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Pathophysiology-based subtypes of individuals at high risk of type 2 diabetes: multi-omics profiles and lifestyle differences across subtypes

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ABSTRACT

Background: Previous studies identified subtypes among individuals at elevated risk of type 2 diabetes mellitus (T2DM), yet the molecular signatures distinguishing these subtypes remain poorly characterized. We aimed to identify and characterize T2DM risk subtypes using routine and non-routine clinical variable lists, and to compare their metabolomic, proteomic, and lifestyle profiles.

Methods: In the Netherlands Epidemiology of Obesity study (median age 56 years; median BMI 29 kg/m^2), we applied partitioning around medoids (PAM) clustering using two variable lists (routine, $N = 5235$; non-routine, $N = 1510$), both including variables derived from a liquid mixed meal challenge. Cox proportional hazards models estimated associations between subtypes and T2DM incidence. Random forest models identified discriminative metabolites and proteins across subtypes.

Results: Each variable list yielded four subtypes ranging from an insulin-sensitive and lean profile (subtype 1) to an obese profile with ectopic fat accumulation and insulin resistance (subtype 4), with a graded increase in T2DM risk (hazard ratios ranging from 1.9 [95% confidence interval (CI): 0.4–9.8] to 19.5 [95% CI: 9.1–41.6]). Both subtyping schemes captured metabolic heterogeneity beyond conventional weight-by-glycemia categories, e.g., redistributing overweight or obese but normoglycemic individuals across subtypes with divergent metabolic profiles and T2DM risks. While multi-omics profiling revealed shared metabolic and proteomic markers across higher-risk subtypes (e.g., glycoprotein acetyls, glucose, hepatocyte growth factor), subtype assignment was predominantly driven by fasting glucose and lipoprotein levels (e.g., very-low-density lipoprotein [VLDL]), with additional subtype-specific molecular signatures (e.g., branched-chain amino acids). Individuals in the higher-risk subtypes also exhibited less healthy lifestyle characteristics, including poorer dietary quality.

Conclusions: Four metabolic subtypes with graded T2DM risk were identified in a predominantly overweight or obese, middle-aged population, revealing metabolic heterogeneity among individuals who appear homogeneous under conventional weight-by-glycemia categories. Although subtype differentiation was largely driven by fasting glucose and lipoproteins, multi-omics profiling uncovered additional molecular signatures, suggesting that data-driven subtyping may complement conventional risk markers and inform targeted prevention.

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1. Introduction

Type 2 diabetes mellitus (T2DM) remains a global health burden that frequently causes multiple complications, such as coronary heart disease and chronic kidney disease, leading to a decrease in life expectancy and quality of life [1]. The progression to T2DM typically passes through a prediabetic phase in which lifestyle interventions can slow or reverse disease development, yet the effectiveness of such interventions varies considerably across individuals [2]. This heterogeneity suggests that at-risk populations may comprise distinct subtypes with divergent metabolic pathophysiology, and identifying these subtypes could enable more targeted prevention.

Data-driven clustering can stratify individuals at increased risk of T2DM into subtypes with distinct metabolic trajectories [3]. Wagner et al. identified six such subtypes in the Tübingen Family Study and Lifestyle Program (TUEF/TULIP) cohort using non-routine measures, including oral glucose tolerance test (OGTT)-derived parameters, magnetic resonance imaging (MRI)-measured body fat distribution, liver fat content, and a genetic risk score for T2DM [4]. These clusters were subsequently replicated in the Whitehall II cohort using routinely accessible anthropometric and glycemic variables [4]. We refer to the different clustering variable lists as the "non-routine variable list" and "routine variable list", respectively (Supplementary Table 1). Despite these findings, several questions remain. First, whether these subtypes generalize to other at-risk populations is unclear, particularly given the reliance on OGTT-derived parameters, which limits replication in cohorts where this test was not performed. A liquid mixed meal challenge, which elicits a more physiological metabolic response [5], represents a pragmatic alternative, yet its utility for subtype identification has not been evaluated. Second, no study has directly compared subtype assignments derived from the non-routine versus routine variable lists within the same population, nor examined how data-driven subtypes relate to conventional subtyping based on body weight and glycemic status. Third, although fasting glucose serves as the clinical standard for T2DM diagnosis, glucose alone may fail to capture the full heterogeneity of metabolic disturbances. Multi-omics approaches, such as metabolomics and proteomics, can characterize the molecular profiles that distinguish different subtypes, and may provide biological insights into subtype-specific risk [6,7].

Here, we address these gaps using the Netherlands Epidemiology of Obesity (NEO) study, a Dutch cohort of middle-aged participants at elevated T2DM risk, the majority of whom were overweight or obese [8]. Our aims are threefold: 1) to identify T2DM risk subtypes using both the non-routine and routine variable lists, incorporating variables derived from a liquid mixed meal challenge; 2) to assess the concordance between non-routine and routine subtype assignments within the same population, and compare both with conventional weight-by-glycemia subtypes; and 3) to characterize the metabolomic, proteomic, and lifestyle profiles across subtypes.

2. Methods

2.1. Study population

The NEO study design has been described previously [8] and in the Supplementary methods. In brief, 6671 participants aged 45–65 years, with an oversampling of overweight or obese individuals, were enrolled between 2008 and 2012. At the baseline visit, participants ingested a liquid mixed meal (400 mL, containing 600 kcal) with 16% of energy derived from protein, 50% from carbohydrates, and 34% from fat. Fasting and postprandial blood samples were collected for measurements of plasma glucose, serum insulin, and serum triglycerides (TG). Participants were excluded from the analyses based on the following criteria: 1) prevalent T2DM; 2) absence of follow-up data; 3) non-fasting status; and 4) incomplete liquid mixed meal challenge. The selection flowchart is shown in Supplementary Fig. 1. The NEO study was approved by the Medical Ethics Committee of the Leiden University Medical Center (LUMC), Leiden, the Netherlands, and all participants provided written informed consent.

2.2. Feature variable lists for clustering analysis

Following the approach of Wagner et al. [4], we selected comparable metabolic parameters derived from the liquid mixed meal challenge and other risk factors for T2DM. All variables in both non-routine and routine lists are presented in Supplementary Table 1. The detailed measurements of the clustering variables are described in Supplementary methods. All clustering variables from the two lists were scaled as z-scores in a sex-stratified manner. To minimize the influence of outliers on clustering, participants whose absolute z-scores exceeded 5 were excluded ($N = 19$ when using the non-routine variable list; $N = 87$ when using the routine variable list, shown in Supplementary Fig. 1).

2.3. Conventional subtyping based on body weight and glycemic status

According to the World Health Organization (WHO) criteria of body mass index (BMI) classification, participants were categorized into three weight groups (normal: 18.5–24.9 kg/m^2, overweight: 25–29.9 kg/m^2, and obese: $\geq$ 30 kg/m^2) [9]. To assess the glycemic status, two major forms of prediabetes (i.e., impaired fasting glucose [IFG] and impaired glucose tolerance [IGT]) were defined by the WHO criteria. IFG was defined as a fasting plasma glucose of 6.1–6.9 mmol/L, while IGT was defined by a 2-h glucose value during an OGTT of 7.8–11.0 mmol/L [10]. Since an OGTT was not performed in the NEO study, we used a postprandial glucose level at 150 min $\geq$ 7.8 mmol/L as a surrogate criterion for IGT. Prediabetes was defined as IFG alone, IGT alone, or both. We further categorized participants into six groups based on body weight and glycemic status: 1) normal weight + normoglycemia; 2) overweight + normoglycemia; 3) obesity + normoglycemia; 4) normal weight + prediabetes; 5) overweight + prediabetes; 6) obesity + prediabetes.

