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## Risk assessment with gut microbiome and metabolite markers in NAFLD development

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**Author contributions:** G.P., W.J., H. Li, and Y.N. conceptualized and designed experiments. X.L. and L.Q. acquired the clinical samples and performed the experiment. H. Leung, Y.N., X.L., E.N., S.L.S., and D.P. processed and analyzed the data. H. Leung, Y.N., and G.P. wrote the original draft. Y.L., A.X., K.C., E.B., R.L., H. Li, and W.J. provided data for analysis and external validation. G.P., W.J., H. Li, Y.N., and K.H. lead and supervised the research work. H. Leung, Y.N., G.P., H.B.N., K.H., Y.L., A.X., K.C., R.L., H. Li, and W.J. reviewed and edited the manuscript.

**Competing interests:** R.L. serves as a consultant to Aardvark Therapeutics, Altimmune, Anylam/Regeneron, Amgen, Arrowhead Pharmaceuticals, AstraZeneca, Bristol-Myer Squibb, CohBar, Eli Lilly, Galmed, Gilead, Glympse Bio, Hightide, Inipharma, Intercept, Inventiva, Ionis, Janssen Inc., Madrigal, Metacrine Inc., NGM Biopharmaceuticals, Novartis, Novo Nordisk, Merck, Pfizer, Sagimet, Theratechnologies, 89 Bio, Terns Pharmaceuticals, and Viking Therapeutics. In addition, his institutions received research grants from Arrowhead Pharmaceuticals, AstraZeneca, Boehringer-Ingelheim, Bristol-Myers Squibb, Eli Lilly, Galectin Therapeutics, Galmed Pharmaceuticals, Gilead, Intercept, Hanmi, Intercept, Inventiva, Ionis, Janssen, Madrigal Pharmaceuticals, Merck, NGM Biopharmaceuticals, Novo Nordisk, Merck, Pfizer, Sonic Incytes, and Terns Pharmaceuticals. R.L. is a co-founder of LipoNexus Inc. K.H. is the founder of the company Afekta Technologies providing metabolomic services. All other authors declare that they have no competing interests.

### SUPPLEMENTARY MATERIALS

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Figs. S1 to S8

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References (98–110)

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## Abstract

A growing body of evidence suggests interplay between the gut microbiota and the pathogenesis of nonalcoholic fatty liver disease (NAFLD). However, the role of the gut microbiome in early detection of NAFLD is unclear. Prospective studies are necessary for identifying reliable, microbiome markers for early NAFLD. We evaluated 2487 individuals in a community-based cohort who were followed up 4.6 years after initial clinical examination and biospecimen sampling. Metagenomic and metabolomic characterizations using stool and serum samples taken at baseline were performed for 90 participants who progressed to NAFLD and 90 controls who remained NAFLD free at the follow-up visit. Cases and controls were matched for gender, age, body mass index (BMI) at baseline and follow-up, and 4-year BMI change. Machine learning models integrating baseline microbial signatures (14 features) correctly classified participants (auROCs of 0.72 to 0.80) based on their NAFLD status and liver fat accumulation at the 4-year follow up, outperforming other prognostic clinical models (auROCs of 0.58 to 0.60). We confirmed the biological relevance of the microbiome features by testing their diagnostic ability in four external NAFLD case-control cohorts examined by biopsy or magnetic resonance spectroscopy, from Asia, Europe, and the United States. Our findings raise the possibility of using gut microbiota for early clinical warning of NAFLD development.

## INTRODUCTION

Since the 1980s, the prevalence of obesity, insulin resistance, type 2 diabetes mellitus, and obesity-associated nonalcoholic fatty liver disease (NAFLD) has grown worldwide (1–3). The occurrence of these interconnected diseases is partly driven by consumption of high-energy food and a sedentary lifestyle, and these diseases are considered critical global health and socioeconomic problems (4). Apart from associations with liver-related diseases, epidemiological studies have associated NAFLD with increased risk of developing extrahepatic chronic diseases, such as type 2 diabetes, cardiovascular disease, and chronic kidney disease (5, 6). A recent cohort study showed that overall mortality risk increases progressively with worsening NAFLD histology, and even simple steatosis increases mortality risk by 71% (7), thus simple steatosis can no longer be considered as benign

as previously thought (8). Although NAFLD affects about 25% of the world's population (9) and has a high disease burden, awareness of NAFLD is low. In a cross-sectional analysis ( $n = 2788$ ) in four U.S. cities, NAFLD prevalence was 23.9%, whereas awareness of NAFLD was 2.4% in study participants with computed tomography (CT)-defined NAFLD (10). One important reason for low awareness is that most patients with NAFLD are largely asymptomatic in the disease course, where disease is mainly detected through an incidental finding of fatty liver on ultrasound or an imaging modality or routine laboratory testing (11, 12). Diagnosis by liver biopsy or imaging is reliable but difficult for large-scale screening and monitoring. Thus, the need to identify individuals who are at high risk of developing NAFLD or are at an early stage of the disease is urgent, as lifestyle interventions can reverse the disease when it is in the first stages (13). According to one study (14), weight loss and healthy diet might be sufficient to reverse simple steatosis, whereas intensified lifestyle intervention coupled with pharmacological treatment might be necessary for more advanced stages of liver diseases. Exercise programs (15), low-carbohydrate diet (16), and various types of gut microbiota-targeted treatments (17) have demonstrated their ability to prevent steatosis development and improve NAFLD outcomes in human or preclinical models. Early diagnosis and interventions to prevent NAFLD progression can also greatly reduce future health care cost, as most economic costs associated with NAFLD are incurred in advanced stages (18). Currently available methods (19–21) for early prediction of NAFLD are limited and use only a few clinical parameters or biomarkers that may not reflect the heterogeneity and complexity of NAFLD (22, 23). Thus, more convenient noninvasive alternatives are needed.

In the last 10 years, the gut microbiome has emerged as a major regulator of host energy homeostasis and substrate metabolism (24–26). The human gastrointestinal tract is colonized with 4644 bacterial species encoding 171 million genes (27). Therefore, it is not unexpected that abnormalities in gut microbiome structure and especially function might affect the brain, adipose tissue, muscle, and liver metabolism. Microbial components or metabolites such as lipopolysaccharides, secondary bile acids, dimethyl- and trimethyl-amines, and compounds derived from carbohydrate and protein fermentation appear to be strongly involved in the gut host-microbiome metabolic axis and the occurrence of metabolic diseases (28–31).

Human cross-sectional studies have delineated the role of gut bacteria in the development of NAFLD. An increased ratio of Bacteroidetes to Firmicutes phyla and a decrease in butyrate-producing *Ruminococcaceae* are suggested to be involved in NAFLD progression; however, the data are not always consistent (32–35). Furthermore, whether NAFLD causes taxonomic and functional changes in the microbiome or the observed dysbiosis in patients with NAFLD leads to progression of the disease is not clear. For a possible causal role in NAFLD development, gut microbiota alteration should take place long before disease is diagnosed, which would suggest prognostic value in evaluating the gut microbiome in individuals with a high risk of developing NAFLD. To assess this potential value, we conducted a 4-year prospective study in a community-based cohort of 2487 Chinese individuals. We profiled 180 matched case-control individuals who were NAFLD free at baseline using well-documented clinical information and comprehensive metagenomic and metabolomic analysis. We developed machine learning models integrating baseline microbial signatures

to classify individuals based on their NAFLD status 4 years after baseline (either remaining disease free or diagnosed with the disease). We also examined whether the selected features in the model were biologically relevant to NAFLD development by exploring the diagnostic power of the model in several case-control cohorts from Asia, the United States, and Europe.

## RESULTS

### Characterization of the study cohort

To develop a microbiome-based prognostic model for long-term development of NAFLD, we designed a nested case-control study within a community-based prospective cohort study of Chinese adults. About 2500 participants were screened in 2014 with ultrasonography, which is recommended as the first-line diagnostic test for NAFLD (36); 1216 participants were determined as NAFLD free using criteria proposed by the Asian Pacific Association for the Study of the Liver (37). Participant enrolment is outlined in fig. S1. Stool and serum samples were obtained from participants at baseline. At the follow-up visit in 2018, after a strict exclusion process, 90 participants (38 males and 52 females) were identified as having NAFLD (NAFLD<sup>-/+</sup>) (Fig. 1). The participants in the NAFLD<sup>-/+</sup> group were matched with 90 controls who did not have NAFLD at baseline or at the follow-up visit (NAFLD<sup>-/-</sup>). The two groups were matched in gender, age, and body mass index (BMI) at both the baseline and follow-up visits and 4-year change in BMI. There were no differences between the two groups in the prevalence of type 2 diabetes, hypertensive disease, metabolic syndrome, and medication usage at both baseline and follow-up in the cohort, apart from a significantly higher metabolic syndrome ratio in NAFLD<sup>-/+</sup> at follow-up as expected (chi-square test,  $P < 0.05$ ; table S1).

