# Role of Patatin-Like Phospholipase Domain–Containing 3 Gene for Hepatic Lipid Content and Insulin Resistance in Diabetes

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## OBJECTIVE

**The rs738409(G) single nucleotide polymorphism (SNP) in the patatin-like phospholipase domain–containing 3 (*PNPLA3*) gene associates with increased risk and progression of nonalcoholic fatty liver disease (NAFLD). As the recently described severe insulin-resistant diabetes (SIRD) cluster specifically relates to NAFLD, this study examined whether this SNP differently associates with hepatic lipid content (hepatocellular lipids [HCL]) and insulin sensitivity in recent-onset diabetes.**

## RESEARCH DESIGN AND METHODS

**A total of 917 participants in the German Diabetes Study (GDS) underwent genotyping, hyperinsulinemic-euglycemic clamps with stable isotopic tracer dilution, and MRS.**

## RESULTS

**The G allele associated positively with HCL ($\beta = 0.36$, $P < 0.01$), independent of age, sex, and BMI across the whole cohort, but not in the individual clusters. Those with SIRD exhibited lowest whole-body insulin sensitivity compared with those with severe insulin-deficient (SIDD), moderate obesity-related (MOD), moderate age-related (MARD), and severe autoimmune diabetes (SAID) clusters (all $P < 0.001$). Interestingly, the SIRD group presented with higher prevalence of the rs738409(G) SNP compared with other clusters and the glucose-tolerant control group ($P < 0.05$). HCL was higher in the SIRD group (median 13.6% [1st quartile 5.8; 3rd quartile 19.1] compared with the MOD (6.4 % [2.1; 12.4], $P < 0.05$), MARD (3.0% [1.0; 7.9], $P < 0.001$), SAID (0.4% [0.0; 1.5], $P < 0.001$), and glucose-tolerant (0.9% [0.4; 4.9), $P < 0.001$) group. Although the *PNPLA3* polymorphism did not directly associate with whole-body insulin sensitivity in SIRD, the G-allele carriers had higher circulating free fatty acid concentrations and greater adipose tissue insulin resistance compared with noncarriers (both $P < 0.001$).**

## CONCLUSIONS

**Members of the SIRD cluster are more frequently carriers of the rs738409(G) variant. The SNP-associated adipose tissue insulin resistance and excessive lipolysis may contribute to their NAFLD.**

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Ectopic triglyceride storage in the liver plays an important role in the development of insulin resistance and contributes to the pathogenesis of type 2 diabetes (1–3). In line, previous cross-sectional analyses showed that patients with type 2 diabetes frequently have increased hepatocellular lipids (HCL) (3), associated with higher insulin resistance. Nevertheless, recent studies indicate that diabetes comprises several subtypes (clusters) with distinct heterogeneous features (4,5), suggesting that the traditional diabetes classification calls for revision. We have shown that the severe insulin-resistant cluster (SIRD) specifically associates with an increased prevalence of nonalcoholic fatty liver disease (NAFLD) and surrogate markers of liver fibrosis (5). The molecular mechanisms underlying this association between HCL and insulin resistance may involve inadequate mitochondrial adaptation to higher lipid flux (6) and subsequent cellular accumulation of lipid metabolites such as diacylglycerols, which inhibit proximal insulin signaling (1), or ceramides, which may act via inflammatory pathways (7).

While the current paradigm infers that acquired insulin resistance of adipose tissue and/or liver primarily leads to hepatic steatosis (3), there is growing evidence that certain gene variants relate to liver fat deposition independently of insulin resistance or even cause NAFLD. Genome-wide association studies revealed an important role of the rs738409 single nucleotide polymorphism (SNP) referring to a missense variant, which alters the protein sequence (I148M) in the patatin-like phospholipase domain–containing 3 (*PNPLA3*) gene in NAFLD (8). The G allele in this SNP associates tightly with increased risk of NAFLD (9) and also favors steatohepatitis and hepatic fibrosis (10), particularly in the context of metabolic diseases (11), subsequently contributing to increased mortality (2,12). *PNPLA3* is expressed predominately in liver and to less extent in adipose tissue and encodes a transmembrane protein composed of 481 amino acid residues. *PNPLA3* expression is controlled by changes in nutrition and energy balance (13). While this protein likely acts primarily as a triglyceride lipase (14), it may also have lipogenic properties (15). In line, the mutation causes a loss of function without excluding the possibility of additional effects of this enzyme (16). The link between *PNPLA3* and insulin resistance remains controversial, as studies show no relationship with insulin resistance (17), suggest an association of the gene expression with hepatic insulin resistance (15), or describe insulin resistance as a consequence of liver damage due to NAFLD progression to fibrosis (18).