2.4. Ascertainment of incident T2DM

After the baseline visit, participants were followed for the occurrence of T2DM. Diagnoses of T2DM were extracted from the electronic health records of the participating general practitioners (GPs) between July 2012 and November 2013, and between October 2017 and July 2018. Health records were screened with the International Classification of Primary Care (ICPC) code T90 and relevant medication prescriptions with the Anatomical Therapeutic Chemical (ATC) code A10 [11]. Details of the extraction process are provided in Supplementary methods. Follow-up time was defined as the number of days between the baseline visit and the date of diagnosis, or censoring due to death, loss to follow-up, or the end of follow-up (extraction date at the GP in 2013 or 2018), whichever came first.

2.5. Metabolomic measurements

Metabolomic measurements were performed in plasma samples with the Nightingale Health high-throughput targeted ^{1}H-nuclear magnetic resonance (NMR) platform, which has been described previously [12]. This platform provides absolute quantifications for 148 metabolites (Supplementary Table 2). Quality control of the metabolomic data involved multiple steps: 1) All metabolite values equal to or below 0 were set to missing; 2) Individuals with >40% missing metabolite values, and metabolites with >10% missing values were excluded; 3) The remaining missing values, assumed to reflect concentrations below the limit of quantification, were imputed with half of the minimum observed values as in the previous study [13]. All metabolite measurements were standardized to z-scores before statistical analysis.

2.6. Proteomic measurements

Proteomic measurements were performed with the Olink Explore Inflammation panel (proximity extension assay) in a subpopulation of the cohort ($N = 1542$). The data are available as Normalized Protein eXpression (NPX) units (an arbitrary unit on a log2 scale). Details of the Olink assay have been reported elsewhere [14]. In the current study, 365 proteins were measured. Two proteins (IL-4 and NFATC3) with all NPX values lower than the limit of detection were excluded, leaving 363 proteins for further analysis. All protein measurements were standardized to z-scores before statistical analysis.

2.7. Lifestyle and psychosocial factors

The following lifestyle and psychosocial factors were compared across subtypes: smoking status (current, former, never), diet quality, physical activity, sleep, and depressive symptoms. Diet quality was characterized by the Dutch Healthy Diet Index (DHDI), a score from 0 to 150 that reflects adherence to the Dutch dietary guidelines [15]. Physical activity during leisure time was characterized by the Short Questionnaire to Assess Health-Enhancing Physical Activity (SQUASH) and expressed in metabolic equivalent of task hours per week (METh/week) [16]. Sleep quality was assessed using the Pittsburgh Sleep Quality Index (PSQI; range 0–21, with a score > 5 indicating poor sleep), which captures sleep duration, disturbances, and use of sleep medication [17]. To characterize chronotype, the midpoint of sleep was calculated as time of waking up − (0.5 * time in bed) [18]. Depressive symptoms were assessed using the Inventory of Depressive Symptomatology (IDS; range 0–84, 30 items) [19].

2.8. Statistical analysis

Statistical analysis was conducted with R (version 4.4.0) and Python (version 3.12.2). As in the prior study [4], complete-case analysis was used in clustering analysis.

2.8.1. Clustering analysis using two lists of variables

For each variable list, clustering analyses were performed separately. The optimal number of clusters was determined using silhouette widths. When silhouette analysis was inconclusive, consensus clustering was used for confirmation, based on inspection of the consensus matrix heatmap, delta area plots, and cluster consensus values. The number of clusters was set from 2 to 8. Consensus clustering was conducted using the *ConsensusClusterPlus* R package (version 1.68.0) with 100 iterations. Clustering analysis was further performed using partitioning around medoids (PAM) with Gower distance and the optimal number of clusters, implemented via the *cluster* R package (version 2.1.8.1).

2.8.2. Concordance between subtypes derived from the two variable lists

For individuals with measurements available in both variable lists, comparison of subtype assignments was visualized by Sankey plots using the *ggalluvial* R package (version 0.12.5), together with crosstabs. Cluster stability was assessed by the Jaccard index with the *clusterboot* function from the *fpc* R package (version 2.2.13) with 100 repetitions [20]. Additionally, both data-driven subtype assignments were compared with the conventional weight-by-glycemia subtypes using Sankey plots and cross-tabulation tables.

2.8.3. Associations between subtypes and T2DM incidence

Kaplan–Meier curves were used to estimate the cumulative incidence of T2DM across subtypes. Cox proportional hazards models were applied to examine the association between subtype assignments and incident T2DM, with subtype 1 as the reference group due to its favorable metabolic profile. Sequential models were constructed. Model 1 was unadjusted. Model 2 was adjusted for age and sex; BMI was additionally included in analyses based on the non-routine variable list but excluded from analyses based on the routine variable list, in which it served as a clustering variable, to avoid overadjustment. The proportional hazards assumption was verified using Schoenfeld residual tests, with no violations detected (global test $P > 0.05$). In sensitivity analyses, 1) models were additionally adjusted for lifestyle factors (i.e., diet quality, total physical activity, sleep quality, smoking status) and baseline clinical metabolic markers (fasting glucose for both lists, fasting TG for the non-routine list only); and 2) the analysis was restricted to participants with at least one year of follow-up, using the same covariate adjustment. Notably, since baseline metabolic markers had already been included as clustering variables, additional adjustment was performed in an attempt to assess whether data-driven subtyping provides information on T2DM risk stratification beyond baseline metabolic traits.

According to the results from Cox regression models, subtypes derived from the non-routine variable list were labelled as relatively low-, intermediate-, intermediate-high-, and high-risk. Of note, these labels reflect relative risk within the study population, not absolute population risk. For clarity, intermediate- to high-risk groups are collectively referred to as “higher-risk” groups.

2.8.4. Metabolic and proteomic characterization underlying each higher-risk T2DM subtype

Random forest models were used to identify the most discriminative circulating metabolites and proteins for each higher-risk subtype using the *ranger* R package (version 0.18.0). A detailed description is provided in Supplementary methods. Feature importance was evaluated with the mean decrease in accuracy metric [21]. The top 10 metabolites and proteins in each higher-risk subtype were selected for visualization. Distributions of key metabolites and proteins across subtypes were illustrated by violin plots. Statistical differences were assessed using generalized linear models (GLMs), with individual metabolites or proteins specified as dependent variables and subtype assignments as independent variables. Multiple testing was corrected by conservative Bonferroni at a significance level of 0.05 divided by the number of tests.

2.8.5. Functional enrichment analysis

To further explore the biological relevance of subtype-specific proteomic signatures, we performed Gene Ontology (GO) enrichment analysis on the top 50 proteins with the highest importance rankings in each higher-risk subtype. The top 10 significantly enriched GO terms were reported. Enrichment analysis was conducted using the *clusterProfiler* package (hypergeometric test, version 4.14.4). Multiple testing correction was applied using the Benjamini–Hochberg method.

2.8.6. Lifestyle and psychosocial characteristics across subtypes

Among subtypes derived from the non-routine variable list, differences in lifestyle and psychosocial factors described above were compared. For the continuous variables, differences across subtypes were evaluated by GLMs, with each lifestyle or psychosocial factor specified as the dependent variable and subtype assignments as the independent variable.

To assess the relative importance of lifestyle and psychosocial factors in distinguishing subtypes, the ordinal logistic regression model was fitted using the *MASS* package (version 7.3.65). Subtype assignments were treated as the dependent variable, and the following variables were included as the independent variables: age, sex, smoking status, diet quality (DHDI), physical activity, sleep quality (PSQI), sleep duration, alcohol intake, and depressive symptoms (IDS). The importance of each variable was quantified as the decrease in McFadden pseudo-R^2 when that variable was removed from the full model.

