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Blood-Based DNA Methylation Models Improve Short-term Cardiovascular Risk Stratification in Individuals With Type 2 Diabetes

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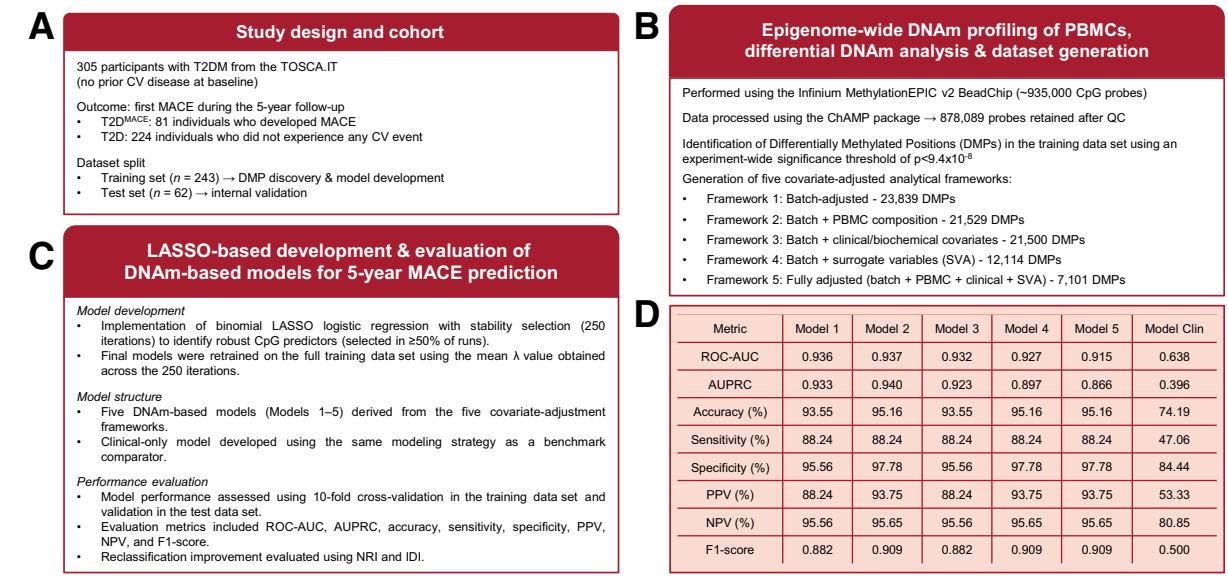

Metric	Model 1	Model 2	Model 3	Model 4	Model 5	Model Clin
ROC-AUC	0.936	0.937	0.932	0.927	0.915	0.638
AUPRC	0.933	0.940	0.923	0.897	0.866	0.396
Accuracy (%)	93.55	95.16	93.55	95.16	95.16	74.19
Sensitivity (%)	88.24	88.24	88.24	88.24	88.24	47.06
Specificity (%)	95.56	97.78	95.56	97.78	97.78	84.44
PPV (%)	88.24	93.75	88.24	93.75	93.75	53.33
NPV (%)	95.56	95.65	95.56	95.65	95.65	80.85
F1-score	0.882	0.909	0.882	0.909	0.909	0.500

AUPRC, area under the precision-recall curve; ChAMP, Chip Analysis Methylation Pipeline; CV, cardiovascular; DNAm, DNA methylation; IDI, integrated discrimination improvement; LASSO, least absolute shrinkage and selection operator; MACE, major adverse cardiovascular event; NPV, negative predictive value; NRI, net reclassification index; PBMC, peripheral blood mononuclear cell; PPV, positive predictive value; QC, quality control; ROC-AUC, receiver operating characteristic-area under the curve; SVA, surrogate variable analysis; T2DM, type 2 diabetes mellitus; TOSCA.IT, Thiazolidinediones Or Sulphonylureas and Cardiovascular Accidents. Intervention Trial.

ARTICLE HIGHLIGHTS

- **Why did we undertake this study?**
  Cardiovascular disease (CVD) risk remains high in individuals with type 2 diabetes mellitus (T2DM), and current clinical risk scores show limited ability to accurately identify those who will develop major adverse cardiovascular events (MACE).

- **What is the specific question we wanted to answer?**
  Can blood-based DNA methylation (DNAm) markers improve risk stratification for MACE over 5 years in individuals with T2DM?

- **What did we find?**
  DNAm analysis identified CpG markers associated with incident MACE and enabled the development of five DNAm-based models sharing 12 CpG loci, showing excellent discriminative performance (receiver operating characteristic area under the curve = 0.915–0.937; area under the precision-recall curve = 0.866–0.940).

- **What are the implications of our findings?**
  DNAm biomarkers may improve cardiovascular risk stratification and support improved identification of individuals with T2DM at higher short-term risk of MACE.

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OBJECTIVE

Cardiovascular complications are the leading cause of death in type 2 diabetes mellitus (T2DM). We investigated whether blood-based DNA methylation (DNAm) biomarkers could improve risk stratification for major adverse cardiovascular events (MACE) in individuals with T2DM.

RESEARCH DESIGN AND METHODS

Epigenome-wide DNAm was profiled in peripheral blood mononuclear cells obtained at baseline from 305 T2DM participants of the Thiazolidinediones Or Sulphonylureas and Cardiovascular Accidents. Intervention Trial (TOSCA.IT) study, including 81 who experienced MACE within 5 years ($T2D^{MACE}$) and 224 who did not (T2D). The population was randomly divided into training and internal test sets for model development and evaluation. Differential DNAm analyses were performed under five frameworks differing by covariate adjustment. Binomial least absolute shrinkage and selection operator (LASSO) logistic regression with stability selection was used to generate DNAm-based models to stratify individuals at higher risk of incident MACE over 5 years.

