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Apolipoprotein CIII and N-terminal prohormone b-type natriuretic peptide as independent predictors for cardiovascular disease in type 2 diabetes

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ABSTRACT

Background and aims: Developing sparse panels of biomarkers for cardiovascular disease in type 2 diabetes would enable risk stratification for clinical decision making and selection into clinical trials. We examined the individual and joint performance of five candidate biomarkers for incident cardiovascular disease (CVD) in type 2 diabetes that an earlier discovery study had yielded.

Methods: Apolipoprotein CIII (apoCIII), N-terminal prohormone B-type natriuretic peptide (NT-proBNP), high sensitivity Troponin T (hsTnT), Interleukin-6, and Interleukin-15 were measured in baseline serum samples from the Collaborative Atorvastatin Diabetes trial (CARDS) of atorvastatin *versus* placebo. Among 2105 persons with type 2 diabetes and median age of 62.9 years (range 39.2–77.3), there were 144 incident CVD (acute coronary heart disease or stroke) cases during the maximum 5-year follow up. We used Cox Proportional Hazards models to identify biomarkers associated with incident CVD and the area under the receiver operating characteristic curves (AUROC) to assess overall model prediction.

Results: Three of the biomarkers were singly associated with incident CVD independently of other risk factors; NT-proBNP (Hazard Ratio per standardised unit 2.02, 95% Confidence Interval [CI] 1.63, 2.50), apoCIII (1.34, 95% CI 1.12, 1.60) and hsTnT (1.40, 95% CI 1.16, 1.69). When combined in a single model, only NT-proBNP and apoCIII were independent predictors of CVD, together increasing the AUROC using Framingham risk variables from 0.661 to 0.745.

Conclusions: The biomarkers NT-proBNP and apoCIII substantially increment the prediction of CVD in type 2 diabetes beyond that obtained with the variables used in the Framingham risk score.

1. Introduction

Cardiovascular disease (CVD) remains a major cause of morbidity and mortality among people with type 2 diabetes (T2D). Currently risk equations such as Framingham are the most commonly used method for assessing an individual's CVD risk [1] though other diabetes specific models have also been shown to have benefit [2]. One potential means to further improve prediction

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and identify people at higher risk is to make use of biomarkers in combination with established clinical risk factors. Among biomarkers shown to be most predictive of CVD in T2D are N-Terminal Prohormone B-type Natriuretic Peptide (NT-proBNP) and high-sensitivity Troponin T (hsTnT) [3]. Recent data also support the potential of Apolipoprotein CIII (apoCIII) as a predictive biomarker in the context of higher triglycerides such as T2D, though whether apoCIII predicts CVD independently of triglycerides in diabetes is unclear [4–6].

Most biomarker studies have tended to focus on single biomarker evaluation, while joint evaluation of panels of biomarkers are relatively uncommon [7,8]. In a previous biomarker discovery study across five cohorts with diabetes that evaluated 42 biomarkers, we identified a subset of biomarkers that together improved prediction for incident CVD events in T2D. These included NT-proBNP, hsTnT, Interleukin-6 (IL-6) and Interleukin-15 (IL-15)- all of which were positively associated with CVD. ApoCIII showed a weak inverse association with CVD, at odds with the direction of effect expected from its known biology [7]. In an assessment of a large panel of potential biomarkers in people with dysglycaemia in the ORIGIN trial, NT-proBNP was also the most predictive biomarker with 10 additional biomarkers and, in a later analysis, Troponin I showed additional predictive information [9].

Here we used samples from the Collaborative Atorvastatin Diabetes Study (CARDS) clinical trial of atorvastatin *versus* placebo for the primary prevention of CVD in T2D[10] to quantify the extent to which prediction of CVD can be improved with NT-proBNP, hsTnT, IL-6 and IL-15, and with apoCIII beyond that obtained with the variables used in the Framingham risk score and also a wider set of potential clinical risk factors.

2. Materials and methods

2.1. Populations

As previously detailed, CARDS (ClinicalTrial.gov registration no. NCT00327418) was a clinical trial of the effects of Atorvastatin in T2D and included adults aged 40–75 years with T2D recruited from across the UK and Ireland with no pre-existing CVD, but at least one CVD risk factor (e.g. smoking, hypertension, albuminuria, or retinopathy) [11]. Patients were randomised between November 1997 and June 2001 and followed up 4 and 3.9 years in the active and placebo arms respectively. Of the 2838 people originally included in CARDS we had serum available, from a pre-randomisation visit, for 2105 people. There was no difference in incident CVD rate between those with and without available serum samples. The study was carried out in accordance with the Declaration of Helsinki and the guidelines on Good Clinical Practice, with each centre obtaining Local Research Ethics Committee approval following approval from the Multi-centre Research Ethics Committee. All patients gave fully informed written consent.