Against this background, this study aimed *1*) at determining whether specific diabetes subtypes, particularly the SIRD cluster, exhibit differences in the prevalence of the *PNPLA3* polymorphism and *2*) at revealing possible relationships of this genotype with metabolic features such as whole-body and tissue-specific insulin sensitivity. We hypothesized that the common *PNPLA3* polymorphism associates with changes in HCL independent of insulin resistance, particularly in patients with SIRD.

## RESEARCH DESIGN AND METHODS

### Participants

All volunteers were recruited from the prospective multicenter German Diabetes Study (GDS), which includes individuals with recently diagnosed diabetes and individuals who are glucose tolerant. The GDS is approved by the ethics committee of the Medical Faculty of Heinrich Heine University Düsseldorf (reference number 4508), registered at ClinicalTrials.gov (identifier number: NCT01055093), and performed according to the Declaration of Helsinki as reported previously (19). Briefly, diagnosis of diabetes was based on current criteria (20). The control group (CON) was comprised of healthy humans presenting neither with dysglycemia, excluded by a 75-g oral glucose tolerance test (20), nor with first-degree relatives with known diabetes. Exclusion criteria comprise poorly controlled diabetes ($HbA_{1c}$ >9%, 75 mmol/mol), pregnancy, acute or severe chronic heart, kidney or liver diseases (clinical signs/transaminases twofold above upper limit of normal range), acute systemic inflammation (clinical signs/hs-CRP >1 mg/dL), immunosuppressive treatment, and alcohol abuse (by questionnaire). Oral glucose-lowering medication was withdrawn 3 days prior to all metabolic tests.

This cross-sectional analysis was performed for 917 Caucasian individuals, of whom 834 had diabetes and 83 were glucose tolerant (CON). Individuals with diabetes were categorized into clusters based on an algorithm including age, BMI, glycemia, homeostasis model estimates (HOMA of insulin resistance and HOMA of β-cell function), and GAD antibodies (4,5). Analyses of main metabolic parameters were also performed upon categorization as to type 1 or type 2 diabetes, as defined by the American Diabetes Association (20).

### Laboratory Analyses

Blood samples were analyzed in in the biomedical laboratory of the center as previously described (19). Adipose tissue insulin resistance index was calculated from fasting concentrations of insulin and free fatty acids (FFA) (21). The fatty liver index (FLI), AST-to-platelet ratio index (APRI), FIB-4 index, and NAFLD fibrosis score were computed from routine laboratory parameters (22). GAD antibodies were measured as described previously (5).

### Modified Botnia Clamp Test

Patients underwent hyperinsulinemic-euglycemic clamp tests with frequent measurements of blood glucose, C-peptide, and insulin (19). The clamp test was preceded by an intravenous glucose tolerance test (1 mL 30% glucose infusion/kg body wt) to assess β-cell function. A priming insulin dose was then applied for 10 min (10 mU * kg body $wt^{-1}$ * $min^{-1}$) followed by constant infusion of 1.5 mU * kg body $wt^{-1} * min^{-1}$ (Insuman Rapid; Sanofi, Frankfurt, Germany). Blood glucose concentration was maintained at 90 mg/dL by a variable 20% glucose infusion. Whole-body insulin sensitivity (M value) was assessed from mean steady-state glucose infusion rates and glucose space correction (19). In a representative subgroup ($n = 218$), endogenous glucose production (EGP) was measured before and during the hyperinsulinemic-euglycemic clamp test using a continuous infusion of [6,6-$^2H_2$]glucose (19). Fasting hepatic insulin sensitivity was computed from EGP and insulin levels after overnight fasting. Insulin-mediated EGP suppression was calculated from the ratio between EGP during clamp and fasted EGP and is given as %.

### MRS

HCL was quantified using $^1$H-MRS on a 3-T magnetic resonance scanner (23). Both water-suppressed and nonsuppressed $^1$H-MRS were taken in the identical voxel within the homogeneous part of liver tissue, avoiding major vessels and gallbladder, with a volume of interest of 25 * 25 * 25 $mm^3$. All liver spectra were processed using jMRUI software. HCL

content was calculated from the methylene peak at 1.3 ppm in water-suppressed spectra, relative to the sum of the methylene and water peaks at 4.7 ppm in water nonsuppressed spectra. Abdominal subcutaneous and visceral adipose tissue volumes were quantified by T1-weighted axial fast spin echo (24).

### Genomics

Genomic DNA was extracted from whole blood, and genotyping was conducted using real-time PCR–based allelic discrimination with probe-based genotyping assay for the rs738409 SNP in the *PNPLA3* gene (Thermo Fisher Scientific, Darmstadt, Germany) (25). The genotype concordance of >99.8% was determined using TaqMan Genotyper software v.1.3 (Thermo Fisher Scientific). The genetic variant was in Hardy-Weinberg equilibrium, and resequencing of 1% of randomly chosen individuals was done for data validation and quality control, confirming genotyping results by 100%.