Detailed baseline anthropometric parameters, glucose homeostasis parameters, serum liver enzymes and renal function, lipid profiles, and cytokines are shown in Table 1. No significant differences ( $t$  test,  $P > 0.05$ ) were seen for most clinical parameters between the NAFLD<sup>-/+</sup> and NAFLD<sup>-/-</sup> groups at baseline. Fasting insulin (FINS), homeostasis model assessment for insulin resistance (HOMA-IR), triglycerides (TGs), and high-sensitivity C-reactive protein (hs-CRP) in the NAFLD<sup>-/+</sup> group were slightly higher than in the NAFLD<sup>-/-</sup> group ( $t$  test,  $P < 0.05$ ); however, their mean or median values were within reference ranges in both groups (FINS: 5.1 to 11.2 uU/ml, HOMA-IR  $< 2.5$ , TG  $< 1.70$  mM, and hs-CRP  $< 1$  µg/ml) (38–41). Only TGs remained significantly different after adjusting for HOMA-IR ( $1.55 \pm 0.90$  mM versus  $1.23 \pm 0.61$  mM; Table 1).

### Modest but distinguishable differences in baseline gut microbiome between NAFLD<sup>-/+</sup> and NAFLD<sup>-/-</sup> individuals

We assessed the gut microbiome structure of the NAFLD<sup>-/+</sup> and NAFLD<sup>-/-</sup> groups at baseline via shotgun metagenomic sequencing, generating 1128 gigabase pairs of high-quality reads with an average of 41,786,187 reads per sample (Fig. 1). Taxonomic profiling with MetaPhlAn2 (42) led to the identification of 405 species. Community alpha diversity measured as richness, and Shannon and Simpson indexes showed no significant differences (Wilcoxon rank-sum test,  $P > 0.05$ ) at the species, genus, or family levels between the two groups (fig. S2A). Bray-Curtis, unweighted UniFrac, and weighted UniFrac distance

comparisons indicated that the NAFLD<sup>-/+</sup> and NAFLD<sup>-/-</sup> groups did not have significant community dissimilarities [permutational multivariate analysis of variance (PERMANOVA),  $P > 0.05$ ; fig. S2, B and C]. The same patterns were observed when using a metagenomic species approach (43) for the taxonomic annotation (table S2).

In addition, we sequenced the baseline gut microbiota from 66 participants who were diagnosed as NAFLD in both 2014 and 2018 (NAFLD<sup>+/+</sup>) and 34 participants who were diagnosed as NAFLD in 2014 but not in 2018 (NAFLD<sup>+/-</sup>). These two groups were also matched with the other two groups described above by age, gender, BMI, and 4-year change in BMI. A thorough comparison of microbiota alpha and beta diversity among the four groups at baseline indicated that the two non-NAFLD groups were distinguishable from the two NAFLD groups ( $P < 0.05$ , Wilcoxon rank-sum test for alpha diversity comparisons and PERMANOVA for beta diversity comparisons using Bray-Curtis distances) (fig. S3). Moreover, the gut microbiota of NAFLD<sup>-/+</sup> subjects was different from that of NAFLD<sup>+/+</sup> and NAFLD<sup>+/-</sup> individuals. This argues that the NAFLD<sup>-/+</sup> group was not already diseased at the baseline because they clustered with NAFLD<sup>-/-</sup> subjects at baseline. Because our focus was to identify gut microbiota signatures in disease-free individuals suggestive of NAFLD predisposition, only the NAFLD<sup>-/-</sup> and NAFLD<sup>-/+</sup> groups were further analyzed.

A compositional analysis found that several of the 10 most abundant genera and species (Fig. 2A) were significantly associated (envfit from R package vegan,  $P < 0.05$ ) with observed variation in the taxonomic profile of the study participants (fig. S2, B and C). However, their relative abundances were not significantly different (zero-inflated Gaussian mixture model,  $P > 0.05$ ) between NAFLD<sup>-/+</sup> and NAFLD<sup>-/-</sup> groups. Nevertheless, the relative abundances of 8 and 21 less-abundant genera and species, respectively, were significantly different (zero-inflated Gaussian mixture model,  $P < 0.05$ ) between the two groups (fig. S2D). *Methanobrevibacter* [false discovery rate (FDR) = 0.01] was decreased in NAFLD<sup>-/+</sup> compared to NAFLD<sup>-/-</sup> (a reduction in *Phascolarctobacterium* was insignificant at FDR = 0.2). Lower abundances of these two genera have been observed in cohort studies in obese individuals compared to lean individuals (44, 45). *Slackia* has been reported to be more abundant in individuals with moderate-to-severe fibrosis than in individuals with absent-to-mild fibrosis (46), and this genus was increased in the NAFLD<sup>-/+</sup> compared to the NAFLD<sup>-/-</sup> group (FDR = 0.06). The relative abundance of *Dorea formicigenerans*, a species that is highly abundant in people with obesity (47), was higher in the NAFLD<sup>-/+</sup> than the NAFLD<sup>-/-</sup> group (FDR = 0.17). Differences in the relative abundances of *Methanobrevibacter*, *Phascolarctobacterium*, *Slackia*, and *D. formicigenerans* between the two study groups remained significant even after adjusting for age, gender, BMI, and HOMA-IR (zero-inflated Gaussian mixture model,  $P < 0.05$ ). Because the NAFLD<sup>-/+</sup> and NAFLD<sup>-/-</sup> groups had no difference in BMI and in the aforementioned cohort studies, the liver status of the obese individuals was not evaluated, and our prospective design suggested that *Methanobrevibacter*, *Phascolarctobacterium*, *Slackia*, and *D. formicigenerans* could be signatures of NAFLD risk in addition to being obesity-related signatures.

We used HUMAnN2 (48) for functional profiling of the microbial communities and identified 458 pathways. Likewise, the taxonomic profile and the microbiota functional potential could not differentiate between NAFLD<sup>-/+</sup> and NAFLD<sup>-/-</sup> groups



by alpha and beta diversity (fig. S4, A and B). Four of the most abundant pathways detected, uridine monophosphate biosynthesis I, uridine diphosphate-*N*-acetylmuramoyl-pentapeptide biosynthesis I and II, and peptidoglycan biosynthesis I (Fig. 2A), were significantly associated with observed variation in the functional profiles of study participants (envfit from R package vegan,  $P < 0.05$ ; fig. S4B). These pathways were proposed to be discriminatory for NAFLD cirrhosis against control groups in a recent U.S. cohort study (49); however, their relative abundances were not significantly different (zero-inflated Gaussian mixture model,  $P > 0.05$ ) between the NAFLD<sup>-/+</sup> and NAFLD<sup>-/-</sup> groups in our prospective study. Nevertheless, we found 19 biosynthetic pathways significantly different in relative abundance between the two groups (zero-inflated Gaussian mixture model,  $P < 0.05$ ) (fig. S4C). We observed a significantly higher relative abundance of geranylgeranyl diphosphate biosynthesis and the mevalonate pathway in the NAFLD<sup>-/-</sup> group. These pathways are dysregulated in mice and humans with nonalcoholic steatohepatitis (NASH) (50). Two genes encoding enzymes involved in these pathways, hydroxymethylglutaryl-coenzyme A (CoA) reductase (EC 1.1.1.34) and mevalonate kinase (EC 2.7.1.36), were significantly enriched in the NAFLD<sup>-/-</sup> group (zero-inflated Gaussian mixture model,  $P < 0.05$ ; table S3). *Methanobrevibacter smithii* was the major contributor of gene expression abundance of hydroxymethylglutaryl-CoA reductase (95%) and mevalonate kinase (40%). In contrast, the NAFLD<sup>-/+</sup> group had a higher relative abundance of phosphatidate metabolism and cholic acid degradation. Cholic acid is a primary bile acid that decreases substantially in rats on a Western diet and is proposed as an early marker of NAFLD development (51). Genes encoding phospholipase D (EC 3.1.4.4) and bile-acid-7- $\alpha$ -dehydratase (EC 4.2.1.106) were also significantly enriched in the NAFLD<sup>-/+</sup> group (zero-inflated Gaussian mixture model,  $P < 0.05$ , table S3). The four significant pathways above (geranylgeranyl diphosphate biosynthesis, mevalonate pathway, phosphatidate metabolism, and cholic acid degradation) remained significantly different (zero-inflated Gaussian mixture model,  $P < 0.05$ ) between the two groups after adjusting for age, gender, BMI, and HOMA-IR, except for cholic acid degradation that was marginally significant ( $P = 0.050$ ).