2.2. Clinical covariates

Clinical covariates were collected at the time of study enrolment and included standard CVD risk factors including fasting total, low-density (LDL-C) and high-density (HDL-C) cholesterol, triglycerides, blood pressure, smoking status, and use of anti-hypertensive drugs along with diabetes specific risk factors such as diabetes duration, HbA_{1c}, serum creatinine, albumin:creatinine ratio (ACR) and insulin use. Estimated glomerular filtration rate (eGFR) was calculated using the MDRD4 equation [11] and albuminuria status was based on ACR at study enrolment with microalbuminuria defined as an ACR 2.5–25 mg/mmol, and macroalbuminuria as an ACR> 25 mg/mmol [10].

2.3. Endpoint definition

Incident CVD in CARDS participants was as defined in the original trial– acute coronary heart disease (a documented myocardial infarction including silent myocardial infarctions detected by electrocardiography, unstable angina, revascularisation or acute coronary heart disease death) or fatal and non-fatal stroke. We also examined associations of the biomarkers with four secondary endpoints: fatal CVD, non-fatal CVD, acute coronary heart disease, and stroke. These outcomes are in line with the ASSIGN CVD event definitions used in the development of risk scores [12].

2.4. Biomarker measurements

Biomarker measurements were made on thawed serum in several batches (see Supplementary Table 1 for quality control data). Three of the biomarkers (apoCIII, IL-6 and IL-15) were measured at the Immunoassay Biomarker Core Laboratory at the University of Dundee, Scotland. For apoCIII. we used the Assaymax ELISA from Assaypro. For the Interleukins we used the V-PLEX Human Interleukin-6 Kit [13] and the V-PLEX Human Interleukin-15 Kit [14] both from Meso Scale Discovery [15]. NT-proBNP and hsTNT were measured at the Glasgow Biomarker Laboratory, University of Glasgow, Scotland, in a sub-set of samples selected to include all events (n = 113), where sufficient sample volume was available to account for dead volume in automated analysers, and a random selection of samples from among those without an event (n = 1456). NT-proBNP was measured with the Elecsys proBNP II assay [16] and hsTnT [17] with the Elecsys Troponin T high sensitivity assay both from Roche Diagnostics (Burgess Hill, UK). For all assays a small number of blinded duplicates were included to assess the intra-class correlation coefficients (ICCs). In addition to the biomarkers measured, we also include measures of C-reactive protein and apolipoprotein B (apoB) concentrations previously measured in CARDS [18,19] as covariates in some analyses.

2.5. Statistical analysis

The data were cleaned, imputed and Gaussianised prior to analysis. Four missing covariate values were imputed to the mean for continuous variables and to the mode for categorical variables. We did not impute the 87 missing ACR observations, but instead ran a sub-analysis when this covariate was included in the model (n = 2018, 138 incident CVD events). Biomarker values missing at random were imputed to the mean (n = 32) but we confirmed that similar results were obtained with these observations dropped from the analyses. We used Cox Proportional Hazards models to assess the association of each biomarker singly and in combination over and above a limited set of clinical covariates based upon the Framingham risk equation (age, sex, total cholesterol, HDL-C, systolic blood pressure and smoking status) and also a wider set of potential predictive clinical covariates. All analyses also adjusted for treatment assignation in the trial. We report the Hazard Ratio (HR) per unit standard deviation of each biomarker when Gaussianised. We also tested for departure from linearity by examining whether including a quadratic term in the models improved model fit, as assessed by a likelihood ratio test. Since many people had no detectable levels of hsTnT, we tested whether modelling it using a term of undetectable, below, and above, median yielded better prediction than treating it as a continuous variable where samples below the detection level were set to 50% of the detection threshold. For IL-6, since raw data plotted into quartiles suggested a threshold effect above the first quartile, we conducted a *post-hoc* test of significance of being above the first quartile overall with CVD risk. Such *post-hoc* analyses are of course suggestive rather than

definitive.

To assess the overall predictive power of the models we used 10 fold cross-validation to calculate the Somers' D statistic (D) from which we calculated the area under the Receiver Operator Characteristic curves (AUROC) using the formula AUROC = 0.5 + 0.5*D. We carried out a series of sensitivity analyses. In one we re-ran all models after excluding eight individuals with very high baseline NT-proBNP (>400 pg/ml). We also re-ran the apoCIII models substituting LDL-C with serum apoB, a marker for the number of LDL particles in the circulation and a predictor of CVD in CARDS [20]. We also calculated the positive predictive value of the test where the probability of being a CVD case is plotted against the percentile of the risk score derived from a logistic regression model inclusive of biomarkers. We did this to demonstrate how using different cut points of the model score to enrich CVD incidence among potential clinical trial enrollees similar to the CARDS population might alter probability of identifying those at risk for CVD i.e. the "predicted event rate enrichment". All statistical analyses were undertaken using R [21].