3. Results

3.1. Characteristics of the study population

Of 5235 individuals with information on the routine measures, 1506

(28.8%) had complete non-routine measures (Supplementary Fig. 1). Characteristics of the population stratified by non-routine measure availability are displayed in Supplementary Table 3. Both subgroups had comparable distributions across key demographic, anthropometric, and metabolic measures, as well as lifestyle factors, including age, BMI, waist and hip circumference, fasting glucose level, TG level, diet quality, and physical activity level.

3.2. Optimal number and stability of subtypes

Silhouette widths were first used to generate elbow plots to identify the optimal number of subtypes (Supplementary Fig. 2A). As no clear optimum emerged from the elbow plots, consensus clustering was applied (Supplementary Fig. 2B–E), which identified four as the optimal number of clusters for both variable lists. While both data-driven clusters showed acceptable cluster stability, the mean Jaccard index was higher for routine-derived clusters than for non-routine-derived clusters (Supplementary Fig. 3).

3.3. Characteristics of subtypes derived from the two variable lists

Among the non-routine-derived subtypes, subtype 1 (N = 364; characterized by an insulin-sensitive and lean profile) had the lowest BMI (median: 26.4 kg/m^2), fasting glucose levels (median: 5.1 mmol/L), abdominal visceral adipose tissue volume (median: 65.1 cm^2), abdominal subcutaneous adipose tissue volume (median: 214.8 cm^2), hepatic triglyceride content (HTGC; median: 1.9%), and highest Matsuda index (median: 9.3). In contrast, subtype 4 (N = 258; characterized by obesity, ectopic fat accumulation, and a marked insulin resistance profile) had the highest HTGC (median: 20.8%), fasting TG levels (median: 1.7 mmol/L), abdominal visceral adipose tissue volume (median: 173.6 cm^2), and subcutaneous adipose tissue volume (median: 320.6 cm^2), but the lowest Matsuda index (median: 2.7). Subtypes 2 (N = 364) and 3 (N = 524) were characterized by intermediate metabolic profiles, with subtype 3 additionally showing the highest genetic predisposition to T2DM. Four comparable subtypes emerged when clustering was performed with the routine variable list in the larger sample (Fig. 1 and Table 1).

3.4. Concordance between data-driven subtypes and weight-by-glycemia categories

We next evaluated the concordance between clusters derived from non-routine and routine variable lists in the subpopulation with complete data for both (N = 1506). Overall, 41% (611 of 1506) of participants were classified into the same subtypes regardless of the variable lists used: 17.8% as subtype 1 and 8.2% as subtype 4 by both approaches. Participants assigned to subtypes 2 and 3, both characterized by intermediate metabolic profiles, were frequently reassigned between these two subtypes depending on the variable list used (Supplementary Fig. 4 and Supplementary Table 4).

To assess whether data-driven subtypes captured metabolic heterogeneity beyond conventional weight-by-glycemia categories, we cross-tabulated subtype assignments with weight-by-glycemia groups. Among participants classified by non-routine subtypes (N = 1510), those with normal weight and normoglycemia, the most favorable metabolic profile, were predominantly assigned to subtype 1 (68%, 129 of 190), whereas those with obesity and prediabetes, the most adverse profile, were largely concentrated in subtype 4 (62%, 88 of 143) (Supplementary Fig. 5A–B). These concordant assignments are consistent with the metabolic characteristics defining subtypes 1 and 4, respectively. However, substantial heterogeneity emerged among overweight or

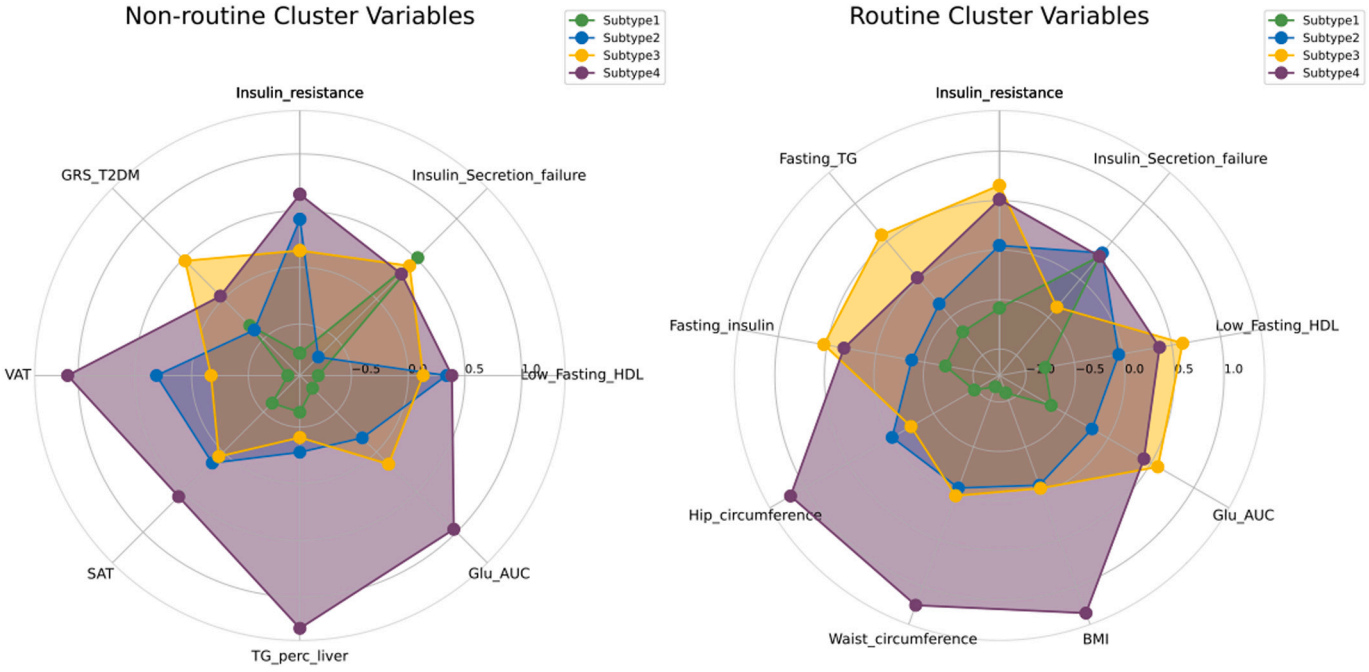

Fig. 1. Radar plots depicting characteristics of the population in each subtype using two lists of clustering variables (non-routine versus routine) separately. Left panel shows the characteristics of identified subtypes 1 to 4 using the non-routine variable list; right panel shows the characteristics of identified subtypes 1 to 4 using the routine variable list. The details of these variables are listed in Supplementary Table 1. Median z-scores are presented for skewed variables; mean z-scores are presented for the others. **Mean** z-score values: BMI, abdominal visceral adipose tissue volume (VAT), abdominal subcutaneous adipose tissue volume (SAT), hip circumference (Hip_circumference), waist circumference (Waist_circumference), glucose AUC (Glu_AUC), fasting HDL, genetic risk score for T2DM (GRS_T2DM). **Median** z-score values: insulin sensitivity (Matsuda$_{\text{mixed-meal}}$), insulin secretion, hepatic triglyceride content (TG_perc_liver), fasting TG (Fasting_TG), and fasting insulin (Fasting_insulin). The z-scores of insulin sensitivity (Matsuda$_{\text{mixed-meal}}$), insulin secretion, and HDL were directionally flipped (−1*z-scores) so that all axes point outward toward a less favorable metabolic profile, corresponding to insulin resistance, insulin secretion failure, and low fasting HDL. The larger size of the polygon area denotes more unfavorable metabolic profiles.

