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Soluble ST2 levels are not associated with secondary cardiovascular events and vulnerable plaque phenotype in patients with carotid artery stenosis

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ABSTRACT

Objective: Soluble ST2 (sST2), a novel biomarker predictive for heart disease, has recently been shown associated with the progression of atherosclerotic disease in a mouse model. The present study was designed to assess sST2 plasma levels in patients scheduled for carotid endarterectomy and relate it with the occurrence of adverse cardiovascular events during follow-up. In addition, sST2 levels were associated to patient clinical data and atherosclerotic plaque characteristics.

Methods and results: Plasma sST2 levels were measured in 391 patients who underwent carotid endarterectomy and were subsequently followed for 3 years. Primary composite endpoint was the occurrence of an adverse cardiovascular event.

At baseline, no differences were observed in sST2 levels between asymptomatic ($n = 75$) and symptomatic ($n = 316$) patients (85 [49–122] versus 90 [58–137] pg/ml, $p = 0.263$). Soluble ST2 plasma levels did not differ between patients who experienced a secondary manifestation of cardiovascular disease and patients who remained free of symptoms (90 [60–129] versus 88 [46–140] pg/ml, $p = 0.519$). There was no association between sST2 levels and any of the following plaque characteristics: size of a lipid core, degree of calcification, number of macrophages or smooth muscle cells, amount of collagen and number of microvessels.

Conclusions: Soluble ST2 plasma levels have no predictive value for future cardiovascular events in patients with significant carotid artery stenosis. In addition, we did not observe an association between plasma sST2 levels and the histopathological features of a rupture prone plaque. This study does not provide supportive evidence that sST2 reflects a progressive state of advanced atherosclerotic disease.

1. Introduction

The aging population leads to an increase of cardiovascular morbidity and mortality. Biomarkers may facilitate the identification of patients at high risk who subsequently can undergo preventive treatment. ST2, the receptor for the Th2 associated cytokine interleukin (IL)-33, is a member of the IL-1 receptor family that has recently gained significant interest in the biomarker research field. As a result of alternative splicing, ST2 is expressed in a transmembrane form (ST2L) and a soluble form (sST2) [1]. Soluble ST2 has been studied extensively as it was shown to be elevated in patients with cardiovascular disease [2,3]. Moreover, sST2 levels were demonstrated to have predictive value for the occurrence of future cardiovascular events in patients suffering from ischemic heart disease [4].

ST2L, expressed on the surface of many inflammatory cells [5], is the receptor trough which signalling of IL-33 has been found to

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exert a cardioprotective role. Administration of IL-33 in animal models of cardiovascular disease resulted in functional improvements and increased survival rates [6–8]. This might explain an unfavourable role of sST2 in cardiovascular disease [6,8,9] since capturing of IL-33 by the soluble form of ST2 from the circulation hampers the beneficial effect of IL-33 [10].

Previous human studies on the predictive value of sST2 for heart failure and mortality mainly focussed on patients with ischemic heart disease [11]. In the present study, we investigated the predictive value of sST2 on future cardiovascular atherosclerotic events in a patient group with significant carotid artery disease: in this patient domain the predictive value of sST2 is still unknown. Soluble ST2 protein levels were assessed in the plasma of patients that underwent carotid endarterectomy and related to the occurrence of adverse cardiovascular events during follow-up.

In patients with coronary and carotid artery disease, acute cardiovascular manifestations are often the result of plaque rupture followed by thrombus formation. Therefore, a particularly interesting observation was that ApoE−/− mice on a high fat-diet receiving IL-33 treatment developed smaller lesions, while sST2 administration resulted in increased plaque size [9]. Experiments showing that administration of IL-33 lowered foam cell formation considerably in vitro and in vivo [12], further support an important role for the IL-33/ST2 pathway in atherosclerotic plaque development. Therefore, in the current study, associations between sST2 plasma levels and the characteristics of a vulnerable plaque have been investigated.

2. Materials and methods

2.1. Study population and design

A total of 391 patients of the Athero-Express were included in this study. The Athero-express biobank involves patients that underwent carotid endarterectomy (CEA) in two Dutch teaching hospitals in Utrecht and Nieuwegein the Netherlands [13]. Indication for CEA was based on recommendations by the Asymptomatic Carotid Atherosclerosis Study, the North American Symptomatic Carotid Endarterectomy Trial and the European Carotid Surgery Trial [14–16]. Patients were operated between March 2002 and August 2008 and randomly selected among those of whom blood plasma samples were available. The local medical ethical boards of both participating hospitals approved this study. The participating patients signed a written informed consent prior to inclusion. The patient's baseline characteristics and medical history were obtained via questionnaires and the patient medical records.