### Metabolomics

Targeted identification and quantification of blood metabolites were performed using gas chromatography–, liquid chromatography–, and flow injection analysis–mass spectrometry and mass spectrometric procedures with quality-control measures as previously described (25). This analysis focused specifically on lipidomics (127 metabolites). Annotations were used to differentiate between acyl-acyl bonds (aa) and acyl-alkyl bonds (ae). None of the studied metabolites were exclusively detected in one specific cluster. No data were available for the glucose-tolerant CON group.

### Statistics

Data are presented as mean ± SD or median (1st quartile; 3rd quartile) for continuous variables and % for categorical variables. Skewed data were log transformed before analysis (M value, alanine aminotransferase, AST, hs-CRP, HCL, triglycerides). The k-means clustering via nearest centroid approach (4) allowed for assigning each patient to a predefined cluster (4,5). Associations between parameters were evaluated using linear regression analyses and were adjusted for age, sex, and BMI. As SIRD was the focus of this analysis, comparisons with other clusters were adjusted using the Dunnett test. In the analysis of metabolites, *P* values were additionally adjusted for multiple comparisons of 127 traits using Bonferroni correction. *P* values <5% were considered to indicate statistically significant differences or correlations. Statistical analyses were performed with SAS (version 9.4; SAS Institute, Cary, NC). Figures were drawn using GraphPad Prism (version 8.1; GraphPad, San Diego, CA).

## RESULTS

### Study Cohort Characteristics

Anthropometric and clinical data of the individuals with recent-onset diabetes, stratified according to cluster analysis (5), and of the glucose-tolerant CON group are shown in Table 1, with further stratification for G-allele carriers and noncarriers in Supplementary Table 1. Patients of the severe autoimmune diabetes (SAID) cluster were younger and had lower body mass and reduced β-cell function. Patients with severe insulin-deficient diabetes (SIDD) had worse glycemic control (mean $HbA_{1c}$ 8.8%) and β-cell function. SIRD patients were characterized by higher body mass and waist-to-hip ratio. Patients with mild obesity-related diabetes (MOD) showed greater body mass at younger age. Patients with mild age-related diabetes (MARD) were older and showed less severe metabolic abnormalities. The CON group was middle-aged, overweight, and—by definition—normoglycemic. Distribution of drug treatment across the study population is shown in Supplementary Table 2. Median alcohol consumption was 5.6 g/day (1st quartile 1.9; 3rd quartile 15.5) (males 6.0 g/day [1.9; 19.0] and females 4.9 g/day [1.6; 15.1]).

In a subgroup ($n = 130$), body composition was assessed showing no difference in adipose tissue volumes between G-allele carriers and noncarriers (subcutaneous 21,980 ± 11,500 vs. 20,517 ± 9,769 $cm^3$ and visceral 2,570 ± 1,763 vs. 2,913 ± 2,254 $cm^3$, respectively).

### Distribution of the rs738409 Variants in the *PNPLA3* Gene Across the Clusters

The common C/C genotype was less prevalent in the SIRD cluster compared with the other clusters (adjusted $P < 0.05$ vs. CON; $P < 0.05$ vs. SAID; $P < 0.05$ vs. MOD; $P < 0.05$ vs. MARD) (Fig. 1), except for SIDD ($P = 0.68$). Genotypes containing the minor alleles, C/G and G/G, were pooled for the subsequent analyses due to the low number of homozygous carriers.

### Insulin Sensitivity in Diabetes Clusters Depending on the rs738409 *PNPLA3* Variants

The G-allele carrier SIRD patients had higher circulating FFA levels than noncarrier SIRD patients ($P < 0.001$) (Fig. 2*A*). In line, adipose tissue insulin resistance was higher in SIRD (median 17.1 [1st quartile 9.3; 3rd quartile 24.5]) than in CON (4.3 [2.4; 6.0], $P < 0.001$), MARD (6.7 [4.1; 11.6], $P < 0.001$), and SAID (5.7 [2.7; 11.2], $P < 0.001$) but comparable with SIDD (11.1 [7.0; 15.5], $P = 0.40$) and MOD (12.8 [8.9; 19.1], $P = 0.42$) (Fig. 2*B*). Triglycerides were higher in G-allele carrier SIDD patients compared with noncarrier SIDD patients ($P < 0.05$) and not different between carriers and noncarriers in other clusters.