### Metabolite enrichment and metabolic shifts in NAFLD<sup>-/+</sup> versus NAFLD<sup>-/-</sup> groups

We next performed targeted metabolomic analysis of serum samples collected at baseline to interrogate whether differences in species and pathway abundance of gut microbiota led to distinct profiles of microbial metabolites in the NAFLD<sup>-/+</sup> and NAFLD<sup>-/-</sup> groups. We detected 123 metabolites grouped into nine metabolite classes (Fig. 2B). We performed enrichment analysis to identify metabolite classes that were significantly overabundant or underabundant in the NAFLD<sup>-/+</sup> or the NAFLD<sup>-/-</sup> group, and found amino acids were significantly elevated in the NAFLD<sup>-/+</sup> group (Wilcoxon rank-sum test,  $P < 0.05$ ; table S4). We further analyzed the untargeted metabolomic data of a European case-control cohort (MICROBARIA) involving 52 obese women including 26 with biopsy-confirmed NAFLD and 26 non-NAFLD (52). Two amino acids positively correlated with NAFLD-related liver enzymes, including the branched-chain amino acid valine with alanine transaminase (ALT) ( $P < 0.05$ , Spearman correlation) and the aromatic amino acid tyrosine with aspartate transaminase (AST) ( $P < 0.05$ , Spearman correlation). Our findings in the Asian prospective and European cohort-based datasets are further supported by recent metabolomic-based

studies, suggesting that perturbations in amino acid metabolism are involved in NAFLD and NASH pathogenesis (53–55).

Of the 15 significantly different metabolites between the NAFLD<sup>-/+</sup> and NAFLD<sup>-/-</sup> groups at baseline (generalized linear model,  $P < 0.05$ ; fig. S5A) and the metabolites that were significantly associated with the observed metabolomic variation (envfit from R package vegan,  $P < 0.05$ ; Fig. 2C), several are reported to be involved in NAFLD in case-control human or animal studies. For example, 3-chlorotyrosine, arachidonic acid, and oxoglutaric acid are markers, respectively, of liver damage and NAFLD development in mouse (56) and rat (57) models and a human NAFLD study (58). Tryptophan was also significantly associated with the metabolome variation (envfit from R package vegan,  $P < 0.05$ ; Fig. 2C) in our cohort, and aromatic amino acids have been associated with NAFLD (54). These metabolites were higher in the NAFLD<sup>-/+</sup> group than the NAFLD<sup>-/-</sup> group. Concentration of a gut microbiota-regulated fatty acid, 8,11,14-eicosatrienoic acid, linked to obesity and insulin resistance (59, 60), was also significantly higher (generalized linear model,  $P < 0.05$ ; fig. S5A) in the NAFLD<sup>-/+</sup> group in our prospective study. Phenyllactic acid, produced by lactic acid bacteria and suggested to reduce reactive oxygen species production in rodents (61), was significantly higher in the NAFLD<sup>-/-</sup> group (generalized linear model,  $P < 0.05$ ; fig. S5A). On the contrary, the direction of concentration differences in the two study groups for isovaleric and docosahexaenoic acids (both higher in NAFLD<sup>-/+</sup>) (generalized linear model,  $P < 0.05$ ; fig. S5A) was inconsistent with proposals in the literature from case-control NAFLD studies about the possible roles of these compounds (62–64). These agreements and discrepancies in metabolite abundances in our prospective study with case-control cohort and mouse studies in the literature should help to narrow the metabolic marker possibilities for NAFLD progression. The concentrations of additional fatty acids were significantly different between the NAFLD<sup>-/+</sup> and NAFLD<sup>-/-</sup> groups (fig. S5A), but the functional significance of these metabolites in NAFLD is relatively unknown. Last, the concentrations of measured serum metabolites such as 3-chlorotyrosine and phenyllactic acid were significantly associated with gut microbiota species composition (Mantel test,  $P < 0.05$ ; fig. S5B and table S5).

### A machine learning prospective model to detect early signatures of NAFLD

We built a noninvasive risk assessment model (random forest algorithm) to classify healthy subjects based on their NAFLD status after 4.6 years, using a combination of baseline metagenomic and metabolomic features. A leave-one-out iterative approach was applied to build and evaluate our model due to the relatively small cohort size ( $n = 180$ ). We built a prospective model using 14 taxonomic, functional, and metabolomic features of the study participants at baseline that enabled classification based on their NAFLD status 4.6 years later with an area under the receiver operating characteristic curve (auROC) of 0.72 (Fig. 3A). The performance of the model was significantly improved to an auROC of 0.79 (DeLong test,  $P$  value for difference  $< 0.05$ ) with the addition of only two more noninvasive clinical features (Fig. 3B). We then slightly improved our model by also including the most accessible anthropometric parameters, BMI and age, to obtain our final model (auROC, 0.80; Fig. 3C and fig. S6).



We evaluated the biological relevance of the selected features by testing the diagnostic ability of components of our model to distinguish between healthy individuals and patients with NAFLD in two publicly available independent external Asian case-control cohorts; one cohort was diagnosed by biopsy and the other was diagnosed by magnetic resonance spectroscopy (MRS). This allowed us to further explore whether the patient diagnosis method had an impact on the model performance. We built a new prospective model using only nine features from the final model that were available in these external cohorts (fig. S6). The new model derived based on our study cohort discriminated healthy and NAFLD groups in the two external cohorts with auROCs of 0.78 and 0.72 (Fig. 3, D and E), showcasing that the features we identified were closely related to NAFLD development or pathophysiology. Besides the Asian cohorts, we further validated our prospective model in other case-control cohorts of different ethnicity. In the European cohort FLORINASH (54), the model (with the same nine features as in the Asian cohorts) reached an auROC of 0.76 (Fig. 3F), whereas in a U.S. cohort (49), the validation auROC (with seven available features) was 0.78 (Fig. 3G). Taking into consideration that only no more than half of features in our original prospective model were available in the external cohorts, we expect that the true accuracy may be higher.

Previous clinical prospective NAFLD studies demonstrated that fibroblast growth factor 21 and BMI (FGF21 + BMI), fatty liver index (FLI), and TG and glucose index (TyG) predict NAFLD development from 3 up to 9 years before diagnosis (auROCs of 0.71 to 0.82) (19–21). We compared the performance of our prospective model with FGF21 + BMI, FLI, and TyG to predict NAFLD occurrence in our cohort with matched baseline characteristics. The performance of our final model (auROC, 0.80) was significantly better than all three clinical models (auROCs of 0.58 to 0.60,  $P$  values for difference  $< 0.01$ ; Fig. 3, H to J). To confirm the importance of metagenomic and metabolomic information in prospective NAFLD prediction, we added metagenomic and metabolomic features from our final prospective model to the clinical models (fig. S6) and observed significant improvements in all (auROCs of 0.73 to 0.75,  $P$  values for difference  $< 0.05$ ; Fig. 3, H to J); however, none of the models reached the auROCs of our final model.

In total, 18 features were used in the final model: two genera, three pathways, nine metabolites, and four anthropometric and clinical parameters (Fig. 4, A and B). Our analysis revealed that the most important feature of our risk assessment model was phenyllactic acid (Fig. 4A). By analyzing the untargeted metabolomic data from the European MICROBARIA cohort (52), we found that phenyllactic acid negatively correlated with ALT, AST, and gamma-glutamyl transferase (correlation coefficients =  $-0.35$ ,  $-0.45$ , and  $-0.45$ ;  $P = 0.004$ ,  $0.14$ , and  $0.053$ ; Pearson's correlation adjusted for age, BMI, fasting glucose, and insulin).

SHapley Additive exPlanations (SHAP) (65) analysis also revealed that *Methanobrevibacter* was associated with NAFLD<sup>-/-</sup>, and *Slackia* was associated with NAFLD<sup>-/+</sup> (Fig. 4, A and B). These genera were differentially abundant in our two study groups (fig. S2D). Furthermore, 8,11,14-eicosatrienoic acid, hydrocinnamic acid, and oxoglutaric acid are associated with type 2 diabetes, obesity, insulin resistance, and NAFLD (58, 60, 61, 66), and our model revealed similar trends (Fig. 4, A and B).

The feature set contribution was also computed by summing the SHAP values per category. Metabolites were the most important in the model, contributing 44.6% to model performance, followed by the microbiome and nonmicrobiome features, with contributions of 31.2 and 24.1%, respectively (Fig. 4C).

Dependence plots were built to reveal the nonlinear correlations of features and risk of NAFLD. The optimal thresholds of each feature were identified (fig. S7). We found that high-density lipoprotein (HDL) was associated with NAFLD occurrence after 4.6 years when  $<1.39$  mM, which is close to the diagnostic criteria for metabolic syndrome when HDL was  $<1.0$  mM (male) or  $<1.3$  mM (female) (67). We also examined the dependence plots of microbial metabolite phenyllactic acid, hydrocinnamic acid, and 8,11,14-eicostrienoic acid (Fig. 4, D to F). Phenyllactic acid was associated with protection against NAFLD at a concentration of  $>0.25$   $\mu$ M. The concentration of 8,11,14-eicosatrienoic acid increased the risk of NAFLD at  $>51.5$   $\mu$ M, and hydrocinnamic acid was associated with NAFLD at a concentration of  $<0.39$   $\mu$ M. Visual inspection of the dependence plots did not indicate any differences by sex. We converted the features into binary variables (or  $<$  thresholds) according to their optimal threshold and found that 12 of 18 features showed significant association with NAFLD progression (chi-square test,  $P < 0.05$ ; table S6). These results demonstrated the importance of including an interpretable machine learning framework, such as SHAP, to provide insights when analyzing microbiome data.