2.2. Follow-up

After CEA patients were followed up to 3 years by questionnaire. Primary outcome was defined as any cardiovascular event including; (non-) fatal stroke, (non-) fatal myocardial infarction, sudden death and other vascular death and any invasive arterial intervention that had not been planned at the time of inclusion (e.g. carotid surgery or angioplasty, coronary artery bypass grafting (CABG), percutaneous transluminal coronary angioplasty (PTCA), peripheral vascular surgery or angioplasty).

2.3. Materials

The carotid plaques used in this study were processed as described previously [13]. In short, after surgical dissection the plaque was cut into segments of 5 mm. The segment with the largest plaque area was fixed in formalin and embedded in paraffin for histology. The two adjacent sections were frozen in liquid nitrogen and used for protein isolation. In addition, blood was drawn prior to CEA procedure and plasma was stored at −80 °C.

2.4. Quantification of sST2 levels in patient plasma

Soluble ST2 levels were measured with an IL-1 R4/ST2 enzyme-linked immunosorbent assay (ELISA, RayBiotech, Norcross, Georgia, USA) according to the manufacturer's instructions. In brief, plasma samples 1:1 diluted with dilution buffer and standards were incubated for 2.5 h in a 96-well plate pre-coated with a capture antibody for human ST2. After the 96-well plate was washed using an automatic washer, a biotin labelled anti-human ST2 antibody was added to the wells. In between washes, the HRP antibody was added, followed by administration of the substrate. After 30 min the stop solution was added and the luminescence was measured at 450 nm with an ELISA reader (Multiskan FC, Thermo Fisher Scientific, Vantaa, Finland). The upper detection limit of the RayBio® human IL-1 R4/ST2 ELISA kit was 1200 pg/ml and the lower limit of detection was 2 pg/ml, with an intra-assay CV<10% and inter-assay CV<12%.

2.5. Plaque protein measurements

To investigate whether sST2 levels are associated with the plaque's inflammatory status, we used expression data of pro-and anti-inflammatory cytokines e.g. IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, INF-γ and TNF-α that were available from measurements of a previous study. These expression levels had been assessed in 211 of the carotid atherosclerotic plaques that were included for the current study by Fluorescent Bead Immunoassay 810FF (Bendermed Systems, Vienna, Austria).

2.6. Kidney function assessment

Kidney function was determined by calculating the glomerular filtration rate (eGFR) expressed in ml/min/1.73 m^2 as previously described [17]. Serum creatinin was measured in blood plasma.

2.7. Immunohistochemistry

Consecutive sections were stained for CD68 (macrophages), smooth muscle cells (alpha actin) CD66 (neutrophils), mast cell tryptase (mast cells) and CD34 (endothelial cells). As previously described, image-analyzing software (Soft Imaging Solutions GmbH, Münster, Germany) was used to determine positive macrophage and smooth muscle cell staining expressed as a percentage of covered plaque area. Microvessels were counted in three hot-spots and were expressed as average microvessel density per hotspot [18]. The presence of intraplaque hemorrhage was assessed with Hematoxylin and Eosin (H&E) staining and fibrin (Mallory's phosphotungstic acid-hematoxylin) [19]. Calcification (H&E) and collagen content (Picrosirius red) were scored semi-quantitatively. The size of the extracellular lipid core (atheroma) was assessed by the H&E and Picrosirius red stain [13]. Overall plaque phenotype was based on the percentage of confluent lipid areas of the total plaque area that were visually estimated (fibrous: <10% fat; fibroatheromatous: 10%–40%; atheromatous: >40% fat).

2.8. Statistics and data analysis

IBM SPSS statistics version 20 was used for all analyses (IBM corporation, Armonk, NY, USA). Soluble ST2 levels are not normally distributed; non-parametrical testing was used to determine differences. The Wilcoxon signed rank test was applied to compare differences in sST2 levels before and after surgery. The Spearman

correlation coefficient was calculated to assess associations between sST2 and all continuous variables in this study. The Mann–Whitney *U* test was used to study sST2 levels as a continuous variable for all risk factors. To assess the independent association between sST2 plasma levels and history of cardiovascular events and interventions, a binary logistic regression model was used in which we corrected for the potential confounders age and diabetes. The relation between sST2 and the occurrence of future manifestations during follow-up was examined using the cox-regression survival analysis. Differences were considered significant with a *p*-value of below 0.05.