3. Results

Serum samples were available for 2105 individuals from CARDS of whom 144 had an incident first CVD event during follow-up. Of the 144 events 51% were due to myocardial infarction, 25% due to stroke, 13% revascularisation procedures, 7% due to unstable angina and 4% due to other acute coronary heart disease death. Demographics and baseline clinical covariates by subsequent CVD event status are shown in Table 1. Individuals with incident CVD events during the study were older, more likely to be male, with a higher BMI, higher total and LDL-C and with macroalbuminuria at study enrolment. Treatment assignment to statin was associated with reduced CVD events as described and treatment assignment was adjusted for in all models. The median time to incident CVD event was 2.1 years (Interquartile range [IQR] 1.0, 3.4 years) and for people who did not have an incident event, median follow-up was 4.1 years (IQR 3.3, 4.7 years).

3.1. Biomarker associations with CVD

For apoCIII, IL-6, IL-15 and NT-proBNP, > 98% of samples had detectable levels but as expected just 43% of samples assayed for hsTnT had detectable levels. On blinded duplicate aliquots ICCs were good for all assays (Supplementary Table 1). Correlations of biomarkers with each other and with clinical covariates are shown in Supplementary Fig. 1. The strongest correlations seen were for apoCIII with triglycerides (r = 0.41) and NT-proBNP with age (0.36). The biomarkers did not show much correlation with each other except for hsTnT and NT-proBNP (r = 0.30). IL-6 was modestly correlated with CRP (r = 0.29). Biomarkers were not associated with HbA_{1c}. NT-proBNP was higher with lower eGFR.

HRs for each biomarker when added to models including either the Framingham covariates or the more extensive set of covariates are shown in Table 2. Modelled as continuous terms, three of the biomarkers were statistically significantly associated with incident CVD; apoCIII, NT-proBNP and hsTnT-while we found no significant association for IL-6 or IL-15. Inclusion of a quadratic term in the model did not significantly improve model fit (data not shown). Associations were robust to both adjusting for Framingham covariates and the more extensive set of covariates including triglycerides. However, when all three biomarkers associated in univariate analysis (apoCIII, NT-proBNP and hsTnT) were included in a model simultaneously, hsTnT was no longer independently associated with CVD (Table 2).

Although there was no significant linear association between IL-6 and CVD, there was some evidence (Table 1) from *post-hoc* analyses that CVD risk was higher in those above the 1st quartile for IL-6 compared to those in the 1st quartile (HR = 1.82; 95% CI 1.13, 2.93; $p = 0.013$ adjusted for extensive covariates). Modelling hsTnT as a categorical variable revealed a very strong association between CVD risk and having values above the median (6.51 pg/ml) compared to having undetectable values (HR 2.36; 95% CI 1.52, 3.67; $p = 0.0001$ adjusted for extensive covariates). However, even when treated as these categorical variables, neither IL-6 nor hsTnT were associated with CVD in a model that included apoCIII and NT-proBNP.

3.2. Prediction performance

In terms of predictive performance for CVD (using AUROCs from a 10 fold cross-validation), the AUROC increased from 0.661 for Framingham covariates alone to 0.729 when including NT-proBNP alone and to 0.676 when including apoCIII alone. The inclusion of both apoCIII and NT-proBNP increased the AUROC from 0.661 (95% CI% 0.615–0.706) to 0.745 (95% CI: 0.701–0.789), a difference that was highly statistically significant ($p = 0.0005$). (Fig. 1A). The AUROC for CVD increased from 0.652 for extensive covariates alone, to 0.732 with the inclusion of apoCIII and NT-proBNP (Fig. 1B).

A useful metric beyond AUROC that summarizes the potential value of biomarkers in selection of patients for a clinical trial is the 'predicted event rate enrichment' achieved by using the biomarkers in a given potential clinical trial population (Fig. 2). This corresponds to the positive predicted value (PPV) computed over a growing proportion of patients, from only the patients most at risk (left side of the plot) to all individuals in the study (right side of the plot). For example, in the subsample from CARDS used in this study, the cumulative incidence of CVD was 6.8%. Thus, without any selection by risk stratification, the expected cumulative incidence of CVD is 6.8% (y-axis value when x = 100%). The plot illustrates that by selecting, say, the most extreme 20% of patients based on their score from a model using the Framingham covariates (red line) would enrich the cumulative incidence of CVD to 11%. At the same 20% threshold, using a model based on Framingham covariates and selected biomarkers (blue line) would enrich the cumulative incidence of CVD to 16%. This illustrates that even small increments in AUROC can yield useful enrichment for events when the extremes of risks scores that include biomarkers are selected.