We further examined whether the features of our risk assessment model could be used to classify subjects of the NAFLD<sup>-/+</sup> group based on different degrees of steatosis. We initially divided the NAFLD<sup>-/+</sup> group based on their liver fat percentage at the time of diagnosis (4.6 years after enrolment). Subsequently, using the values of the 18 features at baseline, we built a model classifying mild and severe steatosis cases. This new random forest model had an auROC of 0.78 (fig. S8A). Similarly as above, we attempted to confirm the biological relevance of the selected features by testing the diagnostic power of our prospective model in an independent external case-control cohort from the United States (49). Despite the lack of absolute quantification of metabolomic data, our model showed an accuracy of 71.4% to correctly identify severe steatosis cases with only gut microbial and clinical features.

Previous work has demonstrated the value of gut microbiome-based diagnostic tests for advanced fibrosis (68). The participants in our cohort were unlikely to develop advanced fibrosis after 4 years, starting as NAFLD free at baseline. Nevertheless, the prospective design of our study enabled us to explore whether the baseline microbiota is associated with the change or deterioration of fibrosis. Grouping our NAFLD<sup>-/+</sup> participants by the change of fibrosis 4 (FIB-4) index from 2014 to 2018, we built a new risk assessment model using five gut microbiota functional pathways, classifying subjects by the fibrosis deterioration with an auROC of 0.72 (fig. S8B). In a U.S. case-control cohort (49), the pathway with the highest importance in our model, phosphopantothenate biosynthesis, was significantly higher (zero-inflated Gaussian mixture model,  $P < 0.05$ ) in the cirrhosis group than in non-NAFLD controls. *Methanobrevibacter*, which was the top taxonomic feature in the prospective model, was also significantly lower (zero-inflated Gaussian mixture model,  $P < 0.05$ ) in patients with cirrhosis.

## DISCUSSION

NAFLD prevalence has rapidly increased over a short time, especially in China (69). China is projected to have the largest number of liver-related deaths among the most economically developed countries by 2030 (70). Accumulating evidence suggests that the gut microbiome may emerge as an active player in NAFLD development (71). Human studies demonstrated different gut microbiota profiles among individuals with NAFLD and those without, as well as in individuals at different stages of NAFLD (68, 72). In the recently proposed concept of metabolic-associated fatty liver disease (MAFLD) that extends beyond NAFLD (23), gut microbiota is suggested to be a major factor related to the heterogeneous phenotype of MAFLD. In both NAFLD and MAFLD, the disease complexity and heterogeneity may be better resolved by the inclusion of omics technologies that integrate patient clinical phenotypes and molecular phenomics and gut microbial features. This approach has shown its potential in the classification of hepatic (73) and, more recently, extrahepatic diseases including ischemic heart disease (74) and coronary artery disease (75). Both studies of cardiovascular diseases suggested that major alterations of the gut microbiome and metabolome might occur earlier than clinical onset of disease, suggesting the utility of gut microbiota-based risk assessment. A recent prospective study extended cross-sectional evidence and demonstrated that gut microbiota composition is predictive of incident type 2 diabetes after 15.8 years (76). Our study comprehensively characterized the gut microbiome of Chinese participants using stool samples taken 4.6 years before the NAFLD diagnosis and matched controls. We assessed the ability of metagenomic and metabolomic features as a risk assessment tool of NAFLD occurrence within 4.6 years and developed a random forest machine learning model that distinguished individuals at risk for NAFLD from controls with a performance of 0.80 auROC. The final model consisted of 18 features of mainly bacterial genera, pathways, and metabolites, with two clinical and two anthropometric parameters. Using subsets of those features available in external case-control cohorts also showed good ability (auROC of 0.73 to 0.78) to classify individuals with and without NAFLD, including in cohorts with the biopsy-confirmed present/absence of NAFLD and of different ethnicities, supporting the biological relevance and generalizability of our prospective model.

Diagnosis of NAFLD requires evidence of hepatic steatosis, either by histology or imaging. Liver biopsies have a risk of severe complications, and the sampling procedure may leave some people with NAFLD undiagnosed if they have unevenly distributed histological lesions (13). Steatosis evaluation based on imaging such as MRS, CT, or ultrasonography has limitations in clinical practice such as high price, radiation exposure, and limited sensitivity. Numerous research efforts have searched for other reliable, cost-effective, non-invasive diagnostic approaches, including using features that are clinical (age, gender, diabetes, and BMI), biochemical (aminotransferases, bilirubin, and ferritin), metabolic (glycated hemoglobin, insulin, and HOMA-IR), or lipid (TG and cholesterol) parameters or other markers such as FGF21 and adiponectin (77–79). A few prospective studies have also attempted to predict the development of NAFLD over the long term (19–21). However, the predictive power of these models was evaluated in study groups with unmatched baseline characteristics, which may have led to overestimation of model performance. In our community-based prospective study, these models showed limited performance (auROC

in the range of 0.58 to 0.60) when our nested case-control design included matching for gender, age, BMI, and 4-year BMI change. This matching is particularly important for removing confounding effects and to uncover microbiome-related risk factors for NAFLD development, given that obesity is a major risk factor for NAFLD. Our microbiome-based model demonstrated a good performance (auROC of 0.72) for predicting the NAFLD status of NAFLD-free individuals after 4.6 years.

Our study has limitations. The classification of patients into two groups was not based on liver biopsy, which remains the gold standard for NAFLD diagnosis. However, this method is impractical in a community study with thousands of participants, as in our study, and is unethical for participants who do not show any sign of the disease (matched controls). Moreover, according to guidelines from the European Association for the Study of the Liver, European Association for the Study of Diabetes, and European Association for the Study of Obesity, ultrasound is the first-line diagnostic test for NAFLD (36), especially for large-scale screening studies. This diagnostic criterion has been extensively used in previous studies, such as the Rotterdam cohort (80), the Golestan cohort (81), and the Kangbuk Samsung Health Study (82). We note that our cohort is of high quality, with relatively comprehensive indexes acquired in a large population. For example, in the measurement of glucose metabolism, oral glucose tolerance tests were conducted for all participants. This test is usually replaced by fasting glucose or FINS tests in many population-based studies. Second, we could not predict the development of more severe outcomes such as fibrosis because of their low incidence. This was mainly due to the nature of our community-based epidemiological investigation. However, using baseline microbiota, we were able to classify subjects by fibrosis deterioration with an auROC of 0.72.

Furthermore, serum ferritin was not measured in our study, although several studies have indicated its relevance in NAFLD (83–85). Therefore adding ferritin in our prospective model could potentially enhance performance. The predictive power of our prospective model (auROC of 0.80) was an advance compared to existing clinical models (auROCs of 0.58 to 0.60). However, further improvements, for example, integrating additional biochemical parameters, will be necessary for clinical applications. Our metabolic signatures and their taxonomic drivers revealed by our prospective model imply but do not prove causality; thus, additional studies are required to clarify the molecular mechanisms involved in NAFLD development.

Integrating bacterial species and functions in machine learning models for predicting host response to treatment or lifestyle interventions and disease progression has shown great potential (86–89). For NAFLD and its complications, gut microbiota changes can independently predict the risk of short-term hospitalizations (90 days) in patients with cirrhosis with an auROC of 0.83 (90). Elucidating the importance of the gut microbiome as a long-term risk assessment tool in NAFLD is important because of the current limited therapeutic landscape for NAFLD and findings that early detection can substantially improve outcomes for patients with NAFLD (91, 92). Our proof-of-concept study identified a microbiome signature in participants at risk of developing NAFLD in the next 4 years and points to the potential of noninvasive diagnostic tests to complement existing clinical screening tools for NAFLD. Moreover, identifying microbiome signatures also opens a

window of opportunities for microbiome-based prophylactic and therapeutic interventions such as the utility of propionic acid as a potent immunomodulatory supplement to multiple sclerosis drugs (93), which is not offered by a clinical predictive model built upon only a few clinical parameters or other features. Evaluation and further improvement of our NAFLD risk assessment model using larger prospective studies that are heterogeneous for ethnicity and lifestyle patterns will increase the model's generalizability and obtain more refined estimations of its accuracy.