Table 1
Baseline characteristics of the study population stratified by different subtypes using two lists of clustering variables (non-routine versus routine).

	Non-routine variable list				Routine variable list			
	Subtype 1[b] (N = 364)	Subtype 2 (N = 364)	Subtype 3 (N = 524)	Subtype 4[c] (N = 258)	Subtype 1[b] (N = 1170)	Subtype 2 (N = 1961)	Subtype 3 (N = 947)	Subtype 4[c] (N = 1157)
Age (years, median, IQR)	56 (51–60)	56 (50–60)	56 (50–61)	57 (51–61)	56 (51–61)	56 (51–61)	57 (51–61)	55 (50–61)
BMI (kg/m^2, median, IQR)	26.4 (23.3–28.4)	29.6 (27.6–31.5)	29.1 (27.4–30.9)	31.5 (29.8–34.2)	25.1 (22.9–26.9)	29.1 (28.0–30.5)	29.5 (28.1–30.9)	34.6 (32.7–37.4)
Waist circumference (cm, mean, SD)	91.1 (11.7)	103.0 (9.7)	99.6 (10.1)	109.5 (8.7)	87.3 (9.6)	100.4 (7.2)	101.8 (7.7)	115.4 (8.7)
Hip circumference (cm, mean, SD)	103.8 (7.8)	109.8 (7.5)	109.2 (7.6)	113.1 (8.5)	100.7 (6.1)	109.7 (5.1)	107.5 (5.2)	120.8 (8.8)
Fasting glucose (mmol/L, median, IQR)	5.1 (4.8–5.4)	5.4 (5.1–5.7)	5.5 (5.2–5.9)	6.0 (5.5–6.4)	5.2 (4.9–5.5)	5.4 (5.1–5.7)	5.7 (5.4–6.1)	5.7 (5.3–6.1)
Matsuda index (modified) (median, IQR)	9.3 (7.5–12.1)	3.7 (2.7–4.7)	5.1 (4.1–6.5)	2.7 (2.0–3.7)	8.5 (6.7–11.4)	5.6 (4.6–7.4)	2.8 (2.1–3.5)	3.4 (2.4–4.6)
Insulin secretion (modified) (median, IQR)	0.2 (0.2–0.3)	0.5 (0.4–0.6)	0.3 (0.2–0.3)	0.3 (0.2–0.4)	0.3 (0.2–0.4)	0.3 (0.2–0.4)	0.4 (0.3–0.6)	0.3 (0.2–0.4)
Fasting triglycerides (mmol/L, median, IQR)	0.9 (0.7–1.1)	1.4 (1.0–2.0)	1.3 (0.9–1.8)	1.7 (1.2–2.3)	0.8 (0.6–1.1)	1.1 (0.9–1.5)	1.9 (1.5–2.5)	1.4 (1.1–1.9)
Fasting insulin (mU/L, median, IQR)	5.6 (4.1–7.2)	13.4 (9.8–17.7)	9.6 (7.1–12.3)	17.3 (12.0–23.0)	5.8 (4.3–7.7)	8.8 (6.7–11.1)	16.8 (13.4–21.1)	14.5 (10.9–20.7)
Total cholesterol (mmol/L, mean, SD)	5.7 (0.9)	5.8 (1.0)	5.8 (1.1)	5.9 (1.1)	5.6 (1.0)	5.8 (1.1)	5.9 (1.1)	5.6 (1.0)
Fasting HDL (mmol/L, median, IQR)	1.6 (1.4–2.0)	1.3 (1.0–1.5)	1.4 (1.1–1.6)	1.2 (1.0–1.5)	1.7 (1.4–2.0)	1.4 (1.2–1.7)	1.2 (1.0–1.4)	1.3 (1.1–1.5)
Liver fat content (%, median, IQR)	1.9 (1.1–3.3)	5.7 (3.2–9.8)	3.9 (2.2–7.2)	20.8 (14.7–26.7)	1.8 (1.1–3.4)	4.2 (2.3–8.0)	9.6 (4.5–18.7)	8.9 (4.2–17.6)
	–	–	–	–	749 (64.0)	1325 (67.6)	620 (65.5)	826 (71.4)
Abdominal visceral adipose volume (cm^2, median, IQR)	65.1 (46.3–94.7)	133.0 (100.2–168.1)	100.9 (74.0–133.9)	173.6 (140.2–209.9)	63.3 (42.8–92.5)	107.3 (79.4–142.2)	131.9 (102.0–172.8)	159.0 (123.1–199.3)
Missing (n, %)	–	–	–	–	671 (57.4)	1188 (60.6)	549 (58.0)	746 (64.5)
Abdominal subcutaneous adipose volume (cm^2, median, IQR)	214.8 (170.6–283.7)	290.0 (231.3–359.7)	291.0 (227.8–372.0)	320.6 (257.1–413.4)	197.2 (159.5–241.2)	292.8 (239.8–353.9)	277.3 (230.2–332.1)	415.5 (338.1–485.0)
Missing (n, %)	–	–	–	–	671 (57.4)	1188 (60.6)	549 (58.0)	746 (64.5)
Genetic risk score for T2DM (mean, SD)	50.1 (0.7)	50.1 (0.6)	50.7 (0.7)	50.4 (0.6)	50.3 (0.7)	50.4 (0.7)	50.5 (0.7)	50.4 (0.7)
Missing (n, %)	–	–	–	–	138 (11.8)	237 (12.1)	111 (11.7)	166 (14.3)
IFG status (n, %)								
IFG	13 (3.6)	32 (8.8)	78 (14.9)	100 (38.8)	53 (4.5)	158 (8.1)	258 (27.2)	286 (24.7)
No IFG	351 (96.4)	332 (91.2)	446 (85.1)	158 (61.2)	1117 (95.5)	1803 (91.9)	689 (72.8)	871 (75.3)
IGT status[a] (n, %)								
IGT	0 (0)	10 (2.7)	36 (6.9)	62 (24.0)	23 (2.0)	71 (3.6)	137 (14.5)	119 (10.3)
No IGT	364 (100)	354 (97.3)	488 (93.1)	196 (76.0)	1147 (98.0)	1890 (96.4)	810 (85.5)	1038 (89.7)
Glucose status (n, %)								
Prediabetes	13 (3.6)	38 (10.4)	104 (19.8)	126 (48.8)	71 (6.1)	213 (10.9)	328 (34.6)	334 (28.9)
Normal glucose	351 (96.4)	326 (89.6)	420 (80.2)	132 (51.2)	1099 (93.9)	1748 (89.1)	619 (65.4)	823 (71.1)

Data are shown as mean (SD) for normally distributed continuous variables, median (interquartile range, IQR: 25th–75th percentile) for non-normally distributed continuous variables, and number (%) for categorical variables.
IFG, impaired fasting glucose; IGT, impaired glucose tolerance.
[a] Since an OGTT was not performed in the NEO study, we used a postprandial glucose level at 150 min $\geq$ 7.8 mmol/L, derived from a standard liquid mixed meal challenge, as a surrogate criterion to define impaired glucose tolerance. Matsuda index was also calculated based on the modified formulas shown in the Supplementary methods.
[b] Insulin-sensitive and lean profile.
[c] Obesity, ectopic fat accumulation, and a marked insulin resistance profile.

obese individuals with normoglycemia, who constituted the majority of the cohort. Specifically, participants with overweight and normoglycemia ($N = 598$) were distributed across subtypes 1 (30%), 2 (26%), and 3 (39%) without clear predominance of any single subtype, indicating distinct underlying metabolic profiles despite similar BMI and glucose levels. Those assigned to subtype 1, who carried favorable metabolic profiles despite elevated BMI, may represent a metabolically healthy obesity phenotype (Supplementary Fig. 5B). A consistent pattern was observed with the routine subtypes. For example, 26% (534 of 2019) of overweight, normoglycemic participants were assigned to subtype 1. Among normal-weight individuals with prediabetes, 75% (36 of 48) were assigned to subtype 1 (Supplementary Fig. 6A–B), suggesting that their metabolic risk was probably lower than conventional BMI or fasting glucose categorization would indicate.

