EXTRACELLULAR VESICLES FOR RISK STRATIFICATION IN CORONARY ARTERY DISEASE
TRASH OR TREASURE

MIRTHE DEKKER

Extracellular vesicles for risk stratification in coronary artery disease
Trash or treasure?

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EXTRACELLULAR VESICLES FOR RISK STRATIFICATION IN CORONARY ARTERY DISEASE
TRASH OR TREASURE

Extracellulaire vesicles voor risico stratificiatie in coronairlijden
Afval of een verborgen schat?

(met een samenvatting in het Nederlands)

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door

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geboren op 29 september 1991
te Dronten

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Aan mijn familie

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CHAPTER 1

General introduction and thesis outline

GENERAL INTRODUCTION

This thesis presents studies on the role of extracellular vesicles in risk stratification of patients with coronary artery disease, involving both the risk of having a disease as well as their future risk for events. In the following introduction, relevant background information and a brief outline of this thesis will be provided.

Communication is key

Communication is essential in life, the evolution of communication goes back to the earliest form of living organisms on our planet. During the Archean Eon, which is over 3.5 billion years ago, primordial single cell organisms developed intercellular communication via chemical signals allowing them to form microbial mats[1]. Proof of this was found in the fossil records formed by the cyanobacterial layers[2]. Although communication is nowadays much broader than between cells it remains the key in our existence. This thesis will focus on a specific form of cell-cell communication by extracellular vesicles (EVs) and their role in coronary artery disease.

Peter Wolf was the first researcher who showed specific interest in this so called "cell dust" in 1960[3]. Since this discovery a lot of knowledge has emerged. EVs are bilayer membrane structures encompassing cell specific content, depending on the origin and current state of their parent cell[4]. There are different types of EVs, although no consensus exists, figure 1 shows three subpopulations (exosomes, microvesicles and apoptotic bodies) most often used for characterization of EVs. They are thought to be involved in different (patho)physiological pathways, working as cell-cell communicators. Most cells are able to produce EVs, they are found in blood, tears, saliva and in conditioned media[5]. EV content originates from the cell of origin and therefore EV content varies with changing circumstances of their parent cell. EVs are therefore considered as "cargo carriers", or, "the liquid biopsy"[6]. Studies have shown that both the total amount as well as the content of EVs are associated with cardiovascular diseases making them potential biomarkers[7,8].

History and epidemiology of coronary artery disease

Coronary artery disease (CAD) is a subgroup of cardiovascular diseases originating in the coronary arteries[9]. CAD is also referred to as: coronary heart disease (CHD) or ischemic heart disease (IHD). CAD is often roughly divided in two groups: acute coronary syndromes (ACS) and chronic coronary syndromes (CCS) with CCS often called: functional relevant CAD, 'stable' CAD or 'stable' angina. Despite tremendous improvement in diagnostic and therapeutic strategies, CAD remains

the number one cause of death globally. The estimated prevalence in 2015 was 110 million people (780.000 in the Netherlands) resulting in 9 million deaths (8500 in the Netherlands) and it has the highest number of disability adjusted life years (DALY's) among the entire world[9,10].

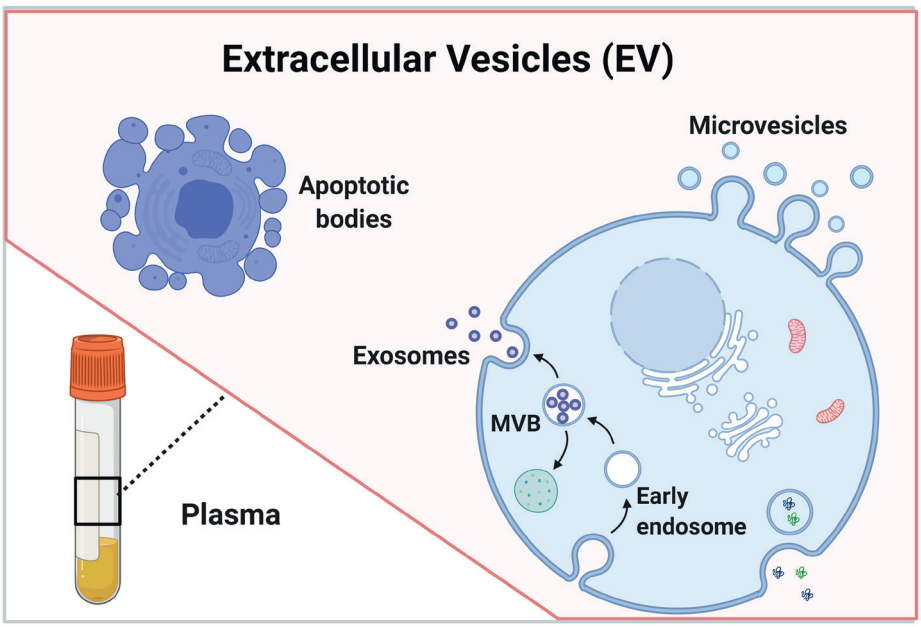

Figure 1. Overview of extracellular vesicle (EV) subpopulations. MVB = microvesicular body. Created with biorender.com

Atherosclerosis and coronary artery disease

The underlying pathophysiological mechanism that causes CAD is atherosclerosis[11]. Atherosclerosis is a long existing disease, even the Egyptian mummies showed atherosclerosis in their arteries[12]. Atherosclerosis starts at a very young age[13], yet typical symptoms emerge decades later, indicating its chronic character. Figure 2 provides an overview of the process of atherosclerosis, starting by the accumulation of plaque material within the vessel wall. At first, plaques consist mainly of fatty material, called fatty streaks (figure 2B). Evidence suggests atheroma lesions can regress up to this point. However, more often these fatty streaks form the precursor of more complex, advanced lesions as a result of fibrosis, thrombosis and calcifications within the plaques (figure 2C)[14,15].

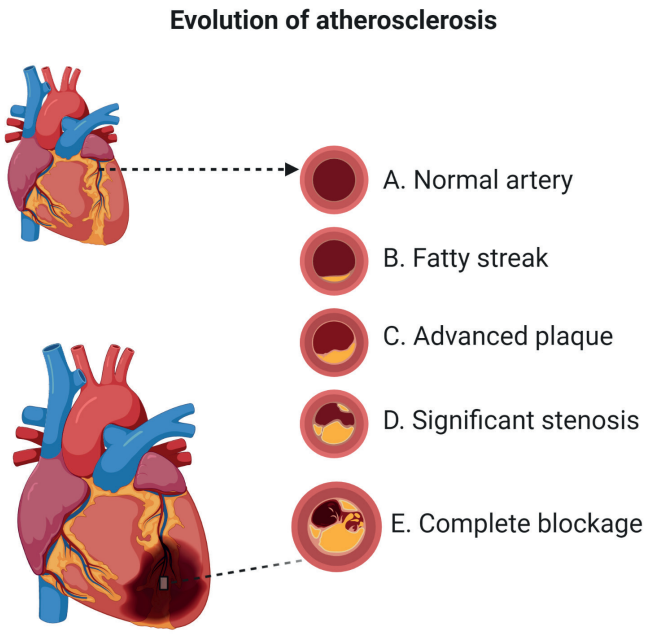

Figure 2. Evolution of atherosclerosis. Created with BioRender.com

Chronic coronary syndrome

The accumulation and formation of plaque material is often characterized by a continuous equilibrium of regression and progression of the plaque. This can be stable for longer periods but might eventually cause impaired patency of the blood vessels[16]. Because coronary vessels facilitate the blood supply to the heart muscle, this impaired patency can result in an oxygen supply-demand mismatch to the heart muscle. Most often symptoms of chest pain or dyspnoea are experienced when the diminished oxygen supply is combined with increased demand of the heart muscle (e.g. with physical exertion or emotional stress). The resulting clinical syndrome is called CCS and can exist either with obstructive or non-obstructive epicardial CAD (figure 2C and 2D)[16].

Acute coronary syndrome

When the chronic atherosclerotic process and the aforementioned equilibrium is disturbed, the plaque can become unstable, causing rupture of the plaque cap and exposure of highly thrombogenic lipid material to the blood stream. This can in turn lead to thrombus formation and acute blockage of the blood vessel lumen causing acute oxygen supply-demand mismatch and subsequently myocardial ischemia (figure 2E). The acute complete blockage of one of the main coronary arteries mostly causes

acute heavy chest pain. This clinical phenomenon is called ACS. ACS can be divided in three entities: STEMI (ST-segment elevated myocardial infarction), NSTEMI (Non-ST-segment elevated myocardial infarction) and UA (unstable angina)[17].

Current diagnostic strategy of CAD

Early recognition of CAD is important to prevent ACS and potentially modify a patients' future risk by addressing pharmacological treatment or lifestyle changes. Although the natural course of atherosclerotic disease can be asymptomatic for a long time, the most typical complaint as a result of the narrowing lumen in coronary arteries is chest pain. To determine a patients a-priori risk of having CAD several factors are investigated. Most important are the classical cardiovascular risk factors, encompassing: smoking, hypercholesterolemia, hypertension, diabetes and (family) history of CAD[18]. Additionally, a thorough anamnesis regarding the exact location, type of pain, triggering factors and presence of radiation is important. Below the current diagnostic strategy will be discussed for both CCS and ACS.

Chronic coronary syndrome

Patients often present themselves at their general practitioners with complaints differing from typical chest pain during exertion, emotion or after dinner to aspecific loss of exercise tolerance or dyspnoea. Approximately 40% of all patients presenting at their GP are referred to the hospital for further evaluation by a cardiologist[19]. The reference standard to determine CAD is a coronary angiography, however considering its invasive character, this is often used with caution[20]. Clinical evaluation by a cardiologist therefore often comprises several diagnostic tests, such as exercise ECG, CT or myocardial perfusion imaging (MPI)[19,20]. However, only ~20% of all referred patients do show stress induced ischemia of the myocardium[21]. This results in an unnecessary high healthcare burden for the patients (radiation exposure, hospital visits) and the health care system (costs, inefficient use of personnel)[22,23]. The use of a biomarker as surrogate for stress-induced ischemia is very attractive and this would serve an unmet need. Until now, no such markers exist.

Acute coronary syndrome

As mentioned, progression of CCS might lead to acute blockage of one of the coronary arteries resulting in an ACS. Timely diagnosis of ACS is essential since "time is muscle". The diagnosis is based on two things; ST-segment-deviations on ECG and/or elevated markers of myocardial damage (troponin/CK-MB). Patients showing pathognomic ST-segment elevations on ECG are referred to as STEMI patients, in case of absence of these ECG abnormalities but elevated cardiac markers a NSTEMI is diagnosed. There is a remaining population with acute chest pain typically showing no

ECG abnormalities nor elevated cardiac markers; i.e. unstable angina (UA), it remains a challenge to identify those patients who truly suffer from UA, and are at high risk for cardiovascular events within the near future[24,25]. The first aim of this thesis was therefore to investigate the role of extracellular vesicles as diagnostic biomarker in CAD.

Bone-heart axis

In addition to diagnosing a patient with either ACS or CCS at a certain time point, it is important to assign them to their appropriate "risk category" concerning future cardiovascular events. Besides traditional risk factors, the coronary artery calcium (CAC) score is the best known and most used marker to establish a patients future cardiovascular risk[26]. The underlying process causing CAC deposition is called vascular calcification (VC). One of the first men describing the process of VC was Virchow in 1863[27,28]. He called it vascular ossification at that time, since he found vascular changes in the arteries that resembles bone formation. Interestingly this process of increased calcification of the vascular wall often coexist with an opposite process occurring in the skeletal bones, called osteoporosis[29]. The association seems to be not only epidemiologically, but also pathophysiologically[30,31]. In both conditions mineralization of the extracellular matrix is observed[32]. Furthermore, in both conditions some degree of disbalance in the process of bone formation and bone degradation leads to the actual disease, which has led to the so called "bone-heart axis". The key-regulator of this process is osteoprotegerin (OPG)[33]. Studies showed that high levels of OPG are associated with a higher risk of future events[34]. In the second part of this thesis we tried to further clarify the bone-heart axis by investigating the association between EV-derived OPG, plasma OPG and CAC scores. Additionally, we used an automated deep learning method to determine CAC scores on already existing non-gated ECG images and objectified their diagnostic and prognostic performance. Ideally, further unravelling the bone-heart axis might lead to a better understanding of the pathophysiology, subsequently leading to earlier detection of patients that will develop VC which might therefore enable us to improve prevention of CAD progression. Additionally, it might also improve the prognostic risk stratification of patients with established CAD enabling the initiation of advanced add-on therapies to prevent secondary events in these patients.

THESIS OUTLINE

The subject of this thesis is coronary artery disease. We aimed to investigate the role of extracellular vesicles (EVs) in risk stratification of patients suspect for a chronic coronary syndrome (CCS). Additionally, we also studied the added value of a coronary artery calcium (CAC) score, derived with a deep learning algorithm in addition to myocardial perfusion imaging (MPI).

Part one: the diagnostic role of extracellular vesicles in coronary artery disease

The current diagnostic strategy to detect CCS often involves MPI. Despite the clear advantage of this imaging modality, they become more and more subject of debate because of costs and the high proportion of patients showing no signs of ischemia. The use of a biomarker strategy as surrogate could help to improve the diagnostic strategy. EVs are considered as potential biomarker source and have been shown to have an important role in CVDs. In **chapter 2** we investigate 6 previously determined EV-derived proteins (EV-Cystatin C, EV-Serpin C1, EV-Serpin F2, EV-Serpin G1, EV-CD14, EV-Plasminogen) and their association with CCS in 450 patients. Another manifestation of CAD is UA, as aforementioned, UA is often hard to recognize and diagnose correctly. Although the number of patients suffering from UA has declined as result of newer, more sensitive troponin assays, it remains an important group of patients who require timely diagnosis and subsequently therapy. We study the same EV-derived proteins as in **chapter** 2 and their association with UA in **chapter 3.** Despite an increasing number of publications on EVs and CVD, EVs are not incorporated in clinical practice. An overview of existing literature is provided in **chapter 4**. This chapter points out the importance of studying large cohorts after a first discovery in a smaller cohort. This will enable valid subgroup analyses and provide more insight into the behaviour and associations of EV-derived proteins in different subgroups and subsequently their potential role in clinical practice. In **chapter 5** we elaborate on some of the abovementioned points by analysing a large cohort. For this chapter we used 1000 randomly selected patients from the Basel VIII cohort study. This is a large ongoing prospective study designed to elaborate on the earlier detection of CAD. In this 1000 patients we studied our hypothesis whether EVs could be used to improve the diagnostic strategy in more detail. Since this cohort was remarkable larger compared to the cohorts used in **chapter 2** and **chapter 3** we were able to perform additional subgroup analyses.

Part two: the bone-heart axis

In the second part of this thesis we focus on investigating the bone-heart-axis. One of the best studied prognostic parameters is the CAC score. The CAC score is often seen as surrogate for VC. Although the CAC score is considered an excellent prognostic marker for future events the exact mechanism of VC remains to be elucidated. Several studies show associations between the key-regulator of this process OPG, and VC, indicating an active process rather than an epiphenomenon. Existing evidence on this topic is mostly based on population studies. In **chapter 6** we investigate the association between OPG and CAC scores in a symptomatic patient cohort. Additionally, we also assess the association between circulating EV-derived OPG and CAC scores. In patients suspected of CCS, often a choice is made for either myocardial perfusion imaging, to assess local perfusion abnormalities, or coronary CT, to assess the CAC score as surrogate of disease burden. It seems obvious that a combination of both would provide additional information, but considering the extra radiation, cost and effort that is required, this is not clinically viable. In part two of this thesis we investigate a deep learning method that derives CAC scores from the low dose CT images acquired during MPI. We assess both the diagnostic role in **chapter 7** as well as the prognostic role in **chapter 8.** A summarizing discussion is provided in **chapter 9**, future perspectives are also discussed in this chapter. The final chapter is **chapter 10**, in which a summary in Dutch is provided.

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PART I

The diagnostic role of extracellular vesicles in coronary artery disease

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CHAPTER 2

Plasma extracellular vesicle proteins are associated with stress-induced myocardial ischemia in women presenting with chest pain

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ABSTRACT

BACKGROUND

Diagnosing stable ischemic heart disease (IHD) is challenging, especially in females. Currently, no blood test is available. Plasma extracellular vesicles (EV) are emerging as potential biomarker source. We therefore aimed to identify stress induced ischemia due to stable IHD with plasma extracellular vesicle protein levels in chest pain patients.

METHODS

We analyzed 450 patients suspected for stable IHD who were referred for ^{82}Rb PET/CT in the outpatient clinic. Blood samples were collected before PET/CT and plasma EVs were isolated in 3 plasma subfractions named: TEX, HDL, LDL. In total 6 proteins were quantified in each of these subfractions using immuno-bead assays.

RESULTS

CD14 and CystatinC protein levels were independent significant predictors of stress-induced ischemia in the LDL and the HDL subfraction and SerpinC1 and SerpinG1 protein levels in the HDL fraction. Subgroup-analysis on sex revealed that these associations were completely attributed to the associations in women. None of the significant EV proteins remained significant in men.

CONCLUSION

Plasma EV proteins levels are associated with the presence of stable IHD in females presenting with chest pain. This finding, if confirmed in larger cohort studies could be a crucial step in improving diagnostic assessment of women with suspected IHD.

INTRODUCTION

Ischemic heart disease (IHD) remains one of the most common causes of death worldwide[1]. IHD comprises two most prevalent clinical syndromes: acute coronary syndrome (ACS) and stable angina/stable IHD. ACS can be quickly diagnosed in most cases by either ST elevation on ECG or elevated troponin levels. Diagnosing stable IHD is more complex with a wide variety of non-invasive tests[1,2]. Although imaging modalities provide reasonable sensitivity and specificity (80-90%) they become more and more subject of debate because of high costs, radiation exposure and increasing use in inappropriate low-risk patients[3,4]. A recently performed cost-effectiveness analysis of non-invasive imaging showed a prevalence of obstructive coronary artery disease (CAD) in only 25% of suspected stable IHD patients[5]. Another concern that merit consideration in IHD is the evolving knowledge regarding sex differences in pathophysiology, symptoms, diagnostic test performance, and prognosis. Women less frequently have obstructive CAD, yet higher mortality rates compared to men within equal age range[6–8]. Since only 25% of patients with suspected IHD appear to have IHD, the need for a novel blood based biomarker to detect stable IHD is evident.

Plasma extracellular vesicles (EVs) are relatively unexplored as biomarker source. EVs have a bilipid membrane layer. Vesicles are ~50-1000nm in size and include exosomes, micro vesicles and micro particles[9]. EVs can be produced by any cell type and consist of proteins, mRNA, miRNA and lipid particles derived from the cell of origin. EVs contain bioactive content that may influence (patho)physiological processes[10,11]. Previous studies showed associations between EV protein levels and future cardiovascular risk[12,13]. It is, however, unknown whether specific EV proteins could be used as biomarker to diagnose stable IHD. We therefore investigated if plasma EV protein levels in 3 subfractions are associated with stress-induced ischemia, in patients presenting with chest pain in the outpatient clinic.

MATERIALS AND METHODS

Study population

The MYOMARKER (MYOcardial ischaemia detection by circulating bioMARKERS) study is a prospective single-centre observational cohort study of consecutively enrolled patients (>18 years) with suspected CAD who presented at the outpatient clinic of the Meander Medical Centre (Amersfoort, the Netherlands) between August 2014 and September 2016. All patients underwent a Rubidium-82 PET/CT. The complete cohort consists of 1265 patients. For the purpose of this study a random sample of 450

patients was selected for the analysis (figure 1). The study (NL5078) was approved by the Medical Ethics Committee-United (MEC_U) and performed in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants.

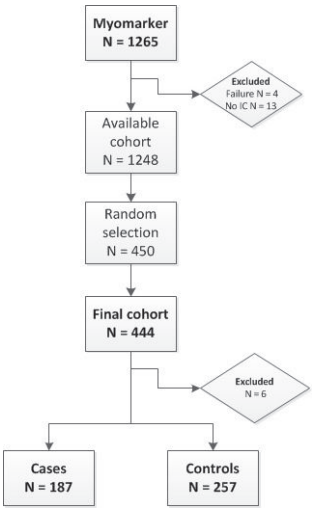

Figure 1. Flowchart patient selection. Original data from myomarker cohort. IC = Informed Consent

Primary outcome

Primary outcome of the study was stress-induced ischemia. The adjudication of the presence of the primary outcome was based on the results of both myocardial perfusion imaging (MPI), and coronary angiography (CAG) data if available. Rubidium-82 PET/CT MPI results were assessed according to the 17-segment model of the American Heart Association[14]. All scans were evaluated by 2 experienced observers. In short, the summed difference score (SDS) was the total difference between the stress and rest score for each of the 17 segments. Cases (patients with stable IHD) were defined as patients with SDS score ≥ 2 and visual agreement by both observers. Patients were considered as control if their SDS score was <2. Based on a previously performed, comparable study, we decided to add available CAG data to the MPI results to improve the diagnostic accuracy of MPI[15]. CAG images were interpreted with quantitative coronary angiography (QCA) by 2 experienced clinicians using Cardiovascular Angiography Analysis System software (CAAS 7.3, Pie Medical Imaging, Maastricht, The Netherlands). CAG data was available in 146 patients. In total 27 (6%) patients were reclassified from stress-induced ischemia to no ischemia and 11 (2%) patients were reclassified from no ischemia into having stress-induced ischemia.

Identification of proteins

Previously performed proteomics analyses was used to select the proteins[12,16]. Selected proteins were Serpin C1 (SC1), CD14, Serpin G1 (SG1), Cystatin C (CC), Plasminogen (PLG) and Serpin F2 (F2). Protein levels were determined in blood plasma and in all three EV subfractions.

Isolation of extracellular subfractions

Venous blood was collected in EDTA tubes directly before MPI from the peripheral intravenous cannula. Blood tubes were centrifuged 10 min at 1850xg at room temperature (RT) within 30 minutes after collection. Plasma was aliquoted and directly stored at -80°C. Plasma extracellular vesicle subfractions were isolated using a modified protocol based on the publication of Burstein et al.[17]. Detailed description of the isolation protocol used can be found in the supplemental materials. In short, a subset of EVs co-precipitated with Low-Density Lipid particles (LDL) while others co-precipitate with High-Density Lipid particles (HDL), which allows separation. In addition, one subfraction is analysed without the LDL and HDL subfractionation and therefore referred to as TEX subfraction. For the sequential isolation of the subfractions Dextran Sulphate (DS) (MP Biomedicals), Manganese (II) Chloride (MnCl2) (Sigma-Aldrich) solutions and Xtractt buffer (1:4) (Cavadis BV) were used (Supplemental figure 1).

Characterization of extracellular vesicles

Both the modified protocol which was used as well as extracellular vesicle characterization are described in detail in two previously published paper (especially in the supplemental materials of Zhang et al.)[16,18]. In short, we used density gradient centrifugation of the 3 plasma subfractions, all density fractions were characterized by CD9 western blot analysis as EV specific antibody. Lipid particles were identified with ApoB in all density gradient fractions. The presence of EVs was confirmed also visually with electron microscopy (EM) showing the typical bilayer EVs separated from lipid particles. The proteins studied in this manuscript (SC1, CD14, SG1, PLG, CC and SF2) were shown in the density gradient fractions that were shown with CD9 western blotting and EM, and, absent in the density gradient fractions with lipid particles. To get easy access to these data an EV-track ID was created: EV200044, in which the data is structured in a uniform way as suggested by Sluijter et al.[19].

Additional to the previous performed experiments to characterize EVs in all three subfractions, we performed a size characterization analysis using Nanoparticle Tracking Analyzer (NTA) (supplemental materials and supplemental figure 2). This

showed relatively small EVs in the TEX fraction (mean 84nm), slightly larger EVs in the LDL fraction (mean 101nm), and the largest particles in the HDL fraction (mean 120nm). All three fraction show much larger particles then LDL (22-29nm) or HDL (7-12nm) particles[20].

Quantification of EV protein levels

EV concentrations of selected proteins were measured using the Bio-plex 200 systems (Bio-Rad). Briefly, magnetic Magplex-C Microspheres (Luminex) were conjugated with antibodies specific for the proteins of interest. 50ml of the protein lysates from the EV subfractions were added to the bead-antibody complexes and were incubated for 45 minutes. Subsequently, biotin-labelled antibodies were added and again incubated for 45 minutes. Dilution of the biotinylated antibodies differed for each of the six proteins, respectively; SG1 1:200, SF2 1:400, SC1 1:100, CC 1:200, PLG 1:400 and for CD14 1:400. Bio-Plex 200 system was used for the sample analysis and calibrated each day before use, in accordance to the specifics of the manufacturer. Streptavidin-phycoerythrin (1:500) (Moss) was used to quantify the concentration. Calibration lines were created for each of the six proteins using recombinant proteins (supplemental figure 3). Washing steps were executed with a Hydrospeed plate washer (Tecan). SC1 and CD14 biotinylated antibodies were diluted in assay buffer 1x (Thermo Fischer Scientific) with human anti mouse antibody (HAMA) blocking reagent (1:90) (Fitzgerald). All other dilutions were prepared in Assay Buffer 1x. Data analysis was performed using Bio-Plex Manager Software version 6.1.1 (Bio-Rad). Antibodies and recombinant proteins are listed in supplementary table 1. Protein concentration were measured in pg/mL. Non-specific binding of the detection antibodies to the bead was verified as follows: capture antibodies of the six proteins were conjugated to six different beads (CD14+Bead 77; SC1+Bead 57; PLG+Bead 67; SF2+Bead 68; CC+Bead 27 and SG1+Bead 55) and mixed. This beadmix was incubated with one of the recombinant proteins (the amount was depended on the protein: CD14 and SF2 = 2222pg/ml; or SC1, PLG and SG1 = 8333pg/ml; or CC = 5555pg/ml) in six wells for each recombinant protein (total 36wells). In each of the six wells with the same recombinant protein one biotinylated detection antibody of the six proteins was added. No non-specific binding of the detection antibodies was found. A signal was only seen in the containing the correct combination of recombinant protein with the detection antibody for that specific protein, while other wells had a value comparable to the blanc (supplemental table 2).

Statistical analysis

Continuous data are expressed as mean ± standard deviation or median ± interquartile range, categorical data as frequencies and percentages. Differences in continuous variables were compared by independent t-test or Mann-Whitney were

appropriate. Dichotomous variables were compared by Chi-square or Fisher's exact test were appropriate. All EV proteins were standardized with the use of synthetic liposomes (SVs) to serve as internal control (detailed description can be found in the supplemental materials). To provide insight in raw data we provided baseline levels of EV proteins as measured with Bio-Plex.

Distribution of all EV proteins were visually inspected with boxplots and histograms. Because of the skewed distribution of all proteins, we logarithmically transformed them to achieve normal distributions. After transformation all distributions were visually inspected again. The SV standardized and logarithmically transformed variables were used for the logistic regression analyses. All EV proteins were tested in a univariable logistic regression model as well as in a multivariable model adjusted for known cardiovascular risk factors (sex, age, smoking, hypertension, hypercholesterolemia, diabetes mellitus and coronary artery disease). Additional adjustment was performed for cardiovascular medication separately.

We performed exploratory subgroup analyses based on sex and history of coronary revascularization, either percutaneously or with coronary bypass surgery. All subgroup analyses were adjusted for known cardiovascular risk factor (were possible). All hypotheses tests were two-sided with a critical significance level of <0.05. We did not correct for multiplicity in our study because of the clear exploratory nature of this study, as suggested by Rothman[21]. Statistical analysis was performed with R software (R software, version 3.5.1).

RESULTS

In total 444 out of the total 450 patients were analysed. Baseline characteristics are summarized in table 1. Sex and generally accepted risk factors were equally distributed between cases and controls. Patients with stable IHD were slightly older compared to patients without stable IHD (67.65 vs. 69.47, p value 0.043). History of CAD and previous complaints of angina were more common among cases (respectively 69.5% vs 37.0%, p value <0.001 and 58.8% vs. 35.4%, p value <0.001).

Raw EV-biomarker levels are shown for cases and controls (supplemental table 3). Univariate analysis showed significant difference between cases and controls for SC1 HDL (odds ratio (OR) 1.31, 95% CI: 1.04-1.66), CD14 HDL (OR 1.46, 95% CI: 1.02-2.11), CD14 LDL (OR 1.54, 95% CI: 1.09-2.16), SG1 HDL (OR 1.37, 95% CI: 1.04-1.80), CC HDL (OR 1.35, 95% CI: 1.04-1.76), CC LDL (OR 1.70, 95% CI: 1.17-2.46). Adjusted

for both cardiovascular risk factors and additional for cardiovascular medication, all biomarkers with statistically significant impact in the unadjusted analysis remained significant. Results can be found next to the univariate analysis in table 2. As can be seen in supplemental figure 4, none of the selected proteins measured in whole plasma would have the ability to distinguish between cases and controls. We performed an exploratory adjusted subgroup analysis based on sex. Raw baseline EV biomarker levels were stratified on sex are provided in the supplemental data (supplemental table 4).