3. Results

3.1. Baseline characteristics

Soluble ST2 plasma levels were measured in a total of 391 patients that underwent carotid endarterectomy. In Table 1 the baseline clinical characteristics of the patients are depicted. With a mean age of 67 years and 67% males, the study population reflects a relatively typical population of patients with cerebral vascular occlusive diseases. The majority of patients was symptomatic (81%), hypertensive (87%) and used statins (74%).

3.2. Soluble ST2 plasma levels and clinically relevant characteristics

Table 1 summarises the associations between sST2 expression levels and relevant clinical characteristics. Plasma sST2 levels correlated with age ($r = 0.240$, $p = 0.037$). Higher sST2 levels were observed in males compared to females (91.8 [62.3–145.0] versus 76.7 [47.1–111.2] pg/ml, $p = 0.002$) and in patients with diabetes mellitus (106.4 [62.8–181.6] versus 88.0 [55.0–125.3] pg/ml, $p = 0.021$). Furthermore, we observed that patients with a history of coronary artery disease that previously experienced a myocardial infarction (MI) or underwent PTCA/CABG had higher sST2 levels (100.8 [69.6–153.4] versus 84.4 [52.6–126.4] pg/ml; $p = 0.007$). However, in a logistic regression model controlling for age and diabetes, sST2 levels were no longer associated with previous MI or coronary interventions. Soluble ST2 levels were not significantly higher in patients that previously underwent a peripheral artery intervention (95.3 [64.9–160.4] versus 88.7 [56.1–126.9] pg/ml; $p = 0.244$).

Clinical presentation was not associated with sST2 levels: no differences were observed in sST2 levels between asymptomatic patients ($n = 75$) and symptomatic ($n = 316$) patients (85 [49–122] versus 90 [58–137] pg/ml, $p = 0.263$). Additionally, no association was found between sST2 levels and the delay between surgery and presentation of symptoms.

Table 1
Baseline characteristics of the patients in relation to sST2 plasma levels.

		sST2 (pg/mL)	*p*-value
Age, mean years (sd)	67 (9)	$r = -0.119$	**0.018**
BMI, mean kg/m^2 (sd)	27 (4)	$r = 0.053$	0.318
Sex			
Male	263/391 (67%)	91.8 [62.3–145.0]	**0.002**
Female	128/391 (33%)	76.7 [47.1–111.2]	
Current smoker			
Yes	133/383 (35%)	90.5 [53.8–137.8]	0.926
No	250/383 (65%)	89.4 [60.4–128.2]	
Diabetes mellitus			
Yes	88/391 (23%)	106.4 [62.8–181.6]	**0.021**
No	303/391 (77%)	88.0 [55.0–125.3]	
Statin use			
Yes	288/389 (74%)	89.5 [55.6–141.8]	0.591
No	101/389 (26%)	88.3 [58.0–124.4]	
Hypertension			
Yes	340/391 (87%)	88.5 [57.5–130.5]	0.588
No	51/391 (13%)	100.9 [57.2–127.4]	
History peripheral intervention			
Yes	71/391 (18%)	95.3 [64.9–160.4]	0.244
No	320/391 (82%)	88.7 [56.1–126.9]	
History coronary artery disease			
Yes	104/391 (27%)	100.8 [69.6–153.4]	**0.007**
No	285/391 (73%)	84.4 [52.6–126.4]	
Clinical presentation			
Asymptomatic	75/391 (19%)	85.1 [48.6–121.5]	0.263*
Symptomatic	316/391 (81%)	90.0 [58.2–136.8]	
Amaurosis fugax	56/316 (18%)	89.0 [59.4–164.1]	
TIA	180/316 (57%)	91.8 [55.3–127.1]	
Stroke	80/316 (25%)	87.7 [63.4–155.4]	

Data are presented as No. (%) and median [IQR] unless otherwise indicated; *r* = Spearman's rank correlation coefficient; sd = standard deviation; IQR = interquartile range; BMI = body mass index; TIA = transient ischemic attack; * *p*-value represents statistical analysis for asymptomatic patients versus symptomatic patients (composed of amaurosis fugax, TIA and stroke).

