

# Novel Biomarkers to Improve the Prediction of Cardiovascular Event Risk in Type 2 Diabetes Mellitus

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**Background**—We evaluated the ability of 23 novel biomarkers representing several pathophysiological pathways to improve the prediction of cardiovascular event (CVE) risk in patients with type 2 diabetes mellitus beyond traditional risk factors.

**Methods and Results**—We used data from 1002 patients with type 2 diabetes mellitus from the Second Manifestations of Atherosclerotic disease (SMART) study and 288 patients from the European Prospective Investigation into Cancer and Nutrition-NL (EPIC-NL). The associations of 23 biomarkers (adiponectin, C-reactive protein, epidermal-type fatty acid binding protein, heart-type fatty acid binding protein, basic fibroblast growth factor, soluble FMS-like tyrosine kinase-1, soluble intercellular adhesion molecule-1 and -3, matrix metalloproteinase [MMP]-1, MMP-3, MMP-9, N-terminal prohormone of B-type natriuretic peptide, osteopontin, osteonectin, osteocalcin, placental growth factor, serum amyloid A, E-selectin, P-selectin, tissue inhibitor of MMP-1, thrombomodulin, soluble vascular cell adhesion molecule-1, and vascular endothelial growth factor) with CVE risk were evaluated by using Cox proportional hazards analysis adjusting for traditional risk factors. The incremental predictive performance was assessed with use of the c-statistic and net reclassification index (NRI; continuous and based on 10-year risk strata 0–10%, 10–20%, 20–30%, >30%). A multimarker model was constructed comprising those biomarkers that improved predictive performance in both cohorts. N-terminal prohormone of B-type natriuretic peptide, osteopontin, and MMP-3 were the only biomarkers significantly associated with an increased risk of CVE and improved predictive performance in both cohorts. In SMART, the combination of these biomarkers increased the c-statistic with 0.03 (95% CI 0.01–0.05), and the continuous NRI was 0.37 (95% CI 0.21–0.52). In EPIC-NL, the multimarker model increased the c-statistic with 0.03 (95% CI 0.00–0.03), and the continuous NRI was 0.44 (95% CI 0.23–0.66). Based on risk strata, the NRI was 0.12 (95% CI 0.03–0.21) in SMART and 0.07 (95% CI –0.04–0.17) in EPIC-NL.

**Conclusions**—Of the 23 evaluated biomarkers from different pathophysiological pathways, N-terminal prohormone of B-type natriuretic peptide, osteopontin, MMP-3, and their combination improved CVE risk prediction in 2 separate cohorts of patients with type 2 diabetes mellitus beyond traditional risk factors. However, the number of patients reclassified to a different risk stratum was limited. (*J Am Heart Assoc.* 2016;5:e003048 doi: 10.1161/JAHA.115.003048)

**Key Words:** biomarker • cardiovascular disease prevention • cardiovascular disease risk factors • risk stratification

Type 2 diabetes mellitus is a growing worldwide health problem, with an estimated 592 million people living with diabetes mellitus by 2035.<sup>1</sup> Patients with diabetes

mellitus are at 2- to 4-fold increased risk of cardiovascular events (CVEs).<sup>2</sup> Formerly, diabetes was regarded as a “coronary risk equivalent,” assuming a 10-year cardiovascular

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Accompanying Data S1, Table S1, and Figure S1 are available at <http://jaha.ahajournals.org/content/5/6/e003048/DC1/embed/inline-supplementary-material-1.pdf>

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risk of  $\geq 20\%$  for every patient with diabetes mellitus.<sup>3</sup> Recent studies indicated that there actually is a wide distribution of risk depending on diabetes mellitus duration, severity, and concomitant risk factors.<sup>4,5</sup> Accurate cardiovascular risk stratification can help clinicians to identify low-risk patients for whom treatment could be postponed or high-risk patients for whom treatment should be initiated or intensified. In the light of an increasing number of patients with diabetes mellitus, an individual patient risk-based approach has the potential to allocate treatment resources more efficiently and effectively.

We previously identified 45 cardiovascular prediction models applicable to diabetes mellitus patients and validated 10 models specifically designed for patients with type 2 diabetes mellitus in different cohorts.<sup>6,7</sup> These risk scores had a reasonable performance with respect to risk stratification (ie, calibration) and a moderate to weak ability to distinguish between patients who did and did not go on to develop a CVE (ie, discrimination). To enhance predictive performance novel markers conveying information on underlying atherosclerotic disease progression could be helpful. Recent studies in mostly healthy populations have suggested a number of biomarkers that might improve CVE risk prediction.<sup>8–10</sup> These include markers of several pathophysiological pathways, such as measures of inflammation, coagulation, collagen degradation, angiogenesis, metabolic regulation, and adipocyte function. In this study, we systematically studied which of the 23 novel biomarkers were associated with CVE risk in 2 separate cohorts of patients with type 2 diabetes. Subsequently, we evaluated whether these novel biomarkers and their combination could improve CVE risk prediction on top of traditional risk factors.

## Methods

### Study Populations

The Second Manifestations of ARterial disease (SMART) study is an ongoing prospective single-center cohort study at the University Medical Centre Utrecht in Utrecht, The Netherlands. Study patients were either newly referred with manifest atherosclerotic disease or for the management of cardiovascular risk factors (ie, hypertension, hyperlipidemia, or diabetes).<sup>11</sup> Patients were screened noninvasively for manifestations of atherosclerotic diseases and risk factors other than the qualifying diagnosis. Exclusion criteria were age  $< 18$  years, malignancy, dependency in daily activities, and insufficient fluency in the Dutch language. For the current study, we used a sample of 1034 SMART patients with type 2 diabetes enrolled between January 1996 and March 2006 to ensure a substantial follow-up duration. Diabetes was defined as a referral diagnosis of type 2 diabetes, self-reported type 2 diabetes, the use of glucose-lowering agents, or a plasma

glucose concentration of  $\geq 7.0$  mmol/L at baseline with initiation of glucose-lowering treatment within 1 year after inclusion. Patients for whom the novel biomarkers could not be assayed ( $n=32$ ) were excluded, resulting in 1002 patients for analyses.

The EPIC-NL cohort is the Dutch contribution to the European Prospective Investigation into Cancer and Nutrition (EPIC) and consists of the Prospect-EPIC and MORGEN-EPIC cohorts.<sup>12</sup> In brief, the Prospect-EPIC study includes 17 357 women 49 to 70 years of age living in Utrecht and the vicinity who participated in the nationwide Dutch breast cancer screening program. The MORGEN-EPIC cohort consists of 22 654 men and women 21 to 64 years of age selected from random samples of the Dutch population in 3 different towns. Participants were recruited in both studies from 1993 to 1997. For EPIC-NL, case-cohort sampling was performed to reduce costs and preserve valuable biological material in participants with confirmed type 2 diabetes at baseline ( $n=526$ ). Three sources of ascertainment of diabetes were used: self-report, hospital discharge diagnoses, and urinary strip test (in the Prospect part of the cohort only). Ascertained cases of diabetes were verified against medical and pharmacy records, and only verified cases were included.<sup>13</sup> From a total of 172 incident CVE cases in patients with diabetes at baseline, 134 cases had available blood samples and were included in this analysis. In addition, a random sample of 218 participants was selected to serve as a subcohort in the case-cohort design. Because of the random selection, 64 participants within the subcohort developed CVE and overlapped between the subcohort and the selection of cases, an inherent feature of case-cohort studies.

