72. Data are in means±SEM. Significances: Kruskal-Wallis test followed by Benjamini-Hochberg correction. AcAc, acetoacetate; β-OHB, β-hydroxybutyrate; HOMA-IR, homeostatic model assessment of insulin resistance; IR, insulin resistance; NAFLD, non-alcoholic fatty liver disease; SAF, steatosis-activity-fibrosis.

perhaps the single most important risk factor for the development of IR and accumulation of IHTG.[2]

DNL produces malonyl-CoA, which is an allosteric inhibitor of carnitine palmitoyltransferase, an essential enzyme transporting fatty acids from the cytosol to the mitochondria.[32] This negative feedback might protect the liver from excessive mitochondrial fatty acid uptake and a subsequent increase in mitochondrial redox state secondary to β-oxidation. Consistent with this interpretation, 'MetComp' was not associated with changes in mitochondrial redox state (Fig. S3).

Simple polygenic risk scores calculated as a sum of risk-increasing alleles have emerged as powerful instruments in the study of NAFLD. A GRS calculated using *PNPLA3, TM6SF2* and *MBOAT7* variants correlated with IHTGs[6] and increased liver damage.[33] In a population-based study, a high GRS consisting of *PNPLA3, TM6SF2* and *HSD17B13* conferred up to 12- and 29-fold risks of cirrhosis and hepatocellular carcinoma, respectively.[9] Here, we calculated a GRS using 5 risk-increasing alleles which have been robustly associated with the full spectrum of NAFLD (*PNPLA3*, *TM6SF2*, *MBOAT7*, *HSD17B13* and *MARC1*). GRSs have the advantage compared to traditional cohort studies that there is no risk of reverse causation and therefore the metabolic effects are secondary to genetic variation, not *vice versa*.

'GenComp' was associated with all features of NAFLD (Fig. 2E-J) but not IR (Fig. 2C, 2Q-R), in keeping with previous reports showing no effect of individual risk-modifying polymorphisms on IR.[4,5,7,34,35] One study associated genetic risk of NAFLD with the risk of type 2 diabetes, but this association was attenuated after adjusting for liver damage, suggesting that diabetes was due to secondary effects of cirrhosis rather than a direct genetic effect.[33] Unlike 'MetComp', 'GenComp' was not associated with circulating substrate surplus of NEFA, glucose, glycolytic intermediates or amino acids, or their flux as measured by stable isotopes (Fig. 2L, Q-S). Instead, 'GenComp' was strongly and dose-dependently associated with a single metabolic variable, the β-OHB/AcAc ratio (Fig. 2M, T), which is a well-established and specific marker of hepatic mitochondrial redox state (*i.e.* $NADH/NAD^+$ ratio).[25] This variable correlated strongly with histological features of NAFLD in 2 independent cohorts, independently of age, sex, BMI and diabetes (Table 3).

In contrast to 'MetComp', individuals with high GRS and IHTG content (Fig. 2N, P) had lower hepatic DNL (Fig. 2S) than those with low GRS. This finding is consistent with a lack of the main drivers of DNL of 'MetComp' (*i.e.* hyperinsulinemia and substrate surplus) in 'GenComp'. The first precursor in DNL is citrate, which is derived from the TCA cycle.[32] Our data are consistent with a model in which individuals with a high GRS have

decreased DNL secondary to increased hepatic mitochondrial redox state (Fig. 2M, T) and subsequent inhibition of the TCA cycle.[26] We have recently shown by a triple tracer technique that a ketogenic diet, which decreases DNL,[36] increases hepatic mitochondrial redox state secondary to high β-oxidation of intrahepatic fatty acids.[16] These data suggest hepatic mitochondrial redox state as a potential mechanism by which genetic risk factors predispose individuals to severe liver disease, and as a novel mechanism regulating DNL.

A high mitochondrial redox state can be secondary to inherited causes such as inborn errors in the mitochondrial electron transport chain,[37] or to acquired causes such as surplus NADH originating from β-oxidation of fatty acids.[16] Since individuals with high GRS had lower hepatic DNL than those with low GRS, they likely had lower hepatic malonyl-CoA concentrations and thereby less inhibition for the entry of fatty acids to mitochondria. Subsequent stimulation of mitochondrial β-oxidation and production of NADH are predicted to increase hepatic mitochondrial redox state and inhibit TCA cycle activity.