3.3. Relation of sST2 and other biomarkers

A correlation was found between sST2 and the acute phase protein CRP mg/l ($r = 0.107$, $p = 0.035$). Kidney function expressed as eGFR (ml/min/1.73 m^2) correlated negatively with sST2 ($r = -0.165$, $p = 0.008$).

3.4. Outcome

A total of 75 out of 391 patients suffered from secondary cardiovascular manifestations during a 3-year follow-up. There was no significant difference in baseline sST2 plasma levels between patients that did and those that did not experience a cardiovascular event (90 [60–129] versus 88 [46–140] pg/ml, $p = 0.519$). Likewise, when divided into two groups by the median, high sST2 levels did not associate with secondary manifestations of cardiovascular disease (39 [20%] in the low level sST2 group versus 36 [19%] events in the high sST2 group; Fig. 1). Median sST2 levels were higher in patients that experienced a cardiac event ($n = 10$), but this did not reach significance ($p = 0.324$). Soluble ST2 plasma levels were significantly higher in patients with all-cause mortality (88.2 versus 111.8 pg/ml; $n = 24$, $p = 0.031$). However in a cox regression

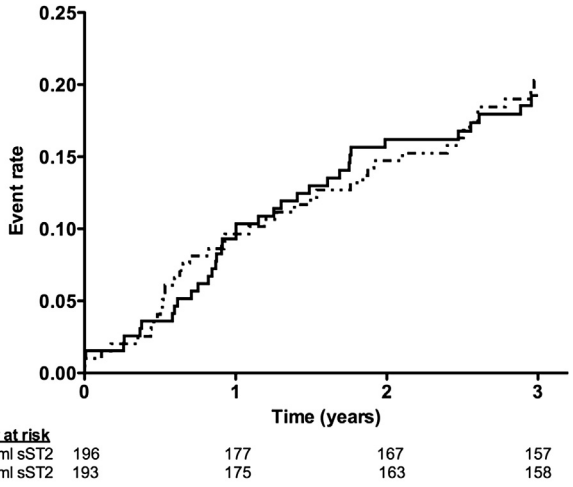

Fig. 1. Kaplan–Meier survival curves for sST2 plasma levels versus combined cardiovascular outcome after carotid endarterectomy. High sST2 levels (continuous line) versus low sST2 levels (dashed line) based on the median (89 pg/ml) as a cut-off value ($p = 0.861$). The numbers of patients at risk for cardiovascular events are shown at 0, 1, 2 and 3 years after endarterectomy.

analysis correcting for age and gender, sST2 was no longer predictive ($p = 0.134$)

3.5. Plasma sST2 levels and plaque characteristics

As depicted in Table 2, sST2 levels were not associated with plaque phenotype categorised in three groups: fibrous, fibroatheromatous, atheromatous (88.2 [48.9–126], 85.5 [53.3–130.9], 92 [67.1–139.8] pg/ml respectively; $p = 0.242$). There was no association between sST2 levels and any of the following plaque characteristics: calcification, collagen, smooth muscle cells, macrophages, neutrophils or mast cells (Tables 2 and 3). In addition, sST2 levels were not related to the plaque protein levels of the anti- or pro- inflammatory cytokines (Table 3). Although no association was found between sST2 levels and microvessel density, an increase of sST2 level was observed in patients with plaques that scored positive for intraplaque hemorrhage (92.6 [61.9–144.9] versus 80.3 [49.4–107.5] pg/ml, $p = 0.004$).

4. Discussion

Soluble ST2 is emerging as a novel biomarker for prediction of mortality and heart failure in patients with established cardiac disease. Multiple studies showed that sST2 is a biomarker that adds value in risk prediction for development of cardiac disease on top of traditional risk factors and other biomarkers in patients with ischemic heart disease [11]. Stenosis or rupture of the atherosclerotic plaque is the underlying cause for myocardial infarction or heart failure of ischemic aetiology. Experimental studies have shown a possible role for the IL-33/ST2 pathway in progression of the atherosclerotic plaque [9,12], but studies revealing an association between sST2 and atherosclerotic lesion phenotype in humans is lacking. Therefore, we aimed to investigate whether sST2 levels would have predictive value for adverse cardiovascular events in a patient group with clinically manifest cerebral artery disease. In addition, the dissection of atherosclerotic plaques in this patient group allowed an association study between sST2 levels and atherosclerotic plaque phenotype.