## MATERIALS AND METHODS

### Study design

The aim of this study was to identify potential predictive signatures for early clinical warning of NAFLD and to develop a prognostic risk assessment model for long-term NAFLD development. For this purpose, we conducted a nested case-control study within a 4.6-year prospective study in 2487 Chinese individuals, and we profiled 180 individuals from 1216 NAFLD-free participants at baseline, including 90 that were diagnosed with NAFLD in the follow-up visit (NAFLD<sup>+/+</sup>), which were matched with 90 controls without NAFLD (NAFLD<sup>-/-</sup>) by gender, age, BMI, and 4.6-year BMI change. We performed comprehensive metagenomic and metabolomic analyses using stool and serum samples taken at baseline, including taxonomic diversity and profiles at family, genus and species levels, microbial enzymes, metabolic pathways, and metabolites. An interpretable machine learning model integrating baseline microbial signatures was built to predict NAFLD development after 4 years. The biological relevance of selected features in the model to NAFLD development was further validated in external cohorts, including three cohorts with the biopsy-confirmed presence/absence of NAFLD. New models were built for validation, given that some features were not available in the external cohorts. All validation models were trained on our cohort and tested in the external cohorts. Further materials and methods details are available in the Supplementary Materials.

### Study participants

All participants were from the Nicheng Diabetes Screening Project (also called the Shanghai Nicheng Cohort Study) previously described (94, 95). This population-based, prospective study was designed to assess the prevalence, incidence, and factors related to cardiometabolic diseases among adults in Nicheng County, a suburb of Shanghai, China. On the basis of the project, we designed a nested case-control study to explore the potential causal role of the gut microbiome in NAFLD in three randomly selected Nicheng communities (involving 2487 participants). Figure S1 outlines study enrolment. Of 2487 participants, 1216 were identified as not having NAFLD at baseline; among them, 524 completed a follow-up visit 4.6 years after baseline and were screened by ultrasonography. Incident cases of NAFLD ( $n = 146$ ) were identified at the 4.6-year follow-up visit, of which 90 participants were eligible for this study involving gut microbiota, according to the following criteria to exclude participants: existed fatty liver, acute infectious disease, biliary obstructive diseases, alcohol abuse (more than 140 g of ethanol/week for men or 70 g of ethanol/week for women), acute or chronic cholecystitis, acute or chronic viral hepatitis, cirrhosis, diarrhea, known hyperthyroidism or hypothyroidism, chronic renal insufficiency,

heart failure, presence of cancer, pregnancy, stroke in acute phase, receipt of any antibiotic treatment within 2 weeks or receipt of any probiotic or prebiotic within 1 week before sample collection, and suffering from chronic or acute gastrointestinal diseases (including diarrhea, gastrointestinal infection, and inflammatory bowel disease) in recent 1 month before sample collection. Controls ( $n = 90$  for a case-control ratio of 1:1) were chosen from the remaining participants who did not develop NAFLD by the follow-up visit. To control for the risk profiles in patients who developed NAFLD and those who did not, controls were matched for age ( $\pm 3$  years), sex (male and female), BMI ( $\pm 3$  kg/m<sup>2</sup>) at both baseline and follow-up, and BMI change ( $\pm 0.5$  kg/m<sup>2</sup>). The study was approved by the ethics committee of the Shanghai Sixth People's Hospital (approval no: 2014–27), following the principles of the Declaration of Helsinki. Written informed consent was obtained from all participants.

### Evaluating the diagnostic ability of the model in external cohorts

To our knowledge, no similar studies have conducted long-term follow-up of NAFLD development in healthy individuals using a combination of gut metagenome, metabolome, and clinical features as a risk assessment tool. Thus, we were unable to test our prospective model directly in an external cohort. Instead, we used external case-control cohorts to examine the ability of our final prognostic model to classify correctly NAFLD and healthy participants. Four cohorts were used, including two cohorts of Chinese: (i) 78 patients with NAFLD and 10 controls without NAFLD, as diagnosed with biopsy (BioProject ID: PRJNA732131), and (ii) 111 MRS-diagnosed NAFLD patients and 8 controls (BioProject IDs: PRJNA703757 and PRJNA414688); and two biopsy-diagnosed cohorts of other ethnicity: (iii) a European cohort of 46 patients with NAFLD and 10 controls (54) and (iv) a U.S. cohort of 26 cirrhosis patients and 54 controls (49). For further additional data (e.g., anthropometric and/or available clinical data) for the two Chinese validation cohorts besides grouping information, please contact the corresponding author.

Because some selected features included in the final model were not available in the external cohorts, we were unable to test our model directly. Instead, we built a new prognostic model based on the NAFLD<sup>-/+</sup> and NAFLD<sup>-/-</sup> groups using a subset of the 18 selected features that were available in the external cohorts. In the model for the two Chinese cohorts and the European cohort, 9 of the 18 selected features were used: two genera, three pathways, two anthropometric parameters, and two noninvasive clinical metadata; whereas 7 of the 18 selected features were used in the model for the U.S. cohort: two genera, one pathway, two anthropometric parameters, and two noninvasive clinical metadata. Performances of models, including ROC curves, precision-recall curves, and confusion matrices (generated with the optimal probability cutoff of the ROC curve), were produced by applying the model to the unseen external cohort data.

### Statistical analysis

Statistical analyses of clinical data were performed with SAS version 9.4 (SAS Institute Inc.). Normally distributed data were expressed as means  $\pm$  SD. Data that were not normally distributed, as determined using the Kolmogorov-Smirnov test, were logarithmically transformed before analysis and expressed as median with lower and upper quartiles. Student's *t* test and chi-square tests were used to assess differences between two groups



for continuous and categorical variables, respectively. In addition, analysis of covariance was used for continuous variables to assess the difference between the two groups after adjusting for HOMA-IR.

Metagenomic data, including taxonomy and functional data, and metabolomic data were analyzed in R software version 3.6.3. Metagenomic data were analyzed with the zero-inflated Gaussian mixture model, using the function `fitZig` from R package `metagenomeSeq` (96) with the default settings; metabolomic data were analyzed using the generalized linear model with inverse gamma distribution. Wilcoxon rank-sum tests were used to test for significant differences in alpha diversity. PERMANOVA was used to analyze beta diversity with `adonis` function from R package `vegan`. A Mantel test, implemented in `mantel` from R package `vegan`, using Spearman's correlation coefficient was used to analyze the associations between microbiome and metabolites. Bray-Curtis dissimilarity matrices based on taxonomic relative abundance and Euclidean dissimilarity matrix for each metabolite were computed to perform this test. The auROCs of different models were compared with the DeLong test, using the `roc.test` function from R package `pROC` (97). Data were considered statistically significant at  $P$  value  $< 0.05$ . The Benjamini-Hochberg procedure was applied to calculate the FDR to adjust  $P$  values for multiple hypothesis testing.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Data and materials availability:

All data associated with this study are in the paper or the Supplementary Materials. Raw metagenomic sequencing data for all samples have been deposited in NCBI Sequencing Read Archive under BioProject IDs PRJNA728908 and PRJNA686835 (only SRR13279648, SRR13279753, SRR13279664, and SRR13279666). Metabolite profiles have been deposited in MetaboLights ([www.ebi.ac.uk/metabolights/](http://www.ebi.ac.uk/metabolights/)) with accession

MTBLS2615. The data used to generate main and supplementary figures are in data file S1. Further information and requests for resources and reagents should be directed to G.P. (gianni.panagiotou@leibniz-hki.de).