RESULTS

Differential DNAm analysis identified 23,839, 21,529, 21,500, 12,114, and 7,101 differentially methylated CpG sites across the five analytical frameworks. LASSO logistic regression was used to develop five DNAm-based models. Stability selection retained 22–26 CpG predictors per model. When evaluated in the internal test cohort, the five models showed high discriminative performance, with receiver operating characteristic area under the curve values ranging from 0.915 to 0.937 and area under the precision-recall curve values from 0.866 to 0.940. Several CpGs were independently associated with either increased or decreased risk of MACE, including 12 CpGs identified across all of the models.

CONCLUSIONS

The DNAm-based models we have generated may improve risk stratification for short-term cardiovascular events in individuals with T2DM, providing a promising approach to refine identification of those at increased risk of MACE.

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The global prevalence of type 2 diabetes mellitus (T2DM) has quadrupled over the past century, largely due to increasing rates of overweight and obesity, and now affects >800 million people worldwide (1). Individuals with T2DM have a two to four times higher risk of developing cardiovascular (CV) disease (CVD) and of experiencing major adverse cardiovascular events (MACE), including sudden cardiac death, myocardial infarction, stroke, or urgent coronary revascularization, and feature more than double the risk of CVD-related mortality compared with those without diabetes (2,3). This increased risk is multifactorial, driven by established factors, including elevated glycated hemoglobin A_{1c} (HbA_{1c}), LDL-cholesterol (LDL-C), systolic blood pressure, smoking, physical inactivity, renal dysfunction, and longer diabetes duration (3). As a result, the combined burden of T2DM and CVD imposes a major public health and economic toll, making prevention of CVD in T2DM a major issue.

Nowadays, several treatments and procedures can reduce CV risk in T2DM (4). However, risk prediction tools are essential to identify high-risk individuals and guide a patient-tailored approach that is economically viable on a population-wide basis (5–11). Commonly recommended calculators, including Systematic Coronary Risk Evaluation 2 (SCORE2)-Diabetes for Europe and Predicting Risk of Cardiovascular Disease EVENTs (PREVENT-CVD) for the United States (10,11), solely rely on conventional risk factors and predict outcomes over a 10-year horizon, a time frame too long to support an incisive and durable precision prevention in clinical practice. Novel approaches are therefore needed to improve risk stratification.

Epigenetics, particularly DNA methylation (DNAm), has been implicated in the pathogenesis of T2DM and its complications (12–15), and specific DNAm patterns have also been associated with CVD and heart failure (16–20). DNAm reflects phenotypic changes induced by risk factors and lifestyle, is readily measured in blood (21,22), and can be leveraged for biomarker development and risk prediction (23,24). DNAm may therefore be regarded both as a mechanistic determinant and as a candidate biomarker of CVD in T2DM, with potential implications for personalized risk prediction and tailored prevention strategies. Supporting this possibility, García-Calzón et al. (25) developed a blood-derived methylation risk score (MRS) based on 87 CpG sites that slightly improved CV risk prediction in individuals with recently diagnosed T2DM. Whether such markers retain predictive utility in individuals with long-lasting T2DM, who have higher CV risk, remains unknown.

Here, we developed five blood-derived DNAm-based models that improve risk stratification for incident MACE over 5 years in individuals with established T2DM. We used baseline blood samples from participants in the Thiazolidinediones or Sulfonylureas Cardiovascular Accidents Intervention Trial (TOSCA.IT), a pragmatic randomized trial comparing pioglitazone versus sulfonylureas as add-on to metformin (26). For the current study, we selected 81 individuals who experienced a first MACE over 5 years and 224 randomly selected individuals free of CV events. At baseline, all of these individuals were free of prior CVD.

RESEARCH DESIGN AND METHODS

Study Design, DNAm Profiling, and Model Development

This study was a secondary analysis of TOSCA.IT using baseline blood samples and follow-up data from participants with T2DM and no previous CVD. The analysis included 81 participants classified as case individuals ($T2D^{MACE}$) if they experienced a first MACE event, defined as all-cause death, nonfatal myocardial infarction, nonfatal stroke, or urgent coronary revascularization, during the 5-year follow-up period, and 224 randomly selected participants who remained event-free as control individuals (T2D). Epigenome-wide DNAm was assessed in peripheral blood mononuclear cells using the MethylationEPIC v2 array. Participants were split into a training set for differential methylation analysis and model development (80%; n = 243) and an internal test set for model evaluation (20%; n = 62). CpG sites associated with first occurrence of a MACE event were identified under five covariate-adjustment frameworks. Five DNAm-based risk-stratification models were then developed using binomial least absolute shrinkage and selection operator (LASSO) logistic regression with stability selection and compared with a clinical/biochemical model. Detailed methods are provided in the Supplementary Material.