Here we show that baseline sST2 levels have no predictive value for future combined cardiovascular events in a patient group with severe carotid stenosis that underwent an endarterectomy. Our results suggest that sST2 is a less potent biomarker for adverse events in the presence of cerebral ischemic disease compared to patients suffering from cardiac ischemic disease. There are several potential explanations for this observation. In the presence of myocardial infarction and subsequent heart failure, severe tissue damage and remodelling takes place in a relatively short time span whereas in the presence of carotid stenosis tissue damage may be minimal or temporary. Furthermore, a significant number of patients suffered from relative minor complications as transient ischemic attack or amaurosis fugax. Another explanation is that blood was drawn prior to surgery which is executed weeks after the index event whereas in cardiac ischemia the intervention is executed mostly in the acute phase.

No difference in sST2 levels was observed between asymptomatic and symptomatic patients at the moment of admission for surgery. Previous studies have shown that sST2 rises to extreme levels within 24 h after myocardial infarction or cardiovascular interventions after which they return to baseline levels within a few days [20–22]. This indicates that the time of blood withdrawal is of great importance since the delay following a cardiovascular event strongly influences sST2 levels. Therefore, we examined whether the delay between operation and the presentation of symptoms was associated with sST2 levels. We showed that the delay did not alter the sST2 levels. However, it merits careful consideration that patients were often scheduled about 10 weeks after presenting clinical symptoms, which might explain the normalisation of sST2 levels. Current guidelines have resulted in much shorter delays between the cerebrovascular event and surgery which is relevant before extrapolating results to current practice. In the current follow-up study we could not select sufficient numbers of patients presenting with stroke who were operated within 1–2 weeks to investigate whether sST2 levels were increased within this few day time frame.

Interestingly, higher sST2 levels were observed in patients with previous cardiac events or coronary interventions, but not in patients with history of peripheral interventions. However, after adjusting for confounders in a logistic regression model, other risk factors were found to be responsible for this effect. Unfortunately, we were underpowered for analysing risk prediction in the subgroup of patients suffering from adverse cardiac events during follow-up. Nevertheless, in line with previous research, a positive correlation between all-cause mortality and sST2 levels was observed [23–28]. We confirmed the previously reported positive association between CRP and sST2 levels [3,24,28–30]. Furthermore, our data concord with previous studies associating sST2 levels with, age, gender and diabetes mellitus [23,26,30–33]. In addition, in our cohort a positive correlation between sST2 and CKD was observed confirming previous observations [34]. This is of interest as CKD has previously been shown one of the most important determinants for future cardiovascular events [17].

Another research question we addressed was whether sST2 levels associated with plaque phenotype. Previously, sST2 administration in ApoE−/− mice resulted in the development of larger atherosclerotic plaques, although collagen content and inflammatory and smooth muscle cell numbers were not altered [9]. We studied advanced atherosclerotic disease and therefore our study cannot address the question if sST2 plasma levels are associated with plaque size in humans. However, we could investigate whether sST2 levels were associated with histological markers of plaque vulnerability. In line with the previously reported mouse data, we showed that sST2 plasma levels in patients with carotid artery disease are not related to most of the established characteristics of the rupture-prone atherosclerotic plaques. Nevertheless, we observed an association with intraplaque bleeding which warrants further research since plaque hemorrhage is considered an emerging determinant of plaque destabilisation.

Signalling of IL-33 trough ST2L has been found to stimulate Th2 associated cytokine production [35], which is abolished by sST2 acting as a decoy receptor for IL-33 [10]. We therefore investigated whether high levels of sST2 would prevent Th2 associated cytokine

Table 2
Soluble ST2 plasma levels with respect to the histological parameters of the plaque.

		sST2 (pg/mL)	*p*-value
Plaque phenotype			
Fibrous	125/391 (32%)	88.2 [48.9–126.0]	0.242
Fibroatheromatous	143/391 (37%)	85.5 [53.3–130.9]	
Atheromatous	123/391 (31%)	92.2 [67.1–139.8]	
Intraplaque haemorrhage			
Yes	272/391 (70%)	92.6 [61.9–144.9]	0.004
No	119/391 (30%)	80.3 [49.4–107.5]	
Collagen			
Minor	76/391 (19%)	106.0 [60.4–142.7]	0.402
Moderate	219/391 (56%)	86.5 [56.2–127.3]	
Heavy	96/391 (25%)	88.1 [57.4–132.4]	
Calcification			
No/minor	168/391 (43%)	86.9 [56.6–143.0]	0.619
Moderate/heavy	223/391 (57%)	89.8 [57.4–127.1]	

Data are presented as Spearman's rank correlation coefficient (r) or median [interquartile ranges].