Table 1. Baseline characteristics

	Control	Case	P value
n	257	187	
Demographics			
Age	67.65 (9.10)	69.47 (9.63)	0.043
%Women	65 (25.3)	46 (24.6)	0.956
BMI	27.47 (4.42)	27.18 (4.49)	0.508
Previous history			
Cardiovascular disease	237 (92.2)	176 (94.1)	0.557
Coronary artery disease	95 (37.0)	130 (69.5)	<0.001
Acute myocardial infarction	54 (21.0)	87 (46.5)	<0.001
Coronary revascularization	93 (36.2)	112 (59.9)	<0.001
Angina pectoris	91 (35.4)	110 (58.8)	<0.001
Kidney disease	4 (3.9)	6 (8.1)	0.393
Risk factor			
Smoking	37 (14.5)	38 (20.4)	0.127
Diabetes Mellitus	55 (21.4)	47 (25.1)	0.419
Hypertension	171 (66.5)	120 (64.2)	0.677
Hypercholesterolemia	151 (59.0)	113 (60.4)	0.835
Family history CAD	75 (29.5)	54 (29.3)	1.000
Medication			
Platelet inhibitors	149 (58.0)	136 (72.7)	0.002
Oral anticoagulants	50 (19.5)	42 (22.5)	0.514
Blood pressure lowering agents	209 (84.6)	170 (92.9)	0.013
Lipid-lowering agents	166 (67.2)	139 (76.0)	0.062

Values are displayed as mean ± SD or frequency(%), Case = patient with a SDS score ≥ 2 on myocardial perfusion imaging (MPI), and/or functionally relevant coronary artery disease on coronary angiogram. CAD = Coronary artery disease. CVD = history of CAD or peripheral vascular disease or history of ischemia CVA, Kidney disease = eGFR<30

Figure 2 shows the OR with corresponding confidence intervals for all EV biomarker levels stratified on sex. In addition to the proteins with significant impact for the complete study population three additional proteins reach significance among women. All proteins became insignificant among men. Supplemental figure 5 shows an additional exploratory subgroup analysis on previous coronary revascularization. Most important differences were found between patients who underwent a coronary artery bypass graft (CABG). Consistent with the results from the complete study cohort SC1 HDL, CD14 LDL and CC LDL remained significant adjusted predictors of stress-induced ischemia.

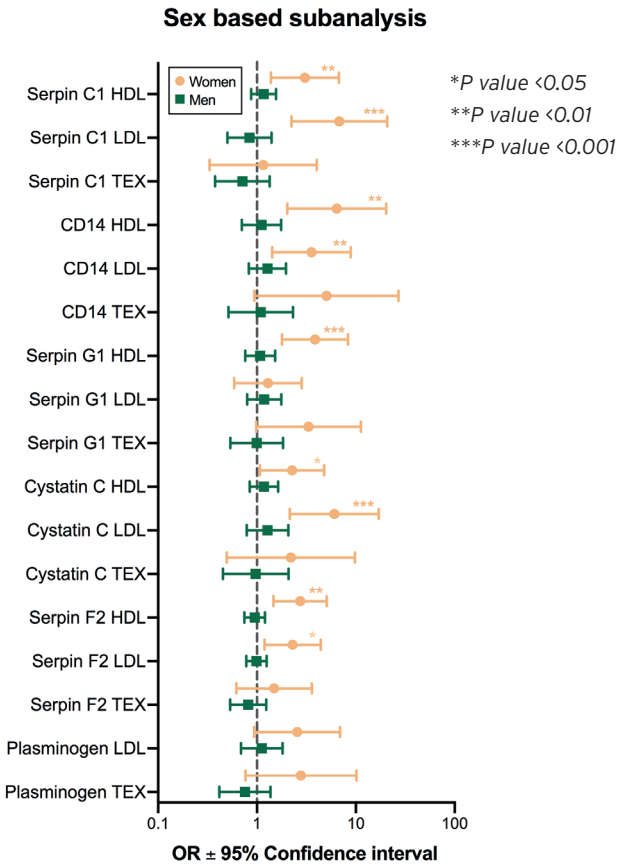

Figure 2. Forestplot of multivariable logistic regression subanalysis stratified on sex

Horizontal bars indicate adjusted+ odds ratios and corresponding 95% CI on ischemia. Biomarker levels are log-transformed and standardized per synthetic vesicle. Original assay units are pg/ml. +Adjusted for: age, hypertension, smoking, hypercholesterolemia, diabetes mellitus and coronary artery disease.

Table 2. Logistic regression analysis for stress-induced myocardial ischemia

	Unadjusted		RF adjusted[1]		RF+Med Adjusted[2]	
Biomarker	OR (95% CI)	P value	OR (95% CI)	P value	OR (95% CI)	P value
Serpin C1 HDL	1.31 (1.04-1.66)	0.024	1.38 (1.05-1.80)	0.017	1.39 (1.06-1.82)	0.017
Serpin C1 LDL	1.35 (0.91-2.01)	0.135	1.33 0.86-2.05)	0.204	1.29 (0.83-2.01)	0.252
Serpin C1 TEX	0.87 (0.52-1.44)	0.581	0.79 (0.46-1.37)	0.400	0.80 (0.46-1.39)	0.428
CD14 HDL	1.46 (1.01-2.11)	0.042	1.56 (1.04-2.35)	0.032	1.62 (1.07-2.45)	0.023
CD14 LDL	1.54 (1.09-2.16)	0.014	1.62 (1.11-2.35)	0.013	1.64 (1.12-2.41)	0.011
CD14 TEX	1.52 (0.83-2.78)	0.172	1.43 (0.74-2.75)	0.288	1.50 (0.77-2.92)	0.230
Serpin G1 HDL	1.37 (1.04-1.80)	0.027	1.42 (1.05-1.92)	0.024	1.41 (1.04-1.92)	0.028
Serpin G1 LDL	1.04 (0.76-1.43)	0.810	1.24 (0.87-1.75)	0.230	1.27 (0.90-1.81)	0.179
Serpin G1 TEX	1.08 (0.68-1.73)	0.737	1.27 (0.74-2.17)	0.393	1.25 (0.72-2.19)	0.432
Cystatin C HDL	1.35 (1.04-1.76)	0.026	1.38 (1.03-1.85)	0.034	1.41 (1.04-1.91)	0.025
Cystatin C LDL	1.70 (1.17-2.46)	0.006	1.69 (1.11-2.55)	0.014	1.70 (1.12-2.60)	0.014
Cystatin C TEX	1.45 (0.81-2.58)	0.210	1.14 (0.59-2.21)	0.694	1.13 (0.58-2.20)	0.726
Serpin F2 HDL	1.00 (0.82-1.22)	0.967	1.07 (0.87-1.32)	0.519	1.07 (0.87-1.32)	0.521
Serpin F2 LDL	0.99 (0.82-1.21)	0.945	1.03 (0.84-1.27)	0.772	1.02 (0.83-1.26)	0.825
Serpin F2 TEX	0.89 (0.63-1.24)	0.480	0.92 (0.63-1.30)	0.582	0.94 (0.65-1.36)	0.738
Plasminogen LDL	1.16 (0.78-1.71)	0.468	1.28 (0.83-1.96)	0.262	1.32 (0.85-2.04)	0.212
Plasminogen TEX	1.02 (0.63-1.64)	0.950	0.92 (0.55-1.54)	0.755	0.94 (0.56-1.57)	0.804

Biomarker levels are log-transformed and standardized per synthetic vesicle and shown as mean ±SD. Original assay units are pg/ml. RF = Risk factor. Med = medication. [1]RF adjusted; age, sex, hypertension, hypercholesterolemia, smoking, diabetes mellitus and coronary artery disease. [2]RF+Med adjusted: platelet inhibitors and blood pressure lowering agents.

DISCUSSION

We showed that EV protein levels in plasma subfractions differ between patients with and without stress-induced ischemia. Exploratory subgroup analysis revealed that the differences in biomarker levels were only seen in women. This finding emphasizes the difference between men and women and the need for sex-specific diagnostic strategies. The role of previous revascularization also needs consideration. Subgroup analysis on CABG imply that EV proteins might have a limited role in CABG patients. This could be the result of the complicated interpretation of MPI results in CABG patients, however, it is an important finding which should be considered for future studies.

Biomarkers to detect stress-induced ischemia are studied extensively. However, only few biomarkers have shown some evidence as potential biomarker. Best known are; high-sensitive cardiac troponin (hs-cTn) and BNP[22-24]. Both have shown significantly higher blood levels in patients with myocardial ischemia compared to those without. Nevertheless, their diagnostic performance remains limited. Differences in EV protein levels depict differences upon cell-level. EV content might change already in a very early stage of the disease, whereas hs-cTn and BNP are end-products of cell damage. This difference might be the explanation for the great potential of plasma EV proteins as diagnostic biomarker. Larger studies are needed to build a reliable plasma EV-based biomarker model to test whether EV proteins are able to improve clinical decision making. However, the differences of protein levels in women observed in this cohort are remarkable and may lead directly to improved patient care for women.

EV proteins levels and ischemic heart disease

Serpin C1, known as anti-thrombin is an anticoagulant protein. Its main function is inhibition of thrombus formation[25]. In our study plasma EV SC1 in the HDL subfraction was associated with stress-induced ischemia. Only one, however not comparable, previously performed study was done on plasma EV SC1, this study showed no effect of statin therapy on plasma EV protein levels of SC1[26]. CD14 is a membrane anchored protein known from its function in the innate immune system as TLR4 co-receptor[27]. Both the HDL and LDL fractionated plasma EV CD14 protein was associated with stress-induced ischemia. This is in line with previous studies on plasma EVs where CD14's role in thrombotic and inflammatory processes during CVD was shown[12,18,28].

Serpin G1, also known as C1-inhibitor, is an acute phase protein which regulates the complement activation[29,30]. Its main function is the inhibition of coagulation and atherosclerotic plaque formation[31]. In our study population plasma EV Serpin G1 in the HDL subfraction was associated with stress-induced ischemia. Previous research

on plasma EV content in cardiovascular disease showed a strong correlation between Serpin G1 and low-grade inflammation, which is known as keystone in atherosclerotic disease[28,32].

Cystatin C is an inhibitor of proteases that play a key role in inflammation. It is produced and secreted by cardiomyocytes and its synthesis is elevated when the myocardium experiences ischemia[33]. Plasma Cystatin C is known as an important marker for renal dysfunction and also for its close relationship with CAD[34–36]. De Hoog et al. showed that plasma EV-Cystatin C was associated with an acute coronary syndrome in the TEX subfraction in male patients[37]. Interestingly we also found an association of EV CC but only in the HDL and LDL subfractions. ACS significantly differs pathophysiologically from stable IHD which might explain this difference. It might also be that plasma EV protein concentrations differ between EV subfractions depending on the atherosclerotic burden and plaque stability. Future studies should provide more insight in this.

Serpin F2, known as alpha-2-antiplasmin, is a protease inhibitor and best known from its function in inhibition of plasmin, which has an important role in fibrinolysis[38,39]. The sex based subgroup analysis revealed F2 in both LDL and HDL subfraction as adjusted significant predictor for stress-induced ischemia. Plasminogen is known as precursor of plasmin, which plays a role in fibrinolysis. No association of PLG with stress-induced ischemia was found. To our knowledge only one study has been performed on EV-plasminogen, which looked into the effect of statin use on plasma EV-plasminogen levels. They found an strong association with EV-plasminogen levels after statin treatment [26].

Sex differences

Our exploratory subgroup analysis on sex showed the differences in biomarker levels between cases and controls were only seen in women. Sex differences in CVD risk are well known, but not well understood[6]. It has been proposed that inflammation, metabolic syndrome and adiposity contribute more significantly to the pathophysiology of CVD in females compared with males[40]. Our results contribute to this statement since plasma EV-Cystatin C, CD14 and Serpin G1 are known to be important proteins within the inflammatory cascade. This was also found in a previous study on EVs which showed their relation with obesity and metabolic complications[28]. The same was seen in 2 studies about the relation between plasma inflammatory markers and the development of CVD in women[41,42]. Recently, E. Lau et al. published an article on sex difference in biomarkers within the CVD field[43]. They performed a large study on >7000 patients (54% female) and examined in total 71 proteins, of

which 61 differed between men and women. The sex differences observed in our study are in line with these results. The associations found in women could be a crucial step in improving the diagnostic assessment of ischemia in this subgroup. However, these findings should be interpreted with caution and seen as hypothesis generating since this based on a subgroup analysis with low numbers. Future studies with large numbers are needed to test this hypothesis and explore the role of an EV based model improves clinical care.

Strengths and limitations

This study was a retrospective single centre analysis with myocardial perfusion analysis as reference standard for stable IHD. Since patients were included in the study after referral for perfusion imaging, indicating relative high suspicion, there will be referral and selection bias. This study has a relatively small sample size and results from subgroup analysis could only be interpreted as hypothesis generating. There are several strengths of this study. The cohort consists of real-world data in a centre with high number of outpatient clinic visits and myocardial perfusion imaging.

CONCLUSION

We showed associations between EV protein levels and stress-induced ischemia. Subgroup analysis on sex showed that all significant associations were completely attributed to women and none of them remained significant in men. Larger studies are needed to confirm our findings, but EV proteins should be considered as promising future tool to improve the diagnostic process for women with suspected stable IHD.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Conflict of interest:

CM has received research support and speakers/consulting honoraria from several diagnostic companies.

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None of the authors had any conflict or financial interest to mention.

Author contributions

MD, FW, AM, LT and DdK contributed to the conception of the design. JB, IB, AS, RS and GP contributed to the performed analysis. MD wrote the manuscript. NT, MS, JW, CM, DG, RW contributed in the first interpretation of the results and critically reviewed the manuscript. All authors reviewed the final manuscript and all authors agreed on this final version of the manuscript.

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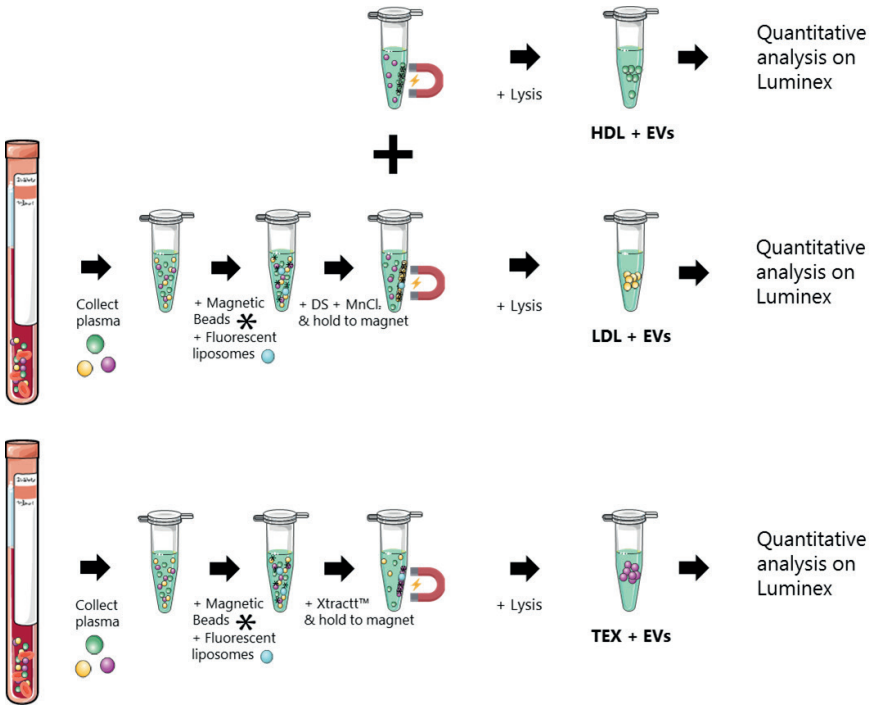
SUPPLEMENTAL MATERIALS



Supplemental Figure 1. Sequential isolation of plasma subfractions and sequential lysis and analysis of extracellular vesicles.

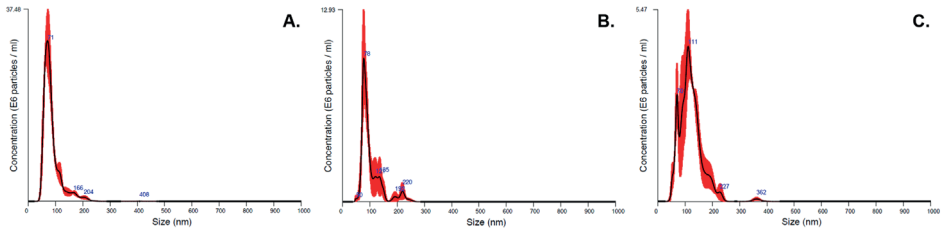
Isolation of extracellular vesicle plasma subfractions

LDL and HDL subfractions can be obtained from plasma by a DS and MnCl2 solution of DS: 0.05%, MnCl2: 0.05M and DS: 0.65%, MnCl2: 0.2M, respectively. For LDL subfraction isolation, 25uL plasma was diluted in 80uL phosphate buffered saline (PBS) (Gibco), followed by addition of 5µL magnetic beads (Nanomag®-D plain, 130mm (1:25) (Micromod)). 15µL of a standard amount of synthetic liposomes, coated with DSG-PEG2000 (Nanocs) and fluorescently labeled with 18:1 liss rhod pe (Merck), was added to each plasma sample to be able to correct for loss of the pellet during isolation. DS and MnCl2 were added into the total volume of 125 µL and were mixed. The mixture was incubated 5 min at room temperature (RT). Subsequently, the samples were placed on a bio-plex handheld magnet (Bio-Rad) and incubated 15 min at RT. The pellets were lysed with 125µL Roche complete lysis-M with protease inhibitors (Roche). To remove magnetic beads and other debris, samples were centrifugated at 3200xg, 10 min. Fluorescence of the synthetic liposomes were measured with

SpectraMax® Multi-Mode Microplate reader (Molecular Devices) directly after completion of the isolation protocol. For HDL isolation, the protocol is repeated when using 115µL supernatant above the LDL pellet. For the TEX subfraction, 25µL plasma was diluted in 80µL PBS, 5µL Nano-mag®-D PEG-OH (1:25) (Micromod) and 15µL of the synthetic fluorescent labelled liposomes. Xtractt buffer was added and the samples were mixed. The protocol is repeated following LDL isolation procedure. The pellet is used as TEX subfraction.

Nanoparticle tracking analyzer

Microparticles were characterized with the Nanoparticle Tracking Analyzer (NTA) N200 (Malvern Panalytical). Directly after EV isolation, samples were resuspended in PBS since a dilution factor was required to be measurable on the NTA (10^{7-9} particles/mL). Data about particle size distribution and concentration were collected for 3 times 30s at room temperature by the NTA Software. Results are shown in supplemental figure 2.



Supplemental figure 2. Nanoparticle tracker analyzer. Size distribution from NTA measurements in A. TEX subfraction, B. LDL subfraction and C. HDL subfraction. Distributiondata are summarized from three measurements.

Supplemental table 1. Antibodies and recombinant proteins to detect selected proteins

Detected protein	Recombinant/antibody used
CD14	- Recombinant human CD14 protein (R&D systems, #383-cd, Minneapolis, MN, USA) - Anti-human CD14 (R&D systems, #MAB3822) - Biotin labelled anti human CD14 (R&D systems, #BAF383)
Serpin C1	- Recombinant human Serpin C1 (R&D systems, #1267-PI) - Anti-human Serpin C1 (Novus Biologicals, #NBP1-05149, Centennial, CO, USA) - Biotin labelled anti-human Serpin C1 (R&D systems, #BAF1267)
Serpin G1	- Recombinant human Serpin G1 (R&D systems, #2488-pi) - Anti-human Serpin G1 (R&D, #MAB2488) - Biotin labelled anti-human Serpin G1 (R&D systems, #BAF2488)
Serpin F2	- Recombinant human Serpin F2 (R&D systems, #1470-pi) - Anti-human Serpin F2 (R&D systems, #MAB1470) - Biotin labelled anti-human Serpin F2 (R&D systems, #BAF1470)
Plasminogen	- Recombinant human Plasminogen (Sunny Lab, #P20401, Maryland, MD, USA) - Anti-human Plasminogen (Hytest, #8F11, Turku, Finland) - Biotin labelled anti-human plasminogen (Novus Biologicals, #NB120-10174B);
Cystatin C	- Recombinant human Cystatin C (R&D systems, #1196-PI-010) - Anti-human Cystatin C (R&D systems, #MAB11962) - Biotin labelled anti-human Cystatin C (R&D systems, #BAM11961)

Supplemental table 2. Non-specific binding of detection antibodies to beads

	Protein					
Bead+recombinant	Serpin G1	Serpin F2	CD14	Serpin C1	Cystatin C	Plasminogen
BM+rCD14	252	162	15221	347	114	235
BM+rSerpin C1	248	174	407	2175	124	239
BM+rSerpin G1	1435	170	431	364	127	246
BM+rSerpin F2	236	8598	422	347	125	247
BM+rPlasminogen	242	346	459	341	135	7341
BM+rCystatin C	239	171	415	347	12115	244
BM+Blanc	236	178	418	352	138	246

BM = Bead Mix; r=Recombinant; Data are given in fluorescence determined in Bioplex 200

Supplemental Table 3. Baseline table biomarker levels

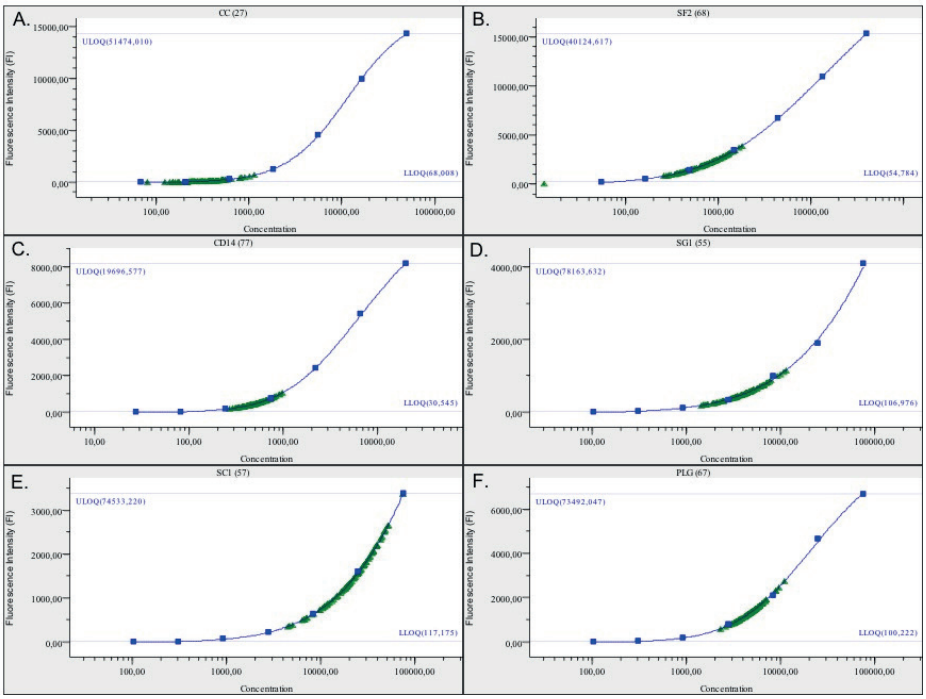
	Controls	Cases
n	257	187
Biomarker		
Serpin C1 HDL	1599700 [874794-2436300]	1615100 [1026650-2880050]
Serpin C1 LDL	7286400 [6227300-8461500]	7031600 [6271400-8438850]
Serpin C1 TEX	63651 [51552-79938]	61126 [51632-80071]
CD14 HDL	6394 [5257-8023]	6515 [5116-8221]
CD14 LDL	21558 [17208-27288]	22888 [18223-29183]
CD14 TEX	24205 [20172-28387]	25530 [21525-31592]
Serpin G1 HDL	1698300 [1250400-2506500]	1837500 [1363750-2454850]
Serpin G1 LDL	2217900 [1296400-4020300]	1986700 [1316450-3629000]
Serpin G1 TEX	155625 [123842-194833]	159038 [127929-203080]
Cystatin C HDL	1653 [1199-2699]	1802 [1312-2779]
Cystatin C LDL	13909 [11638-16432]	15178 [12498-18131]
Cystatin C TEX	44703 [37928-52203]	45561 [38521-57165]
Serpin F2 HDL	171786 [113306-218560]	158221 [107755-209066]
Serpin F2 LDL	16518 [11137-24230]	16694 [11567-23348]
Serpin F2 TEX	182840 [120703-257491]	180735 [126682-263633]
Plasminogen LDL	460528 [367072-570831]	453136 [353731-565912]
Plasminogen TEX	638970 [543764-792345]	652140 [553731-792734]

Raw biomarkers levels in pg/ml. Values are shown as median ± IQR. Case = patient with a SDS score ≥ 2 on myocardial perfusion imaging (MPI), and/or functionally relevant coronary artery disease on coronary angiogram

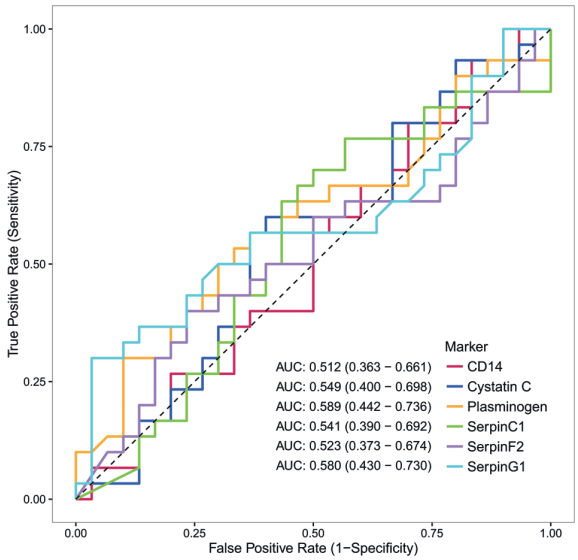
Supplemental Table 4. Sex specific baseline biomarker levels

	Men control	Men case	Women control	Women case
n	192	141	65	46
Biomarker				
Serpin C1 HDL	1470150 [758809-2297725]	1510200 [916266-2413800]	1942700 [1446500-2688500]	2186800 [1254875-3232275]
Serpin C1 LDL	7470250 [6318025-8646225]	7020600 [6149700-8423600]	6831100 [6003300-8029000]	7208350 [6442550-8574875]
Serpin C1 TEX	60637 [50790-78036]	59831 [50950-75966]	67434 [52571-83018]	66142 [54088-85369]
CD14 HDL	6151 [5107-7741]	6234 [4969-7832]	7015 [5775-8817]	7362 [6240-9402]
CD14 LDL	20754 [17155-25077]	22098 [17825-27389]	24311[19240-30468]	25415 [20721-30893]
CD14 TEX	23762 [19820-27651]	24787 [20778-31180]	26239 [21017-29567]	27032 [23258-32670]
Serpin G1 HDL	1677650 [1205000-2558575]	1802500 [1310400-2302300]	1710900 [1335000-2352500]	2184700 [1630000-3157600]
Serpin G1 LDL	2022150 [1184150-3553525]	1986700 [1372100-3540100]	2571300 [1715600-4828600]	2012050 [1121575-3831525]
Serpin G1 TEX	153714 [123521-192380]	154287 [121473-204311]	167343 [127631-204509]	175613 [137774-201934]
Cystatin C HDL	1587 [1173-2637]	1773 [1243-2707]	1940 [1287-2897]	1914 [1485-3140]
Cystatin C LDL	14018 [11868-16293]	15203 [12471-18253]	13562 [11437-16653]	15101 [12605-17992]
Cystatin C TEX	44195 [37549-52912]	45444 [37887-57252]	45088 [38791-51440]	46801 [41711-56144]
Serpin F2 HDL	176112 [113692-221701]	158576 [105260-197965]	164757 [113306-206687]	158205 [124351-228865]
Serpin F2 LDL	16827 [11188-25148]	16766 [11180-22879]	15649 [11079-22302]	16392 [12473-26074]
Serpin F2 TEX	182596 [119966-257389]	173786 [123599-257779]	201913 [133497-257491]	197794 [144200-275583]
Plasminogen LDL	466211 [368607-571308]	461961 [366585-591729]	449127 [362876-560086]	414538 [296258-482069]
Plasminogen TEX	632888 [535633-778020]	642386 [539671-781082]	671643 [569467-822916]	688261 [594694-841418]

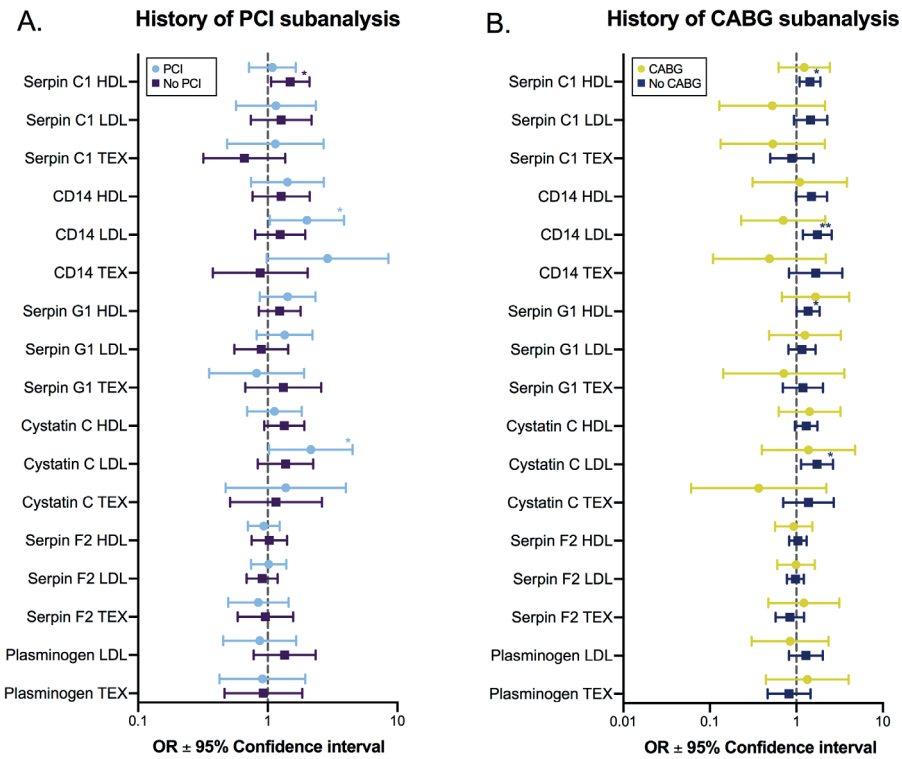
Raw biomarkers levels in pg/ml stratified on sex. Values are shown as median ± IQR. Case = patient with a SDS score ≥ 2 on myocardial perfusion imaging (MPI), and/or functionally relevant coronary artery disease on coronary angiogram



Supplemental figure 3. Calibration lines bioplex 200. Calibration lines for all six proteins; 3A. Cystatin C, 3B Serpin F2, 3C CD15, 3D Serpin G1, 3E Serpin C1 and 3F Plasminogen.



