Use of vascular assessments and novel biomarkers to predict cardiovascular events in type 2 diabetes – the SUMMIT VIP study

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Abstract

Aims: Cardiovascular (CV) disease risk prediction represents an increasing clinical challenge in the treatment of diabetes. We used a panel of vascular imaging, functional assessments and biomarkers reflecting different disease mechanisms to identify clinically useful markers of risk for CV events in subjects with type 2 diabetes mellitus (T2D) with or without manifest cardiovascular disease (CVD).

Research design and methods: The study cohort consisted of 936 subjects with T2D recruited at four European centers. Carotid intima-media thickness and plaque area, ankle-brachial pressure index, arterial stiffness, endothelial function and circulating biomarkers were analyzed at baseline and CV events monitored during a 3-year follow-up period.

Results: The CV event rate in subjects with T2D was higher in those with (n=440) than in those without (n=496) manifest CVD at baseline (5.53 versus 2.15/100 life years, $p<0.0001$). New CV events in T2D subjects with manifest CVD were associated with higher baseline levels of inflammatory biomarkers (interleukin-6, chemokine ligand 3, pentraxin 3 and hsCRP) and endothelial mitogens (hepatocyte growth factor and vascular endothelial growth factor A), while CV events in T2D subjects without manifest CVD were associated with more severe baseline atherosclerosis (median carotid plaque area 34.5 (16.1-92.2) versus 19.5 (9.5-40.5) mm^2, $p=0.01$). Conventional risk factors, as well as measurements of arterial stiffness and endothelial reactivity, were not associated with CV events.

Conclusions: Our observations demonstrate that markers of inflammation and endothelial stress reflects CV risk in T2D subjects with manifest CVD, while the risk for CV events in T2D subjects without manifest CVD is primarily related to the severity of atherosclerosis.

Diabetes is an important risk factor for cardiovascular disease (CVD) and is associated with a two-fold excess risk of acute myocardial infarction (AMI) and stroke (1). A recent large Swedish registry study showed that although the incidence of CV events has declined substantially in subjects with diabetes between 1998 and 2014, it still remains significantly higher than in subjects without diabetes (2). With the worldwide adult prevalence of diabetes rising from 4.7% in 1980 to 8.5% in 2014 the CV complications of diabetes represents a major public health challenge (3). The increased CV risk associated with diabetes remains essentially the same when adjusting for conventional risk factors (1). Accordingly, traditional risk score calculators are less useful in diabetes (4; 5). This has not been a major clinical concern because most guidelines have considered all subjects with diabetes as having high risk based on studies demonstrating that the CV risk is equivalent to non-diabetic subjects with a previous coronary event (6). However, studies that are more recent have shown that the CV risk in type 2 diabetes (T2D) is highly heterogeneous and that many subjects with T2D have much lower risk of CVD than subjects with established CVD and no diabetes (7-10). Hence, there is an urgent need to improve CVD risk prediction in T2D.

The Innovative Medicine Initiative project SUMMIT (SUrrogate markers for Micro- and Macro-vascular hard endpoints for Innovative diabetes Tools) was initiated to identify novel markers for prediction of CV complications in diabetes. Given the poor risk prediction in diabetics based on traditional CV risk factors alone and the still elusive causes behind the increased CV risk in diabetics, we wanted to assess the ability of a panel of non-invasive vascular imaging, functional vascular tests and emerging biomarkers to predict CV risk in subjects with T2D. To meet this end, we carried out the SUMMIT Vascular Imaging Prediction (SUMMIT VIP) study. As there is a growing population of T2D patients with clinically manifest CVD that are at a very high risk for new events (11) we included both subjects with and without prevalent CVD.