The ethics committees of the respective institutions approved both studies, and all participants gave their written informed consent. Detailed descriptions of these studies have been published previously.<sup>11,12</sup>

### Biomarkers and Other Measurements

We measured 23 biomarkers from different pathophysiological pathways involved in the progression to CVE in patients with diabetes, including adiponectin, C-reactive protein, basic fibroblast growth factor, soluble FMS-like tyrosine kinase, soluble intercellular adhesion molecule-1 and -3, matrix metalloproteinase (MMP)-1, MMP-3, MMP-9, N-terminal pro-hormone of B-type natriuretic peptide (NT-proBNP), osteocalcin, osteonectin, osteopontin, placental growth factor, serum amyloid A, E-selectin, P-selectin, tissue inhibitor of MMP, thrombomodulin, soluble vascular cell adhesion molecule, and vascular endothelial growth factor by using single or multiplex assays (Meso Scale Discovery). Heart-type fatty acid binding protein and epidermal-type fatty acid binding protein were measured in plasma by using a 1-step enzyme-linked

immunosorbent assay (FABPulous BV and BioVendor, respectively). Interassay and intra-assay variations were acceptable and are reported in Data S1. Further, participants completed a questionnaire on CVE history, risk factors, and medication use. Venous blood samples were taken to determine serum lipids, glucose, glycated hemoglobin, and creatinine. The techniques used for the laboratory tests have been described previously.<sup>11,12</sup>

## Follow-up and Clinical End Points

SMART participants were biannually asked to complete a questionnaire on hospitalizations and outpatient clinic visits. The outcome of interest was major CVEs as a composite of myocardial infarction, stroke, and vascular death. When a possible event was reported, hospital discharge letters and results of relevant laboratory and radiology examinations were collected. Based on this information, all events were audited by 3 members of the SMART study Endpoint Committee, comprising physicians from different departments. Follow-up duration was defined as the period between study inclusion and first CVE or death from any cause, date of loss to follow-up, or the preselected date of March 1, 2013. Of the 1002 participants, 87 (8.7%) were censored as being lost to follow-up because of migration or discontinuation of the study. For EPIC-NL, follow-up data on CVEs were obtained through linkage with registries. Hospital discharge diagnoses were obtained from the Pharmo Institute, which holds a standardized computerized register of hospital discharge diagnoses. The database was linked to the cohort on the basis of birth date, sex, postal code, and general practitioner with a validated probabilistic method.<sup>14</sup> Information on vital status was obtained through linkage with the municipal registries, and causes of death were collected from Statistics Netherlands. In a subsample of the cohort, cases of coronary heart disease (CHD; defined as *International Classification of Diseases, Ninth Revision* [ICD-9] codes 410–414) obtained from hospital discharge diagnoses were verified against medical records. This showed that 85% of CHD events and 97% of acute myocardial infarctions could be confirmed.<sup>15</sup> Follow-up was complete until January 1, 2008. In EPIC, major vascular events was defined as CHD, congestive heart failure, peripheral arterial disease, stroke, and other CVEs (ICD-9 codes 410–414, 427.5, 428, 415.1, 443.9, 430–438, 440–442, 444, 798.1, 798.2, and 798.9).

## Statistical Analyses

We assessed the independent relation of each biomarker with the outcome in a Cox proportional hazards model adjusting for all variables of the base model composing the traditional CVE risk factors described later. Restricted cubic splines were used to evaluate the relation between the marker and the log

hazard of major CVE and showed that a natural logarithmic transformation was generally the most appropriate functional form. Hazard ratios were presented for the highest versus the lowest quartile of the biomarker. The median follow-up time was 9.2 years in SMART, and we extrapolated the risk estimates through exponentiation to cover a 10-year time period. In EPIC-NL the median follow-up was 11.3 years, and the 10-year estimates were used. Within SMART, we used regular Cox proportional hazards regression models; in EPIC-NL we used Prentice weighting to properly take into account the case-cohort nature of the data.<sup>16</sup>

We evaluated the improvement in predictive performance for each new marker when added to the base model. In addition, we evaluated a multimarker model constituting those markers that were significantly associated with CVE risk and improved predictive performance (defined as an increase in c-statistic of  $\geq 0.1$  and a net reclassification index [NRI]  $\geq 0.20$ ) in both cohorts to avoid the selection of biomarkers performing well by chance in one of the data sets. The base model included predictors of the United Kingdom Prospective Diabetes Study algorithm (age at diabetes diagnosis, duration of diagnosed diabetes, sex, smoking, glycated hemoglobin ( $Hb_{A1c}$ ), systolic blood pressure, total cholesterol/high-density lipoprotein (HDL) cholesterol ratio), and 2 additional variables (previous CVE and urinary albumin:creatinine ratio, the latter not being available in EPIC-NL and replaced by estimated glomerular filtration rate).<sup>17</sup> Variable transformations and model coefficients were reestimated in each study population to ensure optimal fit of the base model. Interactions of the biomarkers with age at diabetes diagnosis and sex were evaluated and retained if the *P*-value for interaction was  $< 0.01$  in both cohorts.

The base model was compared with the expanded biomarker model by using the Wald  $\chi^2$  test, a measure of improvement in global model fit. Next, we examined differences in discrimination between the base and expanded model, using the Harrell c-statistic for censored survival data. The c-statistic indicates the ability to distinguish between patients who will and those who will not have an event. Subsequently, we calculated the continuous and categorical NRI modified for censored survival data.<sup>18</sup> The continuous NRI was reported in the absence of well-established risk categories for patients with diabetes. The continuous NRI defines upward or downward movement by any increase or decrease in probabilities of the outcome.<sup>19</sup> To assess potential clinical impact, we calculated the categorical NRI with the use of 4 clinically inspired 10-year risk categories (low risk [0–10%], intermediate risk [10–20%], high risk [20–30%], and an additional very high risk category [ $> 30\%$ ] given the higher levels of risks in patients with diabetes). CIs for the NRI were obtained by using 1000-fold bootstrap resampling in each imputation set and taking the 2.5th and 97.5th percentiles of the stacked distribution.<sup>20</sup> In addition, a graphic summary of predicted probabilities by the