RESULTS

Baseline Characteristics and Follow-up Outcomes in Individuals With T2DM Stratified by Incident MACE

We aimed at identifying epigenetic biomarkers associated with and potentially informative for MACE risk stratification in individuals with T2DM. To this end, we studied 305 individuals with T2DM previously enrolled in the TOSCA.IT trial. These individuals were aged 50–76 years, had at least 2 years of diabetes duration, and were on stable treatment with full-dose metformin (2–3 $g \cdot day^{-1}$). HbA_{1c} ranged from 7.0 to 9.0% (53–75 $mmol \cdot mol^{-1}$) and BMI was 20.1–43.7 $kg \cdot m^{-2}$. A MACE event occurred in 81 of the 305 patients during the 5-year follow-up ($T2D^{MACE}$; mean time to event: 29.3 months, range: 0.3–60.0 months), whereas 224 remained event-free and were followed up for at least 60 months (mean follow-up: 76.8 months, range: 60–104 months). Clinical and demographic characteristics are reported in Table 1 and Supplementary Table 1. At study baseline, $T2D^{MACE}$ participants were slightly younger ($P < 0.05$), and diabetes was diagnosed at a younger age ($P < 0.05$). Diabetes duration and baseline HbA_{1c} were comparable in $T2D^{MACE}$ and in T2D participants though. $T2D^{MACE}$ individuals were predominantly men ($P < 0.01$) and had a slightly but significantly higher BMI ($P < 0.01$) than T2D. $T2D^{MACE}$ individuals also showed a higher prevalence of smoking ($P < 0.01$), elevated total cholesterol ($P < 0.05$) and LDL-C ($P < 0.05$), lower HDL-C ($P < 0.01$), and higher triglyceride levels ($P < 0.01$). Diastolic blood pressure was higher ($P < 0.05$) in $T2D^{MACE}$, whereas systolic blood pressure did not differ between the groups. Also, the incidence of micro- and macroalbuminuria, estimated glomerular filtration rate, and the use of CV medications, such as antihypertensive, lipid-lowering, and antiplatelet agents, were comparable between the groups. Despite these baseline differences, risk stratification with SCORE2-Diabetes (adapted to 5-year risk) did not discriminate between $T2D^{MACE}$ and T2D. For downstream analyses, the study population was randomly divided into a training set (80%), comprising 243 individuals (n = 179 T2D and n = 64 $T2D^{MACE}$), used for Differentially Methylated Positions (DMP) identification and

Table 1—Baseline characteristics of TOSCA.IT participants, according to MACE occurrence during follow-up

	T2D (n = 224)	T2D^{MACE} (n = 81)	P value*
Age, mean (SD), years	65.0 (6.9)	63.0 (6.5)	<0.05
Male sex, n (%)	120 (53.8)	58 (71.6)	<0.01
BMI, mean (SD), kg·m^{-2}	29.0 (5.0)	30.6 (4.4)	<0.01
CV risk factors			
Smokers, n (%)	35 (16.6)	24 (29.6)	<0.01
Total cholesterol, mean (SD), mmol·L^{-1}	4.67 (0.92)	4.99 (1.06)	<0.05
LDL-C, mean (SD), mmol·L^{-1}	2.69 (0.86)	2.96 (0.84)	<0.05
HDL-C, mean (SD), mmol·L^{-1}	1.26 (0.36)	1.14 (0.30)	<0.01
Triglycerides, mean (SD), mmol·L^{-1}	1.58 (0.87)	1.96 (1.14)	<0.01
Blood pressure			
Systolic, mean (SD), mmHg	133.2 (14.6)	136.7 (16.2)	NS
Diastolic, mean (SD), mmHg	79.0 (9.0)	82.4 (10.6)	<0.05
Microalbuminuria, n (%)	35 (15.6)	19 (23.5)	NS
Macroalbuminuria, n (%)	6 (2.7)	2 (2.5)	NS
eGFR, mean (SD), mL·min^{-1}·1.73 m^2	91.1 (12.5)	93.7 (12.7)	NS
5-year-adapted SCORE2-Diabetes, mean (SD), %	7.9 (2.9)	8.4 (2.8)	NS
Diabetes characteristics			
Age at diagnosis, mean (SD), years	56.6 (9.1)	54.4 (7.3)	<0.05
Diabetes duration, mean (SD), years	8.3 (6.2)	8.6 (5.5)	NS
HbA_{1c}, mean (SD), %	7.66 (0.49)	7.74 (0.54)	NS
HbA_{1c}, mean (SD), mmol·mol^{-1}	60.2 (5.4)	61.1 (6.0)	NS
Use of CV drugs			
Antihypertensive drugs	144 (64.3)	55 (67.9)	NS
Lipid-lowering drugs	118 (52.7)	40 (49.4)	NS
Antiplatelet drugs	72 (32.1)	32 (39.5)	NS
Follow-up			
Add-on therapy to metformin			
+Pioglitazone, n (%)	122 (54.5)	37 (45.7)	NS
+Sulfonylurea, n (%)	102 (45.5)	44 (54.3)	NS
Duration, mean (SD), months	76.8 (10.9)	—	—
Time to MACE event, months	—	29.3 (18.1)	—

Clinical and biochemical characteristics of the individuals with T2DM from the TOSCA.IT study, who did (T2D^{MACE}, n = 81) or did not (T2D, n = 224) experience a MACE during follow-up. Continuous variables are presented as mean (SD) and were compared using two-sided unpaired t tests; categorical variables are shown as n (%) and were compared using χ^2 tests. eGFR, estimated glomerular filtration rate. *Statistical significance ($P < 0.05$) vs. T2D. —, not available; NS, not significant.

model development, and an internal test set (20%), comprising 62 individuals (n = 45 T2D and n = 17 T2D^{MACE}), used for model evaluation, while preserving the proportion of incident MACE events. Characteristics of training and test sets are reported in Supplementary Tables 2 and 3.