Research design and methods

Study population

The baseline study cohort consisted of 458 subjects with T2D and clinically manifest CVD (T2D/CVD) and 527 subjects with T2D but without clinical signs of CVD recruited from existing population cohorts and hospital registers at the university hospitals in Malmö (Sweden), Pisa (Italy), Dundee and Exeter (UK) between November 2010 and June 2013. Diabetes was defined based on contemporary or historical evidence of hyperglycemia (according to WHO 1998 criteria; fasting plasma glucose >7.0 mmol/l or 2-h plasma glucose >11.1mmol/l, or both) or by current medication with insulin, sulphonylureas, metformin or other anti-diabetic drugs. Subjects diagnosed with T2D < 35 years of age or treated with insulin within12 months of diagnosis were not included in the study. Classification of CVD included non-fatal acute MI, hospitalized unstable angina, resuscitated cardiac arrest, any coronary revascularization procedure, non-fatal stroke, transient ischemic attack confirmed by a specialist, lower extremity artery disease defined as ankle brachial pressure index (ABPI) <0.9 with intermittent claudication or prior corrective surgery, angioplasty or above ankle amputation. T2D with and without CVD were matched at each center for gender, age (± 5 years) and duration of diabetes (± 5 years). Exclusion criteria included renal replacement therapy, malignancy requiring active treatment, end-stage renal disease, any chronic inflammatory disease on therapy, previous bilateral carotid artery invasive interventions or atrial fibrillation. T2D subjects with CVD were excluded if the CVD event occurred more than 5 years prior to the diagnosis of T2D. Demographics, clinical characteristics including medication, physical and laboratory examinations were obtained according to a pre-defined study protocol at all 4 participating centers. Study subjects were invited to a follow-up visit after 36 months and information of incident CVD events (same criteria as used for inclusion) recorded. A total of 760 study subjects (81.2%) attended the follow up visit. For those that did

not attend the follow-up visit information regarding clinical events were obtained thorough medical records or telephone interviews. Forty-nine subjects (5.0%) were lost to follow-up. The study was approved by the local ethical review boards and carried out in accordance with the principles of the Declaration of Helsinki. All study subjects provided written informed consent.

Vascular assessments

Intima-media thickness (IMT) in the right and left common carotid artery (CCA) and the carotid bulbs, as well as total carotid plaque area, were determined by ultrasound. Plaques were defined as focal thickenings (≥ 0.8 mm) of the artery wall. The length and height of each individual plaque were measured to calculate plaque area. The inter-observer variability of plaque area measurements was 8.9±4.6%. The total plaque area represents the sum of the area all plaques identified in the left and right carotid arteries. In average, we identified 2.4 plaques per study subject. The median height of the plaques was 1.9mm (IQR 1.5-2.5) and the median length 11.2mm (IQR 8.0-15.9). Segments with plaques were included in the IMT measurements. Endothelial function was measured using an EndoPat device (Itamar Medical, Caesarea Ind. Park, Israel) to estimate the endothelium-dependent post-ischemic hyperemia in response to 5-minutes of arterial occlusion. Arterial stiffness was assessed by calculating carotid-femoral pulse wave velocity (PWV) using a Sphygmocor device (Atcor Medical, Australia). Left and Right Ankle Brachial Pressure Index were calculated. The ankle brachial pressure index was calculated as the ratio between the highest systolic blood pressure value from the foot and the highest blood pressure from the arm on the same side of the body. Detailed information about the methods used for vascular assessments, as well as data

regarding intra- and inter-observer variability and calibration between centres, have been published previously (12).

Biomarker analysis

Plasma levels of biomarkers reflecting inflammation (interleukin (IL)-6, chemokine ligand (CCL) 3, pentraxin 3), endothelial growth activation (hepatocyte growth factor, placental growth factor, vascular endothelial growth factor (VEGF) A), extracellular matrix proteolysis (matrix metalloproteinase (MMP)-3, -7 and -12), apoptosis (Fas, TNF receptor 1, TRAIL receptor 2), as well as other emerging CV risk markers (N-terminal prohormone of brain natriuretic peptide (NT-proBNP), Growth Differentiation Factor (GDF)-15 and fatty acid binding protein (FABP)-4) were analyzed by the Proximity Extension Assay (PEA) technique using the Proseek Multiplex CVD^{96x96} reagents kit (Olink Bioscience, Uppsala, Sweden) at the Clinical Biomarkers Facility, Science for Life Laboratory, Uppsala as previously described.(13) All samples were analyzed in the same run. Data analysis was performed by a preprocessing normalization procedure using Olink Wizard for GenEx (Multid Analyses, Sweden). Values are presented as arbitrary units (AU). Data regarding intra- and inter-assays variations as well as general calibrator curves to calculate the approximate concentrations are available on the OLINK homepage (http://www.olink.com).