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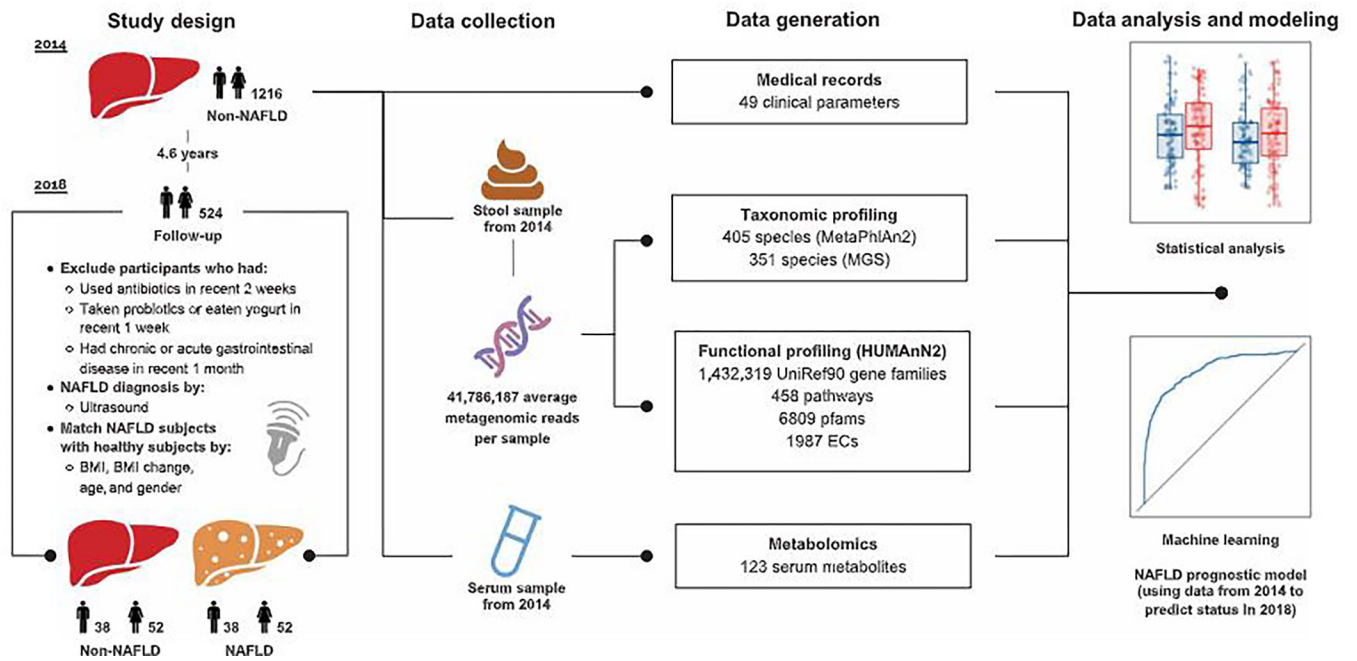
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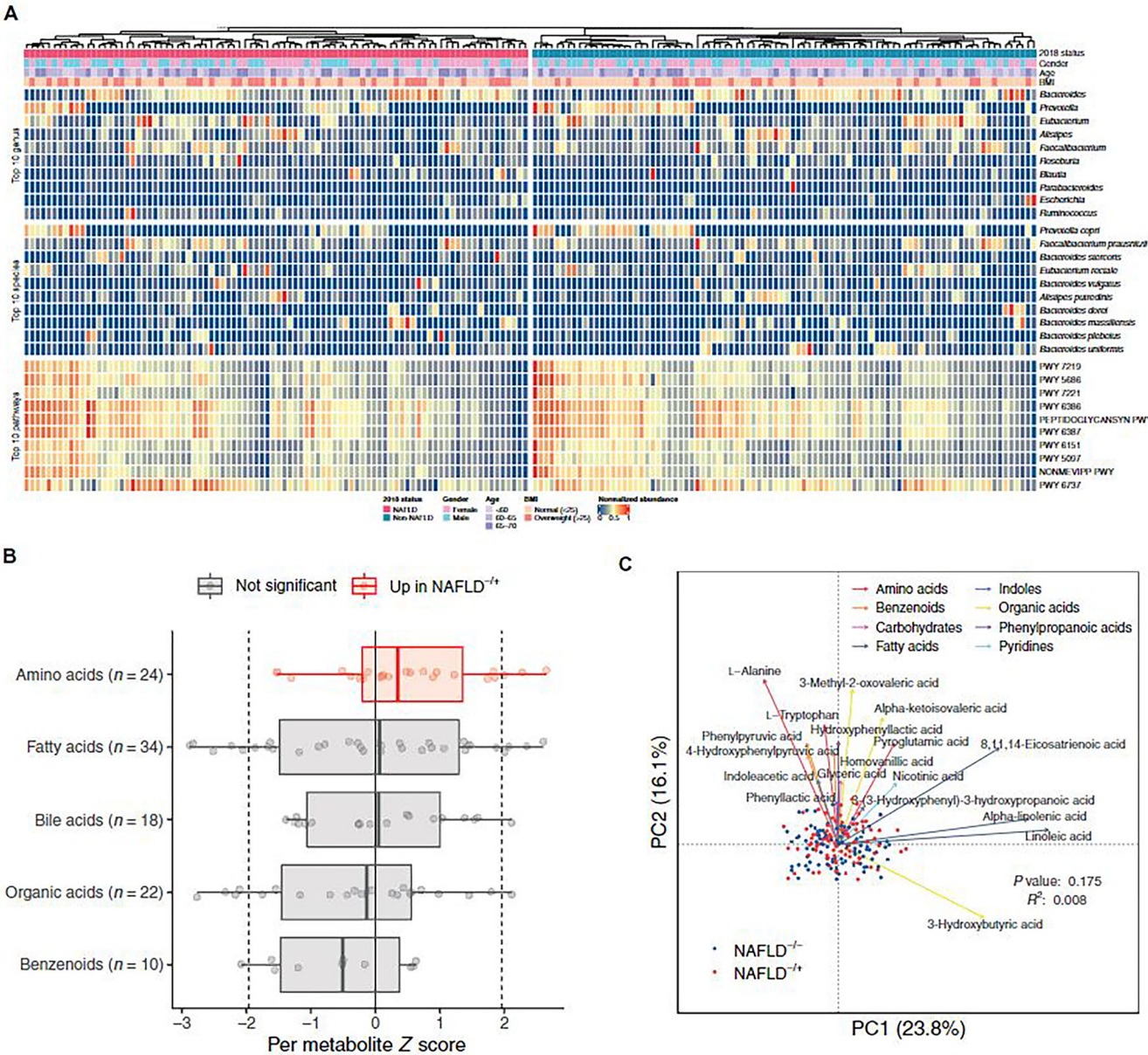
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**Fig. 1. Overview of the prospective study design.**

A graphical representation summarizing the study design, data collection, and the methodologies of data generation and analysis. Further details of the study design can be found in fig. S1.

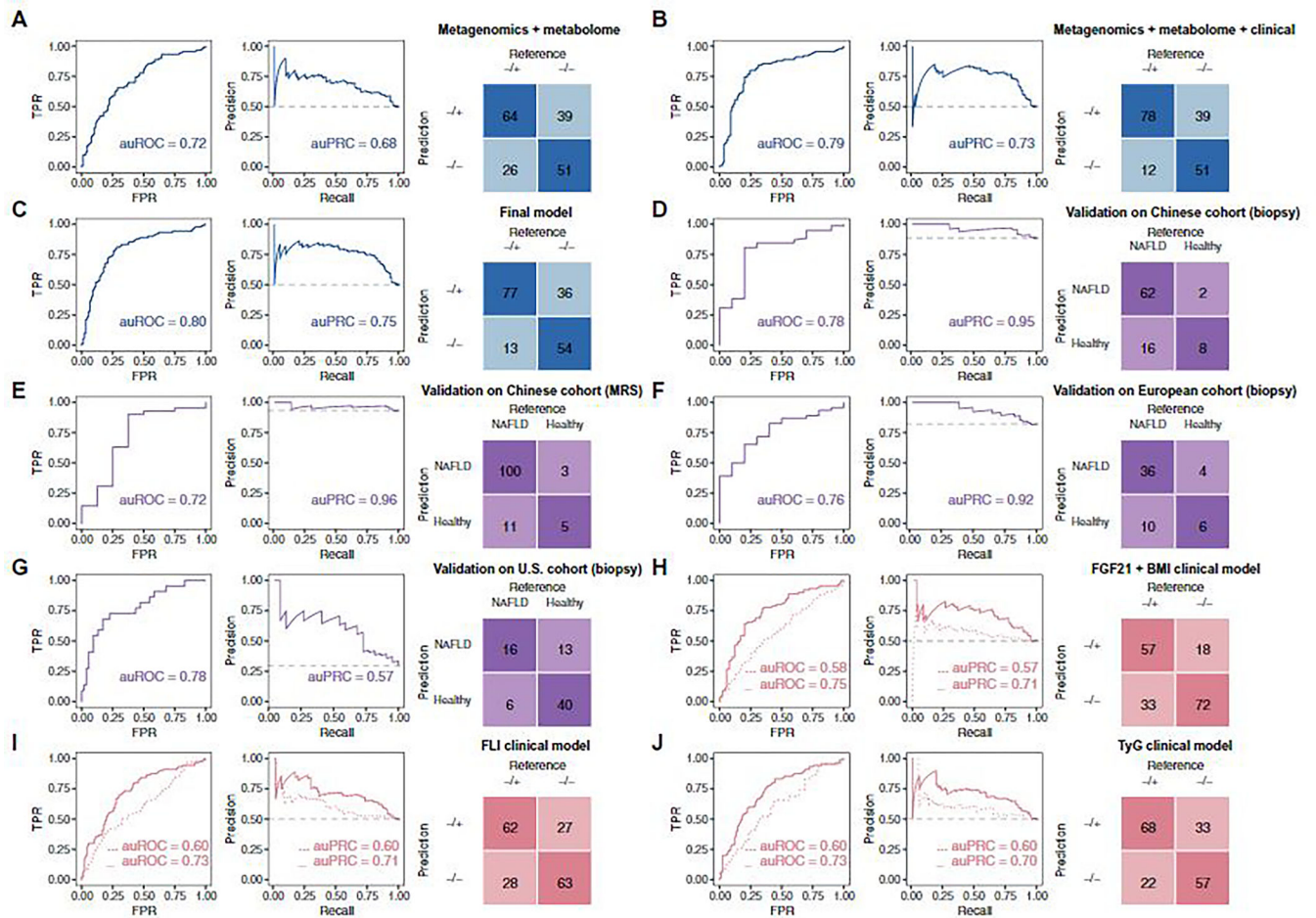




**Fig. 2. Global characteristics of gut microbiome and serum metabolome.** (A) Relative abundance of the 10 most abundant genera, species, and pathways for the 180 participants at baseline, grouped by NAFLD status at the follow-up visit. Anthropometric characteristics of the participants at baseline are also shown. Abundance values are normalized to the range of 0 and 1. (B) Changes of metabolites ( $\mu\text{M}$ ) in metabolite classes containing at least 10 metabolites. Each point represents a metabolite and its z score from Wilcoxon rank-sum test comparing the two groups (negative indicates higher abundance in NAFLD<sup>-/-</sup>; positive indicates higher abundance in NAFLD<sup>-/+</sup>). Dotted lines at  $-1.96$  and  $1.96$  denote the significance threshold. Colors indicate comparisons between z scores of metabolites in a metabolite class against the z scores of metabolites in all other classes. Box plots show median, lower/upper quartiles, and whiskers (the last data points 1.5 times