3.3. Sensitivity analyses

NT-proBNP is strongly associated with heart failure. While all participants were free of CVD at baseline, we undertook a sensitivity analysis excluding all 8 individuals with an NT-proBNP >400 pg/ml at baseline (n = 1501 with 105 incident cases of CVD). There was little difference in the association of NT-proBNP with CVD with these 8 patients excluded (HR = 2.01; 95% CI 1.56, 2.58). The sub-analyses restricted to those in whom albuminuria status was also available (n = 2018, 138 incident CVD events) made little difference to the univariate biomarker associations (apoCIII HR 1.36; 95% CI 1.13, 1.64; NT-proBNP HR 1.99; 95% CI 1.59, 2.49; and hsTnT HR 1.40; 95% CI 1.16, 1.69). Substituting apoB for LDL-C in the models did not appreciably change the associations between biomarkers and CVD (data not shown).

3.4. Secondary end-point analyses

We examined the association of apoCIII and NT-proBNP with fatal and non-fatal events and with stroke and coronary heart disease (CHD) separately. In the model containing both these biomarkers, for apoCIII the HRs per Gaussianized SD were similar for

Table 1
Baseline demographics and biomarker concentrations of CARDS study participants by incident CVD status (n = 2105).

	No CVD event (n = 1961)		CVD event (n = 144)		*p*-value adjusted for age and sex
	Median/frequency	Interquartile range	Median/frequency	Interquartile range	
Age (years)	62.7	55.7, 67.9	65.0	61.1, 69.4	<0.001[a]
Male sex	66.3%	–	85.4%	–	<0.001[b]
Diabetes duration (years)	6.0	3.0, 11.0	7.5	3.0, 12.0	0.267
Systolic blood pressure (mmHg)	143	134, 153	145	135, 155	0.743
Diastolic blood pressure (mmHg)	83	77, 89	84	79, 87	0.598
Height (m)	1.71	1.63, 1.77	1.72	1.65, 1.77	0.011
BMI (kg/m^2)	28.7	26.1, 31.4	29.0	27.1, 31.0	0.032
HbA_{1c} (%) [mmol/mol]	7.7 [60.6]	6.8, 8.7 [50.8, 71.6]	7.6 [59.6]	6.9, 8.4 [51.9, 68.3]	0.768
eGFR ($ml/min/1.73m^2$)	64.2	57.3, 71.0	64.6	57.4, 70.5	0.403
Total cholesterol (mmol/l)	5.4	4.8, 5.9	5.5	4.9, 6.0	0.007
LDL cholesterol (mmol/l)	3.05	2.56, 3.55	3.11	2.69, 3.68	0.007
HDL cholesterol (mmol/l)	1.36	1.17, 1.57	1.35	1.19, 1.52	0.584
Triglycerides (mmol/l)	1.67	1.17, 2.37	1.70	1.20, 2.55	0.456
Smoking status					
Never	34.7%	–	30.6%	–	–
Ex	43.6%	–	41.7%	–	0.214
Current	21.7%	–	27.8%	–	0.171
Insulin therapy	19.7%	–	18.8%	–	0.966
Aspirin use	14.9%	–	18.1%	–	0.604
Antihypertensive use	66.8%	–	63.9%	–	0.428
Lipid lowering drug use	0.2%		0.7%		0.134
Randomised to atorvastatin	52.2%	–	41.7%	–	0.016
Albuminuria status [c]					
Normoalbuminuric	73.2%	–	62.3%	–	–
Microalbuminuric	22.8%	–	29.0%	–	0.150
Macroalbuminuric	4.0%	–	8.7%	–	0.013
Apolipoprotein CIII (μg/ml)	96.4	47.4, 179.1	119.4	56.4, 197.7	0.005
Interleukin-6 (pg/ml)	0.40	0.25, 0.68	0.44	0.32, 0.81	0.261
Interleukin-6 (categorical)					
Quartile 1 (≤0.25 pg/ml)	26.1%	–	14.6%	–	–
Quartiles 2, 3, 4	73.9%	–	85.4%		0.006
Interleukin-15 (ng/ml)	0.83	0.62, 1.12	0.84	0.57, 1.16	0.541
N-terminal prohormone B-type natriuretic peptide (pg/ml) [d]	43.2	25.4, 85.6	85.7	42.7, 160.7	<0.001
High sensitivity Troponin T (pg/ml) [d]	1.5	1.5, 5.5	4.1	1.5, 9.1	0.004
High sensitivity Troponin T (categorical)[d]					
Below detection	58.1%	–	40.4%	–	–
Below median	21.6%	–	18.4%	–	0.819
Above median	20.3%	–	41.2%	–	0.002

[a] Adjusted for sex only.
[b] Adjusted for age only.
[c] Data available for 2018 people (133 cases).
[d] Data available for 1569 subjects (114 cases).

Table 2
Hazard ratios per standardised unit of biomarkers for incident CVD adjusted for clinical covariates.