Identification of DNAm Biomarkers Associated With Incident MACE and Development of Models for Risk Stratification

To identify blood-based CpG biomarkers associated with incident MACE in T2DM, differential DNAm analysis was performed on bisulfite-converted DNA extracted from peripheral blood mononuclear cells of 64 T2D^{MACE} and 179 T2D individuals included in the training set. Five covariate-adjustment frameworks were implemented to assess the impact of technical and biological confounders on the identification of DMPs associated with incident MACE in individuals with T2DM. Specifically, the analytical approaches included *1*) batch-correction (framework 1), *2*) batch and cell composition adjustment (framework 2), *3*) batch and clinical/biochemical covariate adjustment (framework 3), *4*) batch and surrogate variable analysis adjustment (framework 4), and *5*) full adjustment, including batch, cell composition, clinical/biochemical covariates, and surrogate variable analysis (framework 5). A permutation-based, experiment-wide significance threshold of $P < 9.4 \times 10^{-8}$ was applied to minimize the family-wise error rate (27).

DMP analysis revealed 23,839 significant probes in framework 1, 21,529 in framework 2, 21,500 in framework 3, 12,114 in framework 4, and 7,101 in the fully adjusted framework 5.

On the basis of these results, five DNAm-based models for risk stratification of MACE over 5 years in individuals with T2DM were developed using binomial LASSO logistic regression (models 1–5) in the training cohort. Stability selection involved 250 iterations of random subsampling (80% of the training set without replacement), generating 250 LASSO models for each analytical framework. Across the five DNAm-based models, stability selection retained CpG predictors appearing in ≥125 iterations, with the number of predictors ranging from 22 to 26 and the mean optimal λ ranging from 0.00986 to 0.01225, depending on the analytical framework

(Supplementary Table 4). These predictors and the corresponding mean λ were used to define the final LASSO models, refitted on the full training set. The five DNAm-based models showed excellent discrimination for incident MACE over 5 years (Supplementary Table 5), achieving consistently high training receiver operating characteristic area under the curve (ROC-AUC) values across models, estimated as mean performance over internal resampling procedures (bootstrap and 10-fold cross-validation). Consistently, mean predicted probabilities were markedly higher in individuals who developed MACE during follow-up ($T2D^{MACE}$) than in those who remained event-free (T2D) (Supplementary Table 6).

When evaluated on the internal 20% test cohort, the five DNAm-based models achieved high discriminative performance, with ROC-AUC values ranging from 0.915 to 0.937 and area under the precision-recall curve (AUPRC) values from 0.866 to 0.940 (Fig. 1*A* and *B* and Table 2). Calibration and probabilistic accuracy metrics indicated good calibration and low overall error across the five DNAm-based models within the internal test cohort (Supplementary Table 7). A classification threshold of 0.5 showed overall accuracy of the five models ranged from 93.55 to 95.16%, with sensitivity (recall) of 88.24% across all models, specificity between 95.56 and 97.78%, positive predictive value (PPV; precision) between 88.24 and 93.75%, negative predictive value (NPV) between 95.56 and 95.65%, and F1 scores ranging from 0.882 to 0.909 (Table 2). Across all five DNAm-based models, predicted probabilities were consistently higher in $T2D^{MACE}$ than in T2D individuals (Supplementary Table 6), supporting consistent discriminative performance within the internal test cohort.

When clinical and biochemical variables were added to the DMP sets used to build the DNAm-based models, none were retained by LASSO, indicating that the predictive signal was driven entirely by DNAm features (data not shown). Consistently, all DNAm-based models outperformed a model derived using only clinical and biochemical predictors generated using the same LASSO framework (Table 2). The DNAm-based models also demonstrated improved risk classification compared with the clinical model, as well as better calibration and

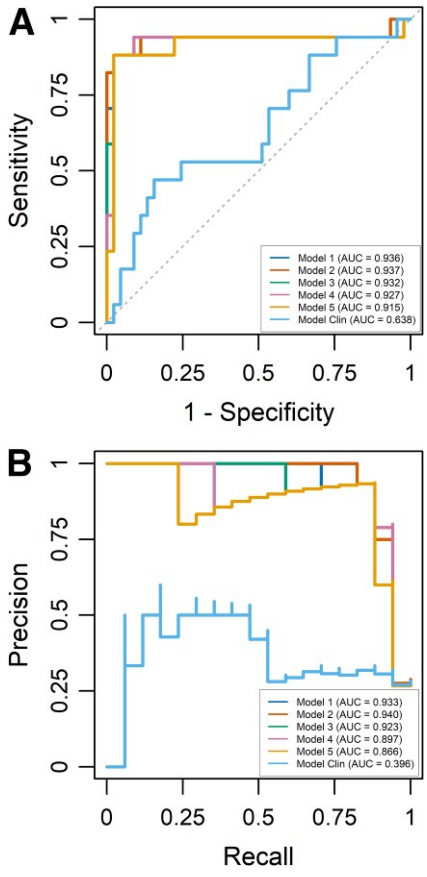

Figure 1—ROC-AUC and AUPRC for models 1–5 and the model based solely on clinical and biochemical variables (Model Clin) in the internal test cohort. ROC-AUC curves (*A*) and AUPRC curves (*B*) for the five DNAm-based models (models 1–5) and the Model Clin, evaluated in the internal test cohort (n = 62; T2D = 45, $T2D^{MACE}$ = 17). Both plots assess each model's ability to discriminate between individuals who did and did not experience MACE over the 5-year follow-up period. Statistical comparisons of model performance were done using appropriate methods for each metric. In the internal test cohort, pairwise comparisons of ROC-AUCs were performed using the DeLong test, and differences in AUPRC were assessed using empirical P values. Statistical significance for pairwise comparisons of ROC-AUCs and AUPRCs: $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. Model Clin.