Whole-body insulin sensitivity (M value) was lowest in SIRD (mean ± SD 4.5 ± 1.5 $mg * kg^{-1} * min^{-1}$) compared with SIDD (6.3 ± 2.5, $P < 0.001$), MOD (5.5 ± 2.3, $P < 0.001$), MARD (7.6 ± 2.7, $P < 0.001$), and SAID (8.6 ± 3.0, $P < 0.001$) (Fig. 2*C*). M value was highest in CON (10.7 ± 3.8 $mg * kg^{-1} * min^{-1}$) compared with all clusters (all $P < 0.05$) (Supplementary Fig. 1). Basal EGP was lower in SIRD (1.7 ± 0.3 $mg * kg^{-1} * min^{-1}$) than in SAID and MARD (both 2.0 ± 0.3, both $P < 0.05$) (Supplementary Fig. 1) and comparable with other clusters. Similar results were observed for fasting hepatic insulin resistance index, whereas hepatic insulin sensitivity (% EGP suppression during clamp) did not differ between groups (Fig. 2*D*). Of note, irrespective of cluster allocation, the rs738409(G) *PNPLA3* variant correlated positively with whole-body insulin sensitivity ($\beta = 0.06$, $P = 0.03$) and liver fat content ($\beta = 0.36$, $P = 0.01$) across the cohort after adjustments for age, sex, and BMI. Examining the effect of the rs738409(G) *PNPLA3* variant in the classic diabetes types (type 1 and type 2 diabetes) revealed an association of the risk allele with higher adipose tissue insulin resistance and higher liver fat content in people with type 2 diabetes but not in people with type 1 diabetes (Supplementary Fig. 2).

### Hepatic Fat Content and Surrogate Measures of Fibrosis Depending on the rs738409 *PNPLA3* Variant

HCL was higher in SIRD (median 13.6% [1st quartile 5.8; 3rd quartile 19.1]) than in CON (0.9% [0.4; 5.0], $P < 0.001$), SAID (0.4% [0.0; 1.5], $P < 0.001$), MOD (6.4% [2.1; 12.4], $P = 0.028$), and MARD (3.0% [1.0; 7.9], $P < 0.001$) and similar to SIDD (2.4% [0.3; 12.8], $P = 0.21$) (Fig. 3*A* and Supplementary Fig. 1). Surrogate estimates of hepatic steatosis (FLI) were higher in SIRD (mean ± SD 90 ± 11 arbitrary units) compared with CON (46 ± 33, $P < 0.001$),

**Table 1—Patients' characteristics**

| | CON | SAID | SIDD | SIRD | MOD | MARD |
|---|---|---|---|---|---|---|
| *n* | 83 | 254 | 25 | 61 | 240 | 254 |
| Genotype frequency (CC/CG/GG), *n* | 54/24/5 | 137/105/12 | 12/13/0 | 22/33/6 | 136/91/13 | 141/95/18 |
| Female sex, *n* (%) | 26 (31) | 117 (46) | 5 (20) | 17 (28) | 108 (45) | 71 (28) |
| Age (years) | 47 ± 14 | 37 ± 12 | 43 ± 12 | 56 ± 10 | 45 ± 10 | 58 ± 7 |
| BMI (kg/m$^2$) | 28.2 ± 5.7 | 25.0 ± 4.3 | 27.1 ± 3.7 | 34.5 ± 3.6 | 34.9 ± 6.3 | 27.4 ± 3.5 |
| WHR | 0.92 ± 0.09 | 0.88 ± 0.09 | 0.94 ± 0.06 | 1.00 ± 0.07 | 0.97 ± 0.08 | 0.95 ± 0.07 |
| Fasting blood glucose (mg/dL) | 91 ± 15 | 136 ± 46 | 187 ± 60 | 117 ± 21 | 130 ± 28 | 123 ± 24 |
| $HbA_{1c}$ (%) | 5.3 ± 0.3* | 6.6 ± 1.2* | 8.8 ± 1.3* | 6.2 ± 0.7 | 6.5 ± 0.9 | 6.3 ± 0.7 |
| HOMA-IR (a.u.) | 1.48 ± 0.71* | 0.99 ± 0.87* | 1.97 ± 0.98 | 4.48 ± 1.21 | 2.77 ± 1.01 | 1.94 ± 0.75* |
| HOMA-B (a.u.) | 122 ± 39 | 46 ± 31* | 39 ± 21* | 170 ± 46 | 98 ± 37* | 83 ± 30* |
| hs-CRP (mg/dL) | 0.1 (0.1; 0.2) | 0.1 (0.1; 0.2) | 0.2 (0.1; 0.4) | 0.4 (0.2; 0.6) | 0.3 (0.2; 0.7) | 0.2 (0.1; 0.3) |
| Total cholesterol (mg/dL) | 205 ± 35 | 185 ± 38 | 206 ± 29 | 197 ± 42 | 203 ± 44 | 201 ± 42 |
| LDL cholesterol (mg/dL) | 129 ± 33 | 109 ± 34* | 131 ± 29 | 123 ± 37 | 130 ± 37 | 129 ± 36 |
| HDL cholesterol (mg/dL) | 61 ± 18* | 62 ± 18* | 51 ± 12* | 42 ± 9 | 45 ± 12 | 51 ± 14* |
| Triglycerides (mg/dL) | 93 (62; 148)* | 73 (53; 106)* | 149 (79; 205) | 176 (119; 239) | 140 (97; 193)* | 117 (83; 161)* |
| ALT (units/L) | 25 ± 11 | 21 ± 8 | 26 ± 16 | 29 ± 13 | 25 ± 11 | 24 ± 10 |
| AST (units/L) | 28 ± 19* | 23 ± 17* | 34 ± 18 | 42 ± 17 | 37 ± 22* | 29 ± 16* |
| GGT (units/L) | 26 ± 18* | 22 ± 23* | 36 ± 24 | 49 ± 44 | 46 ± 62 | 35 ± 33* |