**Table 1.** Baseline Characteristics and Missing Data of the SMART Study (n=1002) and the EPIC-NL Subcohort (n=218)

| Characteristics                                      | SMART               | Missing, % | EPIC-NL             | Missing, % |
|--|---------------------|------------|---------------------|------------|
| Age, y*  | 59±10               | 0          | 58±7                | 0          |
| Female, %  | 31                  | 0          | 82                  | 0          |
| Diabetes duration, y <sup>†</sup>                    | 4 (1–9)             | 8          | 5 (2–10)            | 1.1        |
| Age at diagnosis of diabetes, y <sup>†</sup>         | 53 (46–60)          | 8          | 51 (44–58)          | 1.1        |
| History of vascular disease, %                       | 62                  | 0          | 14                  | 0          |
| BMI, kg/m <sup>2</sup> *                             | 28.8±5.0            | 0.2        | 29.4 (4.9)          | 0          |
| Systolic blood pressure, mm Hg*                      | 147±21              | 0.2        | 142±21              | 0.7        |
| Diastolic blood pressure, mm Hg*                     | 83±11               | 0.3        | 82±10               | 0.7        |
| Current smoking, %                                   | 28                  | 0.8        | 23                  | 0.4        |
| Total cholesterol*                                   | 5.2±1.4             | 1          | 5.2±1.2             | 8.4        |
| HDL cholesterol, mmol/L*                             | 1.1±0.4             | 1.0        | 1.0±0.3             | 12.9       |
| Hb <sub>A1c</sub> , %*                               | 7.4±1.4             | 12.9       | 8.1±1.7             | 8.2        |
| Hb <sub>A1c</sub> , mmol/mol*                        | 57±15               | 12.9       | 65±18               | 8.2        |
| eGFR, mL/min per 1.73 m <sup>2†</sup>                | 80 (67–93)          | 0.6        | 91 (76–98)          | 8.2        |
| Urinary albumin:creatinine ratio, µg/mg <sup>†</sup> | 1.1 (0.6–3.1)       | 8.7        | —                   | —          |
| <b>Medications</b>                                   |                     |            |                     |            |
| Oral glucose-lowering agents, %                      | 61                  | 0          | 64                  | 64         |
| Insulin, %   | 24                  | 0          | 25                  | 65         |
| Lipid-lowering agents, %                             | 49                  | 0          | 4                   | 51         |
| Blood pressure-lowering agents, %                    | 69                  | 0          | 39                  | 0          |
| <b>Biomarkers</b>                                    |                     |            |                     |            |
| Adiponectin, µg/mL <sup>†</sup>                      | 7.2 (4.9–11.1)      | 0          | 8.4 (6.3–11.3)      | 0          |
| NT-proBNP, pg/mL <sup>†</sup>                        | 185.1 (71.8–565.6)  | 0          | 150.7 (73.4–372.6)  | 0          |
| MMP-1, ng/mL <sup>†</sup>                            | 1.8 (1.2–3.0)       | 0          | 2.7 (1.7–3.6)       | 0          |
| MMP-3, ng/mL <sup>†</sup>                            | 13.7 (9.2–19.5)     | 0          | 9.1 (6.9–11.8)      | 0          |
| MMP-9, ng/mL <sup>†</sup>                            | 12.7 (10.1–17.3)    | 0          | 33.3 (24.2–51.4)    | 0          |
| bFGF, pg/mL <sup>†</sup>                             | 3.1 (1.8–6.2)       | 0          | 6.9 (4.3–11.4)      | 0          |
| PIGF, pg/mL <sup>†</sup>                             | 13.3 (11.2–16.2)    | 0          | 16.5 (14.3–18.7)    | 0          |
| sFit-1, pg/mL <sup>†</sup>                           | 134.9 (118.9–154.5) | 0          | 128.2 (106.7–150.1) | 0          |
| VEGF, pg/mL <sup>†</sup>                             | 58.7 (47.5–75.0)    | 0          | 103.6 (76.3–137.7)  | 0          |
| Osteocalcin, ng/mL <sup>†</sup>                      | 28.7 (21.3–40.1)    | 0          | 22.6 (17.1–29.2)    | 0          |
| Osteonectin, ng/mL <sup>†</sup>                      | 58.9 (47.5–79.9)    | 0          | 114.6 (90.0–154.8)  | 0          |
| Osteopontin, ng/mL <sup>†</sup>                      | 18.2 (14.5–23.1)    | 0          | 12.8 (10.1–16.1)    | 0          |
| E-FABP, ng/mL <sup>†</sup>                           | 3.3 (2.0–5.1)       | 0          | 3.0 (2.0–5.0)       | 0          |
| H-FABP, ng/mL <sup>†</sup>                           | 2.0 (1.5–2.6)       | 0.5        | 1.4 (0.9–2.0)       | 0          |
| CRP, µg/mL <sup>†</sup>                              | 2.4 (1.1–5.5)       | 0          | 3.3 (1.8–6.5)       | 0          |
| SAA, µg/mL <sup>†</sup>                              | 2.9 (1.7–5.6)       | 0          | 2.0 (1.3–3.3)       | 0          |
| sICAM-1, ng/mL <sup>†</sup>                          | 253.2 (215.8–306.6) | 0          | 249.9 (209.8–288.3) | 0          |
| sVCAM-1, ng/mL <sup>†</sup>                          | 384.9 (327.2–456.5) | 0          | 357.5 (310.0–421.7) | 0          |
| E-selectin, ng/mL <sup>†</sup>                       | 15.1 (10.9–19.8)    | 0          | 16.7 (12.2–21.3)    | 0          |
| P-selectin, ng/mL <sup>†</sup>                       | 41.5 (32.6–51.1)    | 0          | 46.7 (38.5–54.5)    | 0          |
| sICAM-3, ng/mL <sup>†</sup>                          | 0.8 (0.6–0.9)       | 0          | 1.02 (0.86–1.24)    | 0          |

Continued

Table 1. Continued

| Characteristics                    | SMART               | Missing, % | EPIC-NL             | Missing, % |
|------------------------------------|---------------------|------------|---------------------|------------|
| Thrombomodulin, ng/mL <sup>†</sup> | 3.7 (3.2–4.4)       | 0          | 3.3 (2.8–3.7)       | 0          |
| TIMP-1, ng/mL <sup>†</sup>         | 127.8 (106.5–152.9) | 0          | 120.5 (105.7–145.8) | 0          |

BMI indicates body mass index; bFGF, basic fibroblast growth factor; CRP, C-reactive protein; E-FABP, epidermal-type fatty acid binding protein; eGFR, estimated glomerular filtration rate; EPIC-NL, European Prospective Investigation in Cancer and Nutrition-NL; HbA<sub>1c</sub>, glycated hemoglobin; HDL, high-density lipoprotein; H-FABP, heart-type fatty acid binding protein; MMP, matrix metalloproteinase; NT-proBNP, N-terminal pro-B-type natriuretic peptide; PIGF, placental growth factor; SAA, serum amyloid A; sICAM, soluble intercellular adhesion molecule; sFit, soluble FMS-like tyrosine kinase; SMART, Second Manifestations of ARterial Disease; sVCAM, soluble vascular cell adhesion molecule; TIMP, tissue inhibitor of matrix metalloproteinase; VEGF, vascular endothelial growth factor.