lower overall error (Supplementary Tables 6–8). Decision curve analysis further showed that the DNAm-based models provided a higher net benefit across a broad range of threshold probabilities compared with the clinical model, indicating greater potential clinical utility (Supplementary Fig. 1).

To account for the time-to-event nature of MACE, Cox proportional hazards models were performed using the DNAm-based predicted probability as a continuous predictor. This variable was strongly associated with time to MACE across all models, with hazard ratios of ∼5 per 1-SD increase and high discriminative ability (C-index = 0.839–0.852), whereas the clinical model showed no significant association and poor discrimination (hazard ratio 1.43; C-index = 0.615) (Supplementary Table 9). Kaplan-Meier curves stratified by median predicted probability showed consistent risk separation for DNAm-based models, but not for the clinical model (Supplementary Fig. 2).

Impact of Individual CpG Markers on MACE Over 5 Years in Individuals With T2DM

Twelve CpG sites (cg04341454, cg06976977, cg07075085, cg10581754, cg11094002, cg12015691, cg12853145, cg15728120, cg15985003, cg20452429, cg24508873, and cg24616828) were consistently identified across all five predictive models as stable, model-independent markers for MACE risk stratification over 5 years in T2DM (Supplementary Tables 10 and 11). These loci showed differential DNAm between $T2D^{MACE}$ and T2D individuals in the training and test cohorts (Supplementary Figs. 3–7), with changes ranging from approximately −11.9% (cg07075085) to +11.5% (cg10581754). Univariate Poisson regression analyses further evaluated the association between DNAm levels at each CpG and risk of MACE over 5 years, with relative risks (RRs) expressed per 1% increase in DNAm (Fig. 2). Several CpGs were associated with increased MACE risk, including cg04341454, cg06976977, cg11094002, cg15728120, cg12853145, and cg24616828, with RRs ranging from 1.55 to 3.09 in the internal test cohort. In contrast, cg24508873, cg07075085, cg15985003, and cg20452429 showed protective associations, with RRs between 0.40 and 0.93 in the internal test cohort. Additional CpG sites, although not shared across all models, also showed significant associations with MACE over 5 years (Supplementary Table 12). Collectively, these findings highlight a broader set of CpG methylation markers, including both shared and model-specific loci, showing reproducible associations with short-term CV risk in individuals with T2DM.

CONCLUSIONS

The introduction into diabetes treatment algorithms of drug therapies able

Table 2—Performance metrics of models 1–5 and Model Clin in the internal test cohort of $T2D^{MACE}$ and T2D individuals

Metric	Model 1	Model 2	Model 3	Model 4	Model 5	Model Clin
$ROC\text{-}AUC_{Test}$, mean (95% CI)	0.936*** (0.808–1.000)	0.937*** (0.806–1.000)	0.932*** (0.805–1.000)	0.927** (0.790–1.000)	0.915** (0.779–1.000)	0.638 (0.396–0.808)
$AUPRC_{Test}$, mean (95% CI)	0.933*** (0.818–1.000)	0.940*** (0.824–1.000)	0.923*** (0.791–1.000)	0.897*** (0.720–1.000)	0.866** (0.658–1.000)	0.396 (0.230–0.661)
Accuracy, %	93.55	95.16	93.55	95.16	95.16	74.19
Sensitivity (recall), %	88.24	88.24	88.24	88.24	88.24	47.06
Specificity, %	95.56	97.78	95.56	97.78	97.78	84.44
PPV (precision), %	88.24	93.75	88.24	93.75	93.75	53.33
NPV, %	95.56	95.65	95.56	95.65	95.65	80.85
F1 score	0.882	0.909	0.882	0.909	0.909	0.500

Performance of the five DNAm-based models and the model built solely on clinical and biochemical variables (Model Clin) was evaluated on the internal test cohort (n = 62; n = 17 $T2D^{MACE}$; n = 45 T2D). Reported metrics include the ROC-AUC and the AUPRC, both expressed with 95% CI, along with accuracy, sensitivity (recall), specificity, PPV (precision), and NPV, presented as percentages, and the F1 score, reported as a decimal value (range: 0–1). For DNAm-based models 1–5, performance metrics were estimated using a fixed classification threshold of 0.5. For the Model Clin, metrics were calculated at the optimal threshold of 0.328, determined by maximizing the Youden index. Pairwise comparisons of ROC-AUCs were performed using the DeLong test, and differences in AUPRC were assessed using empirical P values. Statistical significance for pairwise comparisons of ROC-AUCs and AUPRCs: $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. Model Clin.

to reduce the probability of future CV events in high-risk individuals has profoundly changed the current clinical scenario (4). In addition, sophisticated diagnostic techniques capable of detecting early, asymptomatic pathological conditions (e.g., atherosclerotic plaques at high risk of rupture) with high probability to progress to MACE are now available (28). Furthermore, percutaneous coronary intervention can be used in asymptomatic individuals diagnosed with significant, high-risk vascular atherosclerosis to prevent or minimize the risk of MACE (29,30). However, the indiscriminate use of all available tools to reduce MACE risk in the entire population with diabetes cannot be easily afforded by any health care system for financial and organizational reasons (31). Moreover, applying the full range of preventive measures to individuals at low risk of MACE would be unnecessarily burdensome and distressing (31). Therefore, accurate identification of individuals at higher risk of CV events in long-lasting T2DM is of pivotal importance to correctly approach MACE prevention, but, unfortunately, remains a major unmet clinical need.