Statistics

Values are presented as mean and standard deviation for continuous variables with normal distribution and as median and interquartile rage (IQR) for skewed variables. Biomarker values were log transformed when used in statistical analyses. Differences in clinical

characteristics between the groups with or without new CV events were investigated using Chi-square, Student's t-test or Mann–Whitney U tests, as appropriate. Logistic regression was used to test for associations between baseline clinical characteristics and incident CV events (fatal or non-fatal) in subjects with T2D and prevalent CVD at baseline. The additional value of biomarkers to a reference model to predict CV events during follow up was assessed by the integrated discrimination improvement (IDI) and by comparing areas under the receiver operating characteristic (AUROC) curves. Analyses were done using SPSS statistics version 22 and in R version 3.3.0 (using the PredictABEL-package to calculate IDI and the pROC-package to compare AUROCs). All statistical analyses were done in accordance with the original protocol of the study.

Results

The baseline investigation included 458 subjects with T2D and CVD (myocardial infarction, stroke or lower extremity arterial disease) and 527 subjects with T2D but without clinically manifest CVD. The clinical characteristics of the study cohort have been previously published (12). Fatal and non-fatal cardiovascular (CV) events were registered during a 3-year follow-up period. Forty-nine subjects (5.0%) were excluded from the study due to lack of information of clinical events during follow-up. Of the remaining 936 subjects 105 suffered a cardiovascular event during follow-up (3.6 CV events/100 life years). A breakdown of the components of the composite incident CV events in the two groups is shown in the supplemental table. There were also 12 deaths from non-cardiovascular causes and 8 death of unknown cause. Subjects with T2D and manifest CVD at baseline had a more than two-fold higher CV event rate than those free of CVD at baseline (5.5 versus 2.2/100 life years, $p<0.0001$).

Markers for CV events at follow up in subjects with T2D and manifest CVD

There were no difference in major CV risk factors between subjects with or without CV event during follow-up in the two study groups (table 1). Occurrence of a new CV event in the T2D/CVD group was associated with higher baseline HbA1c (table 2). Table 2 also shows CV and antidiabetic medications at the baseline and follow-up visits. Insulin treatment was more common among those with a new event. However, when including both HbA1c and insulin treatment in a binary logistic regression model together with age, sex, duration of diabetes, smoking, BMI, triglycerides, LDL, HDL and eGFR only HbA1c remained significantly associated with a new CV event (hazard ratio 1.03 (95%CI 1.01-1.03). There was no major change in the type of antidiabetic treatment during the study period. Subjects with a CV event during follow-up were more often on statin therapy at the follow-up visit (table 2).

With the exception of an increased IMT in the left carotid bulb there were no significant differences in carotid IMT, total carotid plaque area, pulse wave velocity, endothelial reactivity or ABPI between those with and without a new CV event (table 3). However, baseline plasma levels of endothelial mitogens and biomarkers reflecting inflammation, such as IL-6, CCL3, pentraxin 3 and hsCRP as well as matrix metalloproteinase (MMP)-12, N-terminal prohormone of brain natriuretic peptide (NT-proBNP) and fatty acid binding protein (FABP)-4, were higher in subjects with a new event (table 3). In subjects with T2D and manifest CVD the discrimination slope of a binary logistic regression model with IL-6 and risk factors (age, sex, duration of diabetes, current smokers, total cholesterol, HDL, HbA1c, systolic blood pressure and ethnicity) was significantly improved by 2.7 percentage points compared with a model without IL-6 (IDI 0.027 [95%CI 0.0064-0.048], p=0.010). Similarly,

the discrimination slope of a binary logistic regression model with hsCRP and risk factors was significantly improved by 1.6 percentage points compared with a model without hsCRP (IDI 0.016 [95%CI 0.0025-0.031], p=0.021). The area under the receiver operating characteristic (AUROC) curve was significantly increased with the addition of IL-6 (p=0.02) or hsCRP (p=0.02) to the risk factor model (AUROC of IL-6 and risk factor model 0.68 [95%CI 0.60-0.75], AUROC of hsCRP and risk factor model 0.68 [95%CI 0.61-0.75], AUROC of risk factor model 0.60 [95%CI 0.51-0.69, p=0.02). Addition of hsCRP to the IL-6 model did not significantly increase the AUROC further. Risk reclassification with the addition of IL-6 or hsCRP to the model was mainly downwards (figure 1 A-D).