interquartile range from the lower or upper quartiles). (C) Principal coordinates analysis for 180 participants based on Bray-Curtis distances using baseline serum concentrations of 123 metabolites. For each metabolite class, the top 3 (or fewer) metabolites that were significantly associated with the metabolome variation in the study cohort are shown. PC, principal coordinates.





**Fig. 3. Predictive performance of machine learning models in the study cohort and diagnostic performance of the final model in external cohorts.**

(A to C, in blue) Performance of leave-one-out iterative machine learning models discriminating between NAFLD<sup>-/+</sup> and NAFLD<sup>-/-</sup> groups using features of the following: (A) metagenomics + metabolome, (B) metagenomics + metabolome + 2 clinical parameters (HDL and fasting insulin), and (C) metagenomics + metabolome + 2 clinical parameters (HDL and fasting insulin) + anthropometrics (BMI and age). (D to G, in purple) Diagnostic performances of a model built based on subsets of the selected features to discriminate between participants who were healthy or had NAFLD in four external cohorts: (D) a Chinese cohort in which NAFLD diagnosis was determined with biopsy, (E) a Chinese cohort in which NAFLD diagnosis was based on MRS, (F) a biopsy-diagnosed European NAFLD cohort, and (G) a biopsy-diagnosed U.S. cirrhosis cohort. (H to J, in peach) Leave-one-out iterative machine learning performance to discriminate between NAFLD<sup>-/+</sup> and NAFLD<sup>-/-</sup> groups in models of: (H) FGF21 + BMI clinical model, with and without metagenomics + metabolome features; (I) FLI clinical model, with and without metagenomics + metabolome features; and (J) TyG clinical model, with and without metagenomics + metabolome features. (H to J) Models without metagenomics and metabolome features were trained by logistic regression (dotted lines); models including metagenomics and metabolome features were trained by random forest (solid lines).

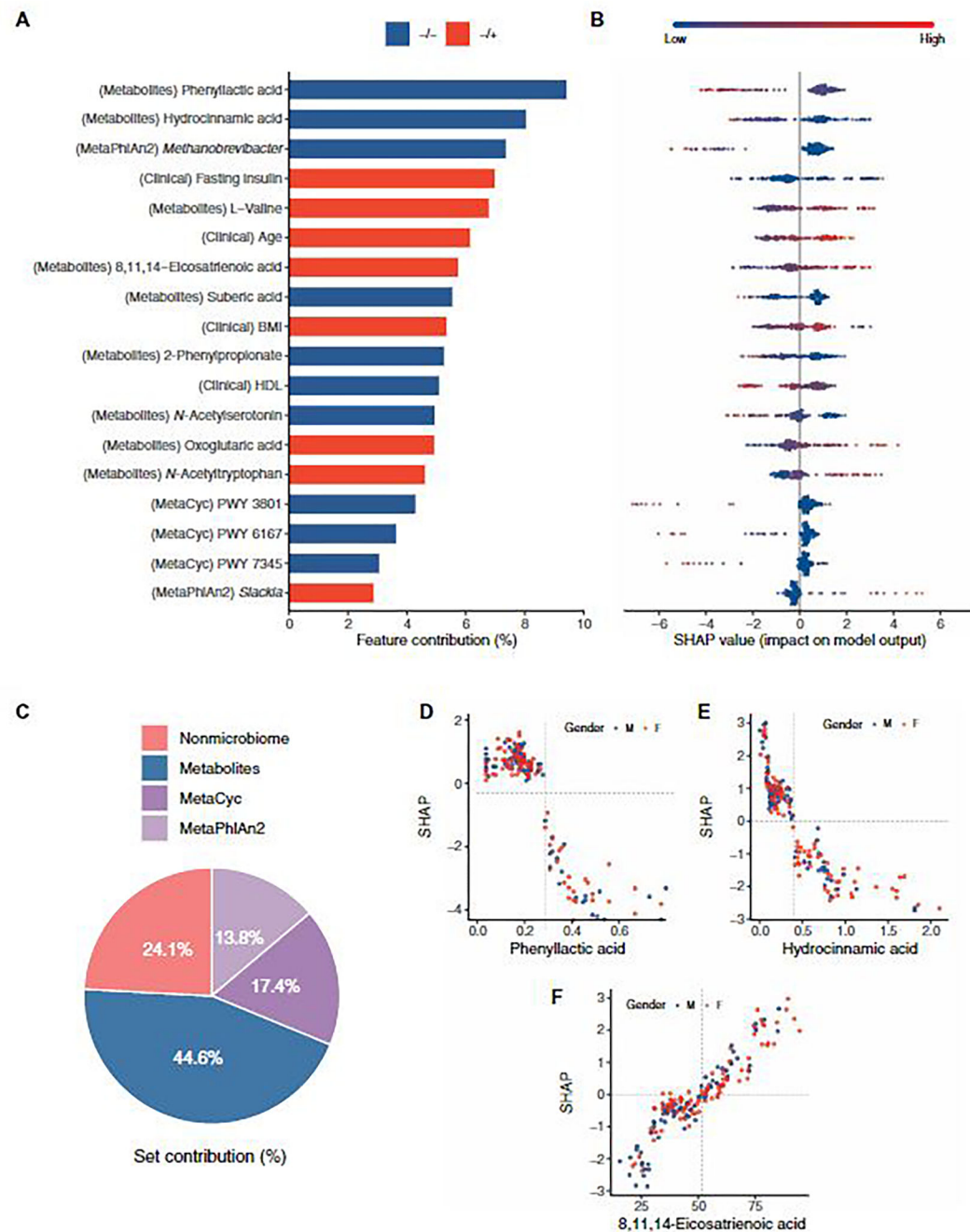
Confusion matrices in (F) to (H) are from models with metagenomics and metabolome features. The figure colors represent the purpose of the model: blue, model construction; purple, external validation in cohorts of different ethnicity; peach, testing performance of previous clinical models in our cohort. Further details of the overall machine learning analysis framework can be found in fig. S6. auROC, area under the receiver operating characteristics curve; auPRC, area under the precision-recall curve; TPR, true-positive rate; FPR, false-positive rate.

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**Fig. 4. SHAP-based model interpretation.**

(A) Bar plot of selected features and their contribution in the NAFLD prediction model. Features are in descending order by contribution (also known as importance) in the model. Blue bar, higher value of the feature for association with NAFLD<sup>-/-</sup>; red bar, higher value of the feature for association with NAFLD<sup>-/+</sup>. Details of associations are shown in (B) a bee swarm plot in which each point represents a participant ( $n = 180$ ). Color indicates the value of the feature, with red higher and blue lower. Negative SHAP value indicates the feature attribution for prediction of NAFLD<sup>-/-</sup>; Positive SHAP value indicates the feature

attribution for prediction of NAFLD<sup>-/+</sup>. **(C)** Feature category contribution calculated by summing the SHAP values per set. **(D to F)** Examples of SHAP dependence plots, showing the effect the feature has on model prediction. Each point represents a participant ( $n = 180$ ). Color indicates sex with blue for male and red for female.  $X$  axis is the feature value, and  $y$  axis is the SHAP value for the feature. The optimal thresholds for features are indicated by the vertical dotted lines.

Baseline characteristic of participants in INAFLD<sup>-/+</sup> and NAFLD<sup>-/+</sup> groups.