Data are shown as absolute numbers, percentages, mean ± SD, or median (interquartile range), as applicable. a.u., arbitrary units; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, γ-glutamyl transferase; HOMA-IR, HOMA of insulin resistance; HOMA-B, HOMA of β-cell function; WHR, waist-to-hip ratio. *$P < 0.05$ vs. SIRD (Dunnett test).

SIDD (53 ± 30, $P = 0.04$), MARD (53 ± 27, $P < 0.001$), and SAID (26 ± 27, $P < 0.001$) and tended to be higher than in MOD (81 ± 25, $P = 0.07$). Among subjects with MOD, carriers of the G allele had slightly lower FLI ($P < 0.05$) (Fig. 3*B*). Surrogate estimates of liver fibrosis (APRI) were similar between all nonautoimmune diabetes clusters and only showed differences between SIRD and SAID (0.20 ± 0.09 vs. 0.28 ± 0.18, $P = 0.002$). In MARD, carriers of the G allele tended to have higher APRI scores ($P = 0.06$) (Fig. 3*C*), whereas FIB-4 (Fig. 3*D*) and NAFLD fibrosis score did not differ between carriers and noncarriers in any cluster. Among people with classical type 2 diabetes, irrespective of cluster allocation, G-allele carriers exhibited higher APRI scores than noncarriers (Supplementary Fig. 2).

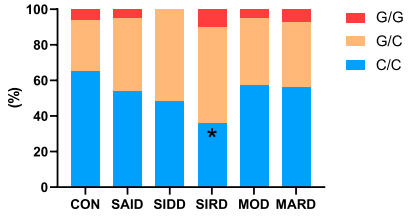


**Figure 1**—Genotype distribution of the variant rs738409 in the *PNPLA3* gene stratified by cluster. Bar graphs show percentages of each variant within a cluster. *$P < 0.05$ vs. CON, MOD, MARD, and SAID.

### Metabolomics

Cluster comparison of the metabolome identified 47 metabolites that differed between SIRD and SAID including the long-chain fatty acids (LCFA) cC18:3w6 and cC20:4w6, sphingomyelins (SM) C18:0 and C18:1, or phosphatidylcholine (PC) aaC38:3 and LCFA-containing PCaaC42:1, C42:4, C42:5, C44:3, C44:4, C44:5, and C44:6 and the lyso-PCs C18:0, C18:1, and C18:2 (Supplementary Fig. 3*A*). Similarly, PCaaC40:6 was lower in SIDD compared with SIRD (Supplementary Fig. 3*B*). While only acylcarnitine C3 was higher in SIRD than in MOD (Supplementary Fig. 3*C*), we identified 12 metabolites, which differed between SIRD and MARD, including SM C18:0, SM C18:1, lyso-PCaC18:1 and C18:2, and PC aaC42:2 and aeC42:3 (Supplementary Fig. 3*D*). Differences for 16 metabolites remained significant after Bonferroni correction.

Metabolites differed further within all clusters between G-allele carriers and noncarriers ($P < 0.05$). Overall, 13 metabolites including LCFA cC20:4w6 and cC22:4w6 as well as the LCFA-containing PCs, PCaaC36:4, C42:0, C42:1, and PCae C42:4, C42:5 C44:3, C44:4, C44:5, and C44:6 differed between carriers and noncarriers within the SIRD cluster. None of the SNP effects remained significant after Bonferroni correction.

## CONCLUSIONS

This study shows that patients of the recently identified SIRD cluster, featuring higher liver fat content and whole-body insulin resistance, are also more frequently carriers of the G allele in the *PNPLA3* gene. The G allele carriers of this cluster also exhibit higher circulating FFA and higher adipose tissue insulin resistance, while the genetic variant was not associated with HCL or insulin resistance within this group. Of note, across the whole study population, the G allele associated with higher HCL as well as peripheral insulin sensitivity, independent of age, sex, and BMI.