Markers for CV events at follow up in T2D subjects without manifest CVD

There were no significant differences in conventional CV risk factors or medication at baseline between those with and without a CV event during follow-up in the T2D/non-CVD group (tables 1 and 2). Subjects with a CV event during follow-up were more often on antiplatelet therapy at the follow-up visit (table 2).

Those with a CV event had increased IMT in both the left and right bulb, the right common carotid artery (CCA), as well as an increased total carotid plaque area (table 3). Pulse wave velocity, endothelial reactivity and ABPI were not associated with the occurrence of CV events. Subjects with CV events also had higher baseline plasma levels of the apoptosis marker TRAIL receptor 2 and of Growth and Differentiation Factor (GDF)-15, but did not demonstrate the same elevation in endothelial mitogens and inflammatory biomarkers as T2D subjects with manifest CVD that suffered a new event (table 4). In the T2D/non-CVD group the discrimination slope of a binary logistic regression model with right CCA IMT and risk factors (age, sex, duration of diabetes, current smokers, total cholesterol, HDL, HbA1c,

systolic blood pressure and ethnicity) was significantly improved by 2.4 percentage points compared to a model without IMT (IDI right CCA IMT 0.024 [95%CI 0.0035-0.045]; p=0.022). (figure 1 E-F). There was no significant difference in AUROC with the addition of right CCA IMT to the risk factor model (p=0.10).

Discussion

Using a panel of conventional risk factors, vascular assessments and emerging biomarkers, we demonstrate in the present study that different markers predict risk for CV events in T2D patients with and without manifest CVD. T2D subjects with manifest CVD that developed a new event had higher baseline plasma levels of hsCRP, pro-inflammatory cytokines, endothelial mitogens, MMP-12, FABP-4 and the cardiac stress marker NT-proBNP, but were not characterized by more severe atherosclerosis as assessed by carotid IMT (except from a marginally thicker IMT in left carotid bulb) or ABPI. The biological process that results in elevated levels of endothelial mitogens remains to be fully characterized, but is likely to involve endothelial stress. Except for a higher HbA1c there were no differences in conventional risk factors between those with and without a new CV event. NT-proBNP is an established marker of CV risk. Notably, NT-proBNP only predicted CV events in subjects with established CVD in the present study. Other studies have identified elevated NT-proBNP as a CV risk factor in subjects with T2D (14), but to our knowledge it has previously not been shown that this primarily is the case for T2D subjects with prevalent CVD. Increased arterial stiffness and endothelial dysfunction as assessed by reduced vasodilatation following transient ischemia are well-established vascular complications in diabetes and have been associated with increased CV risk (15-18). In accordance, subjects with T2D were found to have increased pulse wave velocity and a lower reactive hyperaemia index at the SUMMIT VIP

baseline investigation (12). In spite of this, neither of these measures predicted the occurrence of a new event in subjects with established CVD in the present study.

Development of a CV event in T2D subjects without manifest CVD at baseline was associated with increased carotid atherosclerosis as assessed by the CCA and carotid bulb IMT, as well as by increased total carotid plaque area at the baseline investigation. However, biomarkers were less good predictors with only GDF-15 and the apoptosis marker TRAIL receptor 2 being higher in those with a CV event. Moreover, there were no differences in conventional risk factors between those with and without a CV event.

Our observations are in accordance with previous observations that conventional risk factors are poor predictors of CV events in subjects with T2D, however they suggest some important alternatives. We found that biomarkers reflecting inflammation, as well as endothelial and cardiac stress, are predictors of CV events in subjects with diabetes and manifest CVD, while carotid IMT is a better predictor of risk in diabetic subjects without manifest CVD. Increased carotid IMT is a well-established CV risk factor in the general population (19). In accordance, T2D subjects with manifest CVD at the baseline investigation had significantly greater carotid IMT than those without manifest CVD (12). Hence, there seems to be a clear association between atherosclerosis severity and CV risk in subjects with T2D, but this association diminishes in subjects with manifest CVD. One possible explanation to this could be that a more intense medical intervention in subjects with manifest CVD allows other risk factor mechanisms than those traditionally associated with atherosclerosis progression to become more important as cause of CV events (20). Hence, biomarkers that associate with CV events in this group could provide information regarding such alternative mechanisms. In the present