Currently used CV risk calculators rely on conventional risk factors, are often adapted to diabetes, and typically assess long-term (10-year) risk. They also cannot account for therapy effects, including the initiation or intensification of treatment or medications that modify disease trajectories, which can impair their discriminative performance (5–11,32).

CpG ID	Training RR (95% CI)	p-value	Test RR (95% CI)	p-value
cg06976977	1.97 (1.68-2.29)	8.78×10^{-18}	3.09 (2.00-4.78)	3.85×10^{-7}
cg04341454	2.15 (1.90-2.43)	3.07×10^{-33}	1.55 (1.21-1.99)	6.09×10^{-4}
cg11094002	1.97 (1.71-2.26)	2.10×10^{-21}	1.61 (1.28-2.02)	4.01×10^{-5}
cg15728120	1.78 (1.64-1.93)	1.25×10^{-42}	1.66 (1.39-1.97)	1.22×10^{-8}
cg12853145	1.72 (1.58-1.88)	1.26×10^{-33}	1.56 (1.34-1.81)	9.13×10^{-9}
cg24616828	1.68 (1.54-1.83)	8.25×10^{-31}	1.62 (1.32-1.98)	3.13×10^{-6}
cg12015691	1.22 (1.18-1.27)	1.11×10^{-23}	1.05 (0.99-1.12)	8.38×10^{-2}
cg10581754	1.16 (1.12-1.20)	6.13×10^{-19}	1.15 (1.10-1.20)	8.15×10^{-9}
cg24508873	0.84 (0.81-0.87)	4.10×10^{-22}	0.93 (0.88-0.98)	1.07×10^{-2}
cg07075085	0.83 (0.81-0.86)	9.08×10^{-33}	0.83 (0.75-0.92)	3.94×10^{-4}
cg15985003	0.75 (0.72-0.79)	2.34×10^{-33}	0.77 (0.70-0.84)	1.89×10^{-8}
cg20452429	0.20 (0.14-0.28)	1.81×10^{-21}	0.40 (0.29-0.56)	1.03×10^{-7}

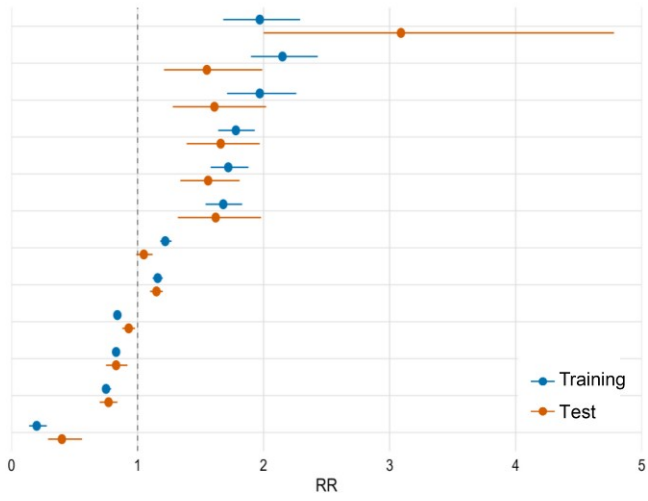

Figure 2—Estimated RRs for the 12 common CpG sites included in models 1–5 in the training and internal test cohort. RRs for MACE over 5 years per 1% increase in CpG methylation for the 12 common CpG sites included in models 1–5 were estimated by Poisson regression for the training (n = 243) and internal test (n = 62) cohorts. Error bars represent 95% CIs. The accompanying table reports RR estimates, 95% CIs, and two-sided Wald test P values. Statistical significance was defined as $P < 0.05$.

Therefore, existing scores cannot target precision prevention, particularly in individuals with established T2DM who feature high CV risk and receive complex treatments and who could benefit from accurate short-term risk stratification to guide timely and individualized interventions (4).

Consistent with these limitations, a head-to-head comparison of 22 risk prediction scores, including diabetes-specific models, in 168,871 U.K. primary care individuals with T2DM recently showed poor discrimination for 10-year CVD events (33). In line with these findings, in 11,224 individuals with T2DM from the Hoorn Diabetes Care System cohort, SCORE2-Diabetes, as well as Atherosclerotic Cardiovascular Disease (ASCVD), Action in Diabetes and Vascular Disease: Preterax and Diamicron Modified Release Controlled Evaluation (ADVANCE), and ADVANCE Observational (AD-ON) models, demonstrated limited discriminative ability for incident CVD (34). Similarly, in a prospective cohort of 752 individuals with newly diagnosed T2DM from the All New Diabetics in Scania (ANDIS) and All New Diabetics in Uppsala (ANDiU) cohorts, established CV risk scores, including SCORE2-Diabetes, UK Prospective Diabetes Study (UKPDS), and ASCVD, showed limited discriminative ability for incident macrovascular events (25). Consistently, in our TOSCA cohort with individuals with long-lasting T2DM, those who developed MACE over 5 years, despite a more adverse baseline profile, could not be distinguished from individuals who remained event-free by the SCORE2-Diabetes calculator (adapted for 5-year risk). This problem highlights the need for novel approaches capable of individualized and precise short-term risk stratification of CV events.