	Adjusted for Framingham covariates[a]			Adjusted for full covariates[b]		
	Hazard ratio	95% Confidence interval	*p*-value	Hazard ratio	95% Confidence interval	*p*-value
Biomarkers assessed singly						
ApoCIII	1.29	1.09, 1.53	0.004	1.34	1.12, 1.60	0.002
Interleukin-6	1.07	0.92, 1.24	0.377	1.07	0.91, 1.25	0.433
Interleukin-15	1.06	0.89, 1.25	0.520	1.05	0.89, 1.24	0.584
N-terminal prohormone B-type natriuretic peptide	1.89	1.54, 2.32	<0.0001	2.02	1.63, 2.50	<0.0001
High sensitivity Troponin T	1.40	1.17, 1.69	0.0003	1.40	1.16, 1.69	0.0004
Biomarkers assessed together						
ApoCIII	1.49	1.23, 1.81	<0.0001	1.55	1.26, 1.90	<0.0001
N-Terminal prohormone B-type natriuretic peptide	1.82	1.46, 2.27	<0.0001	1.94	1.54, 2.45	<0.0001
High sensitivity Troponin T	1.15	0.94, 1.39	0.169	1.10	0.90, 1.33	0.348

[a] Framingham covariates include age, sex, systolic blood pressure, total cholesterol, HDL cholesterol, smoking status and randomisation arm.
[b] Full covariates include the Framingham set plus BMI, triglycerides, LDL cholesterol, diastolic blood pressure, HbA_{1c}, diabetes duration, eGFR, height, insulin use and antihypertensive use (including subgroups of antihypertensive agents).

fatal (HR 1.53 (1.03, 2.26)) and non-fatal events (HR 1.50 (1.21, 1.87)) and were similar for stroke (HR 1.45 (0.99, 2.13)) and CHD (HR 1.53 (1.22, 1.91)). For NT-proBNP, the HR for fatal events (3.03 (1.95, 4.70)) was somewhat larger than for non-fatal events (1.68 (1.33, 2.13)) and was larger for CHD (2.29 (1.80, 2.91)) than for stroke (1.18 (0.78, 1.77)), though confidence limits were wide examining these subsets of events.

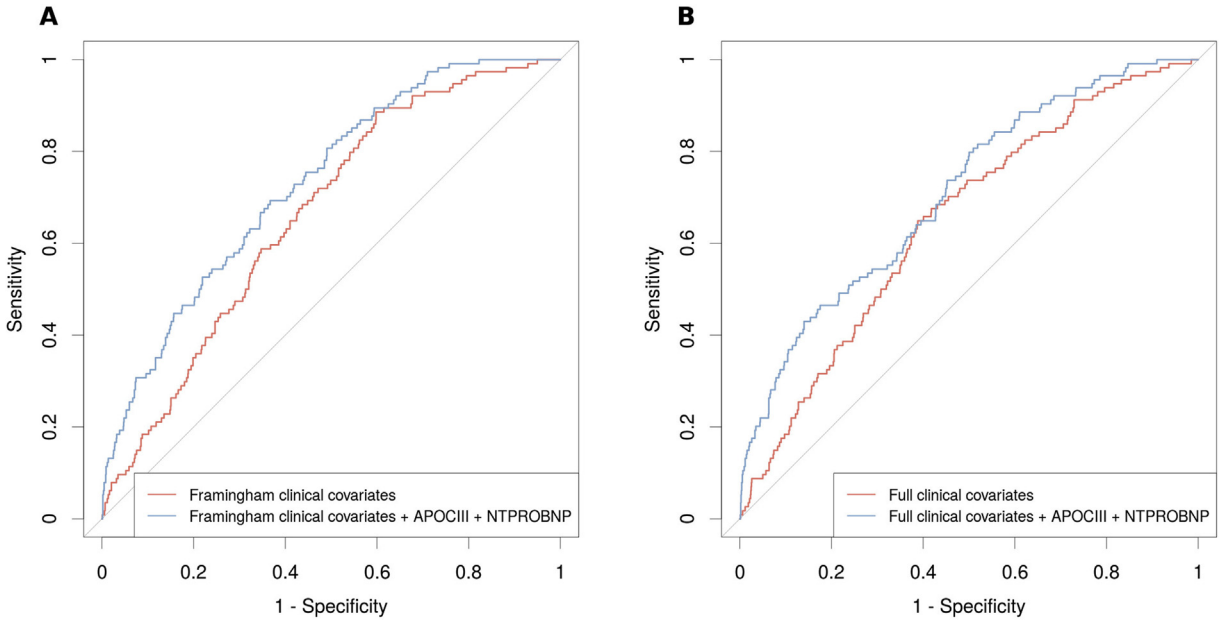

Fig. 1. ROC curves.
(A) Models adjusted for the Framingham clinical covariates, (B) models adjusted for an extensive set of clinical covariates.