studies we found that subjects with new events had higher baseline levels of pro-inflammatory biomarkers and endothelial mitogens suggesting the presence of an inflammatory state involving endothelial stress that persist in the presence of statin treatment. In this context it is interesting to note that the recently published Canakinumab Anti-inflammatory Thrombosis Outcome Study (CANTOS) trial showed that IL-1β antibody treatment lowered the rate of recurrent events in patients with history of myocardial infarction and elevated hsCRP in spite of statin treatment (21). The mechanisms that maintain vascular inflammation in statin-treated patients remains to fully characterized but may involve factors such as altered shear stress over stenotic plaques, intra-plaque accumulation of cholesterol crystals, autoimmune responses against modified plaque antigens and chronic infections (22). It is also possible that the difference in factors predicting CV events in T2D subjects with and without clinically manifest CVD is due to a more advanced stage of vascular disease in the former group.

Our study has both strengths and limitations. The strengths include the comprehensive vascular assessments in combination with a number of established and emerging biomarkers reflecting possible mechanisms responsible for development of cardiovascular complications in subjects with T2M. The study is also unique in that it compares risk assessments in subjects with or without established CVD. The lack of assessments of the coronary arteries and the relatively limited number of cardiovascular events during follow-up, particularly in the group without CVD at baseline, represents important limitations. As we used treatment with antidiabetic medication to define presence of T2D we cannot exclude that some pre-diabetic subjects were included in the study. However, it is unlikely that this should have any major influence on the results of the study. Finally, we used a lower threshold for defining presence of carotid plaques (focal IMT thickenings of $\geq$ 0.8 mm) than used in many other studies.

In conclusion, our observations demonstrate that markers of inflammation and endothelial stress are elevated in T2D subjects with manifest CVD that develop a new event suggesting

that these patients may benefit from novel anti-inflammatory CV therapy. The risk for CV events in T2D subjects without manifest CVD is primarily related to the severity of atherosclerosis.

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Author contributions

ACS, HC, AN, CP, IG and JN designed and supervised the study. GÖ, MP, CK, KA, FC, KG, PEG, SP, EV, MK, FK, HL, FD and JB collected clinical data and performed investigations on patients. JK, HB and JN managed data and performed statistical analyses. ACS and JN drafted the manuscript and all other authors made critical revisions.

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Figure 1: Scatter plots of predicted probabilities of risk factor models with and without biomarkers. Predicted probabilities for models with IL-6 in addition to risk factors (age, sex, duration of diabetes, current smokers, total cholesterol, HDL, HbA1c, systolic blood pressure and ethnicity) in T2D subjects with manifest CVD and with CV event (**A**) or without CV event (**B**) during follow up. Predicted probabilities for models with hsCRP in addition to risk factors in T2D subject with manifest CVD and with CV event (**C**) or without CV event (**D**) during follow up. Predicted probabilities for models with right CCA IMT in addition to risk factors in T2D subject without manifest CVD and with CV event (**E**) or without CV event (**F**) during follow up. The 45 degrees' line designates equal predicted probabilities of the models.

Table 1. Baseline clinical characteristics for subjects with diabetes with or without a cardiovascular event during follow-up

	CVD at baseline (n=440)			**No CVD at baseline (n=496)**		
	No CV event (n=367)	CV event (n=73)	P	No CV event (n=464)	CV event (n=32)	P
Age (years)	69.4±8.5	69.3±8.7	ns	66.5±8.7	68.2±6.1	ns
Sex (% males)	73.4	65.6	ns	62.5	62.5	ns
Current smokers (%)	9.5	16.4	ns	9.1	15.6	ns
Duration of diabetes (years)	12.1±8.6	13.5±8.8	ns	9.1±7.0	11.5±6.3	ns
BMI (kg/m^{-2})	29.9±4.7	30.7±5.6	ns	30.6±5.4	30.4±4.8	ns
Lipids						
LDL (mmol/L)	2.06±0.77	2.08±0.75	ns	2.41±0.93	2.24±0.76	ns
HDL (mmol/L)	1.20±0.36	1.19±0.33	ns	1.32±0.38	1.30±0.41	ns
Triglycerides (mmol/L)	1.42 (1.02-2.08)	1.45 (1.05-1.84)	ns	1.35 (1.00-1.97)	1.40 (0.90-2.43)	ns
Blood pressure						
Systolic (mmHg)	138±20	140±17	ns	136±18	137±17	ns
Diastolic (mmHg)	76±10	74±9	ns	78±10	77±9	ns
Renal function						
eGFR (mL/min^{-1} per $1.73m^2$)	74.8±26.9	78.0±27.6	ns	85.1±20.7	81.0±20.0	ns

Variables with normal distribution are shown as mean±standard deviation and skewed variables as median and interquartile range. Statistical comparisons between subjects with and without events during follow-up were done using Students´ t-test for variables with normal distribution and with Mann-Whitney U-test for skewed variables. Chi-square test was used for categorical variables.