\*Mean±SD.

<sup>†</sup>Median with IQR.

base and expanded model for patients with and without CVE was provided to quantify changes in risk.

Data were missing in up to 12.9% of participants in SMART and EPIC-NL (Table 1). We performed 10-fold multiple imputation by predictive mean matching using the R-library MICE. Results were pooled using Rubin's rule except for variance estimates of the NRI as outlined earlier. All statistical analyses were conducted in R, version 3.0.3 (R Development Core Team), and a level of significance of 0.05 was applied.

## Results

### Baseline Characteristics

Baseline characteristics of the SMART and EPIC population are presented in Table 1. In SMART, 248 major CVEs occurred during a median follow-up of 9.2 years (IQR 7.2–11.1 years), resulting in an average 10-year CVE risk of 25%. Of all SMART participants, 18% were at low, 28% were at intermediate, 24% were at high, and 31% were at very high 10-year CVE risk according to the base model. In EPIC-NL, 134 CVEs occurred during a median follow-up of 11.3 years (IQR 8.0–12.8 years), resulting in an average 10-year CVE risk of 33%. Of all EPIC-NL participants, 1% were at low, 27% were at intermediate, 27% were at high, and 45% were at very high 10-year CVE risk according to the base model. Calibration plots of the base models are shown in Figure S1.

### Associations With Cardiovascular Risk

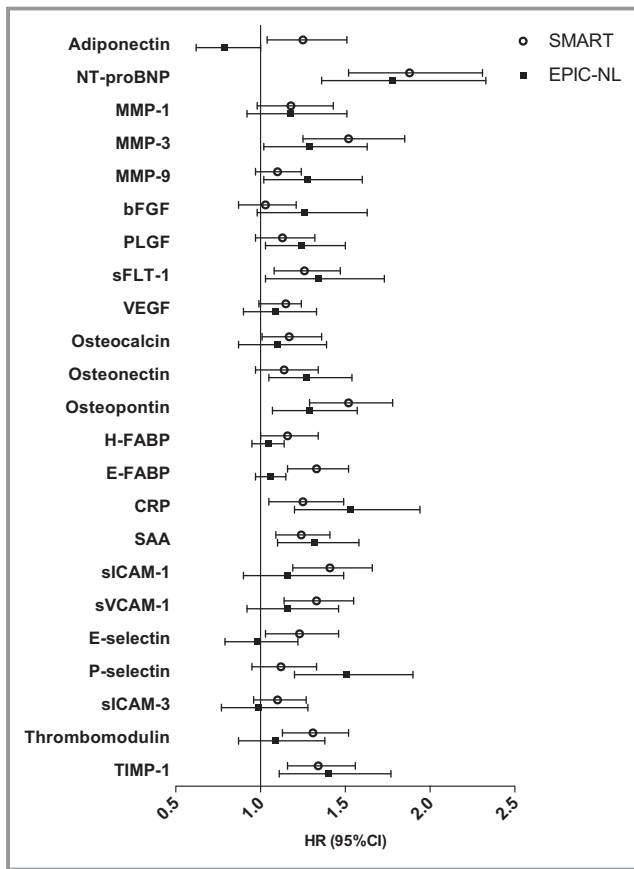
Figure 1 shows the multivariable adjusted hazard ratios (HRs) for major CVEs for each biomarker in both cohorts. In general, the direction of effects was similar between the cohorts, except for adiponectin, which had opposite effects. Significantly higher risks with increasing concentrations were observed in both cohorts for 7 markers (C-reactive protein, serum amyloid A, MMP-3, NT-proBNP, osteopontin, soluble FMS-like tyrosine kinase, and tissue inhibitor of MMP-1). No consistent interactions with age at diabetes diagnosis and sex were found. Detailed statistics of the base models are shown in Table S1.

### Improvements in Predictive Performance

The largest improvement in discriminatory power for a single biomarker was observed for the addition of NT-proBNP with an increase in c-statistic of 0.02 (95% CI 0.00–0.04) in SMART and 0.02 (95% CI 0.00–0.05) in EPIC-NL (Table 2). Slight and consistent improvements in c-statistics were also seen for MMP-3, osteopontin, serum amyloid A, and tissue inhibitor of MMP-1. The multimarker model resulted in a greater improvement of 0.03 (95% CI 0.01–0.05) in SMART and 0.03 (95% CI 0.00–0.03) in EPIC-NL.

In both cohorts, the continuous NRI was substantial with the separate addition of NT-proBNP (SMART 0.27, 95% CI 0.10–0.44; EPIC-NL 0.50, 95% CI 0.26–0.73), osteopontin (SMART 0.32, 95% CI 0.16–0.47; EPIC-NL 0.28, 95% CI 0.06–0.50), and MMP-3 (SMART 0.25, 95% CI 0.09–0.42; EPIC-NL 0.23, 95% CI 0.0–0.45) as shown in Table 3. The multimarker model showed a high continuous NRI in both cohorts (SMART 0.37, 95% CI 0.21–0.52; EPIC-NL 0.44, 95% CI 0.23–0.66).

When assessing reclassification across predefined risk categories, the separate addition of NT-proBNP, osteopontin, and MMP-3 yielded a variable but positive categorical NRI in both cohorts (Table 4). The multimarker model showed an improvement in risk classification with an NRI of 0.12 (95% CI 0.03–0.21) in SMART and of 0.07 (95% CI –0.04 to 0.17) in EPIC-NL. The number of patients reclassified into different risk categories by the multimarker model in the SMART population is shown in Table 5. Of 737 patients without an event, 189 (25.4%) patients were correctly reclassified to a lower risk category and 94 (12.8%) were erroneously reclassified to a higher risk category by the new multimarker model. On the other hand, of 265 patients with an event, 37 (14.0%) of patients were correctly reclassified upward and 46 (18.1%) patients were erroneously reclassified downward. A graphic summary of predicted risks for patients with a CVE and patients without a CVE by the base model versus predicted risks by the multimarker biomarker model in SMART is shown in Figure 2. Most notable were the higher predicted risks among patients who go on to develop a CVE.



**Figure 1.** Multivariable adjusted hazard ratios for risk of major cardiovascular events for the highest vs the lowest quartile of each biomarker in the Second Manifestations of ARterial disease (SMART) study and European Prospective Investigation into Cancer and Nutrition-NL (EPIC-NL) study (adjusted for sex, age at diabetes mellitus diagnosis, duration of diabetes mellitus, HbA<sub>1c</sub>, systolic blood pressure, TC/HDL ratio, urinary albumin/creatinine ratio, smoking status and previous CVE. bFGF indicates basic fibroblast growth factor; CRP, C-reactive protein; CVE, cardiovascular event; E-FABP, epidermal-type fatty acid binding protein; H-FABP, heart-type fatty acid binding protein; MMP, matrix metalloproteinase; NT-proBNP, N-terminal prohormone of B-type natriuretic peptide; PIGF, placental growth factor; SAA, serum amyloid A; sFLT, soluble FMS-like tyrosine kinase; sICAM, soluble intercellular adhesion molecule; sVCAM, soluble vascular cell adhesion molecule; TIMP, tissue inhibitor of matrix metalloproteinase; VEGF, vascular endothelial growth.