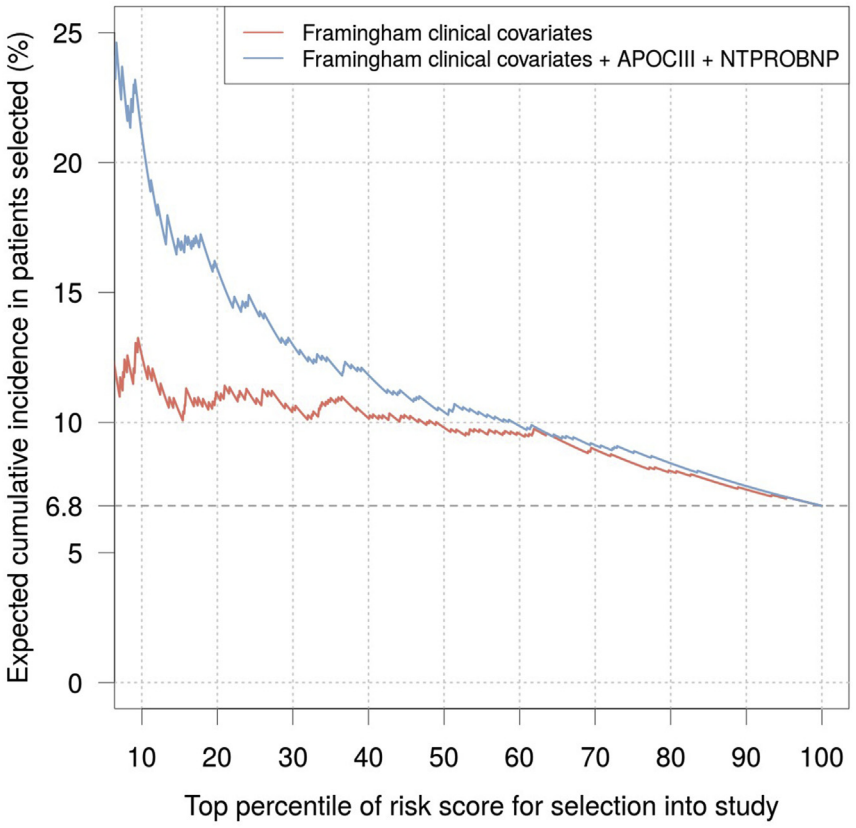

Fig. 2. Expected cumulative event rate.
The figure shows how the expected cumulative event rate would increase from the observed 6.8% (horizontal line) if the trial subsampled the percentage of possible study entrants with a risk score above a given threshold (as denoted on the x-axis) for (i) risk score based on Framingham score variables (in red) and (ii) risk score also including the biomarkers (in blue).

4. Discussion

We have shown that serum apoCIII increments prediction of CVD in type 2 diabetes on top of serum NT-proBNP and an extensive set of clinical risk factors including triglycerides, and factors important in diabetes such as diabetes duration, HbA_{1c} and renal function [20,22]. By selecting those at the extreme of a risk score that includes clinical covariates apoCIII and NT-proBNP, useful enrichment of clinical trial participants for CVD events could be obtained.

Despite the development and ongoing clinical trial assessment of apoCIII-directed therapies, relatively few studies have examined circulating levels of serum or plasma apoCIII as a predictor of CVD. In our study we used the Assaymax ELISA from Assaypro and we demonstrated an association of serum apoCIII with CVD that was independent of triglyceride levels. A previous meta-analysis of the six prospective or retrospective studies available at that time found that total plasma apoCIII was associated with a pooled average relative risks of 1.33 for incident CVD for an increase in apoCIII of 5 mg/dL [5]. However, of the prospective cohort or nested case control studies [23], only one showed an association that was independent of triglycerides; in the Hoorn study, plasma apoCIII measured using an immunoturbidimetric assay (Daiichi) on a Cobas Mira analyzer (ABX Diagnostics) predicted CVD mortality independently of other covariates including triglycerides which were not themselves associated with CVD mortality [24]. Recently, in the EPIC-Norfolk prospective cohort study using an in-house chemiluminescent enzyme-linked immunoassay, plasma apoCIII levels were associated within incident coronary artery disease (CAD) but this was not independent of triglyceride level. Correlations between apoCIII and triglyceride were very similar in that study ($r = 0.39$) to ours ($r = 0.49$). However, in the EPIC-Norfolk cohort very few had overt diabetes and also higher triglycerides were associated with CAD. In contrast all our participants had T2D but among these triglycerides were not strongly associated with CVD. It is possible that in largely non-diabetic cohorts triglycerides predict CAD in part because they are associated with dysglycaemia and prediabetes [25], but that such associations are attenuated when all participants already have diabetes. So whilst both these studies provide supportive evidence for apoCIII as a target of therapy for CVD, the usefulness of apoCIII as a predictive biomarker is only supported by our study. Whether apoCIII is useful for prediction of CVD in diabetes beyond extensive clinical covariate data and other strong biomarkers such as NT-proBNP has not been unequivocally shown previously. We previously reported an unexpected weak inverse association between serum apoCIII and incident CVD in diabetes patients [7]. In that study we drew attention to the unexpected direction of effect. In that study we used the same assay but a different laboratory; the inter-run CV for the assay was double what we assessed in this study (15%, as opposed to 7.3% in this study) and the levels were several fold higher, which may explain the discrepant findings. Of note, the ORIGIN trial, which is one of the few studies to have measured a large number of biomarkers simultaneously, did measure both NT-proBNP and apoCIII using Luminex technology. However, apoCIII was not one of the leading biomarkers selected in their univariate analyses. The inter-run CV reported for apoCIII in that study was higher than in ours at 20% [8]. We note that their analysis did not adjust for any measure of eGFR which may also have influenced which biomarkers are selected first.