3.5. Associations between subtypes and T2DM incidence

Among 5235 participants with complete routine clustering variables, 256 incident T2DM cases occurred over a median follow-up of 6.8 years (interquartile range [IQR]: 5.9–7.9), with an overall incidence rate of

7.4 per 1000 person-years. Among 1510 participants with complete non-routine variables, 64 incident T2DM cases occurred over a median follow-up of 6.7 years (IQR: 5.9–7.9), with an overall incidence rate of 6.4 per 1000 person-years (Supplementary Table 5). Kaplan–Meier curves showed clear separation in cumulative T2DM incidence across subtypes for both variable lists (Fig. 2A), with a graded increase in risk from subtype 1 to subtype 4. For subtypes identified by the routine variable list, subtype $4_{routine}$ had the highest risk compared with subtype $1_{routine}$ (hazard ratio [HR]: 19.5, 95% CI: 9.1–41.6), followed by subtype $3_{routine}$ and subtype $2_{routine}$. Similar associations were observed for the non-routine subtypes, with subtype $4_{non\text{-}routine}$ showing a directionally consistent but less precisely estimated association, reflecting its smaller sample size (HR: 18.0, 95% CI: 4.1–78.6; Fig. 2B and Supplementary Table 5).

In sensitivity analyses, additional adjustment for lifestyle factors and baseline metabolic markers (e.g., fasting glucose and TG) substantially attenuated the subtype–T2DM associations. For the non-routine subtypes, associations with incident T2DM were no longer statistically significant. In contrast, for the routine subtypes, although the effect sizes were attenuated and subtype 2 was no longer statistically significant,

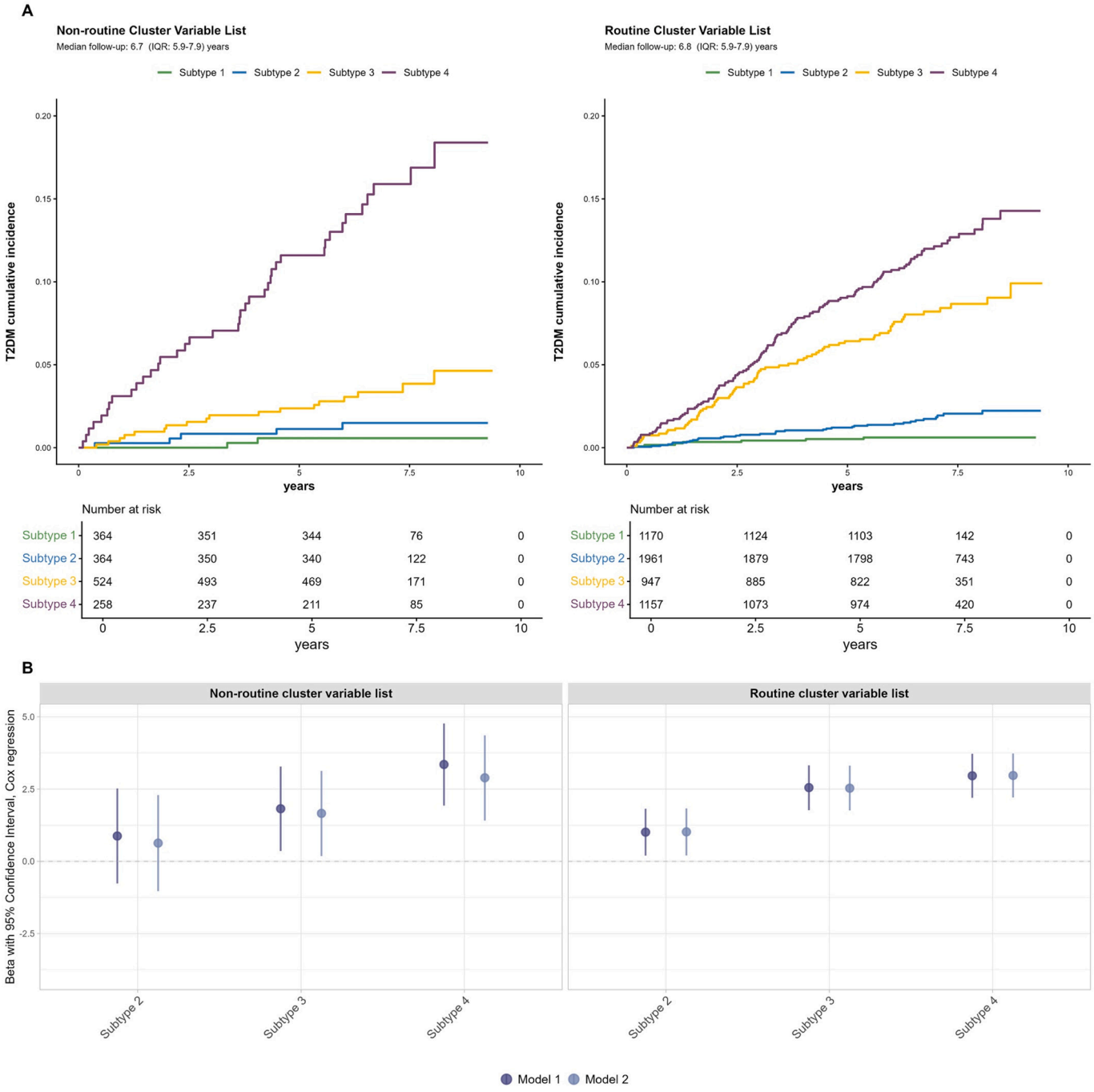

Fig. 2. Associations between subtypes (non-routine versus routine) and T2DM incidence.
A. Kaplan–Meier curves for T2DM cumulative incidence across subtypes. B. Cox regression models quantified associations between subtypes and T2DM incidence. To better present the results, data are shown as log hazard ratios (β values) with 95% confidence intervals (CIs). βs > 0 indicate higher T2DM risk relative to subtype 1 (reference). Model 1: unadjusted; Model 2: when using the non-routine variable list, adjustment for age, sex, and BMI; when using the routine variable list, adjustment for age and sex. The detailed results of the Cox regression models are shown in Supplementary Table 5.

subtypes 3 and 4 retained significant associations with incident T2DM (subtype 3, HR: 4.8, 95% CI: 1.9–12.1; subtype 4, HR: 7.4, 95% CI: 2.9–18.4; Supplementary Table 6; sensitivity analysis 1). This result shows that fasting glucose or TG levels explained a large proportion of the non-routine subtype associations with T2DM, whereas the routine subtypes probably captured additional metabolic risk beyond that defined by individual markers. When analyses were restricted to participants with at least one year of follow-up, results were similar, suggesting minimal influence of reverse causation (Supplementary Table 6; sensitivity analysis 2).