### Discussion

The present study evaluated the improvement in CVE risk prediction with novel biomarkers from different pathophysiological pathways in patients with type 2 diabetes. Of 23 biomarkers evaluated, NT-proBNP, osteopontin, and MMP-3 and their combination resulted in the largest improvement in predictive performance beyond traditional risk factors.

Currently available CVE risk scores for patients with type 2 diabetes were shown to produce reasonable risk stratification and moderate risk discrimination.<sup>7</sup> In the present study, we evaluated novel biomarkers for their potential to improve predictive performance. In particular, the improvement in risk stratification is relevant given that the primary goals of CVE risk algorithms are to inform patients of their risk and to guide treatment decisions.<sup>21</sup> Although the number of patients with diabetes for whom medical cardiovascular risk management is not beneficial is probably small, risk prediction can be useful to decide on intensity of treatment. For example, the

**Table 2.** Differences in c-Statistic After the Addition of Each Biomarker to the Base Model for Both Cohorts

| Biomarker                   | SMART                | EPIC-NL              |
|-----------------------------|----------------------|----------------------|
|                             | c-Statistic (95% CI) | c-Statistic (95% CI) |
| Base model                  | 0.70 (0.67–0.74)     | 0.69 (0.64–0.74)     |
| Adiponectin                 | 0.00 (0.00–0.01)     | 0.01 (0.00–0.02)     |
| NT-proBNP                   | 0.02 (0.00–0.04)     | 0.02 (0.00–0.05)     |
| MMP-1                       | 0.00 (0.00–0.01)     | 0.01 (–0.01 to 0.01) |
| MMP-3                       | 0.01 (0.00–0.03)     | 0.01 (–0.01 to 0.02) |
| MMP-9                       | 0.00 (0.00–0.01)     | 0.01 (0.00–0.02)     |
| bFGF                        | 0.00 (0.00–0.00)     | 0.01 (–0.01 to 0.02) |
| PIGF                        | 0.00 (–0.01 to 0.00) | 0.01 (0.00–0.02)     |
| sFlt-1                      | 0.00 (–0.01 to 0.01) | 0.01 (–0.01 to 0.02) |
| VEGF                        | 0.00 (–0.01 to 0.01) | 0.00 (0.00–0.01)     |
| Osteocalcin                 | 0.00 (–0.01 to 0.01) | 0.00 (–0.01 to 0.01) |
| Osteonectin                 | 0.00 (0.00–0.01)     | 0.01 (0.00–0.03)     |
| Osteopontin                 | 0.01 (0.00–0.03)     | 0.01 (–0.01 to 0.02) |
| H-FABP                      | 0.01 (0.00–0.02)     | 0.00 (–0.01 to 0.01) |
| E-FABP                      | 0.00 (0.00–0.01)     | 0.00 (–0.02 to 0.01) |
| CRP                         | 0.00 (–0.01 to 0.01) | 0.02 (0.00–0.04)     |
| SAA                         | 0.01 (0.00–0.02)     | 0.01 (–0.01 to 0.03) |
| sICAM-1                     | 0.01 (–0.01 to 0.02) | 0.01 (0.00–0.02)     |
| sVCAM-1                     | 0.00 (–0.01 to 0.01) | 0.00 (–0.01 to 0.1)  |
| E-selectin                  | 0.00 (–0.01 to 0.01) | 0.00 (0.00–0.00)     |
| P-selectin                  | 0.00 (0.00–0.01)     | 0.02 (–0.01 to 0.04) |
| sICAM-3                     | 0.00 (0.00–0.01)     | 0.00 (0.00–0.00)     |
| Thrombomodulin              | 0.01 (–0.01 to 0.02) | 0.00 (–0.01 to 0.01) |
| TIMP-1                      | 0.01 (0.00–0.02)     | 0.02 (0.00–0.03)     |
| NT-proBNP+MMP-3+osteopontin | 0.03 (0.01–0.05)     | 0.03 (0.00–0.03)     |

bFGF indicates basic fibroblast growth factor; CRP, C-reactive protein; E-FABP, epidermal-type fatty acid binding protein; H-FABP, heart-type fatty acid binding protein; MMP, matrix metalloproteinase; NT-proBNP indicates N-terminal prohormone of B-type natriuretic peptide; PIGF, placental growth factor; SAA, serum amyloid A; sFLT, soluble FMS-like tyrosine kinase; sICAM, soluble intercellular adhesion molecule; sVCAM, soluble vascular cell adhesion molecule; VEGF, vascular endothelial growth factor; TIMP, tissue inhibitor of matrix metalloproteinase.

**Table 3.** Continuous NRI After the Addition of Each Biomarker to the Base Model for Both Cohorts

| Biomarker                   | SMART                    | EPIC-NL                  |
|-----------------------------|--------------------------|--------------------------|
|                             | NRI (95% CI)             | NRI (95% CI)             |
| Adiponectin                 | 0.122 (−0.043 to 0.281)  | 0.153 (−0.072 to 0.356)  |
| NT-proBNP                   | 0.271 (0.102–0.439)      | 0.509 (0.260–0.731)      |
| MMP-1                       | 0.181 (0.014–0.331)      | 0.043 (−0.192 to 0.263)  |
| MMP-3                       | 0.253 (0.091–0.422)      | 0.226 (−0.001 to 0.446)  |
| MMP-9                       | 0.020 (−0.142 to 0.183)  | 0.224 (0.020–0.456)      |
| bFGF                        | 0.038 (−0.137 to 0.202)  | 0.048 (−0.159 to 0.261)  |
| PLGF                        | 0.172 (0.012–0.342)      | 0.123 (−0.129 to 0.329)  |
| sFlt-1                      | 0.177 (0.022–0.338)      | 0.108 (−0.152 to 0.339)  |
| VEGF                        | 0.056 (−0.096 to 0.215)  | 0.074 (−0.161 to 0.302)  |
| Osteocalcin                 | −0.039 (−0.193 to 0.129) | 0.069 (−0.170 to 0.307)  |
| Osteonectin                 | 0.097 (−0.061 to 0.254)  | 0.270 (0.067–0.484)      |
| Osteopontin                 | 0.316 (0.161–0.472)      | 0.281 (0.060–0.498)      |
| H-FABP                      | 0.161 (−0.006 to 0.332)  | 0.183 (0.006–0.352)      |
| E-FABP                      | 0.095 (−0.075 to 0.248)  | 0.027 (−0.156 to 0.203)  |
| CRP                         | 0.117 (−0.039 to 0.276)  | 0.412 (0.222–0.642)      |
| SAA                         | 0.096 (−0.057 to 0.267)  | 0.312 (0.103–0.503)      |
| sICAM-1                     | 0.209 (0.050–0.376)      | 0.115 (−0.124 to 0.340)  |
| sVCAM-1                     | 0.238 (0.074–0.400)      | 0.124 (−0.098 to 0.353)  |
| E-selectin                  | 0.118 (−0.044 to 0.285)  | −0.003 (−0.221 to 0.236) |
| P-selectin                  | −0.001 (−0.161 to 0.155) | 0.226 (0.008–0.418)      |
| sICAM-3                     | 0.043 (−0.124 to 0.203)  | −0.158 (−0.377 to 0.059) |
| Thrombomodulin              | 0.118 (−0.048 to 0.282)  | 0.097 (−0.138 to 0.342)  |
| TIMP-1                      | 0.113 (−0.054 to 0.276)  | 0.333 (0.122–0.563)      |
| NT-proBNP+MMP-3+osteopontin | 0.368 (0.208–0.522)      | 0.442 (0.230–0.659)      |