ApoCIII is primarily a constituent of triglyceride rich lipoproteins such as Very Low Density Lipoprotein (VLDL) with strong biological evidence for a role in the pathophysiology of CVD. A null mutation of the apoCIII gene is associated with low triglycerides, low LDL-C and high HDL-C along with lower prevalence of CVD [26]. There is considerable interest in apoCIII, not only as a potential biomarker for CVD risk, but also as a potential therapeutic target for preventing CVD [27]. The mechanisms by which apoCIII affects CVD are not fully understood but several pathways may be relevant. In particular, in-vitro studies show apoCIII inhibits lipoprotein lipase leading to hypertriglyceridemia [28] and inhibits hepatic clearance of lipid remnants by disrupting the interaction of apoE with the LDL-C receptor [29]. In some studies despite no association of total apoCIII with CAD, the ratio of total plasma apoCIII to HDL was shown to be higher in CAD patients than controls and inversely correlated with cholesterol efflux capacity [30]. In other studies HDL containing apoCIII was positively associated with CAD risk [31]. Recently the co-existence of apoCIII on HDL with apoE on HDL has also been shown to attenuate the beneficial effect of apoE on reverse cholesterol transport [32].

ApoCIII also has a pro-inflammatory role as it increases the expression of pro-inflammatory adhesion molecules in endothelial cells via the activation of nuclear factor-kappaB and protein kinase C [33]. High apoCIII also increases the susceptibility of LDL-C to hydrolysis and increases the accumulation of lipids in vessel walls. These pro-inflammatory properties require sialyation of apoCIII and this process increases with increased LDL-C apoCIII content [4]. The Epic-Norfolk mediation analysis concluded that much of the CAD risk associated with apoCIII could be attributed to higher levels of triglyceride rich remnant particle concentrations but also by higher LDL particle concentrations.

NT-proBNP has repeatedly been shown to be predictive of CVD [34–36] and more recently it was the leading biomarker identified as a predictor of incident CVD in diabetes in the ORIGIN trial [8]. The cleaving of prohormone BNP separates the active hormone, BNP, from the inactive fragment NT-proBNP. Both are measurable in blood and recognised as biomarkers for heart failure [37]. We had previously identified NT-proBNP as the single strongest biomarker of incident CVD in a case-control study including individuals with diabetes from five European cohorts and we have confirmed that finding here in this independent sample set [35]. An important consideration in diabetes is whether such associations are confounded by reduced eGFR, an important risk factor for CVD but which is also associated with elevated NT-proBNP. No adjustment for eGFR was made in the ORIGIN analysis but here we showed that the HR for CVD associated with NT-proBNP was little changed by adjusting for eGFR. Neither was the association driven by the inclusion of individuals with subclinical incipient heart failure as we obtained very similar results after excluding individuals with elevated NT-proBNP at baseline and since overt heart failure was an exclusion criterion in CARDS. The data are also consistent with reports from the West of Scotland Study Coronary Prevention Study (WOSCOPS) which showed that NT-proBNP is more strongly associated with fatal CVD than non-fatal CVD [38]. NT-proBNP levels have been shown to predict CAD events beyond coronary artery calcium score in the general population [39], though not in those with diabetes [40]. ApoCIII has not been assessed in this context.

Troponin T is produced in cardiac muscle cells and is already used as a biomarker of acute myocardial infarction [41]. The introduction of high sensitivity assays opened the possibility of hsTnT being used as a biomarker for non-acute CVD risk [42]. The combination of NT-proBNP and hsTnT improved risk prediction for CVD among people with T2D in the Atherosclerosis Risk in Communities study [43] and in a nested case cohort from the ADVANCE trial where the combination of biomarkers also predicted all-cause mortality [44]. NT-proBNP and hsTnT also improved prediction of CVD in the Multi-Ethnic Study of Atherosclerosis, a study not restricted to people with diabetes; however, the improvement due to hsTnT was small [35]. In our study with adjustment for NT-proBNP (which is correlated with hsTnT) no useful additional

increment in prediction of CVD was obtained with hsTnT.