Supplemental Figure 4 ROC plasma proteins. Plasma levels of selected proteins and their diagnostic ability with AUC and 95% confidence interval



Supplemental Figure 5. Forestplot of subanalysis on A. history of PCI and B. CABG

Horizontal bars indicate adjusted⁺ odds ratios and corresponding 95% CI on ischemia. Biomarkerlevels are logtransformed and standardized per synthetic vesicle. Original assay units are pg/ml. ⁺Adjusted for: age, hypertension, smoking, hypercholesterolemia, diabetes mellitus and coronary artery disease. PCI = Percutaneous Coronary Intervention. CABG = Coronary Artery Bypass Graft. *Indicates P value <0.05, **P <0.01, ***P<0.001

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CHAPTER 3

Extracellular vesicle cystatin c is associated with unstable angina in troponin negative patients with acute chest pain

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ABSTRACT

BACKGROUND

Despite the use of high-sensitive cardiac troponin there remains a group of high-sensitive cardiac troponin negative patients with unstable angina with a non-neglectable risk for future adverse cardiovascular events, emphasising the need for additional risk stratification. Plasma extracellular vesicles are small bilayer membrane vesicles known for their potential role as biomarker source. Their role in unstable angina remains unexplored. We investigate if extracellular vesicle proteins are associated with unstable angina in patients with chest pain and low high-sensitive cardiac troponin.

METHODS

The MINERVA study included patients presenting with acute chest pain but no acute coronary syndrome. We performed an exploratory retrospective case-control analysis among 269 patients. Cases were defined as patients with low high-sensitive cardiac troponin and proven ischemia. Patients without ischemia were selected as controls. Blood samples were fractionated to analyse the EV proteins in three plasma-subfractions: TEX, HDL and LDL. Protein levels were quantified using electrochemiluminescence immunoassay.

RESULTS

Lower levels of (adjusted) EV cystatin c in the TEX subfraction were associated with having unstable angina (OR 0.93 95% CI 0.88-0.99).

CONCLUSION

In patients with acute chest pain but low high-sensitive cardiac troponin, lower levels of plasma extracellular vesicle cystatin c are associated with having unstable angina. This finding is hypothesis generating only considering the small sample size and needs to be confirmed in larger cohort studies, but still identifies extracellular vesicle proteins as source for additional risk stratification.

INTRODUCTION

An Acute Coronary Syndrome (ACS) remains a major cause of disability and death worldwide[1–3]. ACS comprises three clinical phenotypes: ST-Elevated Myocardial Infarction (STEMI), non-ST-Elevated Myocardial Infarction (NSTEMI) and Unstable Angina (UA). With the use of contemporary high-sensitive cardiac troponin (hsTn) assays, the early and rapid diagnosis of myocardial infarction has improved. As a consequence, the number of patients suffering from UA decreased and the number of NSTEMI patients increased. In 2003, 42% of ACS was due to UA, after implementation of hsTn assays in 2010, this number was decreased to 28%[4]. Recent studies show an incidence of UA of 7-9%[5–8]. In contrast, however, the SWEDEHEART registry showed a relative increase of patients with UA with 13% after the implementation of hsTn[9]. Either way, UA has not (yet) become a rare diagnosis after implementation of newer more sensitive troponin assays.

For a long time NSTEMI and UA were often considered to exist along a continuous spectrum. They are thought to represent the same patient characteristics, underlying pathophysiology and outcome, in therapeutic guidelines they are even considered as one entity[1,2]. However, recent studies show substantial differences between UA and NSTEMI patients with regards to incidence and mortality. Moreover, the non-existence of myocardial injury in UA patients even suggests differences in pathophysiology[10]. Several studies showed higher rates of future MI and coronary revascularisation in UA patients compared with non UA/non ACS patients[10–12]. These findings emphasise the need for additional risk stratification to identify patients with low hsTn levels but true UA, since they have a non-neglectable risk of future adverse cardiovascular events[7].

One way to improve risk stratification, would be the use of biomarkers. A relative unexplored biomarker source are plasma extracellular vesicles (EVs). EVs are approximately 50-1000nm in size, contain a lipid bilayer membrane, and include exosomes, microvesicles and micro particles[13]. All human cells are able to produce EVs. EVs contain a bioactive content (mRNA, miRNA, proteins and lipid particles) reflecting the cell of origin. Previous studies have shown their role in (patho)physiological processes[14,15], as well as associations between specific EV plasma proteins and future cardiovascular risk[16,17]. The role of EV proteins in patients with acute chest pain and low levels of high sensitive cardiac troponin I (hs-cTnI) is unknown. We therefore aimed to investigate whether five selected proteins (serpin C1, CD14, serpin G1, cystatin c and serpin F2) in three different plasma subfractions are associated with UA in patients presenting with acute chest pain and low levels of hsTn.

METHODS

Study population

This study is an exploratory subanalysis of the prospectively collected data from the MINERVA study. Study details are described in detail previously[18]. Shortly, consecutive patients >18 years presenting with acute chest pain at the emergency room (ER) were included. STEMI patients were excluded from participation and additionally, NSTEMI patients were excluded from this sub analysis. Patients were enrolled in the Meander Medical Centre (Amersfoort, the Netherlands) between January 2012 and June 2014. Written informed consent was obtained from all participants. The study has been approved by the Medical Ethics Committee United (MEC-U) and is conform the Declaration of Helsinki. Our primary aim was to compare patients with proven unstable angina (UA) with patients with chest pain but without ACS or proven ischemia (non-UA). The presence of ACS was determined according to the leading ESC guidelines[19]. We performed a nested case-control analysis in which all cases (UA) were selected and twice as much random controls (Fig 1).

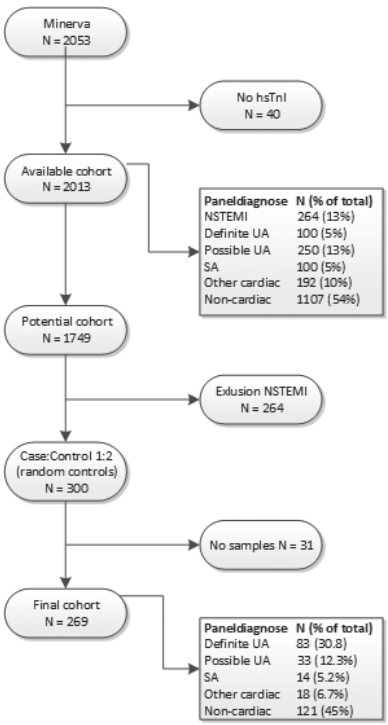

Figure 1 flowchart study population.

NSTEMI=Non ST-elevated Myocardial Infarction. UA = unstable angina. SA = stable angina.

Data collection

Clinical data, e.g. symptoms at presentation, medical history, cardiovascular risk factors, ECG and results from additional testing were all collected and recorded in an online electronic case record form. Clinical decision making was left to the attending cardiologist. Venous blood was collected at presentation at the ER. Blood tubes were centrifuged 10 minutes at 1850xg at room temperature after collection and directly stored at -80°C.

Adjudication of final diagnosis

Adjudication of the final diagnosis was done by two independent cardiologists from the Meander Medical Centre. A third cardiologist was incorporated in case of disagreement. To adjudicate the final diagnosis all available diagnostic information during the index visit (hs-cTnI, CK-MB, ECG, discharge letters) as well as hospital information (e.g. radiology reports, echocardiography, non-invasive ischemia detection, or coronary angiograms) were considered. Non-invasive ischemia comprised evidence of ischemia on rest ECG, exercise tests, myocardial perfusion imaging or coronary CT. To determine whether or not these tests were positive for ischemia, general guidelines were used. Patients were diagnosed in one of the following five groups: (1) Definite UA, (2) Possible UA, (3) Stable Angina, (4) Other cardiac or (5) non cardiac. Definite UA was defined as patients with typical symptoms: AP at rest; deterioration of previous stable angina and proven ischemia in combination with low hs-cTnI (<60ng/L, since this was the clinical cut-off for MI in the referring hospital). Hs-cTnI was measured with the access AccuTnI+3 Troponin I assay on the UniCel DxI immunoassay System (Beckman Coulter, Brea, CA, Limit of detection was 10ng/L, 99th percentile was 42ng/L coefficient of variation <10%). Possible UA was defined as typical symptoms but without objective evidence of ischemia. Patients with definite UA were defined as cases, all the other diagnosis were defined as controls.

Extracellular vesicle protein analysis

Identification of the selected proteins: serpin C1 (SC1), CD14, serpin G1 (SG1), cystatin c (CC) and serpin F2 (SF2) is based on previously performed proteomics analysis [16,20]. All proteins were determined and measured in three subfractions. Detailed description of the isolation and quantification procedure can be found in the supplemental materials (S1 Appendix 1 and S1 Fig 1). In short, for this analysis samples were thawed and EV's were isolated in three subfractions. A subset of EVs are co-isolated with Low-Density Lipid particles (LDL) while others co-isolate with High-Density Lipid particles (HDL), which allows separation. In addition, one subfraction is analysed without the LDL and HDL fractionation and is referred to as TEX. 15µL of a standard amount of synthetic liposomes, coated with DSG-PEG2000 (Nanocs) and fluorescently labelled with 18:1

liss rhod pe (Merck), was added to each plasma sample to be able to correct for loss of the pellet during isolation. For the sequential isolation of the subfractions Dextran Sulphate (DS) (MP Biomedicals), Manganese (II) Chloride (MnCl2) (Sigma-Aldrich) solutions, Xtractt buffer (1:4) (Cavadis BV) and Nanomag®-D plain, 130mm (1:25) (Micromod) or Nano-mag®-D PEG-OH (1:25) (Micromod) in case of TEX were used. Coefficient of variation (CV) was determined for each subfraction, e.g. HDL 9.6%, LDL 6.5% and TEX 6.8%. Full characterization was described previous, to get easy access to this data an EV-Track was created (EVTRACK200044). Quantification of the amount of the selected proteins was performed with an electrochemiluminescence immunoassay (Quickplex SQ120, Meso Scale Discovery, MSD) on specific designed 96-wells plates. Protein concentrations were measured in pg/mL.

Statistical analysis

Continuous data are expressed as mean ± standard deviation or median with interquartile range, according to the distribution of the data. Categorical data are shown as frequencies with corresponding percentages. Differences in continuous data were compared by either independent t-test or Mann-Whitney U. Dichotomous variables were compared by Chi-square or Fisher's exact test where appropriate. All EV proteins were standardised per synthetic vesicles and were transformed to achieve normal distributions. Log transformation was performed for CD14 HDL and LDL subfraction, CC HDL subfraction, SC1 HDL and TEX subfraction, SF2 HDL and LDL subfraction and SG1 HDL subfraction. Square root transformation was performed for CD14, CC and SF2 in the TEX subfraction, SC1 LDL subfraction and SG1 LDL and TEX subfraction. Uni- and multivariable logistic regression analysis were performed to determine associations between the selected proteins and UA. The multivariable logistic regression analysis was adjusted for sex and the HEART score. The HEART score consists of 5 components: History of complaints, ECG abnormalities, Age, Risk factors and Troponin (S1 Table 1). The HEART score was chosen, since this is a validated clinical prediction tool, specifically designed to stratify patients with acute chest pain in the ER and their risk for future cardiovascular events[21]. Since there are no clinical cut-off values known, we additionally determined an optimal cut-off value for the significant proteins in order to dichotomise all patients as either high or low. For this we first created a logistic regression model with cystatin c TEX in it and obtained the predicted probabilities for each patient. These values were used to plot the sensitivity and specificity of the protein against each other to determine the optimal cut point (S2 Fig2). The (adjusted) OR to identify UA patients will be evaluated again with this new variable. All analyses were performed with R Studio (R Software, version 3.5.1)

RESULTS

All patients were included in the Meander Medical Centre in Amersfoort, The Netherlands, between January 2012 and June 2014. The complete cohort included 2053 patients, in 40 patients no hsTnI was available. The incidence of definite UA was 5% and NSTEMI was present in 13%. In this study all N(STEMI) patients were excluded, we compared patients with definite UA with twice as much, randomly selected, non-UA patients. In 31 patients (17 cases and 14 controls) there was not enough plasma obtained to perform the EV analysis. The final cohort for this analysis was therefore 269 patients (Fig 1).

Baseline characteristics of definite UA patients vs. random non-UA controls are summarised in table 1. The mean age of patients with definite UA was 64 and 26.5% were women, both were not statistically different from the non-UA control patients (63 and 24.4%). Patients with definite UA more often had a history of coronary artery disease (59.0% vs. 38.7%, p value 0.003), and coronary revascularisation by percutaneous coronary intervention (44.6% vs. 28.5%, p value 0.015). Patients with UA had more often hypertension (61.4% vs. 46.8%, p value 0.036) had a significantly higher HEART score compared to non-UA patients (4.72 vs. 3.89). Among UA patients the use of aspirin and P2Y12-inhibitors was more common compared to patients without definite UA (62.7% vs. 39.2%, p value 0.001, and 26.5% vs. 13.4% p value 0.015, respectively).

We compared baseline levels of EV proteins among cases and controls. S2 Table 2 shows the results of this comparison. Baseline levels only differed for serpin G1 in the TEX subfraction (7.75 (1.50) vs. 7.39 (1.13), p value 0.049). In addition, we performed a univariate and multivariable logistic regression analysis of which the results can be found in table 2. After adjustment for sex and the HEART score (a clinical prediction tool including traditional risk factors, see also S2 table 2), a significant association between cystatin c in the TEX subfraction and having UA was found (OR 0.93 95% CI 0.88-0.99). We determined the optimal cut-off (S2 Fig 2) and we dichotomised the levels of cystatin c (TEX subfraction) for each patient in either high or low. After dichotomisation, cystatin c in the TEX subfraction remained a significant predictor of unstable angina, we found an adjusted OR of 0.41 (95%CI: 0.22-0.70).

Table 1. Baseline characteristics

	Non-UA	UA	P value
n	186	83	
Demographics			
Age	63 (12)	64 (11)	0.429
%Women	45 (24.2)	22 (26.5)	0.801
BMI	26.90 (4.10)	26.75 (3.65)	0.776
Previous history			
Coronary artery disease	72 (38.7)	49 (59.0)	0.003
Coronary revascularization	62 (33.3)	42 (50.6)	0.011
%CABG	19 (10.2)	10 (12.0)	0.814
%PCI	53 (28.5)	37 (44.6)	0.015
Kidney disease	1 (0.5)	0 (0.0)	1.000
Risk factors			
Smoking	48 (25.8)	20 (24.1)	0.884
Hypertension	87 (46.8)	51 (61.4)	0.036
Hypercholesterolemia	61 (32.8)	38 (45.8)	0.057
Diabetes Mellitus	25 (13.4)	15 (18.1)	0.423
Family history of CAD	59 (31.7)	32 (38.6)	0.340
Heart Score	3.89 (1.27)	4.72 (1.11)	<0.001
Drug therapy			
Aspirin	73 (39.2)	52 (62.7)	0.001
P2Y12-inhibitors	25 (13.4)	22 (26.5)	0.015
Statin	91 (48.9)	52 (62.7)	0.051
ACE/AT-Inhibitor	81 (43.5)	40 (48.2)	0.566
B-blocker	76 (40.9)	45 (54.2)	0.057
Calcium Channel Blocker	29 (15.6)	19 (22.9)	0.203

Values are displayed as mean(sd) or frequency(%), UA = Unstable Angina Pectoris, Non UA contains: other cardiac chest pain CABG = coronary artery bypass graft, PCI = Percutaneous Coronary Intervention, CAD = Coronary artery disease. ACE = Angiotensin Converted Enzyme, AT = Angiotensine.

Table 2. Logistic regression analysis for unstable angina

	Univariate			Multivariable*		
Biomarker	**OR**	**95% CI**	**P value**	**OR**	**95% CI**	**P value**
CD14 HDL	0.94	0.61-1.46	0.787	0.80	0.51-1.28	0.360
CD14 LDL	1.26	0.68-2.32	0.455	1.17	0.60-2.29	0.649
CD14 TEX	0.97	0.90-1.05	0.420	0.95	0.88-1.03	0.234
Cystatin C HDL	0.96	0.82-1.14	0.652	0.85	0.71-1.02	0.085
Cystatin C LDL	0.74	0.42-1.32	0.310	0.63	0.34-1.18	0.15
Cystatin C TEX	0.95	0.90-1.01	0.090	**0.93**	**0.88-0.99**	**0.015**
Serpin C1 HDL	0.99	0.71-1.37	0.935	0.91	0.65-1.28	0.601
Serpin C1 LDL	0.99	0.99-1.01	0.594	0.99	0.99-1.01	0.774
Serpin C1 TEX	0.78	0.44-1.37	0.387	0.93	0.51-1.69	0.799
Serpin F2 HDL	0.43	0.16-1.18	0.101	0.44	0.14-1.32	0.141
Serpin F2 LDL	0.76	0.27-2.14	0.603	0.88	0.28-2.77	0.827
Serpin F2TEX	0.98	0.96-1.01	0.110	0.99	0.96-1.01	0.191
Serpin G1HDL	0.66	0.39-1.12	0.121	0.68	0.39-1.21	0.189
Serpin G1LDL	0.98	0.88-1.11	0.800	0.99	0.88-1.14	0.988
Serpin G1TEX	0.82	0.68-1.01	0.051	0.85	0.69-1.05	0.123

EV protein levels are displayed as mean(sd). Original assay units were pg/mL. Proteins were transformed to achieve a normal distribution and standardized per synthetic vesicle. Log transformation: CD14HDL and LDL, CC HDL and LDL, SC1 HDL and TEX, SF2 HDL and LDL, SG1 HDL. Square root transformation: CD14TEX, CCTEX, SC1LDL, SF2TEX, SG1LDL.and TEX *Adjusted for age, sex and heart score (containing: History, ECG, Age, Risk factors, Tropnin) at admission.

DISCUSSION

In this study we aimed to investigate the potential diagnostic role of EV proteins in patients with acute chest pain and UA but low levels of hs-TnI. We performed a retrospective exploratory analysis measuring EV proteins in plasma subfractions in a large cohort of patients presenting with acute chest pain at the ER. This study showed that EV cystatin c in the TEX subfraction is associated with unstable angina independent of clinical factors represented by the HEART score and sex. In the last years hs-troponine have shown excellent diagnostic accuracy to detect patients with myocardial necrosis/injury[22–24]. The largest disadvantage of hs-troponin measurements is that they often require serial meetings and are therefore time consuming and expensive[25]. Besides, diagnosing patients with low (serial) troponin measurement, but true UA remains a difficult challenge. There seems no benefit of early revascularisation or intensified antiplatelet therapy in terms of mortality in

UA patients, but a considerable amount of UA patients does show obstructive CAD requiring planned revascularisation. They also show higher chances of future MI, indicating the importance of identifying these patients.

Incidence of UA

The incidence of definite UA in our (complete) cohort was 5%. This is in line with recently published results by Puelacher et al., who investigated the incidence and outcomes of UA patients compared to NSTEMI patients in 8992 patients from the international APACE study and patients from a stepped wedge cluster RCT (4739 patients) which is still ongoing (HighSTEACS)[10]. However, wide ranges of incidence rates are reported, most likely as a consequence of an absent universal definition for UA[7]. Most guidelines use the absence of elevated troponin, or absence of rise and fall in combination with typical symptoms[1,26,27]. This is reflected in our cohort as well, since 13% of the patients were adjudicated as "possible UA", meaning typical symptoms, absence of evidence for myocardial injury but non conclusive presence of ischemia.

Plasma EV cystatin c

We found plasma EV protein cystatin c in the TEX subfraction (adjusted for confounders) to be associated with the presence of definite UA among patients with chest pain and low levels of hs-TnI. After determination of an optimal clinical cut-off and dichotomising of patients in either high or low this became even more pronounced. Cystatin c is an inhibitor of proteases that play a key role in inflammation. It is produced and secreted by cardiomyocytes and its synthesis is elevated when the myocardium experiences ischemia[28]. Plasma cystatin c is known as an important marker for renal dysfunction and also for its close relationship with CAD[29–31]. De Hoog et al. also showed that EV cystatin c was associated with ACS in the TEX subfraction in male patients[32]. Our Study has relatively low numbers, prohibiting to investigate whether the association of plasma EV cystatin c differed among sex. Considering the exploratory nature of this study, also no conclusions with regards to diagnostic properties of this marker can be made based on these results. Our findings should be considered as hypothesis generating only.

Biomarkers and ischemic heart disease

Several new biomarkers have been proposed as marker for the early detection of ACS. Best known are; heart-type fatty acid-binding protein (hFABP), Copeptin, hs-CRP, Natriuretic peptides, ischemia modified albumin (IMA) and GDF-15[25]. However, none of them are elevated as early as hs-TnI or as specific as troponin. miRNA's have been studied extensively as well, but also lack sufficient evidence and are often very costly[33]. In contrast to end-products of cell stress (e.g. BNP, Copeptin) differences

in EV protein levels represent differences upon cell-level. EV content might change already in a very early stage of the disease depicting more subtle but also more chronic conditions of disease. This difference might be the explanation for the great potential of EV proteins as diagnostic biomarker. EVs have shown to play an important role in the development and progression of atherosclerotic disease[34-37]. EV proteins have also been shown to be associated with cardiometabolic risk in the Framingham cohort[38] and are independent predictors for future cardiovascular events[16,17]. In this study we have shown that there is a potential role for EV cystatin c in the detection of UA in patients with acute chest pain and low levels of troponin. Although our results are promising, future studies are needed to investigate whether the use of EV cystatin c could also improve clinical decision making and thereby improve patient care.

Strengths and limitations

This study was a sub analysis from a prospective single centre study among acute chest pain patients. Although this was a single centre study, the Meander Medical Centre is known as a large hospital with high numbers of patients which makes the study results easily generalisable to the general 'acute chest pain' patient. A limitation of the study is the relatively small sample size, therefore the results of this study need to be interpreted with caution. A clear strength is the feasibility and clinical potential of the method used. The complete analysis could be performed within 2 hours in any clinical lab and only one droplet of blood is needed. The fractionation is relatively cheap and is suitable for automation. The readout is a standard readout immunobased assay (e.g. ELISA, MSD or Luminex). Thus, considering the use of existing, commonly used products and the possibility for automation of the isolation method we think the clinical implementation of our analysis method would be feasible. But first, larger studies are needed to confirm our findings and determine the diagnostic properties of this new potential biomarker.

CONCLUSION

Despite the use of hsTn, UA remains important and patients require additional therapy. Therefore, additional risk stratification in patients with acute chest pain and low levels of hsTn is needed. In this exploratory, hypothesis generation study, we showed an association between low levels of EV plasma cystatin c and the presence of UA in patient with acute chest pain and low levels of hsTnI. Larger studies are needed to confirm our findings but EV plasma proteins should be considered as a promising tool for additional risk stratification in acute chest pain patients.

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Disclosures
The authors report no conflict of interest.

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None

Supporting information files
1. **S1 Appendix 1** supplemental methods regarding the isolation method
2. **S1 Table 1** Heart score algorithm
3. **S2 Table 2** Baseline Extracellular Vesicle protein levels

The following supplemental material is omitted due to space limitation and can be found at the journal website:
1. **S1 Fig 1 Extracellular vesicle analysis procedure**
   Sequential isolation of plasma fractions and subsequential lysis and analysis of extracellular vesicles
2. **S2 Fig 2 Performance diagram to determine optimal cut-off values**
   Optimal cut-off decision curve for cystatin C and definite UA as determinant

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SUPPLEMENTAL MATERIALS

S1 Appendix

Supplemental method

Isolation of extracellular vesicle plasma subfractions

The isolation process of extracellular vesicles from plasma is shown in S1 Fig1. LDL and HDL subfractions can be obtained from plasma with Dextrane Sulphate (DS) and Manganese Chloride (MnCl2) in different solutions. For DS: 0.05%, MnCl2: 0.05M and DS: 0.65%, MnCl2: 0.2M, respectively. For LDL subfraction isolation, 25uL plasma was diluted in 80uL phosphate buffered saline (PBS) (Gibco), followed by addition of 5µL Nanomag®-D plain, 130mm (1:25) (Micromod). 15µL of a standard amount of synthetic liposomes, coated with DSG-PEG2000 (Nanocs) and fluorescently labeled with 18:1 liss rhod pe (Merck), was added to each plasma sample to be able to correct for loss of the pellet during isolation. DS and MnCl2 were added into the total volume of 125 µL and were mixed. The mixture was incubated 5 min at room temperature (RT). Subsequently, the samples were placed on a bio-plex handheld magnet (Bio-Rad) and incubated 15 min at RT. The pellets were lysed with 125µL Roche complete lysis-M with protease inhibitors (Roche). To remove magnetic beads and other debris, samples were centrifugated at 3200xg, 10 min. Fluorescence of the synthetic liposomes were measured with SpectraMax® Multi-Mode Microplate reader (Molecular Devices) directly after completion of the isolation protocol. For HDL isolation, the protocol is repeated when using 115µL supernatant above the LDL pellet. For the TEX subfraction, 25µL plasma was diluted in 80µL PBS, 5µL Nano-mag®-D PEG-OH (1:25) (Micromod) and 15µL of the synthetic fluorescent labelled liposomes. Xtractt buffer was added and the samples were mixed. The protocol is repeated identical to the LDL isolation procedure. The pellet is used as TEX subfraction.

S1 Table 1 Heart score algorithm

Supplemental table 1. HEART score algorithm

Variable	Description	Score
History	Highly syspicious	2
	Moderately suspicious	1
	Slightly/not suspicous	0
ECG	Significant ST depression	2
	Nonspecific repolarization disturbances	0
	Normal	1
Age	≥65 years of age	2
	45-65 years of age	1
	≤45 years of age	0
Risk factors	≥3 Risk factors', or history of CVD[1]	2
	1 or 2 risk factors	1
	No risk factors	0
Troponin[2]	≥3 times normal limit	2
	1-2 times normal limit	1
	≤ normal limit	0

'Hypertension, Diabetes Mellitus, current smoking, hypercholesterolemia, family history of coronary artery disease and obesity (BMI > 30). [1]CVD = cardiovascular disease: history of myocardial infarction, previous coronary revascularization, stroke or peripheral artery disease. [2]Troponin levels were measured with the Access AccuTnI+3 Troponin I assay on the UniCel DxI Immunoassay System (Beckmann Coulter, Brea, CA). The cutoff for MI was set at >60 ng/L at the coefficient of variation <10%. The limit of detection was 10 ng/L, and the 99th percentile cut−off point of 42 ng/L.