bFGF indicates basic fibroblast growth factor; CRP, C-reactive protein; E-FABP, epidermal-type fatty acid binding protein; H-FABP, heart-type fatty acid binding protein; MMP, matrix metalloproteinase; NT-proBNP indicates N-terminal prohormone of B-type natriuretic peptide; NRI, net reclassification index; PIGF, placental growth factor; SAA, serum amyloid A; sFLT, soluble FMS-like tyrosine kinase; sICAM, soluble intercellular adhesion molecule; sVCAM, soluble vascular cell adhesion molecule; VEGF, vascular endothelial growth factor; TIMP, tissue inhibitor of matrix metalloproteinase.

American College of Cardiology/American Heart Association CVE prevention guideline recommends moderate-intensity statin treatment for diabetic patients with a 10-year atherosclerotic CVE risk <7.5%, whereas high-intensity statin treatment is recommended for patients at higher risk.<sup>22</sup> Further, in case of treatments with a greater potential for side effects, such as intensive versus standard glucose control, risk prediction can help to select low-risk diabetes patients who are less likely to benefit from intensive treatment.<sup>23</sup>

In the current study, NT-proBNP showed the greatest potential to improve predictive performance in patients with diabetes. NT-proBNP is a polypeptide secreted by

**Table 4.** Risk Category-Based NRI After the Addition of Each Biomarker to the Base Model in Both Cohorts

| Biomarker                   | SMART                     | EPIC-NL                  |
|-----------------------------|---------------------------|--------------------------|
|                             | Categorical NRI (95% CI)* | Categorical NRI (95% CI) |
| Adiponectin                 | 0.019 (−0.047 to 0.084)   | −0.025 (−0.085 to 0.036) |
| NT-proBNP                   | 0.074 (−0.020 to 0.163)   | 0.021 (−0.081 to 0.122)  |
| MMP-1                       | 0.017 (−0.044 to 0.075)   | −0.022 (−0.098 to 0.046) |
| MMP-3                       | 0.043 (−0.033 to 0.121)   | 0.055 (−0.028 to 0.131)  |
| MMP-9                       | −0.002 (−0.046 to 0.042)  | 0.023 (−0.050 to 0.105)  |
| bFGF                        | 0.005 (−0.025 to 0.038)   | −0.012 (−0.068 to 0.041) |
| PLGF                        | 0.004 (−0.046 to 0.051)   | −0.004 (−0.102 to 0.098) |
| sFlt-1                      | 0.033 (−0.041 to 0.104)   | 0.003 (−0.107 to 0.104)  |
| VEGF                        | 0.029 (−0.024 to 0.089)   | −0.002 (−0.056 to 0.063) |
| Osteocalcin                 | 0.025 (−0.032 to 0.082)   | 0.005 (−0.036 to 0.049)  |
| Osteonectin                 | 0.010 (−0.047 to 0.075)   | 0.004 (−0.090 to 0.104)  |
| Osteopontin                 | 0.052 (−0.024 to 0.130)   | 0.005 (−0.073 to 0.083)  |
| H-FABP                      | 0.022 (−0.056 to 0.089)   | −0.003 (−0.052 to 0.046) |
| E-FABP                      | 0.007 (−0.049 to 0.063)   | 0.013 (−0.050 to 0.073)  |
| CRP                         | 0.016 (−0.052 to 0.078)   | 0.048 (−0.027 to 0.141)  |
| SAA                         | 0.028 (−0.036 to 0.091)   | −0.003 (−0.085 to 0.085) |
| sICAM-1                     | 0.026 (−0.054 to 0.110)   | 0.004 (−0.043 to 0.054)  |
| sVCAM-1                     | 0.035 (−0.043 to 0.113)   | −0.002 (−0.065 to 0.066) |
| E-selectin                  | 0.020 (−0.043 to 0.088)   | −0.007 (−0.028 to 0.011) |
| P-selectin                  | −0.005 (−0.060 to 0.052)  | 0.024 (−0.097 to 0.152)  |
| sICAM-3                     | 0.006 (−0.052 to 0.065)   | −0.009 (−0.043 to 0.029) |
| Thrombomodulin              | 0.062 (−0.014 to 0.138)   | 0.026 (−0.011 to 0.061)  |
| TIMP-1                      | 0.000 (−0.073 to 0.074)   | 0.085 (−0.015 to 0.184)  |
| NT-proBNP+MMP-3+osteopontin | 0.118 (0.032–0.209)       | 0.067 (−0.043 to 0.169)  |

bFGF indicates basic fibroblast growth factor; CRP, C-reactive protein; E-FABP, epidermal-type fatty acid binding protein; H-FABP, heart-type fatty acid binding protein; MMP, matrix metalloproteinase; NRI, net reclassification index; NT-proBNP indicates N-terminal prohormone of B-type natriuretic peptide; PIGF, placental growth factor; SAA, serum amyloid A; sFLT, soluble FMS-like tyrosine kinase; sICAM, soluble intercellular adhesion molecule; sVCAM, soluble vascular cell adhesion molecule; VEGF, vascular endothelial growth factor; TIMP, tissue inhibitor of matrix metalloproteinase. \*Four 10-year risk categories were constructed: 0% to 10%, 10% to 20%, 20% to 30%, and >30%.

cardiomyocytes in response to increased ventricular stretch and wall tension. A recent meta-analysis summarized the incremental predictive value of measuring NT-proBNP in other populations.<sup>9</sup> The overall adjusted risk ratio for the top third compared with the bottom third of NT-proBNP found in that study was 1.94 (95% CI 1.57–2.39) and is of comparable magnitude to our results. Increments in c-statistics after adding NT-proBNP to conventional risk scores ranged from 0.01 to 0.10 in a variety of populations including patients with elevated CVE risk factors and stable cardiovascular disease at baseline, also in line with the present findings.<sup>9,24</sup> Further, the

**Table 5.** Reclassification Table After the Addition of NT-proBNP, Osteopontin, and MMP-3 to the Base Model in the Second Manifestations of ARterial Disease (SMART) Study