Genetic variation in *PNPLA3* has been shown to associate with both ethnicity-related and individual susceptibility to NAFLD (8). Previous results revealed a synergy between adiposity and genotype indicating that adiposity augments genetic risk of NAFLD at multiple loci that confer susceptibility to hepatic steatosis through diverse mechanisms (26). The current study observed an association of the G allele with increased liver fat content independent of age, sex, and BMI and its higher prevalence in the SIRD cluster, characterized by insulin resistance and increased HCL. This observation extends previous findings associating *PNPLA3* with NAFLD also in individuals with diabetes by

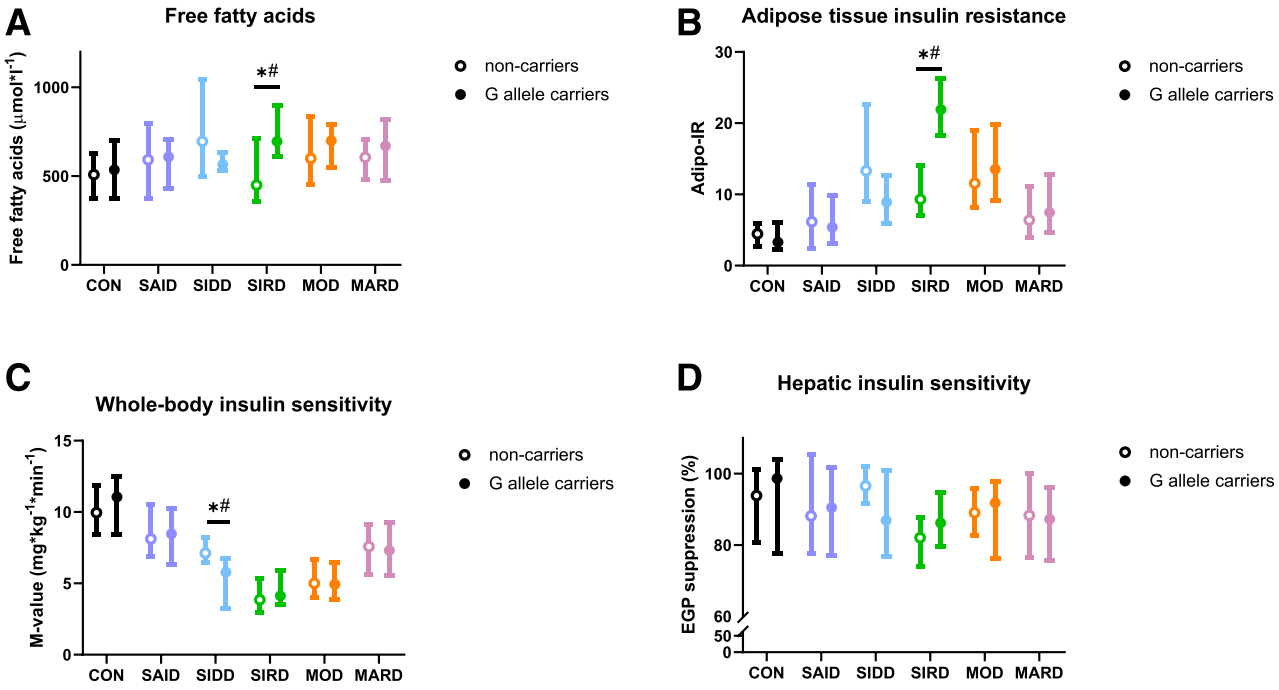


**Figure 2**—Metabolic characteristics of the study population stratified by cluster and *PNPLA3* genotype. Data are presented as median and interquartile range. Depicted are differences in circulating FFA (*A*), adipose tissue insulin sensitivity (*B*), whole-body insulin sensitivity (*C*), and hepatic insulin sensitivity (*D*). *$P < 0.05$; #$P < 0.05$ after adjustments for age and sex. Adipo-IR, adipose tissue insulin resistance index.

describing particularly higher risk of NAFLD in patients with severe insulin resistance. Thus, these findings support the notion that inhibition of the variant PNPLA3 activity may represent a promising target for novel treatment concepts of NAFLD (15).

Furthermore, previous studies suggested that *PNPLA3* is involved not only in hepatic steatosis but also fibrosis. This may occur, as the *PNPLA3* mutation can inhibit retinyl-esterase activity in hepatic stellate cells, where it is involved in retinol metabolism and plays an important role in lipid droplet remodeling (27). Consequently, loss of function in PNPLA3 may associate with reduced release of retinol from lipid droplets and subsequent propensity to liver fibrosis (28). Analysis of the