S2 Table 2 Baseline Extracellular Vesicle protein levels

Supplemental table 2. Baseline Extracellular Vesicle protein levels

	Non-UAP	UAP	P-value
Biomarker	186	83	
CD14 HDL	4.27 (0.56)	4.24 (0.68)	0.788
CD14 LDL	5.53 (0.41)	5.57 (0.44)	0.467
CD14 TEX	16.11 (3.38)	15.74 (3.64)	0.420
CC HDL	3.76 (1.51)	3.67 (1.63)	0.653
CC LDL	6.22 (0.46)	6.16 (0.44)	0.310
CC TEX	23.98 (4.93)	22.89 (4.70)	0.088
SC1 HDL	5.91 (0.75)	5.90 (0.90)	0.935
SC1 LDL	129.08 (35.45)	126.49 (40.07)	0.595
SC1 TEX	4.50 (0.48)	4.45 (0.36)	0.380
SF2 HDL	8.90 (0.27)	8.84 (0.25)	0.100
SF2 LDL	9.21 (0.26)	9.19 (0.23)	0.605
SF2TEX	94.29 (14.68)	91.35 (11.69)	0.108
SG1HDL	3.39 (0.46)	3.28 (0.58)	0.114
SG1LDL	9.24 (2.31)	9.17 (1.94)	0.800
SG1TEX	7.76 (1.50)	7.39 (1.13)	0.049

EV protein levels are displayed as mean(sd). Proteins were transformed to achieve a normal distribution and standardized per synthetic vesicle. Log transformation: CD14HDL and LDL, CC HDL and LDL, SC1 HDL and TEX, SF2 HDL and LDL, SG1 HDL. Square root transformation: CD14TEX, CCTEX, SC1LDL, SF2TEX, SG1LDL and TEX

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CHAPTER 4

Extracellular vesicles in diagnosing chronic coronary syndromes

The bumpy road to clinical implementation

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ABSTRACT

Coronary artery disease (CAD), comprising both acute coronary syndromes (ACS) and chronic coronary syndromes (CCS), remains one of the most important killers throughout the entire world. ACS is often quickly diagnosed by either deviation on an electrocardiogram or elevated levels of troponin, but CCS appears to be more complicated. The most used noninvasive strategies to diagnose CCS are coronary computed tomography and perfusion imaging. Although both show reasonable accuracy (80–90%), these modalities are becoming more and more subject of debate due to costs, radiation and increasing inappropriate use in low-risk patients. A reliable, blood-based biomarker is not available for CCS but would be of great clinical importance. Extracellular vesicles (EVs) are lipid-bilayer membrane vesicles containing bioactive contents e.g., proteins, lipids and nucleic acids. EVs are often referred to as the "liquid biopsy" since their contents reflect changes in the condition of the cell they originate from. Although EVs are studied extensively for their role as biomarkers in the cardiovascular field during the last decade, they are still not incorporated into clinical practice in this field. This review provides an overview on EV biomarkers in CCS and discusses the clinical and technological aspects important for successful clinical application of EVs.

INTRODUCTION

Coronary artery disease (CAD) remains one of the most important killers among the entire world, despite tremendous improvements in diagnostic and therapeutic strategies[1]. CAD comprises acute coronary syndromes (ACS) and chronic coronary syndromes (CCS, e.g., stable angina). The underlying pathophysiology that causes CAD is known as atherosclerosis[1]. This is a longstanding, continuous process of accumulation and progression of plaque material within the vessel wall[2]. Atherosclerotic plaques are often stable for long periods and can eventually cause a diminished oxygen supply to the heart muscle during exertion. This causes ischemia and subsequent chest pain[3]. The resulting clinical syndrome is known as CCS, for which medical or interventional therapies are generally required. Plaque rupture or plaque erosion initiates an acute thrombotic luminal occlusion that can cause acute blockage of one the coronary vessels, resulting in ACS and, subsequently, myocardial infarction[4]. ACS requires immediate revascularization of the affected vessel.

ACS is often quickly diagnosed with either an abnormal electrocardiogram (ECG) or elevated cardiac biomarkers, such as high-sensitive cardiac troponin (hs-cTn), indicating cell damage of the myocardium. Diagnosing CCS appears to be more complicated. Figure 1 provides an overview of the diagnostic workflow of a patient presenting with chest pain at the general practitioner and the diagnostic possibilities once referred to the cardiologist. The reference standard for CCS is still coronary angiography (CAG), but considering its invasive character, this is used with caution[1,5].

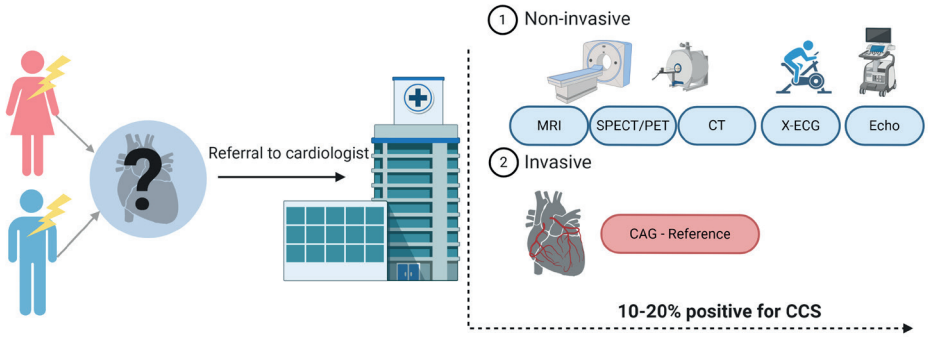

Figure 1. Diagnostic track of patients with chest pain suspected for a chronic coronary syndrome (CCS). Patients suspected of CCS are often referred to a cardiologist. Patients undergo either noninvasive or invasive tests. The choice for one of the tests is based on the pre-test probability of a patient having CCS and availability in the hospital. Created with BioRender.com.

Currently, the most used noninvasive strategies are coronary computed tomography (CT) or myocardial perfusion imaging (MPI). The diagnostic accuracy of these different imaging modalities is relatively high (80–90%), but only 10–20% of symptomatic patients turn out to have CCS[6]. The low number of patients suffering from the actual disease are the result of an increasing use of these test modalities in a low-risk population[7–9]. They are becoming more and more subject of debate because of unnecessary radiation exposure for the patient and high costs. A reliable, blood-based biomarker would therefore be important to improve the diagnostic strategy around patients suspected for CCS. Until now, no such biomarker exists.

Since the early 1960s, there is a growing interest for extracellular vesicles (EVs) as potential biomarker sources[10] EVs are lipid bilayer membrane vesicles containing bioactive contents (e.g., proteins, lipids and nucleic acids)[11]. Almost all cells are able to produce EVs, with their contents changing when the cell of origin changes due to (patho)physiology[12,13]. Due to this, EVs are often referred to as the "liquid biopsy". The ability to study their (variable) contents makes them an interesting source for future biomarkers.

In this review, we first provide an overview of the performance of existing plasma biomarkers in CCS. Second, we review the existing evidence with regards to the additional value that EV biomarkers might have in diagnosing CCS. Last, despite an increasing number of publications regarding EVs as biomarker, the use of EVs in the cardiovascular field is not yet fully established. The use of EVs were recently incorporated into clinical practice in the cancer field of medicine[14–16]. We highlight several clinical aspects that need to be addressed in future studies to accelerate successful clinical implementation of EVs in the cardiovascular field.

CURRENT DIAGNOSTIC PLASMA BIOMARKERS IN CCS

The use of biomarkers to detect CCS are studied extensively. Multiple promising markers were identified using a proteomics or metabolomics approach. However, new markers often fail when applied to an external and/or different population[17–19]. The focus of this review is on proteins and their function as biomarker, however, RNA, DNA or other cell particles in theory could also function as biomarkers.

Single plasma biomarker approach

After the successful implementation of high-sensitive cardiac troponin (hs-cTn) to diagnose ACS, identification of biomarkers with a similar accuracy as hs-cTn for other coronary pathologies such as CCS received a lot attention. Many different markers for

CCS have been proposed, best known are; natriuretic peptides, high-sensitive cardiac troponin (hs-cTn), and C-reactive protein (CRP).

Natriuretic peptides

Natriuretic peptides, both B-type (BNP), and the N-terminal of the prohormone (NT-proBNP) are secreted as result of myocardial stretch[15]. Two studies investigated the diagnostic potential of natriuretic peptides in patients with stable angina who underwent CAG[20,21]. Weber et al. found NT-proBNP as an independent predictor for obstructive CCS in a small cohort study of 94 patients. They found an area under the curve (AUC) of 0.72 at a cutoff level of 214 pg/mL[20]. Additionally, a larger, comparable study performed in 781 patients found the same association but different cutoff points for men (85pg/mL), with an AUC of 0.72, and women (165pg/mL), with an AUC of 0.71[21]. Both studies excluded patients with known heart failure or left ventricular ejection fraction of <60%. A meta-analysis performed in 2009 included 14 studies with a total of 2784 participants. They found a pooled sensitivity for the detection of stress-induced myocardial ischemia of 71% for (NT-pro)BNP, however the pooled specificity was only 52%[22]. The performance of (NT-pro)BNP was consistent throughout different studies but remains limited compared to clinical models[23–27]. Jensen et al. showed an overview of five commonly used clinical risk scores and their performances in a large cohort of 5414 patients[28]. The AUCs of all clinical models varied between 0.68–0.72. Since most BNP studies also showed AUCs of ~0.70, the limited value of BNP on top of clinical models is not surprising. This was also seen when the performance of BNP (AUC 0.66) was compared with a clinical judgement score (AUC 0.66)[29].

High-sensitive cardiac troponin

Hs-cTn is well known for its role in diagnosing ACS, and, since it is a marker of cell damage caused by myocardial ischemia, it might also be helpful in diagnosing CCS. Higher levels of hs-cTn in patients without ACS were observed in patients that were older, had high systolic blood pressure, an increased left ventricular mass and/or renal impairment[30]. Hs-cTn was shown to be associated with the severity of CAD on CAG[31,32]. Moreover, a modest increase in AUC (0.79 to 0.80) to detect CCS in addition to a clinical judgement score was found[33]. However, this finding was not replicated in other large cohorts[29]. Tanglay et al. investigated the incremental value of a single hs-cTn measurement to rule out stress-induced myocardial ischemia and found an AUC to detect stress-induced ischemia with hs-cTn of 0.70 compared to an AUC of 0.69 from their clinical judgement model (*p* value = not significant)[34].

C-reactive protein

CRP is an inflammatory marker, but also an acute-phase protein, and considered to be a nonspecific marker of inflammation[35]. Among all inflammatory biomarkers studied in CAD, CRP requires the most attention; unfortunately, the value of CRP to diagnose CCS appears to be limited[19,36]. The association between CRP and the extend of CAD was studied in a large cohort (>2500 participants) referred for CAG because of typical chest pain[37]. Only very modest correlation coefficients between CRP and CAD severity were found (r: 0.02–0.08). Another study investigating the diagnostic potential of CRP failed to show a statistically significant association between plasma CRP levels and obstructive CAD[38]. Large Mendelian randomization studies analyzing polymorphisms of the CRP gene also did not provide evidence of a causal relationship between CRP and CAD[39–41].

Multimarker approach

After it was recognized that a single biomarker approach might not be able to improve the accuracy of clinical models to detect CCS, multimarker models were introduced. The idea behind a multimarker approach is the ability to combine different markers, all representing different pathophysiological pathways, thereby providing complementary information. Studies investigating a multimarker approach in diagnosing CCS are limited. One study investigated a dual-biomarker strategy to detect CCS[29], comparing the diagnostic accuracy of a clinical judgement score with BNP and hs-cTn. The addition of hs-cTn to the clinical judgement score significantly improved the diagnostic accuracy (AUC: 0.68 to 0.75), however, a dual marker strategy did not further improve the diagnostic accuracy. Although multimarker models are studied in more detail regarding the prognosis of CAD patients, until now, the incremental value of multimarker models in future risk stratification was disappointing. Wang et al. studied 3532 patients from the Framingham Offspring Study and found that a high multimarker score was independently associated with both the outcome death as well as major adverse cardiovascular events (MACE)[42]. The multimarker score for death comprised CRP, NT-proBNP, homocysteine, plasma renin and urine albumin-to-creatinine ration. For MACE, two markers were selected: NT-proBNP and urine albumin-to-creatinine ratio. However, no significant differences in C-statistics were found when comparing a model with clinical predictors (death: 0.80, MACE: 0.76) with a multimarker model (death: 0.82, MACE: 0.77). Another study among >5000 patients without known cardiovascular disease (CVD) analyzed the predictive ability of both single and multimarker models on top of clinical predictors[43]. They analyzed two outcomes, namely, coronary events (selected markers were MR-proADM and NT-proBNP) and MACE (selected markers were CRP and NT-proBNP). Only a very modest increase in C-statistic (0.007 for MACE and 0.009 for coronary events) was found when using a multimarker model compared to a model with clinical predictors. Also,

no significant reclassification of patients into higher or lower risk categories was found. Comparable results were found in studies with patients with manifest CVD[44,45]. Nevertheless, a multimarker approach could be the solution for a future CCS marker, but perhaps from another, relatively unexplored source, such as EVs.

EV ORIGIN

Extracellular vesicles are characterized by a bilayer lipid membrane layer[11]. EVs were reported for the first time in 1946 by Chargaff and West[46], however, they were first recognized by Peter Wolf in 1967[10]. He observed EVs at that time as "platelet dust". Following his endeavor, a lot of knowledge on EVs has emerged since then. Almost all different cell types are able to produce and release EVs. EVs are found systemically and in basically all body fluids, including: blood, urine, cerebrospinal fluid, milk, tears and saliva[47-54]. Characterization and classification of subpopulations has been subject of debate for the last years and still no consensus is reached[55,56]. As a common feature all subpopulations of EVs contain bioactive contents (lipids, proteins, nucleic acids). EV contents originate from the parent cell they are released from[57,58]. Once released into the extracellular space, parts of them can be identified to serve as cell-cell communicators.

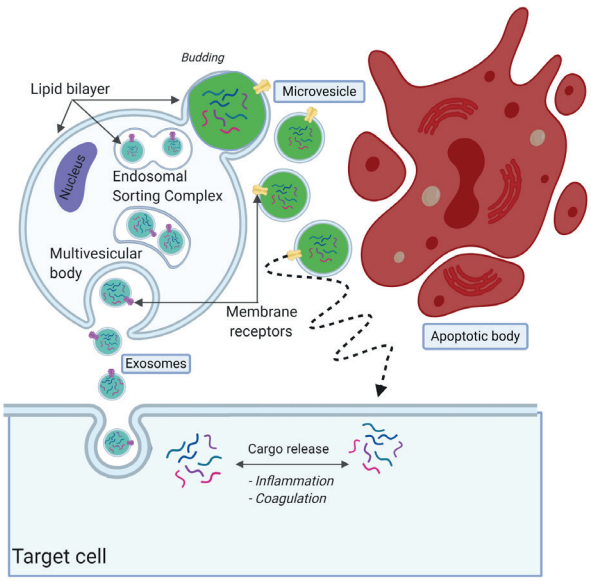

Figure 2. Overview of extracellular vesicle (EV) subpopulations and formation routes. EVs are often divided into three subpopulations, namely, exosomes, microvesicles and apoptotic bodies. Exosomes are considered the smallest population, released by fusion with the plasma membrane. Microvesicles are secreted by blubbing, as can be seen in green. Lastly, apoptotic bodies are fragments released from cells during apoptosis, considered to be the largest in size. Created with BioRender.com.

EV subpopulations

Although it is an ongoing debate regarding how to classify the EV subpopulations, EVs are often divided in three subtypes based on their size and formation route, namely, apoptotic bodies, microvesicles and exosomes[59–61] (Figure 2). There is no consensus on specific identifying protein markers to distinguish between the three subpopulations[62–64]. Exosomes are considered as the smallest particles in the EV family, with a size of 30–150nm[65]. The release and formation of exosomes is via the endosomal sorting complex release transport (ESCRT) pathway[66]. They are formed as intraluminal vesicles and mature into multivesicular bodies (MVBs)[57]. MVBs fuse with the outer plasma membrane to be released within the extracellular space[67]. It was suggested that multiple subpopulations of exosomes exist, potentially providing additional information on their origin and role[65]. Microvesicles are EVs that form by outward budding, sometimes called blubbing, of the cell membrane. Their size is approximately between 100nm and 1000nm[59–61,68,69]. The last subpopulation of EVs are the apoptotic bodies, which are released after cell death. They are >1000nm in size and relatively large compared to exosomes and microvesicles.

EVS AS DIAGNOSTIC BIOMARKERS IN ATHEROSCLEROSIS

Atherosclerosis is considered to be the underlying syndrome of cardiovascular disease. EVs are considered to be key mediators in both the atherosclerotic plaque formation and its progression. EVs are thought to be involved in inflammation and thrombus formation and are therefore thought to carry useful information to serve as biomarkers[70,71]. Clinical risk factors associated with CAD are diabetes mellitus, hypertension, metabolic syndrome, hypercholesterolemia and smoking[72,73]. Several studies showed higher levels of circulating EVs in plasma to be associated with some of these risk factors[74,75]: diabetes[76,77], hypertension[78,79], hypercholesterolemia[80,81], and smoking[82–84]. Moreover, associations between EVs and subclinical atherosclerosis (diagnosed with ultrasound from the femoral artery, carotid artery or abdominal aorta) were also found[85,86]. Within CVD, EVs are most studied as prognostic markers in CAD[87–89]. However, less is known about their diagnostic potential for CCS.

Extracellular vesicle count in CCS

One way to analyze plasma EV levels is to measure the number of circulating EVs, also described as count. There is an increasing number of publications on EVs in the cardiovascular field, however the subject of this review is the presumed role of EVs

specifically in CCS. We focus specifically on their diagnostic potential in CCS patients. Table 1 provides a preselected overview of studies that investigated the role of EV counts of different subpopulations based on cellular origin with regard to CCS. Some of the studies are described below in more detail. Chironi et al. showed that the number of circulating leukocyte-derived EVs (LDEVs) were independently associated with subclinical atherosclerosis[85]. A study among 33 postmenopausal women undergoing coronary calcium scoring on coronary CT showed a positive association between the number of circulating EVs and both the Framingham risk score (FRS) as well as coronary calcium scores[86]. The effect of our circadian rhythm on the levels of circulating EVs was studied by a Scandinavian group in 30 patients, of which 10 had CCS and 20 patients were healthy controls[90]. They found a slight variation in total circulating EV count and the circadian rhythm, but no effect was seen for platelet-derived EVs.

CCS is characterized by stress-induced ischemia. Augustine et al. showed an increase in circulating EVs after dobutamine stress echocardiography, except for the patients with signs suggestive for stress-induced ischemia[91]. Sinning et al.[92], found a similar result of diminished EV release after stress-test imaging in patients with significant CCS, emphasizing a dynamic process of EV release. Several studies showed differences in the number of circulating (subpopulations of) EVs between patients with CCS and healthy controls, but also between patients with CCS and ACS[93–98]. These were however, mainly studies in small cohorts and were often cross-sectional. Mirachi et al. compared levels of two species of endothelial-derived EVs (EDEVs) (CD31+ and CD51+) between 84 patients with CAD (64 ACS and 20 CCS) and 42 healthy controls[93]. Levels of CD31+ EDEVs differed significantly between the ACS, CCS and controls, whereas CD51+ EDEVs only differed between CAD versus control, however no differences between ACS and CCS were observed. Additionally, this study also investigated levels of platelet-derived EVs (PDEVs), showing only elevated levels in patients with ACS. No differences were seen between CCS and ACS or CCS and controls[93]. Another study performed by Biasucci et al. compared levels of EDEVs, PDEVs and circulating EVs (cEVs) in 76 patients[97]. In this study population, 33 patients were diagnosed with CCS and 43 with ACS. All EV subpopulations were found in significantly higher levels in patients with ACS compared to patients with SA. They also investigated whether the levels changed over time, which was seen only for the total amount of circulating EVs[97]. There are contradicting results regarding circulating EV levels and the degree of luminal stenosis. Werner et al. showed a significant (adjusted) correlation between levels of circulating EVs and luminal stenosis[99], whereas two other studies did not[98,100]. Only a few studies investigated the diagnostic or prognostic properties of the number of circulating EVs in CCS patients. The largest study was performed by Nozaki et al[88] showing in 378 CCS patients that endothelial-derived EVs were an independent predictor for MACE (hazard ratio (HR): 1.35; 95% confidence

interval (CI): 1.09–1.65). Their prognostic model had an AUC of 0.73 and included the FRS as a clinical prediction rule and plasma biomarkers (CRP and BNP). After addition of the total count of endothelial-derived EVs, this increased to an AUC of 0.76. These findings were in line with other comparable studies showing the same results[87,89,101].

A different way to analyze subpopulations of EVs is to divide them based on density. This concept of EV separation was derived from a study showing a reduced amount of EVs in patients with familial hypercholesterolemia who underwent LDL apheresis[102]. EV subpopulations are still relatively unexplored and need to be studied in more detail to provide answers on the biological and pathophysiological functions[103]. They could, however, reflect different origins and a better signal-to-noise ratio, thereby providing additional information

Extracellular vesicle content

When the (patho)physiological circumstances of a cell change, not only the number of EVs secreted by this cell changes, but also their content[11,104]. Compared with the count, data on the role of EV content in CCS is limited. Both EV nucleotides as well as protein content were described in CVD[104]. RNA quantification relies on the very sensitive and established technology of qPCR[105]. RNA has, however, the disadvantage of rapid degeneration by RNAse, which is present at high levels in blood. Further, mRNA levels often do not correlate with encoded proteins or reflect the ongoing biological process. Protein levels reflect much closer the ongoing process, and quantification is done by using immunoassays that are commonly used in clinical laboratories. Therefore, we think that proteins have the largest potential in diagnosing CCS[11,104].

One of the first studies that looked into the EV proteome was performed by Vélez et al. The main focus of the study was to explore whether they could study the proteome of EVs by comparing 10 ST-ElevationMyocardial Infarction (STEMI) patients with 10 CCS patients[106]. They found 117 differentially regulated proteins between the two groups, indicating a potential source for protein markers. Another study compared EV-protein levels between STEMI patients and healthy controls[107]. Protein differences were analyzed with a proximity extension assay (Olink, CVD-II panel, N = 92) on the EV lysates and plasma. They identified three proteins (chyotripsin C, tyrosine-protein kinase (SRC) and C-C chemokine ligand 17) that showed differences in levels in EVs but not in plasma. Validation in another set of STEMI patients, CCS patients and healthy controls exposed CRS to be significantly associated with the degree of CAD. This finding was not found in plasma, indicating the additional diagnostic value of EVs.

The myomarker study cohort consisted of consecutive patients presenting with stable chest pain at the outpatient clinic of the Meander Medical Centre in the Netherlands. Details on the study design and study population can be found in a previous publication[108]. For this study, a case control analysis of 44 men suspected of CCS was performed. Cases were defined as patients with stress-induced ischemia determined with MPI. Controls were matched based on age and general cardiovascular risk factors (supplemental Table S1). In this cohort, we performed proteomics on EV subpopulations (rather than a total EV population) based on density since we hypothesized that this would provide a more detailed view of the cell condition. For this, we separated two subpopulations (called the HDL subpopulation and LDL subpopulation, respectively), as described in the study of Wang et al.[109]. We analyzed in the HDL- and LDL subpopulations using both the cardiometabolic panel as well as the cardiovascular III panel (Olink, Proteomics, Uppsala University Sweden). Each panel consisted of 92 proteins known for their associations with CVD. We identified the three most promising proteins (Cathepsin D, CD31 and NT-proBNP) based on literature, their diagnostic properties, and the availability of antibodies (supplemental Table S2). Using the Meso Scale Discovery (MSD) immunoassay, we confirmed our findings. Figure 3A–C shows boxplots of the MSD results for the selected three proteins in the EV-HDL subpopulation. The results for the LDL subpopulation are summarized in Figure 2D-F. It can be appreciated that in the EV-LDL subpopulation, protein levels of Cathepsin D, CD31 and NT-proBNP were significantly higher in cases compared with controls. In the HDL subpopulation, only NT-proBNP-protein levels were found to be significantly different between cases and controls. Our results therefore show the potential of using the Olink technology for the enrichment of EV proteins in EV subpopulations, followed by confirmation in an established immunoassay. For EV-based diagnosis of CCS, not many data exist. A recent study investigated whether a selected group of EV-proteins were associated with CCS[108]. EV-Serpin C1, EV-CD14, EV-Serpin G1, EV-Serpin F2 and EV-Cystatin C (mostly in the HDL-subpopulation) were shown to be independently associated with the presence of stress-induced ischemia. The prognostic value of EV-protein content in a large CVD cohort was for described for the first time by Kanhai et al.[110]. They found EV-Cystatin C, EV-Serpin F2 and EV-CD14 protein levels to be independently associated with future cardiovascular events. A different study found an independent association between the extent of CVD and the levels of EV-CD14[111]. Several other studies investigated the role of EV content in ACS, heart failure, unstable angina and manifest CVD[112–114].

Table 1. Overview of publications on extracellular vesicle counts in chronic coronary syndrome patients including details on subpopulations

Study characteristics			
Name, year	*N (%Male)*	*Design*	*Population*
Jayachandran 2008	33 (0)	Cross	Newly postmenopausal women undergoing CT CAC
Christersson, 2015	30 (53)	CC	CCS pts (CAG+) vs. healthy controls
Augustine, 2014	119 (45)	Co	Consecutive pts undergoing DSE
Sinning, 2016	80 (71)	Co	Consecutive pts undergoing DSE and CAG
Tan, 2009	89 (49)	CC	CCS pts referred for CAG

Design: Cross = Cross-sectional; Co = Cohort; Long = Longitudinal; CC = Case Control. Population: CAC = Coronary Artery Calcium; CCS = Chronic coronary syndrome; CAG = Coronary angiography (+ indicates proven with this modality); DM = Diabetes Mellitus; ACS = Acute Coronary Syndrome; pts = patients; DSE = Dobutamine Stress Echocardiography. Subpopulation: cEV = Circulating EV; PDEVs Platelet-derived EVs;

Extracellular vesicles			Study findings	
Subpopulation	*Identifier*	*Method*		Ref
cEVs	AnnexinV+	FC	Higher in women with high CAC, associated with FRS	[86]
PDEVs	CD61+/CD42a+	FC	Higher in women with high CAC, associated with FRS	
GDEVs	CD11b+	FC	NS, NA	
MDEVs	CD14+	FC	NS, NA	
EDEVs	CD62e+/AnnexinV+	FC	Higher in women with high CAC, associated with FRS	
cEVs	AnnexinV+	FC	Slight circadian variation	[90]
PDEVs	CD41+/CD62+	FC	NA	
EDEVs	CD144+/CD14+	FC	Sign. higher levels in the morning	
cEVs	AnnexinV+	FC	Sign. rise&fall after DSE in patients without ischemia	[91]
PDEVs	CD31+/CD41+	FC	Sign. rise&fall after DSE in patients without ischemia	
EryDEVs	CD235a+	FC	Sign. rise&fall after DSE in patients without ischemia	
EDEVs	CD31+/CD41-, CD62e+,CD106+	FC	Sign. rise&fall after DSE in patients without ischemia	
LDEVs	APC+	FC	NS	
GDEVs	CD66b+	FC	NS	
MDEVs	CD14+	FC	NS	
EDEVs	AnnexinV+/CD31+	FC	Decrease after DSE in patients with ischemia	[92]
MDEVs	AnnexinV+/CD14+	FC	Decrease after DSE in patients with ischemia	
PDEVs	AnnexinV+/CD31+/ CD42b+	FC	NS	
PDEVs	CD61+/CD42b+	FC	Sign higher in CCS, NA with severity of luminal stenosis	[98]

EDEVs = Endothelial-derived EVs; GDEVs = Granolycyt-derived EVs; MDEVs = Monocyte-derived EVs; EryDEVs = Erythrocyte-derived EVs; LDEV = Leukocyte-derived EVs; TFDEVs = TF+-derived EVs. Method: FC = Flowcytometry; PA = Protrombinase Assay. Study findings: FRS = Framingham Risk Score; Sign = Significant p value < 0.05. NR = Not reported; NS = Not significant; NA = Not associated.