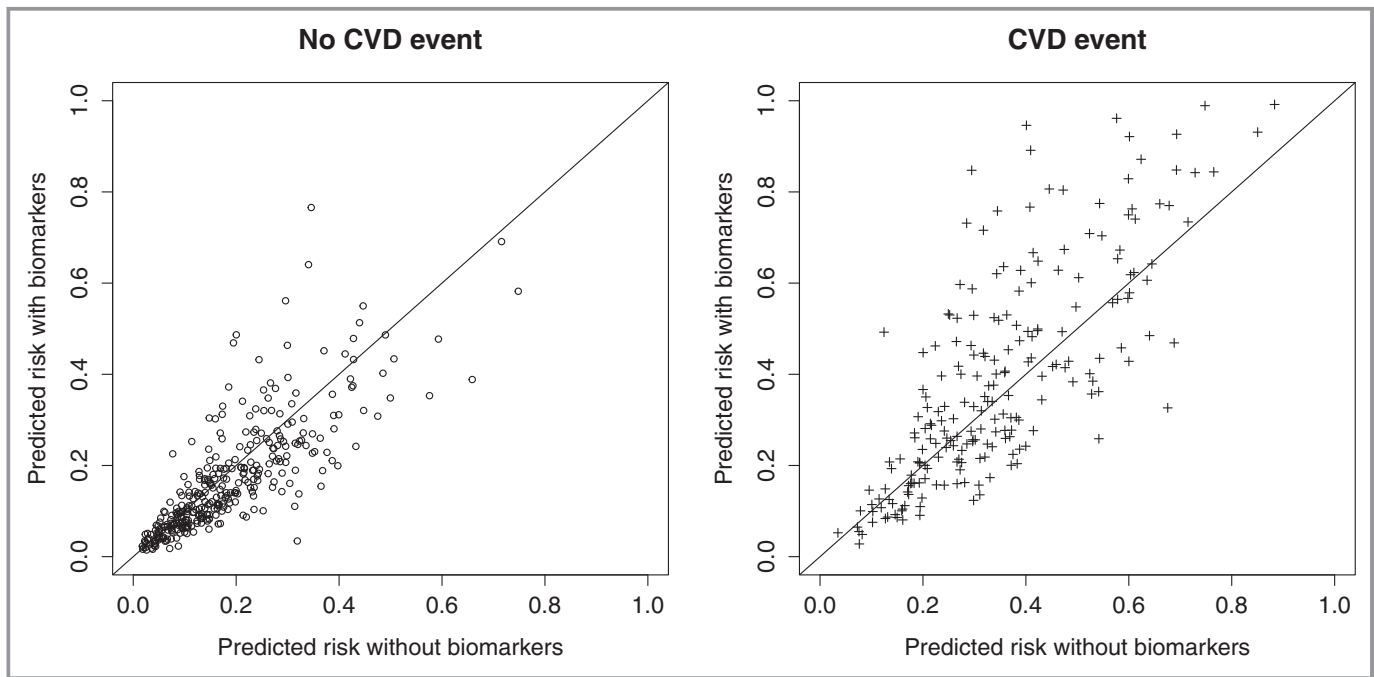
| Base Model            | Base Model+Biomarkers |       |        |      |     | Total | Reclassified Upward* |    | Reclassified Downward* |  |
|-----------------------|-----------------------|-------|--------|------|-----|-------|----------------------|----|------------------------|--|
|                       | <10%                  | 10–0% | 20–30% | >30% |     |       |                      |    |                        |  |
| <b>&lt;10%</b>        |                       |       |        |      |     |       |                      |    |                        |  |
| Persons with event    | 6                     | 2     | 0      | 0    | 8   | 2     | 25.0%                | —  | —                      |  |
| Persons without event | 147                   | 23    | 1      | 0    | 171 | 24    | 14.0%                | —  | —                      |  |
| <b>10–20%</b>         |                       |       |        |      |     |       |                      |    |                        |  |
| Persons with event    | 8                     | 22    | 11     | 3    | 44  | 14    | 31.8%                | 8  | 18.2%                  |  |
| Persons without event | 66                    | 135   | 27     | 8    | 236 | 35    | 14.8%                | 66 | 28.0%                  |  |
| <b>20–30%</b>         |                       |       |        |      |     |       |                      |    |                        |  |
| Persons with event    | 0                     | 10    | 27     | 25   | 62  | 25    | 40.3%                | 10 | 16.1%                  |  |
| Persons without event | 3                     | 65    | 75     | 31   | 174 | 31    | 17.8%                | 68 | 39.1%                  |  |
| <b>&gt;30%</b>        |                       |       |        |      |     |       |                      |    |                        |  |
| Persons with event    | 0                     | 4     | 25     | 124  | 153 | —     | —                    | 29 | 19.0%                  |  |
| Persons without event | 1                     | 11    | 51     | 92   | 155 | —     | —                    | 63 | 40.6%                  |  |

\*Numbers are based on KM-estimators, not actual event numbers and are averaged over 10 imputations sets.

addition of NT-proBNP resulted in an NRI of 0.198 in a large general population study, whereas the NRI increased to 0.386 in a sample of diabetic patients from the Action in Diabetes and Vascular Disease: Preterax and Diamicon MR Controlled Evaluation (ADVANCE) trial.<sup>25,26</sup> The present study extends previous evidence in support of NT-proBNP by demonstrating

the incremental predictive value in a sample of diabetic patients from regular clinical practice not restricted by the selection criteria of a clinical trial.

Other biomarkers also showed significant associations with CVE risk, although the increase in predictive performance varied. Only MMP-3 and osteopontin showed substantial



**Figure 2.** Predicted risks with base model vs predicted risks with four biomarkers (NT-proBNP, osteopontin, MMP-3) added to the base model for patients without CVE events and for patients with CVE events who had available 10-year follow-up (n=551) from SMART. CVD indicates cardiovascular disease; CVE, cardiovascular event; MMP, matrix metalloproteinase; NT-proBNP, N-terminal prohormone of B-type natriuretic peptide; SMART; Second Manifestations of ARterial disease.



improvements ( $\geq 0.20$ ) as measured by the continuous NRI but had modest effects on reclassification across risk categories as measured by the categorical NRI. MMP-3 is an enzyme with the ability to degrade the connective tissue matrix on which atherosclerotic plaque stability depends.<sup>27</sup> MMP-3 polymorphisms and plasma MMP-3 levels were previously shown to be associated with CVEs.<sup>28,29</sup> Osteopontin is a calcium-binding glycoprotein that has been implicated to play a role in cellular immunity and progression of atherosclerosis and was independently related to CVE risk in patients with stable coronary artery disease.<sup>30,31</sup> Further, a recent study in patients with type 1 diabetes demonstrated that osteopontin was associated with CVE risk and improved risk classification on top of Framingham risk factors.<sup>32</sup> The present study is in line with findings from 2 recent reports showing similar directional associations for NT-proBNP, whereas the predictive usefulness of MMP-3 and osteopontin were equivocal, partly due to a shorter follow-up duration and different patient population (eg, also including patients with impaired fasting glucose).<sup>24,33</sup>