3.6. Metabolic and proteomic signatures of higher-risk subtypes

Given the graded associations with incident T2DM, we labelled subtypes 1 through 4 derived from the non-routine variable list as relatively low-, intermediate-, intermediate-high-, and high-risk,

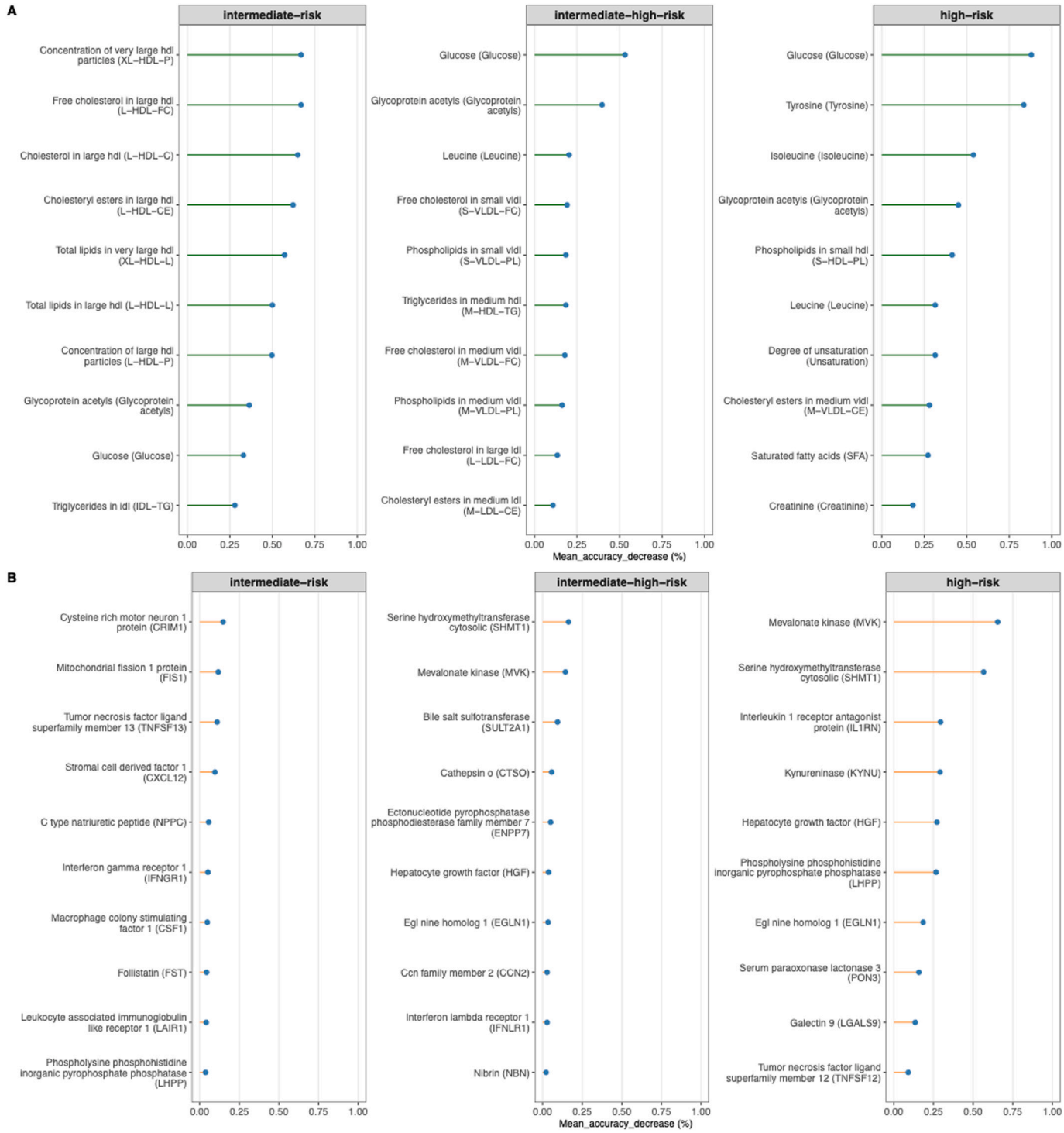

Fig. 3. Top 10 discriminative metabolites and proteins for each higher-risk subtype identified by random forest classification.
A. Top 10 metabolites for the intermediate-risk, intermediate-high-risk, and high-risk subtypes. B. Top 10 proteins for the intermediate-risk, intermediate-high-risk, and high-risk subtypes. Feature importance was ranked by mean decrease in accuracy (%). Subtypes were derived from the non-routine variable list.

respectively. To characterize the molecular profiles of each higher-risk subtype, we performed one-vs-rest random forest classification using circulating metabolites (Fig. 3A) and proteins (Fig. 3B). Across all three higher-risk subtypes, fasting glucose and glycoprotein acetyls (GlycA; a marker of systemic inflammation) were consistently selected as the key discriminative metabolites. At the proteomic level, mevalonate kinase (MVK), serine hydroxymethyltransferase, cytosolic (SHMT1), hepatocyte growth factor (HGF), and egl nine homolog 1 (EGLN1) were recurrently selected for the two highest-risk subtypes (intermediate-high- and high-risk), and phospholysine phosphohistidine inorganic pyrophosphate phosphatase (LHPP) was shared between the intermediate- and high-risk subtypes.

Beyond these shared features, each subtype exhibited a distinct multi-omics signature that aligned with its position along the T2DM risk gradient. The intermediate-risk subtype (subtype 2) was characterized by a reduction in large- and very-large high-density lipoprotein (HDL) subclasses, encompassing the particle concentrations of large and very large HDL (L-HDL-P and XL-HDL-P), and the cholesterol, free cholesterol, and cholesteryl ester content of large HDL (L-HDL-C, L-HDL-FC, and L-HDL-CE). The accompanying signature comprised altered circulating levels of immune- and inflammation-related proteins, such as cysteine-rich motor neuron 1 protein (CRIM1), mitochondrial fission 1 protein (FIS1), and tumor necrosis factor ligand superfamily member 13 (TNFSF13). The intermediate-high-risk subtype (subtype 3) was distinguished primarily by atherogenic features of small and medium very-low-density lipoprotein (VLDL) subclasses, including increased free cholesterol and phospholipid content of small VLDL (S-VLDL-FC and S-VLDL-PL), together with elevated leucine and TG in medium HDL (M-HDL-TG). The corresponding proteomic signature was led by SHMT1, MVK, and bile salt sulfotransferase (SULT2A1). The high-risk subtype (subtype 4) displayed the most pronounced metabolic disturbances. Its discriminative metabolite profile was dominated by elevated branched-chain and aromatic amino acids, such as tyrosine, isoleucine, and leucine, together with saturated fatty acids and a lower degree of fatty acid unsaturation. Its proteomic signature was the most pronounced across the subtypes and was characterized by elevated levels of MVK and SHMT1, along with interleukin-1 receptor antagonist protein (IL1RN), kynureninase (KYNU), and HGF, as well as reduced levels of paraoxonase lactonase 3 (PON3). Comparison of the top-ranked metabolites and proteins revealed differences in concentrations across subtypes (Supplementary Figs. 7–8). For example, fasting glucose, GlycA, VLDL-related lipoproteins, saturated fatty acids, branched-chain and aromatic amino acids, MVK, SHMT1, LHPP, IL1RN, KYNU, and HGF showed a graded increase, reaching their highest levels in subtype 4 (high-risk), whereas the degree of unsaturation, large HDL-related lipoproteins, and PON3 were lowest in this subtype.