Table 3
Soluble ST2 plasma levels with respect to inflammatory markers in the plaque.

	Q1	Q2	Q3	Q4	*p*-value	Spearman (*r*)	*p*-value
sST2 pg/ml	[<57.4]	[57.4–89.4]	[98.5–130.3]	[>130.3]			
Plaque histology							
Patient numbers (*n*)	98	99	97	97			
Microvessel density	8.3 [5.7–11.3]	8.3 [5.2–11.7]	7.7 [5.0–11.1]	7.7 [4.8–11.9]	0.489	−0.028	0.624
Smooth muscle cells	2.01 [0.74–3.83]	2.65 [0.96–4.12]	1.55 [0.52–3.14]	1.69 [0.55–4.02]	0.088	−0.085	0.094
Macrophages	0.72 [0.18–1.50]	0.75 [0.22–2.08]	0.68 [0.17–1.35]	0.65 [0.18–1.54]	0.376	−0.035	0.491
Plaque protein							
Patient numbers (*n*)	49	62	51	49			
IL-2 pg/ml	184 [0–357]	229 [104–533]	153 [4–335]	190 [0–364]	0.104	0.004	0.950
IL-4 pg/ml	144 [0–351]	180 [70–372]	126 [0–283]	168 [0–316]	0.329	0.016	0.819
IL-5 pg/ml	126 [22–324]	153 [65–358]	116 [56–231]	123 [0–304]	0.609	−0.037	0.594
IL-6 pg/ml	53 [16–125]	50 [12–139]	38 [18–81]	31 [14–75]	0.667	−0.098	0.158
IL-8 pg/ml	41 [8–164]	48 [7–230]	60 [6–153]	43 [1–151]	0.888	−0.047	0.497
IL-10 pg/ml	18 [0–64]	22 [8–78]	14 [0–40]	20 [0–51]	0.280	−0.011	0.868
TNF-a pg/ml	12 [0–28]	12 [4–39]	8 [0–22]	15 [0–33]	0.621	−0.030	0.662
IFN-y pg/ml	53 [3–248]	90 [29–318]	78 [14–177]	91 [8–235]	0.473	0.023	0.740

Data are presented as Spearman's rank correlation coefficient (*r*) or median [interquartile ranges].

production and redirected the balance to the Th1 associated cytokines. In this study we did not find an association between sST2 levels and any of the cytokines related to a Th1 or Th2 inflammatory response in the plaque. Taken together, these data suggest that serum sST2 levels do not influence atherosclerotic plaque progression nor their inflammatory profile. Keeping in mind that sST2 levels increase fast and to extreme levels after trauma or surgical procedures, this might indicate that sST2 is just a plain marker for systemic inflammation.

5. Conclusions

The associations found between sST2 and traditional risk factors, other biomarkers and all-cause mortality are in line with previous studies. Soluble ST2 levels have not been found associated with any of the histopathological characteristics of a rupture-prone plaque. Together with the observation that sST2 levels are not elevated in patients that develop secondary cardiovascular events, this study suggests that sST2 levels are not related to progression of atherosclerotic disease following cerebrovascular ischemia. This implies that sST2 levels may have added value in risk prediction for cardiac disease in patients with acute manifestations of myocardial ischemia or severe chronic heart failure, but not in a subgroup of patients with cerebrovascular disease.

Conflict of interest

None declared.

Author contributions

Patient inclusion was performed by G.J.d.B., J-P.P.M.d.V. and F.L.M.; S.W. and G.P. interpreted the data and wrote the paper; P.H.A.Q., I.E.H. and D.P.V.d.K. participated in the analysis, discussion and interpretation of the data; Figures were created by S.W. All authors were involved in finalising the manuscript and approved the final version.

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