Here, using the TOSCA.IT cohort, we identified blood-based DNAm signatures associated with incident MACE and developed five DNAm-based models for risk stratification of MACE over 5 years using binomial LASSO logistic regression. DMPs were selected under five covariate adjustment strategies with stability selection and iterative resampling, yielding 22–26 CpGs per model, with 12 CpGs retained across all models.

In the internal test cohort, all models showed excellent discriminative performance (ROC-AUC = 0.915–0.937; AUPRC = 0.866–0.940; sensitivity = 88.24%, specificity = 95.56–97.78%, PPV = 88.24–93.75%, and NPV = 95.56–95.65%). High F1 scores (0.882–0.909) confirmed balanced classification of events and nonevents. These results support the consistency of the identified DNAm signatures within the study cohort and show that the discriminative contribution of CpG markers was not affected by the covariate adjustment strategies applied in this analysis. Notably, model 1 achieved comparable performance despite not accounting for other covariates, suggesting that within this study cohort, comparable discrimination could be achieved even when complete clinical information is unavailable.

Model predictions were consistently supported by significantly higher mean predicted probability values in patients who developed MACE compared with those who remained event-free, in both the training and the internal test cohorts, underlining the reliability of DNAm-based models as a tool for short-term CV risk stratification in individuals with T2DM.

Exploratory analyses showed that despite the limited number of fatal events (n = 10), individuals who died had higher probability values than those with a nonfatal MACE across all models, suggesting that DNAm signatures may also reflect disease severity. However, larger studies with an adequate number of fatal events and dedicated mortality-specific modeling will be required to confirm this observation.

The DNAm-based probability was also associated with time to MACE, with individuals at higher probability experiencing events earlier during follow-up. However, it is important to note that the DNAm-based models were originally derived within a case-control classification framework. Therefore, the separation observed in survival analyses likely reflects their ability to discriminate between individuals who develop MACE and those who do not, rather than being specifically developed to capture temporal risk dynamics. These findings are consistent with the discriminative performance observed in the main analyses and support the use of DNAm-based probability as a tool for risk stratification within this study setting, while future studies based on longitudinal modeling approaches may further refine its ability to predict the timing of events.

Additionally, in the TOSCA.IT cohort, a LASSO model based solely on clinical and biochemical variables (Model Clin) showed limited discrimination (ROC-AUC = 0.638; AUPRC = 0.396) and lower performance. Reclassification metrics (net reclassification improvement total = 0.5229–0.5451; integrated discrimination index = 0.7405–0.7588) were consistent with improved risk classification by the DNAm-based models. Importantly, when clinical and biochemical covariates were included alongside CpG markers in the DNAm-based models, none were retained, resulting in an identical DNAm-only model. This likely reflects both the feature selection properties of LASSO, which prioritizes the most informative predictors while excluding variables providing overlapping information, and the integrative nature of DNAm profiles, which likely capture the cumulative biological effects of multiple exposures, including metabolic, inflammatory, and lifestyle-related processes not fully reflected by single baseline clinical measurements. In this context, epigenetic features may contribute to capturing CV risk information beyond conventional determinants.

Recently, García-Calzón et al. (25) developed an MRS for predicting incident macrovascular events in 752 individuals with newly diagnosed T2DM, of whom 102 experienced events during follow-up. From 461 outcome-associated CpG sites, they derived an 87 CpG-based MRS with good discrimination (AUC = 0.81). Such an AUC improved to 0.84 when conventional clinical risk factors were added. While the MRS effectively excluded future nonevents (NPV = 95.9%), its PPV remained very low (31.8%), limiting its utility for identifying high-risk individuals for targeted preventive intervention. Although complementary to the findings of García-Calzón et al. (25), our analysis provides strong evidence for the discriminative utility of DNAm markers in assessing CV risk in T2DM. Our DNAm-based models were developed for risk stratification of MACE over 5 years, an extremely relevant clinical horizon for guiding timely intervention, and achieved very high overall discriminative performance. At a comparable NPV (>95%), we report a consistently higher PPV (88.24–93.75%), substantially improving the ability to identify individuals at imminent risk of MACE. Notably, no CpG overlap was observed between the two studies across all DNAm-based models, likely reflecting

differences in both analytical strategies and study design. CpG selection in our study relied on an experiment-wide significance threshold, followed by LASSO with stability selection, whereas García-Calzón et al. (25) applied weighted Cox regression with a predefined effect-size threshold. Cohorts and end points also differed, as our study included individuals with long-standing T2DM and focused on MACE, whereas that by García-Calzón et al. involved newly diagnosed T2DM and broader macrovascular outcomes. Nonetheless, both studies collectively support the potential of blood-based epigenetic biomarkers to improve CV risk stratification in T2D, highlighting the importance of tailoring predictive models to specific clinical settings.

Beyond the overall performance of the five DNAm-based models, another key finding of our study was the identification of 12 CpG sites consistently emerging across all modeling strategies as stable predictors of MACE over 5 years.