Only some patients with steatosis develop NASH. Thus, although IR predisposes to NASH, it is unclear whether IR is merely permissive in genetically susceptible individuals. In the present study, IR and hepatic mitochondrial redox state had additive effects on features of NASH (Fig. 3). This additive effect fits the two-hit model of NASH,[38] in which the development of NASH requires steatosis (first hit) and then a second hit which impairs mitochondrial function. Several human studies have reported decreases in mitochondrial respiration in NASH[13–15] but the underlying mechanisms have remained unclear. Increased hepatic mitochondrial redox state due to 'GenComp' might explain the observed decreases in mitochondrial respiration in NASH, since it inhibits the TCA cycle.[25] Of interest, alcohol consumption also increases the β-OHB/AcAc ratio, which contributes to the development of alcohol-related liver disease.[17] In addition, the β-OHB/AcAc ratio predicts mortality after acute liver failure,[18] alcoholic hepatitis,[19] and liver transplantation.[20]

Strengths of our study include the unbiased metabolomic approach, state-of-the-art metabolic characterization and replication of key findings in independent cohorts. Another strength is the strong relationship between hepatic mitochondrial redox state and liver damage. Our study also has limitations, including use of a simplistic GRS and a relatively small sample size for some genotypes and advanced disease stages. While the stable isotope tracer studies are amongst the largest, they were performed in small subgroups. Furthermore, the dichotomy between 'MetComp' and 'GenComp' can be considered artificial, as these components frequently co-exist (Fig. 3). We do not argue that these components comprise 2 entirely different diseases, but rather aim to provide conceptual insights into the contribution of these components to the pathogenesis of NAFLD. We like to think of this as analogous to hypertension and dyslipidemia: both can co-exist but also contribute independently to the pathogenesis of atherosclerosis.

To conclude, we suggest that 'MetComp' and 'GenComp' contribute to NAFLD via distinct mechanisms. 'MetComp' is characterized by substrate surplus, which may reflect increased energy intake as well as adipose tissue IR and leads to increased IHTG synthesis from glucose and amino acids (DNL) and from NEFA originating from adipose tissue lipolysis. The 'GenComp' lacks substrate surplus and accelerated fluxes but is characterized by increased hepatic mitochondrial redox state and therefore inhibition of hepatic pathways dependent on mitochondrial TCA cycle activity, such as DNL. These findings may have implications with respect to the diagnosis and treatment of NAFLD.

Abbreviations

AcAc, acetoacetate; β-OHB, β-hydroxybutyrate; DNL, *de novo* lipogenesis; GenComp, genetic component; GRS, genetic risk score; HOMA-IR, homeostatic model assessment of insulin resistance; HSD17B13, hydroxysteroid 17-beta dehydrogenase 13; IHTG, intrahepatic triglyceride; IR, insulin resistance; MARC1, mitochondrial amidoxime-reducing component 1; MBOAT7, membrane bound O-acyltransferase domain containing 7; MetComp, metabolic component; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; NEFA(s), non-esterified fatty acids; NMR, nuclear magnetic resonance; PC, principal component; PNPLA3, patatin-like phospholipase domain containing 3; SAF, steatosis-activity-fibrosis; TCA, tricarboxylic acid; TG, triglyceride; TM6SF2, transmembrane 6 superfamily member 2.

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Conflict of interest

The authors declare no conflicts of interest that pertain to this work.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

P.K.L. and H.Y.-J. designed the study. P.K.L. performed clinical studies, analyzed and interpreted data and drafted the manuscript. S.Q., N.A. and K.P. interpreted data. V.M., M.A–K. and J.P. provided validation data. A.J., H.S. and A.K.P. performed liver biopsies. A.H. and T.E.L. analyzed and interpreted MR data. M.G. and A.G. performed glycerol tracer analyses. L.H. performed DNL analyses. M.O.-M. performed genotyping. J.A. analyzed liver histology. H.Y.-J. supervised the study. All authors revised and approved the manuscript.

Data availability statement

Due to the confidentiality of data, the data which support the findings of this study are only available in a redacted form upon request.

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Supplementary data

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Author names in bold designate shared authorship

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