Table 2. Anti-diabetic medication and HbA1c at the baseline and 3-year follow-up investigation

	CVD at baseline (n=440)			**No CVD at baseline (n=496)**		
Baseline	No CV event (n=367)	CV event (n=73)	P	No CV event (n=464)	CV event (n=32)	P
Statin (%)	88.9	80.6	(0.05)	61.3	75.0	ns
ACE inhibitors (%)	54.1	44.4	ns	38.5	34.4	ns
Betablockers (%)	57.4	56.9	ns	17.8	9.4	ns
Antiplatelet (%)	71.9	75.0	Ns	24.6	31.2	ns
Glitazones (%)	6.3	3.0	ns	7.2	15.6	ns
Metformin (%)	65.0	61.4	ns	71.4	81.3	ns
Insulin (%)	29.3	45.7	0.007	15.8	25.0	ns
Sulfonylurea (%)	29.9	20.0	ns	29.7	21.8	ns
DPP-4 inhibitors (%)	11.3	4.3	ns	11.3	6.3	ns
Incretin analogues (%)	5.5	2.9	ns	5.2	3.1	ns
HbA1c (mmol/mmol)	57.7±12.9	62.8±18.7	0.036	56.1±13.6	59.3±12.9	ns
HbA1c (%)	7.43±1.18	7.90±1.71	0.036	7.28±1.24	7.56±1.18	ns
Follow up	No CV event (n=276)	CV event (n=51)	P	No CV event (n=397)	CV event (n=24)	P
Statin (%)	79.7	94.6	0.03	63.1	75.0	ns
ACE inhibitors (%)	47.0	43.2	ns	36.3	55.0	ns
Betablockers (%)	59.9	60.5	ns	19.6	15.0	ns
Antiplatelet (%)	79.1	72.7	ns	26.5	55.0	0.008
Glitazones (%)	4.7	0	ns	5.5	5.0	ns
Metformin (%)	63.5	65.8	ns	68.9	85.0	ns
Insulin (%)	30.0	44.7	ns	21.6	35.0	ns
Sulfonylurea (%)	27.3	21.6	ns	25.4	30.0	ns
DPP-4 inhibitors (%)	13.4	8.1	ns	13.4	15	ns
Incretin analogues (%)	6.5	2.7	ns	5.7	0	ns
HbA1c (mmol/mmol)	46.8±23.8	39.3±23.6	ns	43.1±23.3	50.1±32.2	ns
HbA1c (%)	6.43±2.18	5.75±2.16	ns	6.10±2.13	6.79±2.94	ns

HbA1c values are shown as mean±standard deviation and between-group comparisons are done using Students´ t-test. Chi-square test was used for categorical variables. DPP-4; Dipeptidyl peptidase.

Table 3. Baseline vascular measurements in subjects with diabetes with or without a cardiovascular event during follow-up

	CVD at baseline (n=440)			**No CVD at baseline (n=496)**		
	No CV event (n=367)	CV event (n=73)	P	No CV event (n=464)	CV event (n=32)	P
CCA IMT, right (mm)	0.97±0.25	0.92±0.20	ns	0.89±0.20	1.00±0.23	0.002
Carotid bulb IMT, right (mm)	1.14 (0.96-1.62)	1.38 (1.01-1.84)	ns	1.03 (0.87-1.24)	1.28 (0.85-1.55)	(0.07)
CCA IMT, left (mm)	0.97±0.25	0.87±0.25	ns	0.92±0.24	1.07±0.49	0.001
Carotid bulb IMT, left (mm)	1.13 (0.95-1.47)	1.27 (1.03-1.67)	0.045	1.05 (0.88-1.27)	1.20 (0.95-1.78)	0.04
Total plaque area (mm^2)	30.4 (15.3-61.4)	36.0 (17.6-68.6)	ns	19.5 (9.5-40.5)	30.4 (16.1-92.2)	0.01
Pulse wave velocity (m/s)	11.8±3.2	11.3±2.3	ns	10.9±2.6	11.6±2.5	ns
Reactive hyperemia index	2.10±0.56	2.16±0.55	ns	2.20±0.65	2.04±0.79	ns
ABPI, right	1.11±0.22	1.05±0.28	(0.07)	1.20±0.15	1.20±0.32	ns
ABPI, left	1.11±0.23	1.10±0.28	ns	1.18±0.28	1.18±0.29	ns