The acute-phase inflammatory markers C-reactive protein and serum amyloid A were significantly associated with CVE risk but lacked the ability to improve risk prediction. These findings are in accordance with previous studies, suggesting little additional predictive benefit when traditional risk factors are taken into account.<sup>34</sup> Notably, the only biomarker showing an opposite association with CVE risk in SMART compared with EPIC-NL was adiponectin. Although a protective role of adiponectin was hypothesized based on preclinical data, clinical studies failed to show a relation between adiponectin and CVE risk in patients without preexisting CVE and even showed a positive association in patients with prevalent CVE.<sup>35,36</sup> In SMART, higher levels of adiponectin were associated with increased risk, in patients both with and without symptomatic CVE (data not shown). However, SMART patients without symptomatic CVE had a different risk profile and received more intensive cardiovascular risk management compared with patients from EPIC-NL recruited from the general population, which might explain the observed opposite association.

The combination of NT-proBNP, MMP-3, and osteopontin in a multimarker model resulted in the largest improvement in c-statistic, continuous NRI and categorical NRI, illustrating that these biomarkers convey independent and complementary information related to different biological pathways. Addition of individual biomarkers to the traditional risk model produced higher (ie, more accurate) risk estimates for patients who developed CVEs and lower risk estimates for patients who did not, as measured by the continuous NRI. However, the magnitude of changes in predicted risks was modest and did not result in a significant shift of patients to other predefined risk categories as measured by the categorical NRI. Hence,

while these biomarkers can yield more accurate risk predictions, it is equivocal whether these improved estimates would affect treatment decisions and ultimately clinical outcome.

Strengths of this study included the wide variety in biomarkers from several pathophysiological pathways evaluated, the substantial number of events, and the long follow-up period. Further, this study was conducted in 2 separate populations representing a general population and a hospital-based setting. Moreover, both patients with and without prevalent CVE were included, making the results applicable to the wide range of diabetic patients typically seen in clinical practice. Limitations of our study also merit consideration. The choice of cut-offs to determine incremental predictive performance was difficult, such as the choice of risk thresholds to calculate the categorical NRI given the sample composition and differences in risk threshold used in various guidelines.<sup>22,37,38</sup> Therefore, we primarily used the continuous NRI, which is a marker rather than model descriptive but is less informative on potential clinical consequences.<sup>19</sup> In addition, participants from the EPIC-NL cohort were recruited in the 1990s, and cardiovascular risk management has changed during follow-up, possibly attenuating the association of baseline determinants with CVEs. However, the comparable associations of nearly all biomarkers with CVE risk in both cohorts supported the notion that background treatment rate does not affect the predictive usefulness of these markers. Further, data were partly missing for some predictor variables, although the biomarkers were successfully assayed in the majority of the study populations. We used multiple imputation to reduce bias and increase statistical rigor, and this approach has been shown to adequately handle much larger proportions of missing data.<sup>39</sup>

In conclusion, the present study demonstrated that NT-proBNP, osteopontin, and MMP-3, representing different pathophysiological pathways, were consistently able to improve the prediction of CVE risk beyond traditional risk factors in patients with type 2 diabetes. However, the number of patients reclassified to a different risk stratum was limited.

## Author Contributions

Dr van der Leeuw designed and carried out the data analyses, interpreted the results, and drafted the manuscript. Dr van Dieren contributed to the data analyses and interpreted the results. Drs Beulens, Peelen, and van der Schouw designed the data analyses, interpreted the results, and revised the manuscript for important intellectual content. Drs Schalkwijk, Glatz, Hofker, Verschuren, Boer, van der Graaf, and Visseren revised the manuscript for important intellectual content. Dr van der Schouw is the guarantor of this work.

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## Disclosures

None.

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SUPPLEMENTAL MATERIAL

**Novel biomarkers to improve the prediction of cardiovascular event risk in type 2 diabetes. J. van der Leeuw, et al.**

**Table S1.** Detailed statistics of base models

| Variable                                | SMART $S_0(10) = 0.7964$ |             |         | EPIC-NL $S_0(10) = 0.6906$ |             |         |
|---|--------------------------|-------------|---------|----------------------------|-------------|---------|
|   | HR                       | 95% CI      | p-value | HR                         | 95% CI      | p-value |
| Female sex                              | 0.61                     | 0.45 - 0.83 | 0.002   | 0.76                       | 0.47 - 1.24 | 0.277   |
| Age at diabetes diagnosis (years)       | 1.05                     | 1.04 - 1.07 | <0.001  | 1.39                       | 0.84 - 2.29 | 0.202   |
| Duration of diabetes (years)            | 1.06                     | 1.04 - 1.09 | <0.001  | 1.42                       | 0.96 - 2.10 | 0.082   |
| HbA <sub>1c</sub> (%)                   | 0.78                     | 0.39 - 1.56 | 0.483   | 1.03                       | 0.80 - 1.32 | 0.835   |
| HbA <sub>1c</sub> (squared, %)          | 1.02                     | 0.97 - 1.06 | 0.478   | -                          |             |         |
| Systolic blood pressure (mmHg)          | 0.95                     | 0.90 - 1.00 | 0.034   | 1.32                       | 1.05 - 1.65 | 0.016   |
| Systolic blood pressure (squared, mmHg) | 1.00                     | 1.00 - 1.00 | 0.030   | -                          |             |         |
| TC/HDL ratio (log, mmol/l)              | 1.47                     | 1.03 - 2.13 | 0.036   | 1.45                       | 1.15 - 1.84 | 0.002   |
| UACR (log, µg/mg)*                      | 1.20                     | 1.10 - 1.32 | <0.001  | -                          |             |         |
| eGFR (ml/min)                           | -                        |             |         | 0.97                       | 0.76 - 1.24 | 0.796   |
| Current smoking                         | 1.39                     | 1.04 - 1.84 | 0.025   | 1.72                       | 1.16 - 2.56 | 0.007   |
| History of major macrovascular disease  | 1.65                     | 1.20 - 2.28 | 0.002   | 3.68                       | 2.47 - 5.48 | <0.001  |

SMART: Second Manifestations of ARterial disease study, EPIC-NL: European Prospective Investigation into Cancer and Nutrition-NL subcohort,  $S_0(10)$ = 10-year baseline survival, TC total cholesterol, HDL: high density lipoprotein, UACR: urinary albumin/creatinin ratio, eGFR: estimated glomerular filtration rate.

10-year CVD risk (%) =  $(1 - S_0(10)^{\exp(A - \text{mean LP})}) \times 100\%$ . A is the sum, over all variables in the model, of the patient's specific value times the corresponding coefficient.

**Figure S1.** Calibration plots of base models in Second Manifestations of ARterial disease (SMART) study and European Prospective Investigation into Cancer and Nutrition-NL (EPIC-NL) subcohort.