We did not find any linear associations between either IL-6 or IL-15 and incident CVD in this study. We selected these as candidate biomarkers based on our previous biomarker discovery study across five cohorts with T2D in which we found they were predictive of CVD. Previous studies have identified IL-6 as a risk factor for non-fatal CVD [45], CVD mortality [45,46] and all-cause mortality [46,47] in people with T2D. However, overall concentrations of IL-6 in CARDS were lower compared to previous studies which found evidence of a positive association with incident CVD [7,45–47]. Consistent with the results for IL-6 and IL-15 a prior study in CARDS did not find any significant association with CRP and incident CVD either [18]. This may reflect the younger age of CARDS study population than in some of the previous studies and the strong selection for no prior evidence of any CVD.

Strengths of the current study are that it makes use of samples from a clinical trial with all samples drawn in accordance with clear protocols, and as CVD was the primary end-point of the study all cases had been reviewed and adjudicated according to the trial protocol reducing the risk of misclassification [48]. We measured the biomarkers for this study using commercially available assays which met high QC standards and for three of them, we were able to use identical assays to those used in the earlier SUMMIT CVD study. Limitations of the study are that it is a relatively small sample size, with only 144 cases and a follow-up of under 5 years. This means we cannot make any inferences about the performance of these biomarkers over a longer period such as the more traditional ten years. Moreover, we are underpowered to definitively test whether associations differ with fatal *versus* non-fatal events or with stroke *versus* CHD, with data suggesting that the best biomarker for stroke, say, may well not be the best biomarker for CHD. The study population is also principally Caucasian, limiting us from any assessment of the biomarkers in other ethnicities.

Statistical significance of AUROC improvement and clinical significance of such improvement are not the same thing. Whether or not any given increment in AUROC is useful depends on the context; specifically it depends on the prior probability of disease, what posterior probability of disease one wishes to classify a patient as having in order to reach a given clinical decision, and therefore the threshold value of the likelihood ratio generated by the test needed to achieve this posterior probability. This likelihood ratio is obtained from the ROC curve. We have illustrated that the improvement with NT-proBNP and apoCIII gained here would be useful in stratification into clinical trials if those with extreme values of the biomarkers were selected for inclusion. In terms of clinical decision making, for illustration in the CARDS cohort the observed rate of CVD over the median follow up of 3.7 years was 6.8%. If say in a cohort of patients with a similar overall event rate (i.e. prior probability of disease) one wanted to identify the subset of individuals with a risk of at least 20% over 10 years, this would require any test result threshold to have a likelihood ratio of 3 for favouring case over control status. At this cut-off it can be shown the model including the biomarkers would have twice the sensitivity of the Framingham model alone at a slightly lower specificity.

In conclusion, we find evidence that both NT-proBNP and apoCIII may have use in improving prediction of incident CVD in individuals with T2D and no evidence of existing CVD. Even when using all available clinical risk factor data including measures of diabetes control and renal function, these biomarkers were associated with an improvement in prediction. The PONTIAC trial showed that risk stratification into trials using NT-proBNP was useful in identifying high-risk patients with diabetes for intensive primary prevention trial for CVD [49]. Here we provide data suggesting that, combined with NT-proBNP, apoCIII can also be useful in clinical trial event rate enrichment in diabetes.

Conflicts of interest

HMC received research support, travel expenses and honorarium and is also a member of the advisory panels and speaker's bureaus for Sanofi Aventis, Regeneron, and Eli Lilly. HMC is also a member of the Advisory Panel and receives institutional fees from Novartis Pharmaceuticals. HMC also receives or has recently received research support from Roche Pharmaceuticals, Pfizer Inc., Boehringer Ingelheim and AstraZeneca LP. HMC receives research support, travel expenses and is on the Steering Committee for Novo Nordisk. HMC is a shareholder of Roche Pharmaceuticals and Bayer. HMC has received speaker fees from Pfizer. All other authors declare that there is no duality of interest associated with their contribution to this manuscript.

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Author contributions

MC carried out the primary statistical analyses and reviewed/edited the manuscript, HCL wrote and revised manuscript, BF researched data and reviewed/edited the manuscript, MJB researched data and reviewed and edited the manuscript, FA contributed to the design of the study and revised the manuscript, PW and NS undertook the biomarker assays and reviewed/edited the manuscript, SJL,PND, DJB researched data and reviewed/edited the manuscript, PMM and HMC designed the study, researched the data and revised the manuscript. All authors have approved the final version of the manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.atherosclerosis.2018.05.014.

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