Table 1. Continued

Study characteristics			
Name, year	*N (%Male)*	*Design*	*Population*
Stęogonekpień, 2012	30 (73)	CC	CCS pts vs. ACS vs. control pts no CCS criteria defined
Biasucci, 2012	76 (74)	Obs	CCS pts referred for CAG vs. ACS
Mizrachi, 2003	39 (69)	CC	CCS pts vs. ACS vs. controls
Mallat, 2000	52 (69)	NR	CCS (CAG+) vs. ACS vs. Non cardiac controls
Werner, 2005	50 (68)	Co	CCS (CAG+), acetylcholine
Song, 2015	73 (45)	Co	CCS pts undergoing CAG
Nozaki, 2009	378 (61)	Long	CCS pts (CAG+ or >2riskfactors)
Sinning, 2011	200 (70)	Long	CCS pts (CAG+)

Design: Cross = Cross-sectional; Co = Cohort; Long = Longitudinal; CC = Case Control. Population: CAC = Coronary Artery Calcium; CCS = Chronic coronary syndrome; CAG = Coronary angiography (+ indicates proven with this modality); DM = Diabetes Mellitus; ACS = Acute Coronary Syndrome; pts = patients; DSE = Dobutamine Stress Echocardiography. Subpopulation: cEV = Circulating EV; PDEVs Platelet-derived EVs;

	Extracellular vesicles			**Study findings**	
	Subpopulation	*Identifier*	*Method*		Ref
	PDEVs	CD42+	FC	CCS vs. control NS. ACS vs. CCS Sign	[96]
	LDEVs	CD45+	FC	CCS vs. control NS. ACS vs. CCS Sign	
	MDEVs	CD14+	FC	CCS vs. control NS. ACS vs. CCS Sign	
	EDEVs	CD31+,CD34+,CD51+/ CD61+	FC	CCS vs. control NS. ACS vs. CCS Sign	
	TFDEVs	CD142+	FC	CCS vs. control NS. ACS vs. CCS Sign	
	cEVs	CD31+/AnnexinV+	FC	CCS vs. ACS Sign. Sign decrease over time	[97]
	PDEVs	CD31+/CD42b+	FC	CCS vs. ACS Sign. NS decrease over time	
	EDEVs	CD31+/CD42b-	FC	CCS vs. ACS Sign. NS decrease over time	
	EDEVs	CD31+, CD51+	FC	CCS vs. control Sign	[93]
	PDEVs	CD42+	FC	NS (any subgroup)	
	cEVs	AnnexinV+	PA	CCS vs. Control Sign. ACS vs. CCS Sign	[95]
	NR	CD3+	NR	NS	
	NR	CD11a+	NR	NS	
	NR	CD31+	NR	NS	
	NR	CD146+	NR	CCS vs. Control Sign. ACS vs. CCS Sign	
	NR	GP-Ib+	NR	NS	
	EDEVs	CD31+/AnnexinV+	FC	Sign. (adjusted) correlation with luminal stenosis	[99]
	EDEVs	CD144+/AnnexinV+	FC	Intermediate lesion vs. no lesion Sign. Not correlated with degree of stenosis	[100]
	EDEVs	CD144+	FC	Independently associated with MACE HR1.35 (95% CI 1.09-1.65)	[88]
	EDEVs	CD31+/AnnexinV+	FC	Independently associated with MACE HR 2.3 (95% CI 1.3-3.9)	[87]

EDEVs = Endothelial-derived EVs; GDEVs = Granolycyt-derived EVs; MDEVs = Monocyte-derived EVs; EryDEVs = Erythrocyte-derived EVs; LDEV = Leukocyte-derived EVs; TFDEVs = TF+-derived EVs. Method: FC = Flowcytometry; PA = Protrombinase Assay. Study findings: FRS = Framingham Risk Score; Sign = Significant p value < 0.05. NR = Not reported; NS = Not significant; NA = Not associated.

Table 1. Continued

Study characteristics			
Name, year	*N (%Male)*	*Design*	*Population*
Koga, 2005	234 (57)	CC	CCS pts (CAG+) +DM vs. control
Hu, 2014	33 (48)	CC	CCS pts (CAG+) vs. control

Design: Cross = Cross-sectional; Co = Cohort; Long = Longitudinal; CC = Case Control. Population: CAC = Coronary Artery Calcium; CCS = Chronic coronary syndrome; CAG = Coronary angiography (+ indicates proven with this modality); DM = Diabetes Mellitus; ACS = Acute Coronary Syndrome; pts = patients; DSE = Dobutamine Stress Echocardiography. Subpopulation: cEV = Circulating EV; PDEVs Platelet-derived EVs;

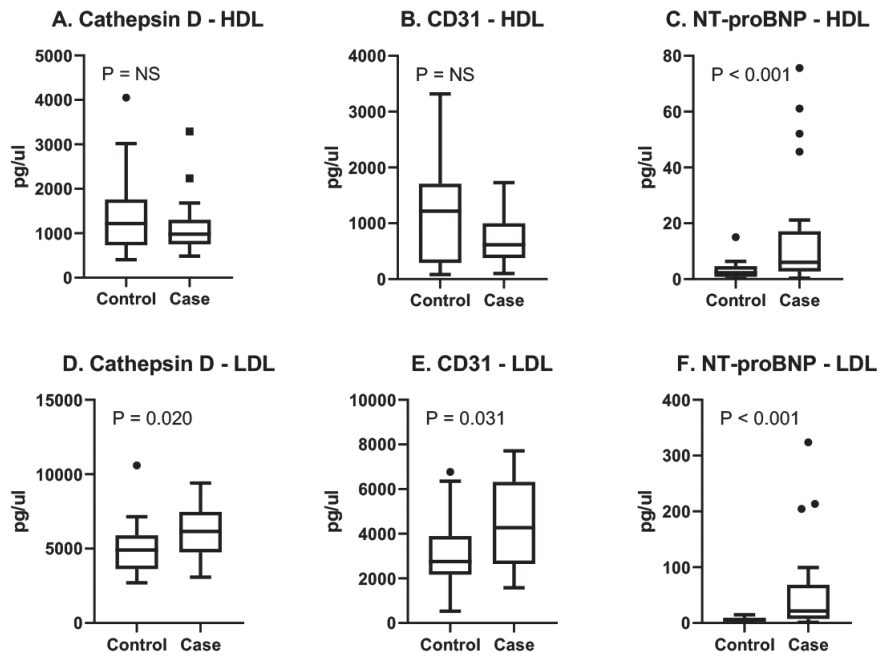

Figure 3. Boxplots of three selected proteins measured with MSD. Assessment of reproducibility of Olink results with a clinically available immunoassay. HDL and LDL indicate EV-subpopulations. Cases were 22 male patients with proven CCS and controls were 22 age- and risk-factor-matched patients who were symptomatic without CCS. Original assay units are pg/uL.

Extracellular vesicles			Study findings	
Subpopulation	*Identifier*	*Method*		Ref
EDEVs	CD144+/CD42b-	FC	CCS+DM vs. control Sign. predictor of presence CCS (OR 4.1 095%CI 2.20-7.70)	[89]
EDEVs	CD31+/CD42b-	FC	NS	[101]
	CD62e+	FC	CCS vs. Control Sign. Diagnostic accuracy AUC: 0.80	
PDEVs	CD41+	FC	NR	

EDEVs = Endothelial-derived EVs; GDEVs = Granolycyt-derived EVs; MDEVs = Monocyte-derived EVs; EryDEVs = Erythrocyte-derived EVs; LDEV = Leukocyte-derived EVs; TFDEVs = TF+-derived EVs. Method: FC = Flowcytometry; PA = Protrombinase Assay. Study findings: FRS = Framingham Risk Score; Sign = Significant p value < 0.05. NR = Not reported; NS = Not significant; NA = Not associated.

CLINICAL ASPECTS OF CCS DIAGNOSIS USING (EV) BLOOD TESTS

The population suspected of CCS is very heterogenous, ranging from patients presenting with clear symptoms and obstructive CAD to patients with nonspecific chest pain without obstructive CAD and everything in between. The current ongoing search towards a biomarker for more accurate detection of CCS is being developed to apply to all patients suspected for CCS but, considering the heterogeneity in this population, this should raise questions. A study performed by Ouellete et al. found clear differences in the clinical profile of patients with respectively normal, near normal, nonobstructive CAD and obstructive CAD[115]. These differences in clinical profiles between the groups seem obvious but are important in the development of a future biomarker. Moreover, considering the fact that EV content enables us to look at cellular level, one could imagine the EV content of a patient with a known history of CAD is not comparable to a patient with new-onset disease.

Another point that merits consideration are sex differences. Although differences are well known in clinical symptoms and pathophysiology, exact underlying mechanisms are barely understood[116]. Evolving knowledge supports the differences in pathophysiology, diagnostic test performance and also prognosis[117–119]. Women tend to have less obstructive CAD and more often a preserved ejection fraction, yet higher mortality rates and more extensive myocardial ischemia[118]. Women often present with more complex signs and symptoms. It was suggested that this is due to

a more complex and multifactorial pathophysiological process compared to men[119]. A large study investigating biomarkers within CVD showed a difference in protein profile between men and women of almost 85%[120]. Research performed in EVs also showed differences in the associations of both EV count as well as content with clinical outcomes stratified on clinical factors[103,108,112].

These data and hypotheses raise the question whether future studies on biomarkers should focus on predefined subgroups of patients rather than the entire "suspected CCS" group. It emphasizes the need to incorporate clinical aspects associated with CCS into future studies with EVs. From our point of view, the most important clinical aspects that merit attention are sex, age and the cardiovascular status of a patient. This cardiovascular status refers to whether or not a patient is already known with atherosclerotic disease or if a patient previously received (invasive) treatment. Until now, EV studies in CCS did not have enough power to perform reliable subanalyses to reveal different associations within this heterogenous group. It might be possible that we need to develop different biomarkers, or cut-offs, within the entire group of patients suspected of CCS.

FUTURE PERSPECTIVES

Despite great efforts of the international society of extracellular vesicles (ISEV) to standardize EV research and improve reproducibility, it remains difficult to compare results between studies[56]. This is mainly because studies still use numerous different techniques for isolation and quantification[121]. There are various protocols for sample preparation, processing and centrifugation, which are known to cause different results[122]. Currently, flow cytometry is the most used method to quantify EVs (see also Table 1). This method is standardized and accepted for the identification and detection of different cell types, however, is most reliable for particles >200nm[123]. Considering the fact that most EVs are around 100–120nm in the blood on average, it is questionable whether this is the best method to count circulating EVs. Also, it does not enable measuring EV contents besides proteins stained on the EV membrane.

Automation

One reason why the use of EVs in clinical practice is hampered is the inability to use high-throughput isolation techniques[122]. Currently, ultracentrifugation is often used to isolate EVs from whole plasma, however, this is time-consuming, labor intensive and requires many manual steps[107]. Before clinical implementation of EVs is considered, large confirmatory trials are needed[104]. Considering the current time effort, costs and the amount of precious clinical blood used for the isolation and quantification of

EVs, this is a disillusion. Future studies should therefore focus on development of an automated method for EV isolation, purification and downstream analysis[124], ideally using very small sample volumes to improve chances for clinical implementation.

Internal standard

The use of a reliable internal standard would also increase the chances for clinical implementation. As rightly opposed by Loyer et al., despite efforts to identify specific subpopulations of EVs with specific membrane markers, very few studies report the purity of their obtained subpopulations[13]. Improvement can be obtained with an internal standard for the number of EVs per milliliter plasma in a sample. For this, a housekeeping protein present in all EVs (e.g., beta-actin) might be a way of developing such a standard. Alongside this, an internal control to visualize the loss of EVs during isolation is needed. Labeled synthetic beads or liposomes might be used for this. Already, these two standards could improve reproducibility and accuracy of EV count and content and measurements in precious clinical samples.

Future directions

Future studies should focus on clinical applicability by developing internal standards and introduce automation and standardization of EV isolation and quantification[56]. Larger cohorts are warranted in order to derive valid clinical prediction models that enable the added value of EV contents as biomarkers to be shown, particularly when taking the heterogeneity within CCS patients into account.

Since EV protein content are based on established immunoassays and are increasingly showing merit in the diagnosis and prognosis of CVD, including the potential for automation and standardization, we expect this to prevail in this field in the next few years.

Although technical challenges still have to be resolved, we anticipate that EVs will be used as a reliable source for research into the diagnosis and prognosis of CCS in the next few years. This could potentially contribute to more personalized medicine and a more efficient use of our healthcare system.

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Conflict of interest

The authors have nothing to declare.

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SUPPLEMENTAL MATERIALS

Supplemental table 1. Baseline characteristics of the Myomarker study cohort

	Control	Case	P value
n	22	22	
Age	63.32 (10.27)	67.86 (16.23)	0.273
BMI	29.79 (4.20)	27.97 (4.31)	0.174
Risk factors			
Smoking	5 (22.7)	2 (9.1)	0.410
Diabetes	6 (27.3)	6 (27.3)	1.000
Hypertension	12 (54.5)	14 (63.6)	0.759
Hypercholesterolemia	12 (54.5)	12 (54.5)	1.000
Family history of CAD	8 (36.4)	10 (45.5)	0.759
Medical history			
Cardiovascular disease	16 (72.7)	19 (86.4)	0.455
Coronary artery disease	9 (40.9)	12 (54.5)	0.546
Coronary revascularization	8 (36.4)	10 (45.5)	0.759
Medication			
Anti hypertensive drugs	15 (68.2)	18 (81.8)	0.486
Lipid lowering drugs	13 (59.1)	14 (63.6)	1.000
Anticoagulans	2 (9.1)	5 (22.7)	0.410
Antiplatelet	11 (50.0)	13 (59.1)	0.762

Values are shown as mean (SD) or number with corresponding frequency. Case is defined as stress-induced ischemia objectified with myocardial perfusion imaging. CAD = Coronary Artery Disease.

Supplemental table 2. Diagnostic performance selected proteins

Biomarker	Controls	Cases	p value	AUC	Sens(%)	Spec(%)	PPV(%)	NPV(%)
LDL-Cathepsin D	0.44 [0.38-0.51]	0.55 [0.45-0.64]	0.007	0.74	68.2	81.8	78.9	72
LDL-CD31	1.50 [1.28-1.90]	2.05 [1.64-2.81]	0.004	0.75	68.2	72.7	71.4	69.6
LDL-NT-proBNP	1.22 [1.04-1.41]	1.98 [1.35-2.85]	<0.001	0.81	68.2	95.5	93.8	75
HDL-Cathepsin D	0.24 [0.23-0.29]	0.26 [0.23-0.30]	0.573	0.55	68.2	54.5	60	63.2
HDL-CD31	0.80 [0.71-0.85]	0.92 [0.81-1.15]	0.015	0.72	68.2	72.7	71.4	69.6
HDL-NT-proBNP	0.99 [0.86-1.22]	1.30 [1.16-1.66]	0.001	0.78	86.4	63.6	70.4	82.4

Sens = Sensitivity, Spec = Specificity, PPV = positive predictive value, NPV = negative predictive value. Case = symptomatic patient with proven CCS, control = symptomatic patients without CCS.

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CHAPTER 5

Extracellular vesicles derived proteins improve the detection of functional relevant coronary artery disease in adjunct to clinical judgement

In preparation

ABSTRACT

BACKGROUND

The simple and inexpensive detection of functional relevant coronary artery disease (fCAD) is a major unmet clinical need. Plasma extracellular vesicle (EV) proteins may serve as novel biochemical signatures of fCAD.

METHODS

Consecutive patients suspected for fCAD work-up were enrolled in this ongoing study. Presence of fCAD was centrally adjudicated by independent cardiologist using both myocardial perfusion single photon emission tomography (MPI-SPECT) and coronary angiography. Clinical judgement (CJ) for the presence of fCAD was quantified by the treating cardiologist. Blood samples were collected before stress testing. EVs were isolated in two subpopulations; LDL and HDL. We quantified 4 proteins in both subpopulations using an immuno-bead assay.

RESULTS

Among 1034 patients 451 (44%) were adjudicated to fCAD. EV-derived Cystatin C and CD14 levels were higher in patients with fCAD versus those without. A biomarker model encompassing the best combination of extracellular vesicle derived proteins (Cystatin C-LDL and CD14 in both subpopulations) increased the diagnostic accuracy of CJ alone to an area under the curve 0.68 (95% CI 0.64-0.71, P-value for comparison 0.017).

CONCLUSION

EV-derived Cystatin C and CD14 may have incremental value when used in conjunction with clinical judgement in the evaluation of patients with suspected fCAD.

INTRODUCTION

Cardiovascular diseases (CVD) remain the most important cause of death worldwide with coronary artery disease (CAD) as the main contributor[1-3]. CAD comprises a broad spectrum of clinical syndromes ranging from asymptomatic patients, sometimes after being treated for an acute coronary syndrome (ACS), to patients with severe disabling complaints during daily life as result of exercise-induced myocardial ischemia due to functional relevant CAD (fCAD). Early detection and adequate risk stratification of patients with fCAD is important to improve clinical decision making and appropriately initiate treatment[1]. Coronary revascularization and/or intensification of anti-ischemic medication will improve symptoms and quality of life only in the vast majority of patients in case symptoms are the result of fCAD, and not due to noncardiac causes. Exercise stress testing (EST) and cardiac stress imaging including myocardial perfusion imaging (MPI) are commonly used to diagnose fCAD[1,4]. MPI has a sensitivity and specificity of about 80-90%, whereas both are <75% with the use of conventional EST[5]. Although the diagnostic performance of MPI is good, its use remains subject of debate as a result of high costs, radiation exposure and an increasing inappropriate use in low risk patients[6,7].

We are facing an unmet need for a widely available diagnostic test strategy that resembles the diagnostic performance of MPI, but without the disadvantages of radiation exposure and high health care burden. One way to improve the diagnostic accuracy without using advanced imaging modalities is the use of biomarkers, eventually in combination with easily available EST results. In this study, we focus on plasma extracellular vesicles (EVs) as a specific biomarker source. EVs are bilipid membrane layered vesicles including exosomes, microvesicles and microparticles[8]. Almost all cells are able to produce them, and they consist of proteins, mRNA, miRNA and lipid particles. Depending on the cell of origin and the condition of the cell the amount and content of the vesicles might vary[9,10]. Earlier studies suggest that EV-derived proteins are associated with future cardiovascular risk, and the presence of (un)stable CAD[11-14]. This study aimed to test the hypothesis that EV-derived proteins may help in the detection of fCAD in all-comers with suspected fCAD.

METHODS

Study design

The current analysis is part of a large ongoing prospective diagnostic study (NCT01838148, clinicaltrials.gov) designed to further elaborate the early detection of fCAD[15-18]. The study was approved by the local ethics committee and was carried out according to the principles of the Declaration of Helsinki. All patients provided written informed consent.

Population

Patients with suspected fCAD, who were referred for rest/stress myocardial perfusion single-photon emission tomography/computer tomography (MPI-SPECT) to the University Hospital Basel, Switzerland, were consecutively enrolled from 2010-2016. During this period, MPI-SPECT/CT was the preferred imaging modality in patients with a wide range of pre-test probabilities for fCAD. Patient with an ACS in the past 21 days were excluded from this analysis.

Blood sample preparation

Venous blood was collected in EDTA tubes at 4 different time points, directly before the stress test, immediately after and 2 and 4 hours after the stress test. Samples were processed immediately and stored at -80°C until further use. For this analysis, samples were transported to the Netherlands for the isolation of the EVs and subsequently quantifying 4 different EV-derived proteins.

EV isolation

The isolation procedure is described in detail in previous studies, especially in the supplemental materials of these manuscripts[13,19,20]. In short, EVs were separated based on co-precipitations of the different particles with different densities, a subpopulation of EVs co-precipitated with Low-Density Lipid particles (LDL) while others co-precipitate with High-Density Lipid particles (HDL). Before precipitation magnetic beads are added to allow separation after the precipitation. For the sequential isolation of the subpopulations Dextran Sulphate (DS) (MP Biomedicals) and Manganese (II) Chloride (MnCl2) (Sigma-Aldrich) solutions were used. After separation lysis buffer was added to enable the analysis of the EV content. To get easy access to the data regarding the isolation and characterization of the EVs we created an EV-track ID: EV200044. In this track-ID the information and data are provided in a structured and uniform way[21].

EV quantification

Once the EV subpopulations are created and lysed we quantified 4 preselected proteins (Cystatin C, CD14, Serpin G1 and Serpin C1. These proteins were selected as result from a previously performed proteomics analysis[11,19]. Concentrations of Cystatin C, CD14, Serpin G1 and Serpin C1 were quantified in the LDL- and HDL subpopulation with an electrochemiluminescence immunoassay (Quickplex SQ120, Meso Scale). According to the manufacturer's protocol, a specific U-Plex Development Assay was developed and validated for these 4 proteins. For Cystatin C, standard antibody sets of Meso Scale were used (supplemental table 1). The antibody set for Serpin C1, Serpin G1 and CD14 were custom-made and are displayed in supplemental table 1. SoftMax Pro 7 Software (Molecular Devices) was used to analyze the data. Protein concentrations were expressed in pg/mL. If protein levels were below the calibration curve, 0.5 times the lower limit of detection was used.

Adjudication of the primary outcome

The presence of functional relevant CAD was adjudicated by two independent cardiologists (one general and one interventional cardiologist). For this the result of stress testing (EST and/or MPI) was used and if available combined with findings from an additional coronary angiogram (CAG)[15–18]. All patients underwent a routine rest/stress dual isotope (201T1 for rest, 99mTc sestamibi for stress) or single isotope (99mTc sestamibi for stress and rest) according to MPI-SPECT protocol as described previously[22]. MPI-SPECT images were scored semi-quantitatively using a 17-segment model with a 5-point scale (0 =normal, 1=mildly reduced tracer uptake, 2=moderately reduced uptake, 3=severely reduced uptake, 4=no uptake). The 17 segments in the stress and rest images were used to calculate the summed stress score (SSS), and summed rest score (SRS), of which the difference yielded the summed difference score (SDS). An SDS score of at least 2, or positive transient ischemic dilation ratio (TID) was considered indicating inducible myocardial ischemia and thereby fCAD. Two readers derived SSS and SRS by visual assessment and compared with the software (QGS) result. Difference in the visual assessment by the two readers were resolved by finding consensus. In case of equivocal findings from MPI-SPECT and coronary angiography, two independent cardiologist (one interventional, one general) that were blinded to the biomarker results reviewed the case. A positive perfusion scan was overruled if CAG (within 3 months) revealed a high-grade coronary lesion (>75% or fractional flow reserve (FFR) <0.80). In total 14 patients (1.4%) were reassigned to the ischemic group after CAG and 36 (3.5%) were reassigned as not having ischemia after CAG.

Adjudication of the clinical judgment score

For all patients a clinical judgement (CJ) score was determined before the stress test. The score was given by the treating cardiologist on a visual analogue scale with a range from 0% - 100%. The CJ score takes the pre-test probability as determined by a patients age, sex, previous cardiac history, risk factors and symptoms during the index visit into account. This score was chosen instead of an actual pre-test probability score as it was thought to obtain a more holistic view of the patient, which is important in this all comers population. The CJ score was obtained before and after exercise testing. The cardiologist was blinded for the MPI results and biomarker measurements at the time of scoring.

Statistical methods

Distribution of the proteins were first visually inspected with boxplots and histograms. Since the distribution of all proteins was skewed they were all logarithmically transformed. To enable comparison between the proteins and improve the interpretation of the results all proteins were standardized after transformation. Baseline levels of the EV proteins were compared between patients with and without fCAD. Univariable logistic regression analysis were done for the entire cohort but also for pre-defined subgroups based on a patients' sex and history with cardiovascular disease, and exercise modality (physical exercise versus pharmacological), based on prior data suggesting that these may impact on the diagnostic utility of biomarkers[23-26]. In addition to the univariable analysis we performed multivariable logistic regression analysis to correct for all potential confounders. A list of confounders was selected based on previous literature, encompassing: sex, age, BMI, smoking, hypertension, hypercholesterolemia, diabetes mellitus, coronary artery disease and cardiovascular disease. For all analysis an available (biomarker) case analysis approach was used. To assess the potential discriminatory ability three different models were created: 1. A model containing only the CJ score (as described before), 2. A model with the best combination of EV proteins. For this we started with a full model and reduced it based on the AIC. The third model combines the clinical judgement score and the biomarker model to assess if there is any additional value in this combined assessment. Additionally, the analysis was repeated for the subgroup of patients undergoing physical exercise testing only; with model 1 containing the CJ score + the results of the exercise test and model 2 in which the best combination of EV-derived proteins was added additional to the CJ score and stress test results. All hypotheses tests were two-sided with a critical significance level of <0.05. Analysis were performed with R software (R Software, version 3.5.1).

RESULTS

The entire Basel VIII study included over 4000 patients, the cohort used for this study comprises 1138 patients randomly selected patients of which 11 (1%) patients were excluded from the primary analysis because of an acute coronary syndrome or revascularization within the past 21 days. Another 93 (8.2%) patients were excluded because of incomplete biomarker values. Mean age was 66 years, 313 (30.3%) patients were women and 591 (57.2%) had a history of cardiovascular disease, and physical exercise only was the stress modality used in 552 (53.4%) patients. In total 451 (44%) of all patients were adjudicate to have fCAD (table 1).

Table 1. Baseline characteristics

	All patients	Control	Case	P value	Missing
n	1034	583	451		
Demographics					
Age	66 (11)	65.09 (11.67)	67.54 (10.82)	0.001	0.0
% Women	313 (30.3)	228 (39.1)	85 (18.8)	<0.001	0.0
BMI	27 (5)	27.40 (4.72)	28.06 (4.56)	0.024	0.0
Previous history					
Cardiovascular disease	591 (57.2)	239 (41.0)	352 (78.0)	<0.001	0.0
Coronary artery disease	524 (50.7)	195 (33.4)	329 (72.9)	<0.001	0.0
Chronic pulmonary disease	92 (8.9)	53 (9.1)	39 (8.6)	0.890	0.0
Terminal kidney disease	11 (1.1)	5 (0.9)	6 (1.3)	0.668	0.0
Risk factor					
Smoking	204 (19.8)	119 (20.5)	85 (18.9)	0.568	0.4
Diabetes Mellitus	254 (24.6)	111 (19.0)	143 (31.7)	<0.001	0.0
Hypertension	848 (82.0)	441 (75.6)	407 (90.2)	<0.001	0.0
Hypercholesterolemia	750 (72.5)	372 (63.8)	378 (83.8)	<0.001	0.0
Family history CAD	294 (28.4)	142 (24.4)	152 (33.7)	0.001	0.0
Medication					
ASA use	666 (64.4)	302 (51.8)	364 (80.7)	<0.001	0.0
Platelet inhibitors	167 (16.2)	59 (10.1)	108 (30)	< 0.001	
Oral anticoagulants	123 (11.9)	70 (12.0)	53 (11.8)	0.977	0.0
ACE inhibitor	343 (33.2)	152 (26.1)	191 (42.4)	<0.001	0.0
AT-II antagonist	313 (30.3)	168 (28.8)	145 (32.2)	0.276	0.0
Lipid-lowering agents	628 (60.7)	287 (49.2)	341 (75.6)	<0.001	0.0

Values are displayed as mean ± SD or frequency (%), Case = patient with a positive ergometry or a SDS score ≥ 2 on myocardial perfusion imaging (MPI), and/or functionally relevant coronary artery disease on coronary angiogram. CAD = Coronary artery disease. CVD = history of CAD or peripheral vascular disease or history of ischemia CVA, Terminal kidney disease = on dialysis, ASA = Aspirin

History of CAD and most cardiovascular risk factors were more common among patients with fCAD compared to those without. The same was seen for the use of cardiovascular medication.

Raw baseline values were compared between cases and controls in table 2. Levels differed between the two groups for Cystatin C (P value for comparison 0.032) and CD14 (P value for comparison 0.016) in the HDL subpopulation and Cystatin C (P value for comparison 0.026) in the LDL subpopulation. A detailed logistic regression analysis for all proteins is summarized in the supplemental materials. Supplemental table 2 summarizes the univariable and multivariable odds ratios of having fCAD for the entire group. Higher levels of Cystatin C in the HDL subpopulation (OR 1.16 95% CI 1.03-1.32) and in the LDL subpopulation (OR 1.17 95% CI 1.02-1.34) were significantly associated with the presence of functional relevant CAD. After adjustment for all potential confounders Cystatin C in the LDL subpopulation remained significantly associated with fCAD (OR 1.17 95% CI 1.01-1.35).