CCA; common carotid artery, IMT; intima-media thickness, ABPI; ankle brachial pressure index. Variables with normal distribution are shown as mean±standard deviation and skewed variables as median and interquartile range. Statistical comparisons between subjects with and without events during follow-up were done using Students´ t-test for variables with normal distribution and with Mann-Whitney U-test for skewed variables.

Table 4. Baseline biomarkers in subjects with diabetes with or without a cardiovascular event during follow-up

	CVD at baseline (n=440)			**No CVD at baseline (n=496)**		
	No CV event (n=367)	CV event (n=73)	P	No CV event (n=464)	CV event (n=32)	P
Inflammation						
IL-6	42.8 (29.8-68.1)	58.5 (42.1-93.5)	0.00005	34.1 (23.8-52.7)	39.5 (24.2-58.0)	ns
CCL3 (MIP-1α)	4.8 (3.9-5.9)	5.1 (4.2-6.7)	0.008	4.6 (3.9-5.9)	4.7 (3.9-5.4)	ns
Pentraxin 3	2.1 (1.7-2.6)	2.3 (2.0-2.7)	0.043	2.1 (1.7-2.6)	2.1 (1.8-2.6)	ns
hsCRP (mg/L)	1.46 (0.69-3.30)	2.74 (1.30-4.68)	0.00005	1.48 (0.66-2.95)	2.20 (0.70-4.38)	ns
Endothelial mitogens						
Hepatocyte growth factor	122 (95-148)	134 (107-169)	0.029	110 (88-135)	112 (89-146)	ns
Placental growth factors	189 (153-253)	207 (156-250)	ns	167 (138-204)	184 (143-223)	(0.08)
VEGF A	1520 (1199-1934)	1624 (1246-2131)	0.045	1409 (1136-1783)	1558 (1199-1824)	ns
Matrix proteolysis						
MMP-3	2.6 (2.1-3-5)	2.6 (2.2-3.3)	ns	2.4 (1.9-2.9)	2.2 (2.0-2.6)	ns
MMP-7	517 (333-780)	545 (342-750)	ns	410 (282-580)	539 (347-691)	ns
MMP-12	172 (11-249)	204 (147-289)	0.025	125 (92-180)	130 (102-234)	(0.09)
Apoptosis						
TNF receptor 1	7231 (5743-9153)	7033 (5873-9793)	ns	6295 (5220-7591)	6451 (5433-7899)	ns
TRAIL receptor 2	3.9 (2.7-5.3)	4.2 (2.8-5.4)	ns	3.3 (2.5-4.1)	4.0 (3.1-4.4)	0.039
Fas	231 (186-274)	218 (179-276)	ns	210 (175-247)	212 (169-254)	ns
Other						
NT-proBNP	26.2 (14.3-43.6)	38.6 (20.5-58.9)	0.001	14.3 (9.8-26.0)	16.2 (10.3-22.7)	ns
GDF-15	1458 (1044-2154)	1541 (1143-2073)	ns	1121 (830-1632)	1483 (1180-1898)	0.005
FABP-4	10.7 (7.8-14.9)	13.7 (8.5-19.8)	0.01	9.6 (7.3-12.6)	10.6 (7.6-17.2)	ns

CCL; chemokine ligand 3, MIP1-α; macrophage inflammatory protein 1-α, hsCRP; high sensitive C-reactive protein, VEGF A; vascular endothelial growth factor A, MMP; matrix metalloproteinase, TNF; tumor necrosis factor, TRAIL; tumor necrosis factor-related apoptosis-inducing ligand, NT-proBNP; N-terminal prohormone of brain natriuretic peptide, GDF-15; growth differentiation factor-15, FABP-4; fatty acid binding protein-4. All values except hsCRP are arbitrary units shown as median and interquartile range. Statistical comparisons between subjects with and without events during follow-up were done on log2-transformed values using Students´ t-test.