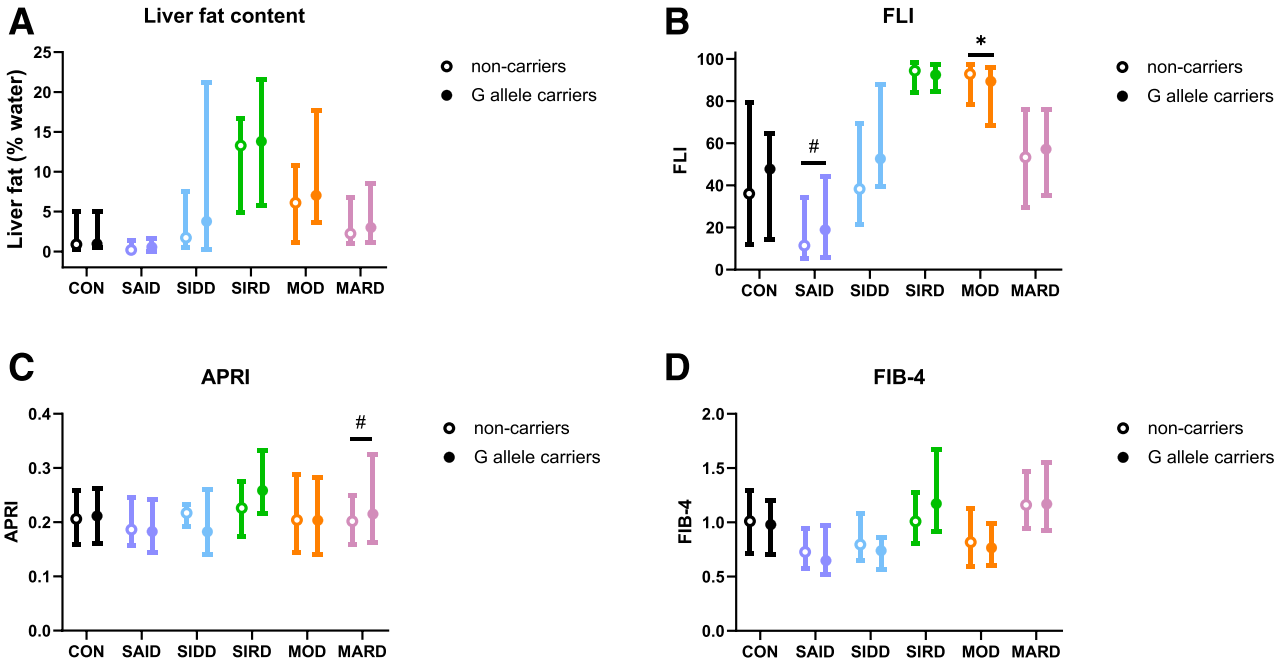


**Figure 3**—Liver parameters of the study population stratified by diabetes cluster and *PNPLA3* genotype. Data are presented as median and interquartile range. Depicted are differences in liver fat content (*A*), FLI (*B*), and liver fibrosis indices APRI (*C*) and FiB-4 (*D*). *$P < 0.05$; #$P < 0.05$ after adjustments for age and sex.

clusters separately revealed no association of any liver fibrosis surrogate with the *PNPLA3* mutation, whereas higher APRI scores associated with the G allele among the entire type 2 diabetes cohort. Of note, our study population exclusively comprised patients with recently diagnosed diabetes. While this allowed us to monitor early metabolic abnormalities without interference from long-term hyperglycemia and dyslipidemia, these recently diagnosed patients are likely at low risk of liver fibrosis.

The current study detected an association of the *PNPLA3* genotype with increased peripheral insulin sensitivity across the whole cohort but not within the individual clusters—except for lower insulin sensitivity along with higher serum triglycerides in the G-allele carriers in the SIDD group, possibly due to insulin deficiency. The relationship between the risk allele and insulin resistance remains controversial. The present data confirm previous reports on a disassociation between insulin resistance and fatty liver in G-allele carriers (17,29). The underlying mechanisms may involve lower availability of (monoun)saturated fatty acids—despite higher fat content—for activation of the hepatic diacylglycerol/protein kinase Cε pathway, which would prevent lipid-induced whole-body insulin resistance (15). Indeed, *PNPLA3*-associated NAFLD has previously been associated with increases in hepatic polyunsaturated triglycerides (8,29). PNPLA3 may sequester α/β-hydrolase domain–containing 5 (ABHD5) (also known as CGI-58), and prevents it from activating PNPLA2, a major intracellular adipose and hepatic triglyceride lipase, triggering triglyceride accumulation in adipose tissue and liver (30). Along these lines, previous studies suggest that the higher liver fat content in G-allele carriers is caused by reduced mobilization of triglycerides from lipid droplets, rather than by increased triglyceride synthesis from either endogenously synthesized or circulating fatty acids (31). Our study detected differences in FFA and adipose tissue insulin resistance in noncarriers compared with G-allele carriers of the SIRD cluster. SIRD patients in turn showed highest liver fat content, independently of *PNPLA3* genotype. Nevertheless, adipose tissue insulin resistance and lipolysis might mutually accelerate NAFLD and diabetes progression.