GO enrichment analysis of the top 50 discriminative proteins for each higher-risk subtype showed distinct biological process enrichments despite all proteins being derived from an inflammation-targeted panel (Supplementary Fig. 9). The intermediate-risk subtype (subtype 2) was enriched for TNF-related signaling and leukocyte differentiation. In the intermediate-high-risk subtype, the enriched pathways shifted to hemopoiesis and positive regulation of leukocyte activation, along with interleukin-related signaling. Overall, the high-risk subtype (subtype 4) displayed the broadest inflammatory landscape, in which cytokine-mediated signaling and chemotaxis dominated.

3.7. Lifestyle and psychosocial characteristics across subtypes

Beyond molecular signatures, subtypes identified by the non-routine variable list differed in lifestyle and psychosocial characteristics (Supplementary Table 7). Subtype 1 (relatively low-risk) had the most favorable lifestyle profile, including better adherence to a healthy diet, higher physical activity level, and fewer depressive symptoms. In contrast, the higher-risk subtypes, particularly subtype 4, showed a less favorable profile, with lower adherence to a healthy diet, reduced physical activity level, and more depressive symptoms. When we evaluated the relative importance of these factors in distinguishing between subtypes, the PSQI, physical activity, depressive symptomatology score (IDS), and DHDI were the top four contributors to the variance in subtype discrimination (Supplementary Fig. 10).

4. Discussion

4.1. Main findings

In this study of a predominantly overweight or obese, middle-aged population, data-driven clustering incorporating variables from a liquid mixed meal challenge identified four T2DM risk subtypes, reflecting a progressive gradient from an insulin-sensitive, metabolically favorable profile to a high-risk profile characterized by obesity, ectopic fat accumulation, and marked insulin resistance. These subtypes were reproducible with either a non-routine or routine clustering variable list, though concordance between the two assignments was moderate, with greater agreement at the extremes of the risk spectrum. Notably, subtype assignments captured metabolic heterogeneity beyond that shown by conventional weight-by-glycemia categories, redistributing overweight individuals or those with early-stage glycemic abnormalities across subtypes with metabolic profiles that conferred divergent T2DM risk. In addition to the well-known metabolic traits (e.g., fasting glucose, inflammation-related GlycA, and TG-related lipoproteins), multi-omics profiling revealed that each higher-risk subtype harbored distinct metabolomic and proteomic signatures. These signatures progressed from altered HDL metabolism to broad atherogenic lipoprotein remodeling, dysregulated amino acid metabolism, and pro-inflammatory proteomic activation. Our findings indicate that data-driven subtyping stratifies T2DM risk and reveals distinct molecular signatures that may inform subtype-targeted prevention strategies.

4.2. Comparisons with previous studies

Compared with previous findings from Wagner et al. [4], we identified four instead of six subtypes with varying T2DM risk. The discrepancy may reflect several factors. First, we used data from a different population with a higher risk of T2DM, characterized by high BMI, rather than selecting participants based on glycemic status. Second, instead of an OGTT, we derived glycemia-related parameters from a liquid mixed meal challenge. Although the mixed meal challenge has demonstrated validity as a predictive tool for T2DM [5], it has not been standardized in routine clinical practice, and since the two tests provide different stimuli for insulin secretion, further studies are warranted to determine whether subtypes derived from each test perform equivalently for T2DM risk stratification. Third, our study used more recent GWAS summary statistics to calculate the genetic risk score for T2DM, which may influence subtype clustering.

Despite these differences, when we mapped our subtypes (Fig. 1) to the six established Tübingen subtypes on the basis of baseline characteristics of participants, subtypes 2 to 4 were each associated with increased T2DM risk, consistent with previous findings [4]. Among the six subtypes identified by Wagner et al., C3 and C5 conferred the highest diabetes risk. Specifically, C3 was characterized by an elevated genetic risk and impaired insulin secretion, while C5 was characterized by higher liver fat and insulin resistance. In our study, subtypes 3 and 4 derived from both variable lists showed similar metabolic profiles to the Tübingen C3 and C5, respectively, and were likewise associated with increased T2DM incidence. However, the effect estimates (HRs with 95% CIs) associated with T2DM incidence for the intermediate-high-risk (subtype 3) and high-risk (subtype 4) subtypes in our study (ranging from 5.2 [95% CI: 1.2–22.8] to 18.0 [95% CI: 4.1–78.6]) were larger than those reported for C3 and C5 subtypes (ranging from 1.5 [95% CI: 1.2–2.0] to 6.6 [95% CI: 5.1–8.7]). This difference may be attributable to two factors. First, the baseline risk of T2DM among our participants

was already substantially elevated owing to their high BMI and chronological age. Second, the small number of incident T2DM cases within our subtypes may have limited the precision of effect estimates, as reflected by the wide CIs. Further studies with a larger sample size and longer follow-up are needed to validate these findings.

4.3. Data-driven subtyping compared with weight-by-glycemia categories

A central question of the subtyping approach is whether assigning individuals to metabolic clusters offers greater clinical utility for predicting disease risk than categorization based on routinely measured clinical features alone. Prior work in patients with established T2DM suggests this added value may be modest. One study reported that although data-driven clusters showed differences in glycemic progression, age at diagnosis alone explained a comparable amount of variation in disease trajectory, and simple clinical features outperformed clusters for selecting individual-level therapy [22]. Whether this limitation extends to the pre-diabetes setting, where subtyping might capture early metabolic heterogeneity not yet reflected in clinical diagnoses, remains unclear. We therefore examined whether data-driven subtypes provided additional predictive value beyond conventional weight-by-glycemia categories. Data-driven subtyping did capture more granular metabolic heterogeneity, particularly among overweight or obese individuals with normoglycemia, indicating that these subtypes can discriminate metabolic risk profiles that conventional traits alone may not resolve. Nevertheless, after additional adjustment for fasting glucose and/or TG levels, the subtype–T2DM associations were substantially attenuated, underscoring that the two routinely measured biomarkers account for much of the information embedded in the data-driven subtypes. This attenuation was more pronounced for non-routine subtypes, likely reflecting a stronger correlation between non-routinely measured traits and fasting glucose; the smaller sample size of that analysis may also have reduced precision. The interpretation is corroborated by the random forest analysis, which identified fasting glucose and TG-related lipoproteins as the key features distinguishing high-risk T2DM subtypes. These findings suggest that data-driven metabolic subtyping reveals heterogeneity that conventional categories may miss, yet much of the subtype-associated T2DM risk can be captured by crucial metabolic markers, e.g., fasting glucose. However, whether subtypes offer advantages over conventional metabolic traits for selecting individualized intervention strategies and identifying individuals who respond well remains a question that our study cannot address, and warrants investigation in prospective interventional studies.