Their convergence across models supports the consistency of these CpG markers across different analytical approaches, suggesting that these CpG markers may capture disease-related epigenetic variation. Notably, these loci demonstrated strong predictive value, with consistent RR estimates reproduced in both training and internal test sets, encompassing both risk-enhancing and protective effects.

Among the 12 CpGs, 7 mapped to genes. Notably, cg07075085, annotated to *ADAMTS9-AS1*, a long noncoding RNA implicated in vascular remodeling and cardiac hypertrophy (35), showed the largest methylation differences between the T2D and $T2D^{MACE}$ groups ($\Delta\beta$ = ~−11.9%). *ADAMTS9-AS1* can bind *miR-206*, upregulating β-actin and promoting pathological remodeling, particularly in hypertrophic cardiomyopathy (35). Although direct evidence linking cg07075085 DNAm to CV outcomes in humans is lacking, our finding of a protective effect of increased DNAm (testing RR = 0.83) may reflect an epigenetic mechanism limiting *ADAMTS9-AS1* expression and attenuating prohypertrophic and proatherogenic signaling. Another locus, cg06976977, is located near *COX18*, which encodes a mitochondrial protein essential for cytochrome c oxidase assembly. Mitochondrial dysfunction is a recognized contributor to cardiometabolic disease, and *COX18* mutations have been linked to mitochondrial disorders with cardiac involvement (36). Consistent with this biology, cg06976977 was hypermethylated in $T2D^{MACE}$ individuals ($\Delta\beta$ = ~+0.95%) and conferred increased risk across models (testing RR = 3.09). While evidence directly connecting *COX18* DNAm to human CV outcomes remains to be established, our findings suggest that epigenetic variation at this locus may influence CV risk in T2D by altering mitochondrial function and energy metabolism.

Beyond the gene-annotated loci, the other five CpGs were located in intergenic or poorly annotated regions. Although lacking immediate gene assignment, these sites are biologically relevant. CpGs in open sea/low-density regions are among the most variable in the genome and often coincide with distal regulatory elements such as enhancers, capturing environmental and genetic influences with disease relevance (37,38). Indeed, disease-associated differential methylation occurs predominantly outside CpG islands, particularly in shores and other distal regions, underscoring the functional potential of these sites (37). Clinically, predictive utility does not require full mechanistic resolution (39). Thus, the strong and reproducible associations observed for these loci indicate that they provide reliable biomarkers of CV risk in T2DM and may reveal previously unrecognized epigenetic pathways deserving mechanistic investigation.

The strengths of this study include the use of a well-characterized cohort from the TOSCA.IT trial, with a 5-year follow-up and adjudicated CV outcomes, and the design choice of splitting participants into training and test sets, enabling both model construction and internal validation. Methodologically, we applied robust epigenome-wide analyses with adjustment for multiple covariates and stringent significance thresholds.

Furthermore, LASSO logistic regression with stability selection provided an unbiased approach to identify the most informative CpG sites while minimizing overfitting, enabling us to derive parsimonious and reproducible models whose discriminative performance was less likely to be inflated by sample-specific noise.

Several limitations should also be acknowledged. First, the study was conducted in a single clinical cohort of European ancestry, which might limit generalizability to other, diverse populations. In addition, the sample size, although adequate for epigenome-wide analyses (25,40), is relatively limited, particularly with regard to the number of CV events. This may affect the stability of model estimates and limit the generalizability of the findings. However, the use of stringent thresholds and robust modeling mitigated the risk of false-positives. Furthermore, the models were validated only through internal training/test splits, and external validation in independent cohorts will be necessary to confirm their broader applicability. Additionally, while peripheral blood DNAm provides accessible biomarkers, it might partially capture tissue-specific epigenetic changes relevant to CV pathology. However, emerging evidence suggests a degree of cross-tissue concordance of epigenetic signals. Meder et al. (17) demonstrated that a number of CpG sites exhibit concordant DNAm changes between myocardial tissue and peripheral blood, while García-Calzón et al. (25) showed that several blood-derived CpG sites are also differentially methylated in vascular tissues, including atherosclerotic plaques, and are associated with gene expression changes in diseased tissues.

Another limitation is that all T2DM participants were treated with metformin, and none received newer cardioprotective agents such as sodium–glucose cotransporter 2 inhibitors or glucagon-like peptide 1 receptor agonists as add-on therapy. Therefore, the potential impact of these therapies on DNAm profiles and on DNAm-based risk stratification could not be assessed in the current study and should be evaluated in appropriately designed cohorts. Also, the observational design does not allow causal inferences between CpG methylation changes and incident MACE. In addition, given the case-control–like sampling of the study subset, the proportion of events does not reflect true population incidence. Therefore, although calibration metrics indicated good agreement between predicted and observed outcomes in the internal test set, this reflects internal calibration only and does not enable accurate estimation of absolute risk

in external clinical populations. Accordingly, the predicted probabilities generated by the present models should be interpreted as tools for risk stratification within the study setting, rather than as calibrated estimates of absolute population-level risk. Estimation of absolute risk will require validation and recalibration in prospective population-based cohorts.

In conclusion, we reported the development and internal validation of blood-based DNAm models for risk stratification of MACE over 5 years in individuals with long-lasting T2DM. Across five modeling strategies, all based on epigenome-wide DNAm profiling, the discriminative performance was consistently high, outperforming conventional clinical and biochemical risk factors. The DNAm-based models we propose in the current study may improve CV risk stratification and provide a promising approach to refine identification of individuals at higher short-term risk of MACE.

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