Three different models were obtained and area under the curves were determined (table 3). Selected biomarkers for the final model were CD14 in both the LDL and the HDL subpopulation and Cystatin C in the LDL subpopulation. The CJM had a moderate AUC of 0.66 (0.62-0.69), the model with only EV proteins in it showed an AUC of 0.58 (0.54-0.62), and finally the combined model showed an AUC of 0.68 (0.64-0.71). The difference between the clinical judgement model (CJM) and CJM+biomarker model was statistically significant (P value for comparison 0.017).

Supplemental figure 1 compares the AUC between a model containing the results of the physical exercise + CJ scores with a model in EV-derived proteins were added (the same combination as in the entire cohort). Addition of the EV-derived biomarkers significantly improved the AUC in the subgroup of patients undergoing only physical exercise testing (AUC 0.73 vs 0.75, P value for comparison 0.031).

We performed 3 predefined stratified subgroup analysis based on sex, history with CVD and test modality of stress induction (physical versus pharmacological). The results for the univariable regression analyses are summarized in supplemental table 3A (sex) 3B (history of CVD) and 3C (exercise modality). The sex based subanalysis revealed Cystatin C in the HDL subpopulation (OR 1.26 95% CI 1.09-1.46) and LDL subpopulation (OR 1.19 95% CI 1.02-1.38) as significant predictor for fCAD in males. None of the proteins remained significant in the female subgroup. In patients without a history of CVD higher levels of Cystatin C in the LDL subpopulation were associated with the stress-induced ischemia (OR 1.39 95% CI 1.04-1.86). For those with established

CVD Cystatin C levels in the HDL subpopulation were associated with ischemia (OR 1.19 95% CI 1.01-1.40). In patients undergoing physical stress induction Cystatin C levels in the LDL subpopulation were associated with fCAD (OR 1.28 95% CI 1.04-1.58). In the patients with a combination of physical stress and pharmacological stress or pharmacological stress only none of the proteins remained significant.

Table 2. Levels of biomarkers

	Control	Case	P value
n	583	451	
HDL subpopulation			
Cystatin C	6053 [2123- 11719]	6510 [3079- 13658]	0.032
CD14	4979 [2969-8023]	5658 [3082-9024]	0.016
Serpin G1	20434 [12139-35740]	21836 [12986- 38847]	0.114
Serpin C1	126725 [56674-220528]	122172 [55226-225474]	0.970
LDL subpopulation			
Cystatin C	33561 [24038-52048]	38924 [246901-55292]	0.026
CD14	24044 [16639-34039]	22849 [16770-33412]	0.511
Serpin G1	57278 [37988-86154]	61399 [40817-86333]	0.180
Serpin C1	2044283 [1288333-2773487]	1962582 [1312195-2705587]	0.534

Values are displayed as median with interquartile range. Case = patient with a positive ergometry or a SDS score ≥ 2 on myocardial perfusion imaging (MPI), and/or functionally relevant coronary artery disease on coronary angiogram.

Table 3. Diagnostic accuracy by the area under the curve (AUC) of a clinical judgement model versus a clinical judgement + biomarker model

	AUC for ischemia	P values compared to CJM
Clinical judgement model	0.66 (0.62-0.69)	*Comparator*
Biomarker model	0.58 (0.54-0.62)	0.001
CJMb + biomarker model	0.68 (0.64-0.71)	0.017

CJM = Clinical judgement model. CJMb = Clinical judgement model + biomarker combination. Biomarker model encompasses: HDL CD14, LDL Cystatin C and LDL CD14. All values were first logarithmically transformed and standardized afterwards.

DISCUSSION

This large study tested the hypothesis whether EV-derived proteins might help in the detection of fCAD in unselected patients referred for fCAD work-up. We showed that higher levels of EV-derived Cystatin C and CD14 in the HDL subpopulation and Cystatin C in the LDL subpopulation were associated with fCAD. After full adjustment for all potential confounders Cystatin C in the LDL subpopulation remained an independent predictor for the presence of fCAD. Addition of EV-derived proteins significantly improved the diagnostic performance compared to a model with only the CJ score (clinical model 0.66 vs EV model 0.68, P value 0.017). A subgroup analysis in patients who underwent physical stress alone the diagnostic performance increased from 0.73 (a model with CJ and exercise ECG information) to 0.75 (P value 0.031) after addition of EV-proteins.

The diagnostic utility of EV-proteins

Although the combined use of EV-derived proteins in addition to clinical parameters, in this study summarized as CJ score, led to a significantly improved model, the diagnostic accuracy remains low. Sensitivity and specific of noninvasive imaging modalities (SPECT, PET or Coronary CT) to detect fCAD range between 80-95%[27]. With the use of EVs in addition to clinical parameters we were able to improve the diagnostic utility but with an AUC of 0.68 this remains moderate. Before clinical implementation of an EV-based strategy further improvement is a necessity.

Subgroup analysis in patients undergoing physical stress

In patients undergoing physical stress only the reported accuracy is often relatively moderate around 0.65%[27]. However, as for all choices for additional testing, if the correct population is selected and the test is carried out and interpreted in right way, it can be very useful. Especially since the cost are relatively low, there is no radiation exposure and a referral to a cardiologist might be prevented[1]. We determined the diagnostic accuracy of EVs in adjunct to CJ score and the results for the physical stress test in a subanalysis. This revealed an increased diagnostic utility, measured by AUC from 0.73 towards 0.75 after the addition of EV-derived proteins to a CJ score and ECG findings. Although 0.75 is still not high enough to be used in clinical practice but it is clearly better compared to 0.68 in the entire group. This raises the question whether EVs should be used in adjunct to physical stress testing and if EV release is influenced by the modality of myocardial stress induction.

Not much is understood about the pathophysiological difference on myocardial cell level between pharmacological and physical stress, but is seems that pharmacological stress enables identification of flow disabilities rather than actual myocardial

ischemia[26]. A previous performed study that investigated the role of BNP in fCAD showed an increased release of BNP in patients with ischemia undergoing physical exercise, whereas in patients with ischemia a decrease of BNP release was found[26]. One study compared echocardiographic pressure and volume parameters in healthy individuals between patients undergoing physical versus pharmacological stress testing[28]. They revealed a complete different physiological response of the left ventricle in response to stress induction. The aforementioned different physiological response together with previous findings of differences between biomarker release as a result of stress modality are interesting for further investigation. A more sophisticated combined model based on physical exercise data together with EV-derived protein measurement might be a useful tool to improve the diagnostic strategy to detect fCAD. Especially considering the wide availability and low costs of both tests. Such a diagnostic model might have immediate clinical impact by reducing the number of referrals to a cardiologist and the unnecessary use of MPI and will subsequently reduce the amount of unnecessary radiation exposure. Our finding furthermore suggests that EV-derived proteins may have clinical utility if used within a multimarker approach combining several biochemical signals possibly involved in the pathophysiological processes underlying fCAD.

EV-derived proteins in fCAD

Our findings also extend and corroborate to prior pilot studies evaluating EVs as diagnostic tool, most studies on EVs in CAD are investigating the effect of total count[29]. We analyzed 4 different proteins (content) in this study: Cystatin C, CD14, Serpin G1 and Serpin C1. Cystatin C is known as a protease inhibitor playing an important role in inflammation. Cardiomyocytes are able to produce them and a previous study showed the synthesis of Cystatin C increases when the myocardium experiences ischemia[30]. Plasma derived Cystatin C has been previously mainly studied as an important marker for renal dysfunction[31–33]. As a single biomarker it is likely that Cystatin C is sensitive to detect any form of atherosclerosis/CVD, but less likely to reach high specificity. In this study, we also consistently found Cystatin C to be associated with functional relevant CAD. Even after complete adjustment for potential confounders. The second protein we investigated was CD14, which is a membrane anchored protein and best known from its role in the innate immune system[34]. In this study, CD14 levels were not independently associated with the outcome, but on top of clinical parameters it remained a strong predictor in our final models in both the LDL and HDL subpopulation in combination with Cystatin C in the LDL subpopulation. Serpin C1, better known as anti-thrombin, and its main function is to inhibit thrombus formation[35]. We hypothesized that during the atherosclerotic process the formation and degradation in a continues ongoing process, which we thought might be reflected

by this protein. However, this was not found. The last investigated protein was Serpin G1, which is a C1-inhibitor[36]. It regulates the activation of the complement system[37]. We found no association with fCAD. This was in contrast to a previous study in which a strong correlation was observed between Serpin G1 and low-grade inflammation, which is known as an important keystone in CVD[38,39].

Limitations

The following limitation should be considered when interpreting these findings. First, experimental details may have contributed to e.g. the different finding regarding sex-specific aspects. In the previous pilot study synthetic vesicles were used as internal control for EV loss during the isolation procedure[13]. The quality of these synthetic beads was considered not optimal and therefore no longer used. However, we could therefore not adjust for potential loss of EVs during the process, neither did we have another internal control. This underlines the need for a more sophisticated method for the isolation and quantification procedure. A second important issue occurred after the entire isolation procedure was finished, as a result of the COVID pandemic we unexpectedly had to close our laboratory. Since it was unclear how long the situation would last, we first stored the samples in a -20°C freezer, but once it became clear that the closure would at least take 6 week we transferred them back to a -80°C. It is unknown whether this could have impacted our results. Third, although based on a large cohort of unselected patients with suspected fCAD, as a single center study it still may have introduced selection bias.

CONCLUSION

EV-derived Cystatin C was independently associated with fCAD. EV-derived Cystatin C and CD14 may have incremental value when used in conjunction with clinical judgement in the evaluation of patients with suspected fCAD. Further studies are warranted.

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SUPPLEMENTAL MATERIALS

Supplemental table 1. Antibodies used for quantification of protein levels in EVs

	Coating	Detection	Set
Cystatin C			MSD Mescoscale R-plex antibody set (F21YW)
CD14	Novus (NBP1-05149)	R&D Systems (AF1267)	
Serpin G1	R&D Systems (MAB2488)	R&D Systems (AF2488)	
Serpin C1	R&D Systems (MAB3833)	R&D Systems (AB383)	

Antibodies were the same for the LDL and HDL subpopulation.

Supplemental table 2. Logistic regression analysis for stress-induced myocardial ischemia

	Unadjusted		RF adjusted[1]	
	OR (95% CI)	P value	OR (95% CI)	P value
HDL subpopulation				
Cystatin C	1.16 (1.03-1.32)	0.018	1.09 (0.95-1.26)	0.210
CD14	1.13 (0.99-1.28)	0.065	1.08 (0.94-1.25)	0.261
Serpin G1	1.10 (0.97-1.25)	0.125	1.09 (0.95-1.25)	0.222
Serpin C1	0.99 (0.88-1.13)	0.951	0.98 (0.85-1.12)	0.721
LDL subpopulation				
Cystatin C	1.17 (1.03-1.34)	0.020	1.17 (1.01-1.35)	0.040
CD14	0.97 (0.86-1.10)	0.674	1.02 (0.89-1.17)	0.818
Serpin G1	1.08 (0.95-1.22)	0.235	1.08 (0.94-1.24)	0.308
Serpin C1	0.96 (0.85-1.08)	0.489	1.04 (0.90-1.19)	0.600

Biomarker levels are log-transformed and standardized. Original assay units are pg/ml. RF = Risk factor. [1]RF adjusted; age, sex, BMI, known coronary artery disease/cardiovascular disease and the standard cardiovascular risk factors.

Supplemental table 3A. Univariable logistic regression analysis for stress-induced myocardial ischemia stratified on sex

	Male		**Female**	
N	793		345	
	OR (95% CI)	P value	OR (95% CI)	P value
HDL subpopulation				
Cystatin C	1.26 (1.09-1.46)	0.002	1.03 (0.78-1.34)	0.855
CD14	1.12 (0.97-1.30)	0.111	1.29 (0.97-1.72)	0.084
Serpin G1	1.06 (0.92-1.22)	0.426	1.24 (0.94-1.64)	0.130
Serpin C1	1.01 (0.88-1.17)	0.877	0.96 (0.74-1.26)	0.764
LDL subpopulation				
Cystatin C	1.19 (1.02-1.38)	0.028	1.12 (0.84-1.50)	0.441
CD14	0.97 (0.84-1.12)	0.652	1.11 (0.84-1.46)	0.474
Serpin G1	1.05 (0.92-1.21)	0.474	1.26 (0.94-1.69)	0.123
Serpin C1	0.99 (0.86-1.14)	0.906	0.91 (0.70-1.19)	0.500

Biomarker levels are log-transformed and standardized. Original assay units are pg/ml.

Supplemental table 3B. Univariable logistic regression analysis for stress-induced myocardial ischemia stratified on a history with cardiovascular disease

	Without CVD		**With known CVD**	
N	485		653	
	OR (95% CI)	P value	OR (95% CI)	P value
HDL subpopulation				
Cystatin C	1.06 (0.85-1.33)	0.620	1.19 (1.01-1.40)	0.044
CD14	1.08 (0.85-1.37)	0.543	1.14 (0.97-1.34)	0.109
Serpin G1	1.05 (0.83-1.33)	0.688	1.11 (0.95-1.31)	0.200
Serpin C1	1.07 (0.85-1.34)	0.590	0.94 (0.80-1.11)	0.475
LDL subpopulation				
Cystatin C	1.39 (1.04-1.86)	0.027	1.13 (0.96-1.34)	0.141
CD14	1.13 (0.88-1.45)	0.354	0.95 (0.81-1.12)	0.553
Serpin G1	1.11 (0.87-1.42)	0.385	1.05 (0.89-1.24)	0.536
Serpin C1	1.18 (0.93-1.50)	0.173	0.90 (0.76-1.06)	0.186

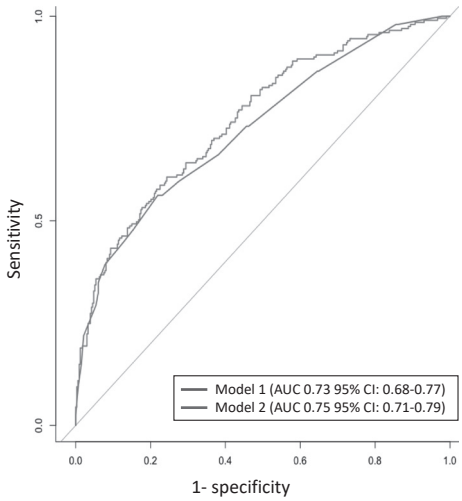
Biomarker levels are log-transformed and standardized. Original assay units are pg/ml.

Supplemental table 3C. Univariable logistic regression analysis for stress-induced myocardial ischemia stratified on modality of exercise induction

	Physical exercise only		Pharmacological or combined exercise	
N	552		482	
	OR (95% CI)	P value	OR (95% CI)	P value
HDL subpopulation				
Cystatin C	1.13 (0.96-1.33)	0.157	1.12 (0.93-1.36)	0.243
CD14	1.12 (0.93-1.33)	0.230	1.09 (0.91-1.31)	0.331
Serpin G1	1.21 (1.01-1.46)	0.038	0.99 (0.83-1.18)	0.909
Serpin C1	0.92 (0.78-1.09)	0.321	1.06 (0.88-1.28)	0.522
LDL subpopulation				
Cystatin C	1.28 (1.04-1.58)	0.018	1.02 (0.84-1.23)	0.858
CD14	1.01 (0.84-1.20)	0.946	0.91 (0.76-1.09)	0.310
Serpin G1	1.11 (0.93-1.32)	0.242	0.99 (0.81-1.20)	0.901
Serpin C1	1.07 (0.89-1.28)	0.475	0.87 (0.73-1.03)	0.109

Biomarker levels are log-transformed and standardized. Original assay units are pg/ml.

Supplemental figure 1



Supplemental figure 1. ROC Curves diagnostic models

ROC Curves with corresponding AUC values comparing model 1 (red) comprising the clinical judgement score + stresstest results with model 2 (blue) containing model 1 + EV-derived Cystatin C (LDL) and EV-derived CD14 (LDL+HDL). P value for difference in AUC between model 2 and 3 was 0.031

PART II

The Bone-Heart axis

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CHAPTER 6

High levels of Osteoprotegerin are associated with coronary artery calcification in patients suspected of a Chronic Coronary Syndrome

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ABSTRACT

BACKGROUND

Plasma osteoprotegerin (OPG) and vascular smooth muscle cell (VSMC) derived extracellular vesicles (EVs) are important regulators in the process of vascular calcification (VC). In population studies, high levels of OPG are associated with events. In animal studies, however, high OPG levels result in reduction of VC. VSMC-derived EVs are assumed to be responsible for OPG transport and VC but this role has not been studied. For this, we investigated the association between OPG in plasma and circulating EVs with coronary artery calcium (CAC) as surrogate for VC in symptomatic patients.

METHODS

We retrospectively assessed 742 patients undergoing myocardial perfusion imaging (MPI). CAC scores were determined on the MPI-CT images using a previously developed automated algorithm. Levels of OPG were quantified in plasma and two EV-subpopulations (LDL and TEX), using an electrochemiluminescence immunoassay.

RESULTS

Circulating levels of OPG were independently associated with CAC scores in plasma; OR 1.39 (95% CI 1.17-1.65), and both EV populations; EV-LDL; OR 1.51 (95% CI 1.27-1.80) and EV-TEX; OR 1.21 (95% CI 1.02-1.42).

CONCLUSION

High levels of OPG in plasma were independently associated with CAC scores in this symptomatic patient cohort. High levels of EV-derived OPG showed the same positive association with CAC scores, suggesting that EV-derived OPG mirrors the same pathophysiological process as plasma OPG.

INTRODUCTION

Osteoprotegerin (OPG) is a glycoprotein of the tumor necrosis factor receptor family[1,2]. The main function of OPG is to inhibit osteogenesis by preventing the binding of the receptor activator of nuclear factor-kB ligand (RANKL) to its natural receptor activator nuclear factor-kB (RANK)[3]. The RANKL/RANK complex normally results in differentiation of osteoclasts and osteogenesis[3,4]. OPG is therefore important in maintaining the balance between bone formation and resorption[5].

Additional to its function in bone metabolism, OPG is also implicated in cardiovascular diseases (CVDs)[6]. OPG is thought to be involved in the process of vascular calcification (VC). Experimental studies showed the presence of OPG within the vessel wall in the media and intima, and also in the fibrous cap of atherosclerotic lesions[7]. Animal studies showed more VC in mice lacking OPG than mice without OPG-deficiency[8,9]. Furthermore, administration of OPG to atherosclerotic mice deficient for the LDL receptor led to less calcified plaques compared to the placebo mice[10]. In contrast, human studies show that high levels of plasma OPG are associated with a higher risk of future events[11,12]. Although the exact role remains unclear, it might be that plasma OPG is produced as response to VC to protect against progression rather than preventing it[13,14].

The discrepancy between the presumed role of OPG in mice studies compared to large population studies could potentially be found in extracellular vesicles (EVs). EVs are bilayer lipid membranes containing bioactive content (nucleic acid, proteins and lipids)[15]. EVs are often referred to as "the liquid biopsy", and considered as cell-cell communicator [16]. Almost all cell types are able to produce EVs[16].

EVs derived from vascular smooth muscle cells (VSMCs) are thought to be involved in VC, and contain calcification inhibitors such as OPG and matrix GLA-protein to regulate the micro-environment[17,18]. In pathological circumstances VSMC-derived EVs become microvascular calcified structures that form the start of advanced calcified plaques. In these calcified plaques OPG was found near these VSMC-derived EVs suggesting a role in transportation of OPG by EVs. EVs are often analyzed by total number of EVs, however their content might be also informative[19]. We previously found that EVs can be separated based on size and density, potentially reflecting pathophysiological phenomenon. Despite OPG is associated with EVs, levels of plasma OPG and/or EV-derived OPG and its association with VC has not been studied.

Coronary artery calcium (CAC) score measured with coronary CT is used as surrogate for VC[20]. The CAC score has been shown to be an excellent predictor of major adverse cardiovascular events (MACE), as well as a risk stratifying tool in patients suspected of chronic coronary syndrome (CCS)[21–23]. The relationship between OPG and CAC is studied in asymptomatic population-based studies as well as patients with renal failure or diabetes mellitus[24–29]. However, little is known about the association between CAC scores and levels of OPG in a symptomatic cohort. Neither do we know if OPG in circulating EVs provides additional information to plasma levels. In this study, we investigate the association between CAC, plasma OPG and circulating EV-derived OPG in two subsets of EVs in patients suspected of CCS.

METHODS

Study cohort

We will perform a retrospective analysis on the prospectively collected MYOMARKER study cohort. The MYOMARKER (MYOcardial ischemia detection by circulation bioMARKERs) is a prospective single center cohort study of consecutively enrolled patients who underwent myocardial perfusion imaging (MPI) with ^{82}Rb-PET/CT because of chest pain suspected for CCS. All patients were aged >18years and included between August 2014 and September 2016 in the Meander Medical Center, the Netherlands. The study (NL5078) was approved by the Medical Ethics Committee-United (MEC U), in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients, more details on the study protocol have been published previously[30]. For the purpose of this study all patients with a history of a percutaneous coronary intervention or coronary artery bypass grafting were excluded.

Studyprotocol

Levels of OPG were measured in previously collected blood samples. Venous blood was collected in EDTA tubes just before the MPI was performed. The samples were centrifuged 10 minutes at 1850xg at room temperature (RT) within 30 minutes after they were collected. After centrifugation all samples were aliquoted and directly stored at -80°C. Additional to the protein measurements CAC scores were obtained. For an overview of this study protocol see also figure 1.

Study protocol - extracellular vesicles isolation

Levels of OPG were measured in both plasma as well as in EVs. For this, two EV subpopulations were isolated. The isolation was performed as described in previous publications[30,31]. In brief, a subset of EVs co-precipitate with low-density lipid particles

(LDL) which allows separation. Magnetic beads were therefore added for both subpopulations (nanomagÒ-D plain voor LDL and nanomagÒ-D PET-OH for TEX). For the sequential isolation of the EV-LDL subpopulation Dextran Sulphate (DS, 0.05%, MP biomedicals) was used in combination with Manganese II Chloride (MnCl2, 0.05M, Sigma-Aldrich) (EV-TEX). The TEX subpopulation was precipitated with Xtractt buffer (1:4, Cavadis BV). Subsequently, a bio-plex handheld magnet was used. The remaining pellet containing the EV subpopulations was separated from magnetic bead debris with centrifugation, after removal lysis buffer was added to study the OPG levels carried within the EV subpopulations.

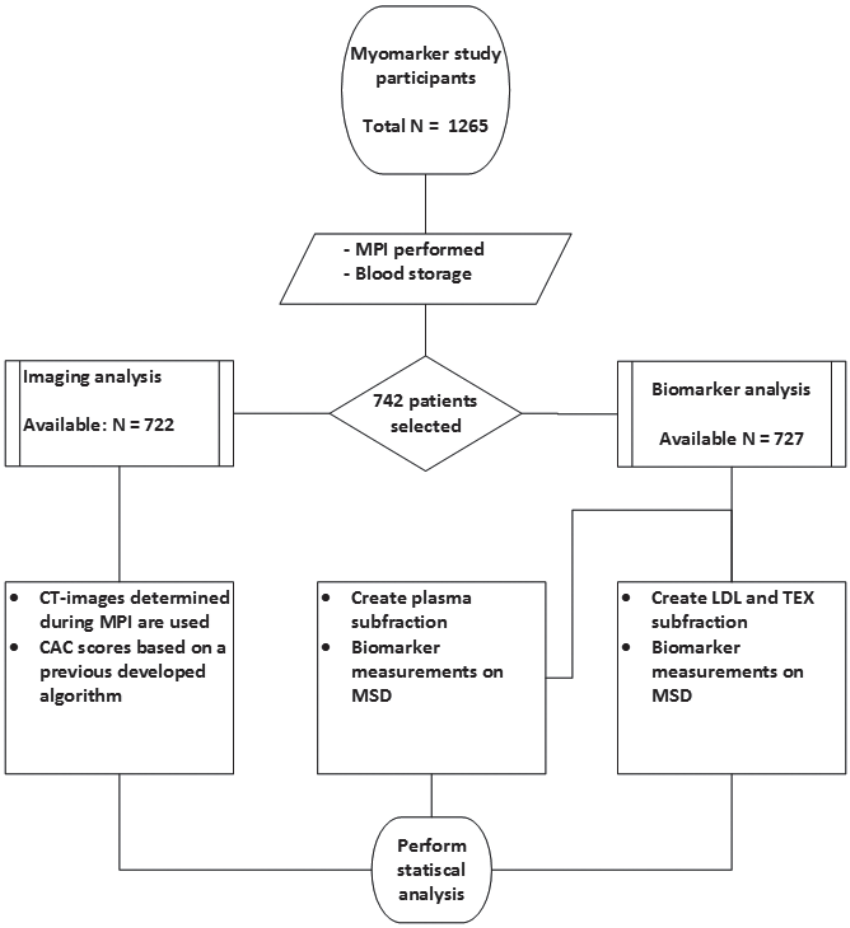

Figure 1. Study protocol

MPI = myocardial perfusion imaging. CAC = coronary artery calcification. MSD = MesoScaleDiscovery platform. LDL and TEX refer to extracellular subfractions.

Study protocol - extracellular vesicle quantification

Levels of OPG were quantified in plasma and the EV-LDL and EV-TEX subpopulations using an electrochemiluminescence immunoassay (Meso Scale Discovery, MSD) following manufacturers protocol. In short; MSD GOLD Small Spot Streptavidine plates were coated O/N at 4ºC with OPG antibodies (MSD R-Plex human OPG antibody set, F21ZK). After washing three times with 150µL wash buffer (0.2% Tween-20 in PBS) per well, the coated plates were blocked with blocking buffer A (MSD) for 1 hour at RT. Subsequently, plates were then washed as described before and 50µL diluted plasma or protein lysate (2-fold) from the EV subfractions, blancs or calibrators were added to designated wells and incubated for 2 hours at RT. After washing the plates, detection antibody was added to all wells and incubated for 1 hour. Plates were then washed and filled with 100µL Reading Buffer (MSD) before analysis on the MSD Instrument (Quickplex SQ120, MSD). Protein concentration were measured as pg/mL. Data analysis was performed using MSD Discovery Workbench 4.0 software (Meso Scale Diagnostics).

Study protocol - extracellular vesicle characterization

Both the modified protocol which was used as well as extracellular vesicle characterization are described in detail in two previously published papers (specifically in the supplemental materials of Zhang et al.)[32,33]. Easy access to this data in a nice structured way can also be obtained via the EV-track that was created with EV-ID: EV200044.

Study protocol - coronary artery calcium scoring

A previously developed algorithm was used to determine CAC scores on the low-dose, non-ECG-triggered, attenuation correction CT (LDACT) images acquired during MPI[34]. Scans were acquired with 120kVp on a hybrid scanner (Biograph CT Flow 64-Slice scanner, Siemens Healthcare, Knowxville, Tennessee). Detailed information on both the scanning protocol for MPI as well as CAC score measurements have been published before[30,35]. In short, the developed algorithm first detects and excludes the lungs to identify the region of interest, on the LDACT. In the identified volume, the algorithm analyzes voxels above the standard intensity level threshold of 130 Hounsfield Units using two subsequent convolutional neural networks. The first network identifies candidate CAC voxels and assigns them a label of the coronary artery they reside in, while the second network identifies true CAC among the candidate CAC voxels. Finally, the identified CAC voxels are quantified using the per artery and total Agatston scores. As this method is not (yet) able to distinguish between CAC and a coronary stent, all patients with a history of coronary revascularization were excluded.

Statistical analysis

Continuous variables are summarized as mean ± standard deviation (SD) or median with interquartile range [IQR] depending on the distribution. Categorical variables are shown as number with corresponding frequencies. The distribution of all potential confounding variables[36], CAC scores, and biomarkers were assessed and transformation was performed achieve normal distributions. Levels of OPG were standardized after logarithmic transformation. Patients with levels of OPG >3SD were considered as influential outliers and removed from the dataset.

For informative purpose and to correct for all possible confounders, associations between a wide range of cardiovascular risk factors and levels of OPG in plasma and both EV subpopulations were assessed. The continuous association between levels of OPG and the (logarithmically transformed) CAC score were assessed with Spearman's correlation coefficient. Spearman's correlation was used instead of Pearson since not all assumptions for Pearson's correlation were met since no linearity and homoscedasticity was found between the variables. To assess this association in more detail we performed an ordered regression analysis between OPG levels and categories of CAC scores. For this CAC scores were divided in 5 commonly used categories: 0-9; 10-99; 100-399; 400-999 and >1000[26,37]. Next to the univariable associations, adjusted ordered regression analysis were performed with 2 sets of confounders: 1. age + sex and 2. a parsimonious set of variables. For this parsimonious set of variables we selected general cardiovascular risk factors and variables that were significantly associated with OPG in the regression analysis. The full set of variables contained: age, male sex, smoking, diabetes mellitus, hypercholesterolemia, a family history of CAD, known history of CAD, use of aspirin, statins or betablockade. After model reduction, using a stepwise backward method based on AIC this resulted in a final parsimonious model containing: age, male sex, hypertension, smoking, diabetes mellitus and a history of CAD. In addition, to assess if OPG in plasma and both EV subpopulation showed complementary information they were added all together in a final model.