The heterogeneity in insulin sensitivity among diabetes cohorts can be at least partly attributed to differences in abdominal fat volume (32). Particularly, abdominal visceral obesity is associated with adipose tissue insulin resistance and impaired glucometabolic control (21). The subgroup analysis of the current study did not detect any difference in adipose tissue volumes between G-allele carriers and noncarriers, further suggesting that *PNPLA3* could affect adipose tissue function rather than mass. Lipotoxic FFA, mainly saturated fatty acids, diacylglycerols, and ceramides, have been identified as possible causal factors underlying obesity- and diabetes-related insulin resistance and NAFLD (1,3,7). The current study found that SIRD patients have certain higher PC and SM species, which have been proposed to link NAFLD and diabetes onset (33). Of note, lyso-PCa18:2 differed between SIRD and SAID as well as MARD, two clusters that had lower HCL compared with SIRD. Lyso-PCa18:2 associates with diabetes progression (33) but was not affected by the carrier status in this study. On the molecular level, the PCaa lipids are major components of human lipoproteins and involved in the release of triglyceride-enriched VLDLs (34). In contrast, PCae lipids inhibit lipid oxidation and have cell-protective properties (35), while SM may interfere with reactive oxygen species and decrease insulin secretion and signaling (36). In SIRD, the presence of the SNP affected arachidonic acid cC20:4w6. Along these lines, cC20:4-containing PC species have been shown to correlate negatively with insulin sensitivity (37). Taken together, results show that the SIRD cluster presents with alterations in the circulating metabolome consistent with patterns linked to insulin resistance, which specifically differ compared with other clusters.

Furthermore, there is evidence that adipose tissue dysfunction and insulin resistance may prevail during the early stage of type 2 diabetes without relevant hepatic insulin resistance (1). Adipose tissue insulin resistance would favor hepatic triglyceride accumulation and NAFLD in the absence of inadequate adaptation of mitochondrial function (6) before hepatic insulin resistance would occur at later disease stages. One might speculate that the *PNPLA3* variant could directly affect adipocyte function and thereby induce adipose tissue insulin resistance. Indeed, mRNA expression of *PNPLA3* was originally detected in murine adipocytes and can also be upregulated during human adipocyte differentiation despite overall lower expression compared with in liver (38). In this context, changes of the saturation of FFA and triglycerides as observed in liver (39) might differently affect the FFA composition of circulating FFA and in turn whole-body and hepatic insulin resistance.

Previous findings indicate that NAFLD is associated with an increased risk of cardiovascular events (1,2). In line, cluster analysis of a Swedish population revealed increased cardiovascular risk specifically in patients with SIRD (4). This process may occur through the systemic release of proatherogenic mediators from the diseased liver or through the contribution of NAFLD to insulin resistance and dyslipidemia, which are important risk factors for cardiovascular disease (1,2). This is particularly relevant for patients of the SIRD cluster who present with high triglycerides, increased FFA, and high whole-body and tissue-specific insulin resistance. Consequently, it is paramount for clinicians to consider intensified cardiovascular risk factor management in patients with SIRD and NAFLD.

The current study benefits from the comprehensive phenotyping applying gold standard methods to assess insulin sensitivity and HCL and from the comparison with a metabolically healthy CON group. In addition, the recruitment of patients with recent-onset diabetes allows for metabolic studies avoiding interference from chronic metabolic dysregulation or long-term treatment effects. On the other hand, while using state-of-the-art noninvasive measures to quantify liver fat, we had to rely on liver fibrosis indices, as liver biopsies were not available for the current study. Of note, MRS techniques have proven to have lower reliability due to limited spatial coverage and higher variability in obese persons due to specific hardware requirements. MRI-based assessment of proton density fat fraction combined with MR elastography will likely overcome current limitations and provide additional information on hepatic fibrosis in future studies (40). The one-step clamp test is optimized for the assessment of whole-body insulin sensitivity but has lower sensitivity for detecting smaller differences in hepatic and adipose tissue insulin sensitivity. Furthermore, the comprehensive phenotyping approach and the exclusion criteria of the GDS (19) may lead to a selected population, which will require further studies in population-based cohorts. Finally, the low absolute number of SIRD patients is another limitation but also reflects the lower prevalence among the other

subtypes, which is in good agreement with that of other larger cohorts (4).

In conclusion, patients of the SIRD cluster are more frequently carriers of the G allele in the *PNPLA3* gene, which associates with increased circulating FFA levels and adipose tissue insulin resistance. They also present with the highest hepatocellular lipid content and whole-body insulin resistance, which did not associate with the carrier status. These results are suggestive of a lipotoxic milieu in carriers of the G allele, which may accelerate whole-body and tissue-specific insulin resistance and NAFLD. Collectively, these observations warrant the screening of persons at highest risk of NAFLD and targeting reduction of adipose tissue insulin resistance for the prevention and treatment of NAFLD in people with diabetes.

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**Author Contribution.** O.P.Z. wrote the manuscript and researched data. K.S. performed the statistical analyses. B.K. and J.K. performed the genetic analyses. Y.Ku. and J.-H.H. performed and analyzed the metabolic imaging data. Y.Ka., M.W., K.B., D.F.M., V.B., H.A.-H., J.S., and M.R. researched data, contributed to the discussion, and reviewed and edited the manuscript. All authors critically reviewed the manuscript. All authors read and approved the final manuscript. M.R. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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