4.4. Molecular profiles across subtypes with higher risk of T2DM

Using the non-routine variable list, our study revealed distinct metabolic and proteomic signatures for subtypes with higher risk of developing T2DM. Beyond fasting glucose, GlycA, an inflammatory marker, was identified as an important metabolite shared by all three higher-risk subtypes, aligning with previous studies documenting the role of inflammation in T2DM [23–25]. A cross-sectional study reported that GlycA was associated with insulin resistance and other features of the metabolic syndrome, independent of C-reactive protein [26]. Additionally, metabolites including branched-chain amino acids (BCAAs, e.g., leucine and isoleucine), aromatic amino acids (tyrosine), lipids related to large and very large HDL particles, subclasses of VLDL particles, saturated fatty acids, and the degree of unsaturation in fatty acids were the most discriminative across subtypes. The associations between these metabolites and T2DM risk have been well documented in prior studies [24,25,27–30]. In line with our findings of higher levels of VLDL-related lipoproteins (e.g., cholesteryl esters in medium VLDL) but lower levels of HDL-related lipoproteins (e.g., cholesterol in large HDL) in higher-risk T2DM subtypes, studies have reported that lipids in small, medium, or large VLDL particles showed positive associations with T2DM incidence, while lipids in large and very large HDL particles showed inverse associations [27,29]. Existing evidence has shown that all lipid components in VLDL subclasses exhibited progressive elevation with deteriorating glucose tolerance. Conversely, HDL concentrations, especially those of large HDL particles, showed a significant reduction in different categories of abnormal glucose tolerance [29,31]. Regarding amino acids, we observed higher levels of tyrosine, leucine, and isoleucine in the higher-risk subtypes. In agreement with our results, previous studies have shown that circulating amino acids were associated with insulin resistance, potentially through disruption of insulin signaling in skeletal muscle [32,33]. BCAAs have been implicated in impairing insulin signaling by interacting with mTOR kinase, and accumulation of these metabolites has been linked to increased insulin secretion and pancreatic β-cell exhaustion [34]. Interestingly, the highest-risk subtype (subtype 4) was also distinguished by creatinine levels, which may reflect altered renal metabolism associated with a combination of insulin resistance, atherogenic dyslipidemia, and inflammation. This is consistent with prior evidence linking creatinine to T2DM risk [35]. This finding reinforces the notion that subtype 4 represents the broadest metabolic disruption extending beyond atherogenic dyslipidemia and inflammation.

Proteomic profiling revealed that several proteins (e.g., IL1RN and HGF) were recurrently selected across higher-risk subtypes, consistent with prior evidence linking these inflammatory markers to T2DM risk [36–42]. Notably, both the intermediate-high-risk subtype (subtype 3) and the high-risk subtype (subtype 4) featured increased MVK and SHMT1, enzymes in the mevalonate and one-carbon metabolism pathways, respectively. Supporting our findings, SHMT1 has been associated with lower insulin sensitivity and been proposed as a biomarker for insulin resistance [43]. The high-risk subtype was also marked by the highest levels of KYNU and HGF, both previously associated with T2DM risk [44,45]. Conversely, it showed the lowest levels of PON3, an antioxidant enzyme inversely associated with incident T2DM [37,42]. Our GO enrichment analysis further revealed that the high-risk subtype had the broadest inflammatory landscape, dominated by cytokine-mediated signaling. In line with this finding, a recent study from the KORA F4/FF4 cohort also reported subtype-specific inflammatory profiles, which demonstrated differences in subclinical inflammation between pathotype-based clusters in older adults before T2DM diagnosis, with the highest inflammatory burden in clusters with higher cardiometabolic risk [46]. Beyond inflammatory proteins, differences in blood coagulation markers across prediabetic clusters have also been reported [47], further underscoring the molecular heterogeneity of subtypes at elevated T2DM risk. Despite accumulating evidence, it is noteworthy that the causal associations of circulating metabolites and proteins with T2DM risk are not fully established; therefore, any mechanistic interpretation should be made with caution.

4.5. Implications for clinical practice and future research

We identified divergent lifestyle and psychosocial characteristics across subtypes, with sleep quality, physical activity, depressive symptomatology, and dietary quality emerging as the top four contributors to subtype differentiation. These observations raise the hypothesis that modifiable factors, including sleep, physical activity, and dietary habits, may represent putative targets for subtype-tailored T2DM prevention, although interventional evidence is still lacking. Supporting the concept of subtype-dependent treatment response, a recent study indeed found that participants classified into a subtype with higher risk of developing T2DM derived greater benefit from bariatric surgery regarding insulin resistance, β-cell function, and prediabetes remission, whereas those in a low-risk subtype did not [48]. Comparable findings were previously reported for T2DM subtypes, where individuals in the severe insulin-resistance diabetes cluster benefited more from bariatric surgery for T2DM remission and improvement in renal function than those from the mild obesity-related diabetes or severe insulin-deficient diabetes clusters [49]. Whether tailored interventions similarly differ in effectiveness

across the subtypes identified in the current study warrants further investigation.

4.6. Strengths and limitations

The current study has several strengths. First, the liquid mixed meal challenge design more closely mimics real-life metabolic conditions, allowing us to derive clustering variables in a setting that reflects normal digestive processes. Second, analyzing multi-omics data alongside detailed lifestyle and psychosocial factors provided deeper insights into the molecular and lifestyle heterogeneity across subtypes. Several limitations should also be acknowledged. First, as not all participants had non-routine measurements performed, the reduced sample sizes limited the statistical power of these analyses. Second, since the Nightingale platform measures only a restricted range of metabolites, additional untargeted metabolomics platforms could provide broader insights into the metabolic heterogeneity underlying T2DM risk. A similar limitation applies to protein measurements, as only inflammation-related proteins were included. Third, the present study was conducted in an overweight- and obesity-enriched Dutch cohort of middle-aged, predominantly White individuals. The subtypes identified here may not generalize to populations with lower baseline metabolic risk or different ethnic backgrounds. Fourth, the current study considered only incident T2DM as the outcome; associations between subtypes and other diabetes-related complications (e.g., chronic kidney disease) warrant further investigation.

5. Conclusion

In conclusion, we identified four metabolic subtypes with graded T2DM risk in a predominantly overweight or obese, middle-aged Dutch population, using routine or non-routine clustering variables. This subtype scheme reveals heterogeneity among individuals who appear homogeneous under conventional weight-by-glycemia categories. Although fasting glucose and lipoproteins were central to subtype differentiation, multi-omics profiling uncovered broader metabolomic and proteomic signatures, both shared and subtype-specific, highlighting that these subtypes reflect biologically distinct dimensions of T2DM risk that are not fully captured by conventional metabolic traits. These findings suggest that this metabolic subtyping approach, combined with multi-omics characterization, may help inform subtype-targeted strategies for T2DM prevention.

Prior presentation

A part of the results in this manuscript was presented at the 60th European Association for Study of Diabetes (EASD) conference in Madrid, Spain, 2024.

CRediT authorship contribution statement

Keyong Deng: Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Formal analysis, Conceptualization. **Annoeska Hameete:** Writing – original draft, Visualization, Formal analysis. **Patrick Schrauwen:** Writing – review & editing, Funding acquisition. **Robert Wagner:** Writing – review & editing. **Astrid van Hylckama Vlieg:** Writing – review & editing, Supervision. **Frits R. Rosendaal:** Writing – review & editing, Supervision, Funding acquisition. **Dennis O. Mook-Kanamori:** Writing – review & editing. **Saskia le Cessie:** Writing – review & editing, Methodology. **Ko Willems van Dijk:** Writing – review & editing. **Renée de Mutsert:** Writing – review & editing, Resources, Funding acquisition, Conceptualization. **Ruifang Li-Gao:** Writing – review & editing, Supervision, Resources, Investigation, Funding acquisition, Conceptualization.

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Declaration of competing interest

R.W. reports honoraria during the past 36 months for lectures/presentations/speaker's bureaus from Daiichi-Sankyo, Eli Lilly, Boehringer Ingelheim, Novo Nordisk, Sanofi-Aventis and Synlab; travel support from Eli Lilly, Novo Nordisk, Daiichi-Sankyo and Sanofi Aventis; honoraria for advisory boards from Eli Lilly, Boehringer Ingelheim and Sanofi-Aventis. The other authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.metabol.2026.156687.

Data availability

The NEO study data used in this study were obtained by the Department of Clinical Epidemiology at LUMC, the Netherlands. The code used to analyze the data in this work is available via link: https://github.com/Keyong-bio/Subtypes_Validation.

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