Data are expressed as means  $\pm$  SD or median (lower quartile and upper quartile) for continuous variables, and *n* represents percentage for categorical variables. BMI, body mass Index; SBP, systolic blood pressure; DBP, diastolic blood pressure; FBG fasting blood glucose; hs-CRP, high sensitivity C-reactive protein; TC, total cholesterol; TG, triglycerides; HDL-C high-density lipoprotein cholesterol; FGF-21, fibroblast growth factor 21; HbA1c, hemoglobin A1C; HQMA-IR, homeostasis model assessment insulin resistance; apo, apolipoprotein; PG30, 30-min postprandial plasma glucose; PG120, 120-min postprandial plasma glucose; INS30, 30-min postprandial insulin; INS120, 120-min postprandial insulin; FINS, fasting insulin; Cr, creatinine; UA1b/Ucr, urinary albumin tocreatinine ratio; UA uric acid; FFA, free fatty acid; TBIL total bilirubin; GA, glycated albumin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma glutamyl transferase.

| Characteristic                 | Total (n = ISO)      | NAFLD <sup>-/+</sup> (n=94) | NAFLD <sup>-/+</sup> (n = 90) | P value <sup>*</sup> | P value <sup>‡</sup> |
|--------------------------------|----------------------|-----------------------------|-------------------------------|----------------------|----------------------|
| Anthropometric parameters      |                      |                             |                               |                      |                      |
| Sex (male)                     | 76 (42.22%)          | 33 (42.22%)                 | 36 (42.22%)                   | -                    | -                    |
| Age (years)                    | 62.51 $\pm$ 3.81     | 62.03 $\pm$ 3.78            | 62.99 $\pm$ 3.81              | 0.0921               | 0.1481               |
| Weight (kg)                    | 62.82 $\pm$ 8.07     | 62.47 $\pm$ 7.53            | 63.16 $\pm$ 8.6               | 0.5688               | 0.3617               |
| BMI (kg/m <sup>2</sup> )       | 24.55 $\pm$ 2.13     | 24.35 $\pm$ 2               | 24.75 $\pm$ 2.25              | 0.2059               | 0.0681               |
| SBP (mmHg) <sup>‡</sup>        | 130 (130, 140)       | 130 (120 140)               | 130 (120 140)                 | 0.2854               | 0.3363               |
| DBP (mmHg) <sup>‡</sup>        | 80 (80, 86)          | 80 (80, 84)                 | 80 (80, 86)                   | 0.7579               | 0.7304               |
| Glucose homeostasis parameters |                      |                             |                               |                      |                      |
| FBG (mM) <sup>‡</sup>          | 5.93 (5.57, 6.36)    | 5.86 (5.54, 6.33)           | 6.02 (5.67, 6.39)             | 0.2866               | 0.8033               |
| PG30 (mM) <sup>‡</sup>         | 10.29 (9.3, 11.45)   | 10.12 (9.19, 11.16)         | 10.34 (9.37, 11.76)           | 0.2219               | 0.4460               |
| PG120(mM) <sup>‡</sup>         | 7.72 (16.63, 9.3)    | 7.49 (6.17, 8.96)           | 7.85 (7.12, 9.58)             | 0.0612               | 0.2829               |
| FINS(uU/ml) <sup>‡</sup>       | 5.2 (4.03, 7.11)     | 5.06 (3.99, 6.06)           | 5.42 (4.11, 8.08)             | 0.0266               | 0.8033               |
| INS30 (uU/ml) <sup>‡</sup>     | 38.82 (26.11, 57.25) | 36.25 (25.73, 50.64)        | 40.39 (26.77, 60.31)          | 0.2956               | 0.5751               |
| INS120 (uU/ml) <sup>‡</sup>    | 37.62 (25.13, 57.42) | 34.59 (21.78, 53.46)        | 41.65 (29.18, 60.03)          | 0.0562               | 0.3623               |
| GA (%) <sup>‡</sup>            | 0.61 (0.57, 0.67)    | 0.62 (0.57, 0.68)           | 0.61 (0.57, 0.67)             | 0.6013               | 0.1853               |
| HbA1c (%) <sup>‡</sup>         | 5.5 (5.2, 5.9)       | 5.4 (5.2, 5.7)              | 5.6 (5.3, 6)                  | 0.0604               | 0.2707               |

| Characteristic                                 | Total (n = ISO)         | NAFLD <sup>-/-</sup> (n=94) | NAFLD <sup>-/-</sup> (n = 90) | P value* | P value <sup>†</sup> |
|--|-------------------------|-----------------------------|-------------------------------|----------|----------------------|
| HOHA-IR <sup>‡</sup>                           | 1.35 (1.05, 1.99)       | 1.33 (1.02, 1.69)           | 1.5 (1.09, 2.21)              | 0.0266   | -                    |
| HOMA-β <sup>‡</sup>                            | 40.23 (31.6, 54.5)      | 37.68 (31, 52.44)           | 42.13 (32.75, 56.04)          | 0.1038   | 0.8752               |
| Serum liver enzymes and renal function indexes |                         |                             |                               |          |                      |
| ALT (IU/liter) <sup>‡</sup>                    | 15 (12, 18)             | 15 (12, 17)                 | 15 (13, 18)                   | 0.2112   | 0.2477               |
| AST (IU/liter) <sup>‡</sup>                    | 21 (19, 23)             | 21 (19, 23)                 | 21 (19, 23)                   | 0.9994   | 0.8621               |
| GGT (IU/liter) <sup>‡</sup>                    | 18 (15, 25)             | 17 (14, 22)                 | 20 (16, 27)                   | 0.2764   | 0.2087               |
| TBIL (μM) <sup>‡</sup>                         | 10.7 (9, 14.4)          | 10.9 (9, 14)                | 10.7 (9, 14.5)                | 0.9516   | 0.7712               |
| Cr (μM) <sup>‡</sup>                           | 64 (56, 73)             | 64 (57, 73)                 | 54 (55, 76)                   | 0.8085   | 0.4501               |
| UA/b/Ucr <sup>‡</sup>                          | 6.79 (5.12, 12.59)      | 6.62 (5.16, 13.03)          | 6.84 (5.02, 11.91)            | 0.5568   | 0.4701               |
| UA (μM) <sup>‡</sup>                           | 295 (249, 341)          | 269.50 (243, 334)           | 302 (258, 342)                | 0.1618   | 0.0913               |
| Lipid profiles                                 |                         |                             |                               |          |                      |
| TG (mM) <sup>‡</sup>                           | 1.20 (0.86, 1.66)       | 1.07 (0.80, 1.53)           | 1.34 (0.90, 1.80)             | 0.0051   | 0.0193               |
| TC (mM) <sup>‡</sup>                           | 4.97 (4.44, 5.58)       | 4.80 (4.41, 5.57)           | 5.01 (4.46, 5.58)             | 0.4808   | 0.7310               |
| FFA (μM) <sup>‡</sup>                          | 497 (377, 666)          | 497.5 (371,688)             | 497 (395, 650)                | 0.3526   | 0.5783               |
| HDL-C (mM) <sup>‡</sup>                        | 1.35 (1.14, 1.53)       | 1.40 (1.19, 1.64)           | 1.30 (1.09, 1.48)             | 0.0895   | 0.1451               |
| LDL-C (mM)                                     | 3.08 ± 0.72             | 2.99 ± 0.74                 | 3.17 ± 0.69                   | 0.0965   | 0.1969               |
| apoA-1 (g/liter)                               | 1.49 ± 0.26             | 1.51 ± 0.28                 | 1.46 ± 0.24                   | 0.2124   | 0.3083               |
| apoB (g/liter)                                 | 0.91 ± 0.16             | 0.9 ± 0.17                  | 0.93 ± 0.16                   | 0.1899   | 0.3731               |
| apoE (mg/dl) <sup>‡</sup>                      | 3.92 (3.23, 4.67)       | 3.84 (3.22, 4.56)           | 4.09 (3.4, 4.87)              | 0.0609   | 0.1709               |
| Lipoprotein (a) (mg/dl) <sup>‡</sup>           | 14.05 (5.81, 25.07)     | 15.33 (6.12, 25.89)         | 12.51 (5.78, 23.24)           | 0.7654   | 0.6452               |
| Cytokines                                      |                         |                             |                               |          |                      |
| FGF21 (pg/ml) <sup>‡</sup>                     | 302.06 (180.19, 429.52) | 268.84 (171.83, 389.11)     | 332.27 (236.74, 452.81)       | 0.9538   | 0.9304               |
| hs-CRP (μg/ml) <sup>‡</sup>                    | 0.63 (0.35, 1.17)       | 0.53 (0.28, 1.17)           | 0.72 (0.43, 1.16)             | 0.0351   | 0.0758               |

\* P value denotes differences between NAFLD<sup>-/+</sup> and NAFLD<sup>-/-</sup> analyzed by *t* test without adjustment.



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$P$  value denotes differences between NAFLD<sup>-/+</sup> and NAFLD<sup>-/-</sup> analyzed by analysis of covariance with HOMA-IR adjusted.

$^{\#}$  Log-transformed before analysis.