To provide insight in the potential clinical use we investigated the discriminative ability of levels of OPG in plasma, EV-TEX and EV-LDL to detect significant CAC defined as a CAC score >10 with logistic regression analysis[20]. After internal validation with bootstrapping techniques C-statistics with corresponding confidence intervals were obtained. All analyses were performed with R Studio Version 1.1.456 (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

In total 1265 patients were included in the MYOMARKER cohort study. Seventeen patients were incorrectly included in the study and therefore removed, another 500 patients were excluded because of a history of coronary revascularization, and in 6 patients there was not enough blood left to perform the analysis. This led to a study population of 742 patients (mean age 67 years, 50.5% male) who are the subject of this manuscript (table 1). Many patients were overweight with a mean BMI of 27.6, 62% were known with hypertension and half of the patients suffered from hypercholesterolemia, previous CVD was seen in 75.6%. With regards to medication use, aspirin, statins, betablockade and ACE- or angiotensin-II-inhibition was seen in nearly 50% of the patients. Supplemental table 1 shows the distribution of cardiovascular risk factors between sexes, despite age (women tend to be slightly older in this study) no statistical differences were seen.

Association between OPG and cardiovascular risk factors

Bivariate correlations between levels of OPG in plasma and both EV-subpopulations and cardiovascular risk factors are provided in table 2. In all three groups higher age, diabetes, hypertension and betablockade use were associated with higher levels of OPG, while male sex, and a family history of CAD were associated with lower levels of OPG. Hypertension was associated with higher OPG levels in plasma and the EV-LDL subpopulation.

Association between OPG and CAC

Valid CAC scores were derived in 720 patients. There were 184 patients with no significant CAC <10 (25.6%), 124 (17.2%) had mild CAC (10-99), 159 (22.1%) moderate (101-399), 110 (15.2%) severe (>400) and 143 (19.9%) had extensive CAC (>1000). Supplemental table 1 shows CAC scores were significantly higher in men compared to women. Across the OPG-plasma, LDL and TEX measurements in total 15 measurements failed and were therefore reported as missing values. None of the values were considered as influential outliers. In supplemental table 2 the untransformed levels of OPG are summarized for each predefined category of CAC. In general levels of OPG gradually increased with increasing CAC scores. Supplemental figure 1 provides boxplots comparing levels of OPG for EV-LDL, EV-TEX and plasma between the different categories. No significant differences were found between moderate, severe and extensive CAC.

Table 1. Baseline characteristics

	Overall
n	742
Demographics	
Age, years	67±10
Male sex (%)	375 (50.5)
BMI	27.6 (5.2)
Risk factors	
Current smoking	145 (19.5)
Diabetes Mellitus	139 (18.7)
Hypertension	460 (62)
Hypercholesterolemia	376 (50.7)
Familial coronary artery disease	179 (24.1)
Medical history	
Cardiovascular disease	561 (75.6)
Coronary artery disease	53 (7.1)
Heart failure	37 (5.0)
Atrial fibrillation	119 (16.0)
Ischemic CVA	31 (4.2)
Drug therapy	
Aspirin	302 (40.7)
P2Y12-inhibitors	53 (7.1)
Anti-coagulants	140 (18.9)
Statin	349 (47.0)
ACE/AT-inhibitor	326 (43.9)
Betablockade	336 (45.3)

Values are shown as mean±SD or frequency with corresponding percentages. CVA = Cerebrovascular accident, AT = Angiotensin II

We assessed the associations between levels of OPG in plasma, EV-LDL and EV-TEX and CAC scores. Figure 2 shows that this association was significant for plasma and both EV-subpopulations, all with *p* values <0.001. The strongest associations were found for EV-LDL with R: 0.3, and plasma, R: 0.29, compared to the weaker association for EV-TEX, R: 0.19.

Table 2. Bivariate correlations between OPG levels and cardiovascular risk predictors

	EV-LDL OPG		EV-TEX OPG		Plasma OPG	
	Beta	*p value*	*Beta*	*p value*	*Beta*	*p value*
Variable						
Age	0.05	**<0.001**	0.03	**<0.001**	0.05	**<0.001**
Male sex	-0.32	**<0.001**	-0.26	**<0.001**	-0.33	**<0.001**
Smoking	-0.07	0.42	0.03	0.70	0.01	0.88
Diabetes	0.26	**<0.01**	0.32	**<0.001**	0.44	**<0.001**
Hypertension	0.22	**<0.01**	0.10	0.14	0.22	**<0.01**
Hypercholesterolemia	0.04	0.56	0.07	0.27	0.12	0.09
Familial CAD	-0.29	**<0.001**	-0.21	**<0.01**	-0.24	**<0.01**
History of CAD	0.09	0.51	-0.08	0.54	0.07	0.59
Ascal use	0.04	0.59	0.04	0.60	0.03	0.69
Statin use	0.14	**0.04**	0.01	0.89	0.10	0.16
Betablockade use	0.28	**<0.001**	0.19	**<0.01**	0.32	**<0.001**

All biomarkers were transformed depending on their original distribution and standardized. EV = Extracellular vesicle. EV-LDL and EV-TEX indicate both different subpopulation of EVs. CAD = Coronary Artery Disease.

More in depth analysis was performed with an ordered regression analysis to find associations between levels of OPG and categorical CAC scores, the results of this analysis can be found in table 3. EV-LDL (odds ratio (OR) 1.81, 95% confidence interval (CI) 1.56-2.10), EV-TEX (OR 1.40; 95% CI 1.21-1.62) and plasma levels of OPG (OR 1.66; 95% CI 1.44-1.91) were significantly associated with categorical CAC scores. The associations remained significant after adjustment for only age and sex as well as full adjustment including cardiovascular risk factors (EV-LDL OR 1.51; 95% CI 1.28-1.80, EV-TEX OR 1.19; 95% CI 1.02-1.40 and plasma OPG OR 1.38; 95% CI 1.16-1.63).

Table 3. Ordered regression analysis of OPG levels and categorical CAC scores

	Univariable	***p* value**	**Age+Sex adjusted**	***p* value**	**Full adjusted**	***p* value**
EV-LDL OPG	1.85 (1.60-2.15)	<0.001	1.60 (1.35-1.91)	<0.001	1.51 (1.27-1.80)	<0.001
EV-TEX OPG	1.42 (1.23-1.65)	<0.001	1.27 (1.08-1.49)	<0.001	1.21 (1.02-1.42)	0.024
Plasma OPG	1.69 (1.47-1.96)	<0.001	1.49 (1.27-1.77)	<0.001	1.39 (1.17-1.65)	<0.001

Full adjusted model included: age, sex, smoking, hypertension, diabetes mellitus and a previous history of coronary artery disease. Outcome variable was categorized in 5 classes: 0; 1-99; 100-399; 400-999 and >1000. EV = Extracellular Vesicle. EV-LDL and EV-TEX indicate different subpopulations of EVs.

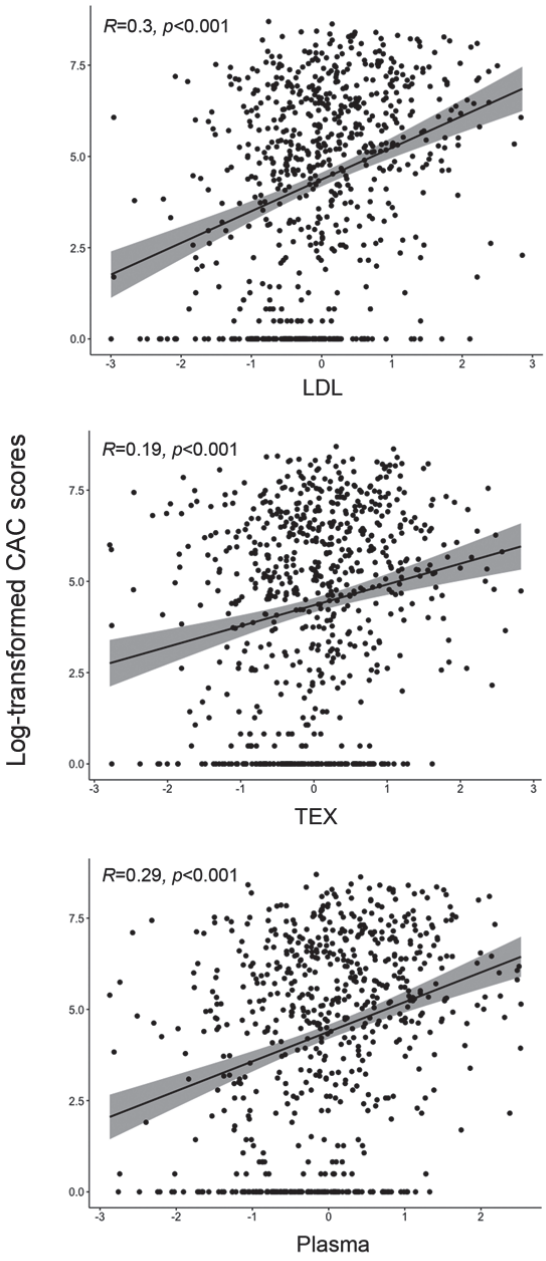

Figure 2. Correlations between standardized levels of OPG and CAC scores in EV-LDL, EV-TEX and plasma CAC scores were logarithmically transformed. *p* values correspond to spearman's coefficient.

Discriminatory ability of OPG to detect significant CAC

To assess if OPG plasma levels and EV-derived OPG levels provide additional information OPG levels in plasma and EVs were added together in the parsimonious model. In this model only EV-derived OPG in the LDL-subpopulation remained significantly associated with CAC (OR 1.18; 95%CI 1.05-1.17), while EV-derived OPG in the TEX subpopulation (OR 0.98; 95%CI 0.79-1.21) and OPG plasma levels (OR1.18; 95%CI 0.93-1.52) were no longer significant predictors of CAC. To gain more insight in the potential discriminatory ability of OPG C-statistics were obtained (table 4). Modest discrimination to predict presence of CAC (defined as CAC>10) was seen for both plasma OPG and EV-LDL (C-statistics both 0.67), C-statistic for EV-TEX was lower with 0.63.

Table 4. C-statistics for OPG to detect significant CAC

	CAC>10
	C statistic (95% CI)
EV-LDL OPG	0.67 (0.63-0.71)
EV-TEX OPG	0.63 (0.59-0.65)
Plasma OPG	0.67 (0.65-0.70)

CAC>10 refers to the clinical scenario with a binary outcome for the coronary artery calcium score defined as <10 or >10. EV = Extracellular vesicle. EV-LDL and EV-TEX refer to different subpopulations of EVs.

DISCUSSION

In this study the association between levels of OPG in EVs and plasma were studied in a large symptomatic population suspected of CCS. We report 3 major findings, (1) we showed plasma levels of OPG as an independent predictor of CAC scores in a symptomatic population (OR 1.39; 95% CI 1.17-1.65). (2) We reported the first data on EV-derived OPG and the relation with CAC. Additional to plasma OPG, high levels of both EV-LDL OPG (OR 1.51; 95%CI 1.27-1.80) as well as EV-TEX OPG (OR 1.21; 95% CI 1.02-1.42) were independently associated with a higher CAC score category. (3) We assessed the clinical potential of plasma OPG, and EV-derived OPG to correctly stratify patients to their corresponding CAC risk-category. The C-statistics to predict significant CAC (>10) were only modest.

OPG and cardiovascular risk factors

We found a positive association between age and OPG, which was also observed in a substudy of the CLARICOR trial[38]. Since atherosclerosis itself is highly age-dependent, the association between OPG and age might be explained by this. Higher levels of OPG

are consistently found in women compared to men, the CLARICOR trial showed that women had, irrespective of diabetes or statin use 15% higher levels of OPG. A nested case-control study in the EPIC Norfolk trial showed higher levels of OPG in women compared to men for both cases (coronary event or death from coronary cause) and controls (healthy individuals)[39]. Both studies suggest biological difference in the levels of OPG between sexes but not directly in their association with disease, in our study we found the same result. Other associations with sex, diabetes, hypertension, family history with CAD and the use of beta blockade, were all consistent with existing literature[38,40]. Interestingly, we did not find an association between statin use and OPG, whereas other studies did[38,41,42]. The studies of Kadoglou et al.[41] and Davenport et al.[42] were both small studies involving different study populations compared to our study which might explain the difference. However, the CLARICOR trial was also a large study >3000 participants and the percentage of patients on statin therapy was comparable to our study, approximately 40%. We have no clear explanation for this difference.

OPG and VC

We found OPG to be an independent predictor of CAC scores. This result is in agreement with previous studies showing the same association in the general population[28,29] as well as asymptomatic patients with established CVD[26,43-45]. We now show that high levels of OPG are also associated with CAC in a symptomatic chest pain cohort. Additionally, we investigated the association of circulating EV-derived OPG with CAC for the first time. High levels of EV-derived OPG were also associated with high CAC scores, just as plasma levels of OPG. The positive association for both plasma OPG and circulating EV-derived OPG with CAC suggests that they probably both just mirror the same underlying processes[13,18,28]. Two decades ago Jono et al. was the first to report that levels OPG were associated with the degree of CAD measured with coronary angiography[46]. OPG was also found as predictor for progression of carotid atherosclerosis[13]. Several animal studies also showed that in the absence of OPG more VC of the vessel wall is seen[8-10]. Interestingly, Morony et al. showed that administration of OPG (and thus high levels of OPG) in mice deficient for the LDL receptor, led to less calcification of plaques[10]. However, they did not find a decrease in number of plaques, nor lower cholesterol levels in these mice. This might indicate OPG could have a specific role in calcification of the plaques rather than preventing the atherosclerotic process itself. This finding is in contrast to the consistent finding of high levels of OPG in human and its association with MACE. Other groups have also suggested that high levels of OPG are a simple epiphenomenon of inflammation as result of atherosclerosis[47-49].

Previous studies showed VSMC derived EVs to be present in calcified plaques and they also showed OPG in these EVs[50]. In normal physiological conditions VSMCs are contractile and secrete EVs that regulate the micro-environment which enable phenotypical change [17]. The secreted VSMC-derived EVs contain calcification inhibitors, such as OPG and matrix GLA-protein[18]. As a result of cellular stress and mineral imbalance VSMCs become less contractile and start to form calcified EVs[51]. Calcified EVs tend to aggregate, form microcalcification and increase calcification in existing plaques[52]. In vulnerable plaques this could lead to rupture, whereas it could also further stabilize a calcified plaque under a thick fibrous cap[52]. We did not find a different association between EV-derived OPG levels and CAC scores compared to plasma OPG. This suggests that EV-derived levels of OPG have a similar function as plasma OPG. The correlation between the EV-derived OPG levels and plasma OPG were statistically significant (LDL R: 0.75 P<0.001 and TEX R: 0.66, P<0.001) emphasizing this hypothesis (data not shown). Circulating EV-derived OPG represents a more general view of atherosclerosis itself rather than being involved in the actual process of VC like VSMC-derived EVs are.

Discriminatory ability of OPG

We showed a moderate correlation between levels of OPG and CAC, additionally, we assessed whether a single OPG measurement in plasma or EVs has the clinical potential to predict the presence of CAC (defined as CAC>10). A cut-off value of 10 was used, since the aim of this analysis was to see if OPG levels have potential to distinguish between patients at risk for future event (defined as presence of CAC) and those without (CAC <10). If so, this could have immediate clinical implications. After internal validation with bootstrapping techniques, we found a C-statistic to detect CAC>10 for plasma OPG of 0.67, values for EV-derived OPG were comparable (EV-LDL: 0.67 and EV-TEX: 0.63). One other study investigated the discriminatory ability of a single OPG measurement to predict high CAC scores (>400) in healthy participants [53]. With C-statistics to detect CAC >1, CAC>10 and CAC>400 of 0.5 they concluded OPG was not able to identify healthy participants with significant CAC scores. Although our C-statistics to detect CAC>10 were also modest, the discriminatory ability of OPG was clearly better in symptomatic patients compared to healthy participants. To assess the added value of EV-derived OPG, we analyzed EV-OPG and plasma OPG together in the final parsimonious model, no added value was observed. It is therefore likely that EV-derived OPG represents the same process as plasma derived OPG. The modest correlation as well as the cross-sectional nature of this study makes it hard to determine the potential clinical importance of our findings. It remains to be elucidated what the exact role of OPG in the pathophysiology of vascular calcification is. For this, the etiologic relation should be studied in more detail and eventually a clinical impact study would be the next step.

Limitations

Several limitations merit consideration. Because of the retrospective character of the study, we were not able to investigate whether levels of OPG would have changed clinical decision making by the treating physician. The study remains limited to only one center, which might influence the generalizability of the results. In this study patients with previous coronary revascularization were excluded but the clinical relevance of CAC determination in these patients is limited anyway. Compared to general population studies the sample we studied is only modest (N=742).

CONCLUSION

Increased levels of OPG in plasma were independently associated with CAC scores. High plasma OPG and EV-OPG levels were associated with high CAC scores. Our findings suggest that plasma and EV-derived OPG seem to mirror the same underlying pathophysiological process.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of interest

None of the authors declare any potential conflict of interest.

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Author contributions

MD, FW, MjmS, and DdK contributed to the conception of the design. JB and AS contributed to the performed analysis. II, NL and BkV contributed to the automated algorithm used for the study. MD wrote the manuscript. All authors contributed in the interpretation of the results and critically reviewed the manuscript. All authors agreed on this final version of the manuscript.

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SUPPLEMENTAL MATERIALS

Supplemental table 1. Cardiovascular risk factors stratified on sex

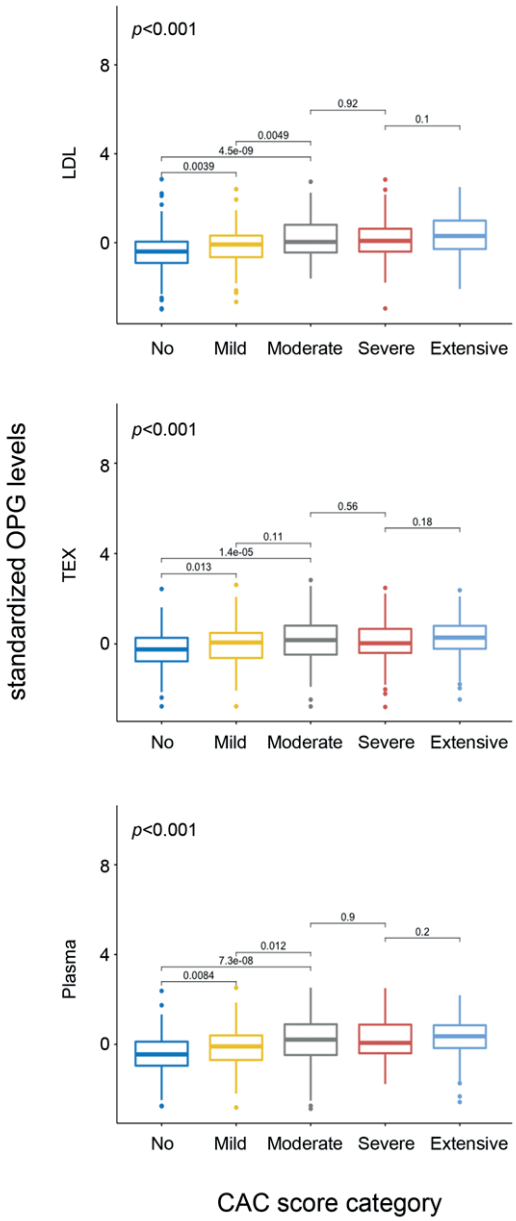
	Men = 375	**Women = 367**	*P-value*
Age, years	66.21 (10.24)	68.57 (10.05)	0.002
BMI	27.79 (4.76)	27.38 (5.68)	0.276
Known coronary artery disease	34 (9.1)	19 (5.2)	0.056
Risk factors			
Current smoking	70 (18.7)	75 (20.4)	0.606
Diabetes Mellitus	76 (20.3)	63 (17.2)	0.323
Hypertension	235 (62.7)	225 (61.3)	0.760
Hypercholesterolemia	183 (48.8)	193 (52.6)	0.338
Familial coronary artery disease	82 (21.9)	97 (26.4)	0.172
Coronary calcium			
Coronary artery calcium score	825.91 (1139.03)	308.72 (528.41)	<0.001
Log transformed CAC score	4.98 (2.65)	3.75 (2.58)	<0.001

CAC = Coronary artery calcium.

Supplemental table 2. Levels of osteoprotegerin stratified on CAC categories

	CAC 0-9	**CAC 10-99**	**CAC 100-399**	**CAC 400-999**	**CAC >1000**
n	184	124	159	110	143
EV-LDL OPG	69 [57-82]	78 [63-92]	82 [68-111]	83 [69-103]	91 [73-119]
EV-TEX OPG	135 [107- 170]	155 [114-187]	163 [122-215]	153 [127-202]	172 [137-218]
Plasma OPG	666 [560-809]	753 [614-900]	824 [659-1052]	792 [674-1050]	888 [737-1047]

Levels of osteoprotegerin are shown as median [Interquartile range]. Assay unit is pg/ml. EV=Extracellular Vesicle, EV-LDL and TEX respresent different subpopulations. CAC=Coronary artery calcium



Supplemental figure 1. Standardized levels of OPG per category of CAC score. No = CAC score 0-9, Mild = CAC score 10-99; Moderate = CAC score 100-399, Severe = CAC score 400-999; Extensive = CAC score >1000. *p* values <0.05 were considered significant.

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CHAPTER 7

Automated calcium scores collected during myocardial perfusion imaging improve identification of obstructive coronary artery disease

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ABSTRACT

BACKGROUND

Myocardial perfusion imaging (MPI) is an accurate noninvasive test for patients with suspected obstructive coronary artery disease (CAD) and coronary artery calcium (CAC) score is known to be a powerful predictor of cardiovascular events. Collection of CAC scores simultaneously with MPI is unexplored.

AIM

We aimed to investigate whether automatically derived CAC scores during myocardial perfusion imaging would further improve the diagnostic accuracy of MPI to detect obstructive CAD.

METHODS

We analyzed 150 consecutive patients without a history of coronary revascularization with suspected obstructive CAD who were referred for 82Rb PET/CT and available coronary angiographic data. Myocardial perfusion was evaluated both semi-quantitatively as well as quantitatively according to the European guidelines. CAC scores were automatically derived from the low-dose attenuation correction CT scans using previously developed software based on deep learning. Obstructive CAD was defined as stenosis >70% (or >50% in the left main coronary artery) and/or fractional flow reserve (FFR) ≤0.80.

RESULTS

In total 58% of patients had obstructive CAD of which seventy-four percent were male. Addition of CAC scores to MPI and clinical predictors significantly improved the diagnostic accuracy of MPI to detect obstructive CAD. The area under the curve (AUC) increased from 0.87 to 0.91 (p: 0.025). Sensitivity and specificity analysis showed an incremental decrease in false negative tests with our MPI + CAC approach (n = 14 to n = 4), as a consequence an increase in false positive tests was seen (n = 11 to n = 28).

CONCLUSION

CAC scores collected simultaneously with MPI improve the detection of obstructive coronary artery disease in patients without a history of coronary revascularization.

INTRODUCTION

Angina pectoris (AP) is a clinical syndrome characterized by episodes of retrosternal complaints, usually induced by exercise or other stress factors with quick relieve after discontinuation of exercise or stress. AP is often caused by myocardial ischemia due to the presence of obstructive coronary artery disease (CAD) and/or microvascular dysfunction[1,2]. The diagnostic assessment of patients with suspected obstructive CAD is challenging and one of the most common aspects of cardiology nowadays. Since the presence of obstructive CAD often requires coronary intervention, accurate diagnostic tests are of great importance. Myocardial perfusion imaging (MPI) with positron emission tomography (PET)/computed tomography (CT) is an accurate noninvasive test for patients with suspected obstructive CAD[3,4]. It provides measurements on myocardial perfusion, myocardial blood flow (MBF) and coronary flow reserve (CFR). The coronary artery calcium (CAC) score on the other hand is a powerful predictor for cardiovascular events[5–9]. Recent studies have demonstrated additional diagnostic power of the CAC score on top of perfusion imaging in patients with suspected obstructive CAD[10–13]. For these studies an additional ECG triggered CT-scan was acquired for manual assessment of CAC scores instead of using the attenuation correction CT images gathered during MPI. Several studies compared manual CAC scoring on an ECG triggered CT with manual CAC scoring on attenuation correction CT images and showed encouraging results[14–16]. Recently, two studies performed in our center compared manual CAC scoring on ECG triggered CT images with automated CAC scoring in low dose chest CT and attenuation correction CT[17,18]. Both studies used a previously developed algorithm based on deep learning and showed that this is a reliable and accurate method of calculating the CAC score. Therefore, the aim of our study is to assess whether automatically derived CAC scores simultaneously collected with MPI on attenuation correction CT images improve the diagnostic accuracy of MPI in patients with suspected obstructive CAD.

MATERIALS & METHODS

Study population

The MYOMARKER (MYOcardial ischaemia detection by circulating bioMARKERs) study is a prospective single-center observational cohort study of consecutively enrolled patients (>18 years of age) with suspected CAD who presented at the outpatient clinic of the Meander Medical Center (Amersfoort, the Netherlands) between August 2014 and September 2016. All patients underwent a Rubidium-82 PET/CT scan as part of their diagnostic work-up. The complete cohort consists of 1265 patients. For

the purpose of this study only patients who underwent coronary angiography (CAG) within 90 days prior to or after MPI were selected. After exclusion of patients with previous coronary artery bypass grafting (CABG) or previous percutaneous coronary intervention (PCI), and exclusion of five patients with incomplete MPI results, the final cohort consisted of 150 patients (Appendix A, Fig. A1). The study was approved by the regional medical ethics committee and performed in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants.

PET-CT imaging

A detailed description of MPI imaging protocol is provided in the supplemental materials (Appendix B). Briefly, patients were asked to discontinue caffeine- or methylaxanthine-containing food/drinks and theophylline and dypiridamol 48h prior to the PET/CT scan. Rubidium-82 PET/CT scans were acquired using a hybrid scanner (Biograph CT Flow 64-slice scanner, Siemens Healthcare, Knoxville, Tennessee). Rest and stress cardiac PET/CT images were acquired on the same day, pharmacological stress was administered intravenously with regadenoson. The estimated effective radiation dose for this protocol to the patients was 3.7 mSv. Heart rate, systolic blood pressure and 12 lead ECG were recorded at baseline, 1 min after regadenoson administration and after imaging. Rate-pressure product was calculated for manual correction of rest flow values.

PET image analysis

Myocardial perfusion was evaluated according to the European guideline in two ways: semi-quantitative and quantitative[19]. All scans were evaluated by 2 experienced observers. Semi quantitative analysis was performed with the use of the 17-segment model of the American Heart Association[20,] in short; the summed difference score (SDS) is the difference between the perfusion deficit score in stress and rest, a SDS score≥4 was defined as stress induced ischemia. Quantitative analysis of myocardial perfusion was assessed by the myocardial blood flow (MBF, mL/g/min) and coronary flow reserve (CFR). MBF was computed from the dynamic rest and stress imaging series with commercially available software (Siemens Syngo Dynamic PET). A global MBF was calculated for the left ventricle as well as regional MBF for each of the three coronary vessel territories. Resting MBF was manually adjusted for the patient-specific rate-pressure product at rest. Global and regional coronary flow reserve was defined as the ratio of hyperemic to (adjusted) baseline MBF. MPI scans were considered as normal if a patient had a normal MBF, CFR and a SDS score of 0. Normal MBF refers to normal MBF at a threshold of 2.0 ml/g/min. Normal CFR was set 1.6. MPI Scans were considered as suspect for obstructive CAD if either a SDS≥4 was measured, or patients with an SDS between one and three but with abnormal MBF and/or wall motion abnormalities (WMA).

Calcium scoring

CAC scores were determined from the low-dose attenuation correction CT scan, which were derived during MPI using a previously developed algorithm[17]. This software was originally developed for fully automated calcium scoring in low dose chest CT scans. We therefore manually annotated coronary calcifications in 200 consecutive CT scans from the present study and retrained the software with a combination of low dose chest CT and low dose attenuation correction CT scans. Briefly the software first detects the lungs to identify a region of interest in the image and then automatically detects CAC above the standard threshold of 130 Hounsfield Units using a deep learning approach. Detected calcifications are labeled according to the affected coronary vessel (left anterior descending including left main coronary artery, left circumflex artery and right coronary artery. CAC scores were calculated for all three coronary vessels[21]. Since this new method is not able to distinguish previously placed coronary stents from coronary calcium we excluded patients with coronary stents from the analysis. CAC scores were categorized according to previous literature as 0, 1-100, 101-300 and 301 or more [5,22].

Coronary angiography

All lesions were measured by quantitative coronary angiography (QCA) by a blinded trained clinical physician (MD), using Cardiovascular Angiography Analysis System software (CAAS 7.3, Pie Medical Imaging, Maastricht, The Netherlands). In case of uncertainty a board-certified interventional cardiologist (GL) was asked to measure the lesion as a second observer. Uncertainty was mostly based on doubts regarding the second frame in which the lesions should be measured. Lesions were considered hemodynamically important if: 1. FFR positive ≤0.80 or 2. A luminal stenosis >70% (or >50% in case of left main) measured with QCA. In case of discrepancy between FFR measurement and QCA measurement, FFR result was considered more reliable and therefore used. In total, 15 lesions were measured with FFR.

Statistical analysis

Continuous data are expressed as mean ± standard deviation (SD), categorical data as frequencies and percentages. Differences in continuous variables were compared by independent t-test. Dichotomous variables were compared by Fisher's exact test. Univariable and multivariable logistic regression were used to analyze predictors for the presence of obstructive CAD. Univariable logistic regression was performed for all variables that were considered possible clinical predictors based on previous literature or differences in baseline characteristics. All variables with a P value <0.20 in the univariable analysis were used for the multivariable logistic regression. We used the likelihood ratio test statistic with a backward stepwise method to determine

which combination of clinical predictors performed best in prediction of obstructive CAD. This resulted in the first of three models used for the final analysis. The other two models were derived to compare the diagnostic accuracy of MPI alone versus MPI in combination with automated CAC scores on top of clinical predictors. Benjamini–Hochberg test was used to correct for multiple comparison testing. The natural logarithm of the CAC score (ln_CAC+1) was used because of a wide range and right skewness of the CAC scores. Receiver operator characteristic (ROC) areas under the curve (AUC) were calculated for all three models to determine their ability to predict obstructive CAD. We calculated sensitivity, specificity and predictive values for MPI only, CAC scores only and the combination of both. For this analysis ischemia was analyzed as a dichotomized variable. For the CAC score a cut off value of 300 was used since this was considered clinically relevant[6]. All hypotheses tests were two-sided with a critical significance level of <0.05. Statistical analyses were performed with SPSS version 25.0 (SPSS, Chicago, IL) and R software (R software, version 3.4.1).

RESULTS

Clinical characteristics are shown in table 1. The mean age was 68±12 years, and the majority of patients was male (64%). Patients had a mean BMI of 28.1kg/m^2. Already 57% of the patients were using platelet aggregation inhibition (aspirin, clopidogrel or ticagrelor) in accordance with their previous medical history of atherosclerotic disease. Patients with obstructive CAD were more often male (74% vs. 48%, p value 0.001), and had more often a history of CAD (24% vs. 5%, p value 0.002) or previous myocardial infarction (18% vs. 3%, p value 0.008). There were no differences in age, BMI, or any of the other known risk factors for cardiovascular disease between patients with and without obstructive CAD. The left ventricular ejection fraction was above normal limit during stress and rest in all patients. As expected, the average SDS score in patients with obstructive CAD was higher compared to those without (6 vs. 1, p value <0.001). No difference between (un)corrected rest MBF, hyperemic MBF and CFR were observed. There were in total 8 patients with a CAC score of 0. In patients with obstructive CAD the majority of patients had CAC scores >300 (71%).

Table 1. Clinical characteristics of all patients with and without obstructive CAD

	All N = 150	No obstructive CAD N = 63	Obstructive CAD N = 87	P value*
Demographics				
Age in years	67.6 (11.5)	66 (10.39)	68 (12.23)	0.470
Male sex	96 (64%)	29 (48%)	67 (74%)	0.001
BMI	28.1 (5.4)	28.7 (6.3)	26.9 (4.6)	0.291
Medical history				
History of CVD	111 (74%)	41 (68%)	70 (78%)	0.196
History of CAD	25 (17%)	3 (5%)	22 (24%)	0.002
History of MI	18 (12%)	2 (3%)	16 (18%)	0.008
History of PAD	10 (7%)	3 (5%)	7 (8%)	0.504
Diabetes mellitus	35 (23%)	14 (23%)	21 (23%)	0.575
Hypertension	97 (65%)	36 (60%)	61 (68%)	0.329
Dyslipidemia	87 (58%)	37 (62%)	50 (56%)	0.458
Current smoker	39 (26%)	15 (25%)	24 (27%)	0.820
Family history of CAD	45 (30%)	19 (32%)	26 (30%)	0.783
Medication				
Platelet aggregation inhibitors†	85 (57%)	29 (48%)	56 (62%)	0.093
Anticoagulants	30 (20%)	11 (18%)	19 (21%)	0.667
Beta-blockers	81 (54%)	31 (52%)	50 (56%)	0.640
Statins	87 (58%)	37 (62%)	50 (56%)	0.458
ACE inhibitor or ARB	57 (38%)	19 (32%)	38 (42%)	0.192
Calcium channel blockers	34 (23%)	16 (27%)	18 (20%)	0.339
Loopdiuretics	22 (15%)	9 (15%)	13 (14%)	0.925
Nitroglycerin	57 (38%)	22 (37%)	35 (39%)	0.784
^{82}Rb PET-CT findings				
Rest LVEF	58 (16)	61 (17)	57 (16)	0.670
Stress LVEF	61 (17)	61 (17)	69 (16)	0.947
SDS	4 (5)	1 (2)	6 (4)	<0.001
RPP	11024 (3068)	10341 (2258)	11501 (3458)	0.227
Rest MBF uncorrected	1.15 (0.38)	1.12 (0.33)	1.17 (0.42)	0.511
Rest MBF corrected	0.86 (0.25)	0.89 (0.24)	0.83 (0.25)	0.170
Stress MBF	2.41 (1.88)	2.61 (0.77)	2.28 (2.35)	0.313
CFR	3.19 (0.99)	3 (1)	3 (1)	0.584

Table 1. Continued

	All N = 150	No obstructive CAD N = 63	Obstructive CAD N = 87	P value*
CAC results				
0	8 (5%)	5 (8%)	3 (3%)	0.038
1-100	27 (18%)	17 (27%)	10 (12%)	0.007
101-300	26 (17%)	14 (22%)	12 (14%)	0.043
>300	89 (59%)	27 (43%)	62 (71%)	<0.001

Continuous variables are presented as mean (SD), categorical variables as n(%). * P value for comparison between groups with and without obstructive CAD. CVD = cardiovascular disease, CAD = coronary artery disease, MI = myocardial infarction, PAD = peripheral artery disease. †Aspirin, clopidogrel or ticagrelor, LVEF = Left ventricular ejection fraction. SDS = Summed Difference Score, RPP = rate pressure product, MBF = myocardial bloodflow, CFR = Coronary flow reserve, CAC = Coronary Artery Calcium.

Predictors of obstructive CAD

In the univariable analysis (Appendix C, Table C1) male sex (OR 3.11, 95% CI 1.56–6.23), history of cardiovascular disease (CVD) (OR 1.62, 95%CI 0.78–3.39), history of CAD (OR 6.15, 95% CI 1.75–21.60), previous myocardial infarction (MI) (OR 6.27, 95% CI 1.39–28.37), use of an ACE-inhibitor and/or ARB (OR 1.58, 95% CI 0.80–3.13) and use of a platelet aggregation inhibitor (OR 1.76, 95% CI 0.91–3.41) were considered as significant clinical predictors of obstructive CAD. These variables were used for multivariable analysis, finally after model reduction with the likelihood ratio test male sex and history of CAD remained significant predictors of obstructive CAD.

Table 2. Odds ratios calculated with logistic regression comparing diagnostic performance of MPI and CAC score

	Model 1 Clinical predictors	
	OR (95% CI)	P value
Predictor		
Male sex	2.94 (1.44-6.12)	0.003
History of CAD	5.70 (1.60-20.46)	0.008
Ischemia	-	-
Ln_CAC	-	-

MPI = myocardial perfusion imaging, CAC score = coronary artery calcium score, Ischemia = dichotomized with a cut off SDS score of 4 and/or abnormal myocardial bloodflow/coronary flowreserve, Ln_CAC = natural logarithm of coronary artery calcium score +1

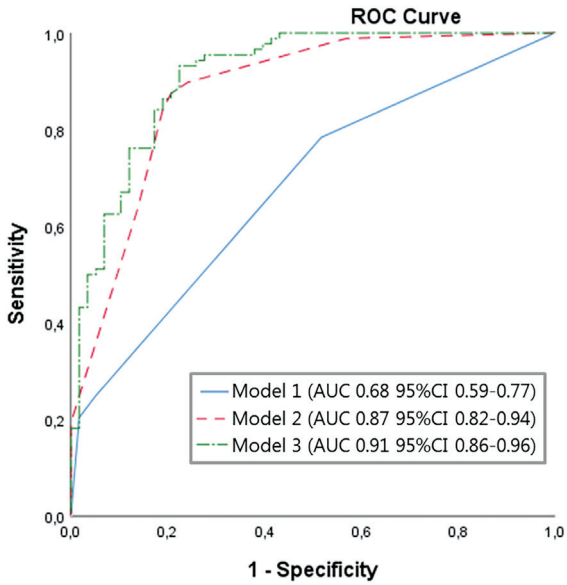

Figure 1. ROC Curves diagnostic models

ROC Curves with corresponding AUC values for all three models in complete cohort. Model 1 contains the clinical predictors, in model 2 the presence of ischemia is added, the third model consist of the clinical predictors, presence of ischemia and the CAC score. P value for difference in AUC between model 2 and 3 was 0.025.

	Model 2 **Model 1 + MPI**		**Model 3** **Model 2 + CAC score**	
	OR (95% CI)	P value	OR (95% CI)	P value
	2.33 (0.91-6.0)	0.078	1.90 (0.70-5.11)	0.212
	11.0 (2.35-51.53)	0.002	10.75 (2.05-56.53)	0.005
	27.7 (10.60-72.26)	<0.001	26.49 (9.45-74.24)	<0.001
	-	-	2.47 (1.40-4.34)	0.002

Diagnostic performance of combined models

Table 2 shows the final three constructed models. The first model contains the clinical predictors (male sex and history of CAD) for the presence of obstructive CAD selected with multivariable logistic regression. In the second model the presence of ischemia on MPI (SDS ≥4, or SDS 1-3 and abnormal MBF/WMA) was added to the first the model. CFR was considered as possible MPI-derived predictor for the presence of obstructive CAD, but it showed no additive effect on the model performance (OR 1.10 95%CI 0.78-1.58 p value 0.501 appendix C1). In the third model, CAC score was added on top of the second model. Both the presence of ischemia on MPI (OR 26.49, 95%CI 9.45-74.24) and the CAC score (OR 2.47, 95%CI 1.40-4.34) were significant predictors for the presence of obstructive CAD in addition to the clinical predictors.

Corresponding ROC curves with AUC values are shown in figure 1. The diagnostic accuracy of MPI to detect obstructive CAD improved with 4% when adding the automatically derived CAC scores (0.87 vs. 0.91). This difference in AUC between model 2 and 3 was statistically significant, p value 0.025.

Table 3 provides an overview of the estimated diagnostic parameters in clinical practice for three single parameters comparing the use of the presence of ischemia alone, CAC scores (dichotomized as either <300 or >300) alone and presence of ischemia and/or a CAC scores above 300. When comparing MPI result on its own (AUC 0.83, 95%CI 0.76-0.90) with only CAC scores (AUC 0.69, 95% CI 0.60-0.78), both sensitivity (0.84 vs. 0.74) and specificity (0.82 vs. 0.63) were better in the model with only MPI, according to existing literature. Addition of CAC scores to MPI data substantially reduced the number of false negative tests (from n=14 to n=4 patients), which leads to a remarkable increase of the sensitivity and negative predictive value. As a consequence, the number of false positive tests is increased (from n=11 to n=28), which affects the specificity and positive predictive value of the tests.

Table 3. Estimated diagnostic performance to predict obstructive CAD in clinical practice

Measure	TP	TN	FP	FN
Ischemia*	76	49	11	14
CAC score†	67	38	22	23
Ischemia_CAC score‡	86	32	28	4

TP = True positive, TN = True negative, FP = False positive, FN = False negative, PPV = Positive Predictive value, NPV = Negative predictive value, *Ischemia = dichotomized with a cut off SDS score of 4 and/or abnormal myocardial bloodflow/coronary flowreserve,

DISCUSSION

This study was a proof-of-concept to see whether our algorithm could automatically determine CAC scores on low-dose CT images gathered during MPI. We showed that presence of ischemia and CAC scores were both significant predictors of obstructive CAD in addition to clinical parameters. We have shown that addition of these CAC scores increased the diagnostic accuracy of MPI to detect obstructive CAD (AUC increase 4%, p value for difference 0.025). The increased diagnostic yield is mainly due to the reduction of false negative test results (N = 14 to N = 4). Important counterpart to this finding was the increased number of false positive tests (N = 11 to N = 28), this needs further research.

Predictors of obstructive coronary artery disease

In line with previous studies, history of CAD, ischemia and the CAC score were significant predictors for the presence of obstructive CAD[3,5,7,9]. In contrast, none of the generally accepted risk factors (smoking, diabetes, hypertension and dyslipidemia) for obstructive CAD were significant predictors in our population[2]. This might be the result of our high risk study population, namely only patients referred for CAG were included. The same is seen in previous comparable studies[12,13].

In our results CFR did not contribute to the prediction of the presence of obstructive CAD (OR 1.10 95% CI 0.78–1.58). Several studies established an association between low CFR and adverse cardiac outcomes[23-26]. However, they did not use CFR as a predictor of obstructive CAD. Taqueti et al. showed that impaired CFR is not only a marker of epicardial disease but especially a marker of diffuse nonobstructive CAD and microvascular dysfunction[27]. They state that CFR might be especially useful in women and diabetic patients. The limited added diagnostic value of CFR in our study was therefore not surprising.

Sensitivity	Specificity	PPV	NPV	AUC (95%CI)
0.84	0.82	0.87	0.78	0.83 (0.76-0.90)
0.74	0.63	0.75	0.62	0.69 (0.60-0.78)
0.96	0.53	0.75	0.89	0.74 (0.66-0.83)

†CAC score = coronary artery calcium score as dichotomous variable, <300 or >300. ‡Ischemia_CAC = dichotomized as either ischemia and/or CAC score >300.

Diagnostic performance of MPI and automated CAC score

Diagnostic performance of MPI and automated CAC score alone were in agreement with previous literature[2]. Existing literature on the added value of CAC scoring in addition to MPI is limited. Bybee et al. analyzed patients with a negative MPI and found subclinical atherosclerosis in 22–30% of the patients with the use of CAC scores[28]. Thompson et al. showed 17% reclassification of patients with normal MPI results into having obstructive CAD after adding CAC scores[29]. Schepis et al. observed the added value of CAC scores in patients with suspected obstructive CAD[11]. They showed an increased sensitivity of MPI after adding CAC scores from 76% to 83%. In our study an even larger beneficial effect was observed (increase of 84% to 96%). Zampella et al. showed an AUC of a combined model with CAC score and MPI (without clinical parameters) of 0.79[13]. Regardless of our much more heterogeneous population we showed similar results (AUC combined model 0.74, 95% CI 0.66-0.83). Danad et al. showed that the incremental value of a combined assessment of PET with coronary CT also depends on which nuclear tracer is used[30]. An important difference between existing literature and our study is the use of a fully automated CAC scoring algorithm, which makes acquisition of extra CT-images and manual scoring unnecessary. These results are therefore more directly applicable in clinical practice because only already available information from PET/CT is used. The clear benefit of our method is the reduction in false negative test results, since this would be of great importance for patientcare. However, the overall performance of our combined model showed slight reduction of diagnostic performance compared to a model with only MPI (AUC 0.74 vs 0.83). This is due to the increased number of false positive test results leading to poor specificity. Special caution for the interpretation of a newly positive tests results after addition of CAC scores is therefore necessary. Future research should focus on this.

Strengths and limitations

This study was a single center retrospective analysis on perfusion imaging data. Patients with a previous CABG were excluded because MPI often yields positive results just above the level of the anastomosis and correlation with epicardial coronary artery disease is notoriously complicated in post CABG patients. We did not use core lab evaluations for the coronary angiography results, however we did perform QCA analysis on all lesions. As in all MPI studies with CAG as reference, there will be referral bias. We observed an increase of false positive tests as a result of the decrease in false negative test results, which has an impact on the specificity. Most patients with negative tests results are not referred for angiography, this might have induced biased assessment of the true negative fraction. However future studies

should focus on reducing the amount of false positive test results to make this method trustworthy in clinical practice. There are several strengths of this study. This is real world data from a center with high numbers of rubidium PET imaging. To our knowledge this is the largest study on the simultaneous assessment of ischemia and CAC scores on MPI images for the detection of obstructive CAD. Another important strength is the algorithm which is used to calculate the CAC scores which is fully automated and easily applicable to data that is already acquired for another purpose. Currently this software is not (yet) free available, however it is possible to purchase a license and use the algorithm.

CONCLUSION

We found that automatically derived coronary calcium scores simultaneously collected with MPI improve the diagnostic accuracy of MPI for the detection of obstructive CAD in patients with suspected myocardial ischemia without previous coronary revascularization.

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Disclosures
None of the authors had a relationship with the industry to declare

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SUPPLEMENTAL MATERIALS

Appendix A Supplementary figure

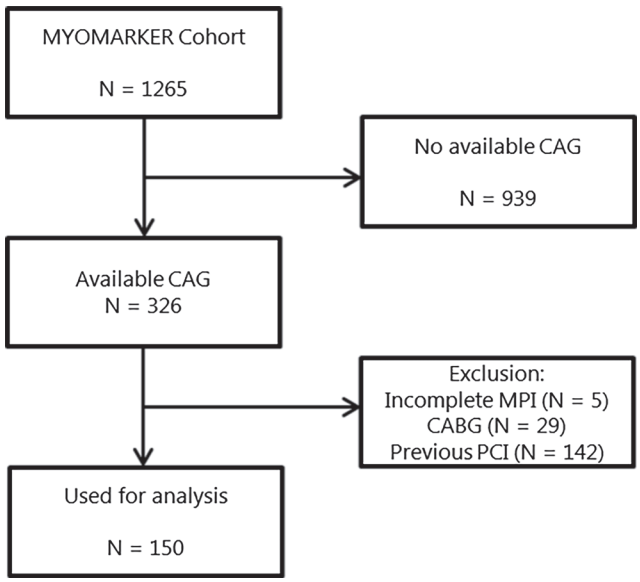

Figure A1. Flowchart patient selection

Original data from MYOMARKER cohort. Selected cohort based on available CAG (coronary angiography) prior to or within 90 after MPI (myocardial perfusion imaging). Patients with CABG (Coronary artery bypass graft) and or previous PCI (Percutaneous coronary intervention) were excluded.

Appendix B supplementary methods

Patients were asked to discontinue caffeine- or methylaxanthine-containing food/drinks and theophylline and dypiridamol 48 hours prior to the PET/CT scan. Rubidium-82 PET/CT scans were acquired using a hybrid scanner (Biograph CT Flow 64-slice scanner, Siemens Healthcare, Knoxville, Tennessee). Rest and stress cardiac PET/CT images were acquired as follows. After scout CT acquisition (120kV; 35mA) to check the patient position, a low-dose CT (120kV; 36mA) was performed for attenuation correction during normal breathing before PET acquisitions. A dose of 1110 MBq of ^{82}Rb was injected intravenously in rest, and a 7-minute 3-dimensional list-mode PET study was acquired. Pharmacologic stress was then administered using regadenoson 0.4mg given intravenously over at least 10 seconds followed by a 10mL saline flush. Approximately 30s after regadenoson injection, a second dose of 1110MBq of ^{82}Rb was administered intravenously and emission images were acquired. Both rest and stress dynamic images were reconstructed into 18 time frames (1x10sec, 8x5sec, 3x10sec, 2x20sec, 4x60sec ; total, 6 minutes) and static images were reconstructed from the myocardial uptake period (150sec-420sec) using ordered-subset expectation maximization 3D reconstruction (2 iterations, 21 subsets) with 6.5mm Gaussian post-processing filtering to reconstruct images. The estimated effective radiation dose for this protocol to the patients was 3.7mSv. Heart rate, systolic blood pressure and 12 lead ECG were recorded at baseline, 1 minute after regadenoson administration and after imaging. We performed rate pressure product (RPP) adjustment by adjusting to an assumed normal resting RPP of 8000. The calculation of the correction factor was [measured resting RPP/8000] and correction was performed as [measured resting MBF/(measured resting RPP/8000)]. The CFR presented in our manuscript was based on the adjusted resting MBF value.

Appendix C Supplementary tables

Table C1. Univariate analysis on clinical predictors of obstructive CAD

	Univariate analysis		
	Odds-Ratio	**95% CI**	**P-Value**
Predictor			
Male sex	3.11	1.56-6.23	0.001
History of CVD	1.62	0.78-3.39	0.198
History CAD	6.15	1.75-21.60	0.005
History of MI	6.27	1.39-28.37	0.017
History of PAD	1.60	0.40-6.46	0.507
Family history CAD	0.91	0.44-1.84	0.783
Diabetes mellitus	1.00	0.46-2.17	1.00
Hypertension	1.40	0.71-2.77	0.330
Dyslipidemia	0.77	0.40-1.51	0.460
Current smoker	0.82	0.52-2.31	0.820
ACE inhibitor or ARB	1.58	0.80-3.13	0.193
Platelet aggregation inhibitor	1.76	0.91-3.41	0.094
CFR	1.10	0.78-1.58	0.581
Stress MBF	0.90	0.72-1.13	0.372

MI = Myocardial infarction, PCI = percutaneous coronary intervention, CVD = cardiovascular disease, CAD = coronary artery disease, PAD =Peripheral artery disease, CFR = coronary flow reserve, MBF = myocardial bloodflow

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CHAPTER 8

The prognostic value of automated coronary calcium derived by a deep learning approach on non-ECG gated CT images from 82Rb-PET/CT myocardial perfusion imaging

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ABSTRACT

BACKGROUND

Assessment of both coronary artery calcium (CAC) scores and myocardial perfusion imaging (MPI) in patients suspected of coronary artery disease (CAD) provides incremental prognostic information. We used an automated method to determine CAC scores on low-dose attenuation correction CT (LDACT) images gathered during MPI in one single assessment. The prognostic value of this automated CAC score is unknown, we therefore investigated the association of this automated CAC scores and major adverse cardiovascular events (MACE) in a large chest-pain cohort.

INTRODUCTION

We analyzed 747 symptomatic patients referred for ^{82}RubidiumPET/CT, without a history of coronary revascularization. Ischemia was defined as a summed difference score≥2. We used a validated deep learning (DL) method to determine CAC scores. For survival analysis CAC scores were dichotomized as low (<400) and high (≥400). MACE was defined as all cause death, late revascularization (>90 days after scanning) or nonfatal myocardial infarction. Cox proportional hazard analysis were performed to identify predictors of MACE.

RESULTS

During 4 years follow-up, 115 MACEs were observed. High CAC scores showed higher cumulative event rates, irrespective of ischemia (nonischemic: 25.8% vs 11.9% and ischemic: 57.6% vs 23.4%, P-values <0.001). Multivariable cox regression revealed both high CAC scores (HR 2.19 95% CI 1.43-3.35) and ischemia (HR 2.56 95% CI 1.71-3.35) as independent predictors of MACE. Addition of automated CAC scores showed a net reclassification improvement of 0.13 (0.022-0.245).

CONCLUSION

Automatically derived CAC scores determined during a single imaging session are independently associated with MACE. This validated DL method could improve risk stratification and subsequently lead to more personalized treatment in patients suspected of CAD.

INTRODUCTION

The prognostic and diagnostic role of myocardial perfusion imaging (MPI) in patients suspected of coronary artery disease (CAD) has been very well established during the past decades[1]. However, this functional test modality is not able to detect subclinical atherosclerosis, or nonflow-limiting coronary stenosis. Additional to this, one of the most thoroughly studied test modalities in the establishment of coronary artery disease (CAD) is the coronary artery calcium (CAC) score[2–5]. CAC is seen as a highly specific manifestation of atherosclerosis and therefore considered to be an excellent anatomic measure of plaque burden[5–7]. There is a growing body of evidence showing that quantitative assessment of CAC scores improves the prognostic ability of MPI in the detection of major adverse cardiovascular events (MACE)[8–10].

Considering this clear complementary value of both MPI and CAC scores, it would be ideal to combine both in preferably one imaging session. Most existing studies performed an additional scan to obtain CAC scores. This leads to a higher effective radiation dose for the patient and often requires manual assessment by a trained physician, which is unfavorable in an era of growing interest for algorithms based on machine learning leading to improved accuracy[11].

We used a previously validated deep learning (DL) method to automatically determine CAC scores on non-ECG-gated low-dose attenuation correction CT (LDACT) images during one single imaging session, without additional scanning[12]. A previously performed study investigated the diagnostic role of this DL method in symptomatic patients undergoing Rubidium-82 (^{82}Rb) PET/CT scanning[13]. The AUC to detect obstructive CAD with MPI improved from 0.87 to 0.91 after the addition of the automated CAC score. Whether this automated CAC score is also associated with MACE is unknown. The aim of this study was therefore to determine the association of automated CAC scores (derived during MPI in one single imaging session) with MACE.

METHODS

The MYOMARKER (MYOcardial ischemia detection by circulating bioMARKERs) study is a prospective single-center observational cohort study of consecutively enrolled outpatient clinic patients aged>18 years with suspected CAD. Patients were included between August 2014 and September 2016 at the Meander Medical Center (Amersfoort, the Netherlands). All patients underwent ^{82}Rubidium PET/CT as part of their diagnostic work up. For the purpose of this study only patients without a history

of coronary revascularization were included. The study (NL5078) was approved by the regional Medical Ethics Committee and performed in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants. Patients were not involved in the design or recruitment of the study.

A detailed description of the MPI protocol has been published previously[13]. In short, patients were asked to discontinue caffeine- or methylaxanthine-containing food/drinks and theophylline and dypiridamol 48 hours prior to the PET/CT scan. Scans were acquired using a hybrid scanner (Biograph CT Flow 64-slice scanner, Siemens Healthcare, Knoxville, Tennessee). Standard acquisition parameters were used e.g. 120kVp and 35mA. Both rest and stress images were acquired in the same session, regadenoson was administered intravenously as pharmacological stress. The estimated effective radiation dose for the patients was 3.7mSv. Rate-pressure product was calculated for manual correction of rest flow values.

^{82}RbPET/CT MPI results were assessed according to the 17-segment model of the American Heart Association[14]. Semi-quantitative analysis was performed using the summed difference score (SDS) for each patient. Patients were assigned to either positive or negative for ischemia on ^{82}RbPET/CT, using SDS≥2 as a threshold for ischemia[15].

CAC scores were determined from the LDACT scan, a standard part of MPI for attenuation correction, using a previously developed algorithm[16,17]. No additional ECG-gated CT images were obtained, nor was the MPI protocol adapted to enable the measurement of CAC scores. Briefly, the lungs are excluded first by the software to identify a region of interest. The software then automatically detects voxels above the standard threshold of 130 Hounsfield Units as CAC using a deep learning approach. No adaptation for the threshold of CAC scores was needed since a protocol with 120kVp, typically used for CAC scoring was used [18]. Calcifications were first labelled according to the presumed affected coronary vessel (left anterior descending including left main coronary artery, left circumflex artery and right coronary artery and then the CAC scores were calculated[19]. Since this method is not (yet) able to distinguish previously placed coronary stents from coronary calcium, patients with prior coronary revascularization were excluded from the analysis.

Patients received a questionnaire to inquire information about cardiac events or other medical procedures after 30 days, 1 year and 3 years. Collection of follow-up was performed per batch of approximately 300 patients and started if the last patients reached the 3 year timepoint. As result slight differences in follow up-time occurred.

If patients did not respond to the questionnaire they received a reminder by mail, in case of no response their general practitioner was contacted, or their hospital records were used. This thorough attempt to collect as much follow-up details as possible resulted in follow-up details that were received up to 1 year after the initial invitation to deliver the required questionnaire.

The primary outcome, MACE included all cause death, myocardial infarction and coronary revascularization (percutaneous coronary intervention (PCI) and/or coronary artery bypass graft (CABG))[20]. Early revascularization within 90 days after MPI was considered to be triggered by the MPI result and therefore excluded[21]. In patients with multiple events, only the first event was considered for survival analysis. All endpoints were adjudicated by two members of the research team, in case of disagreement or uncertainty a third member was involved.

Continuous data are presented as mean ± standard deviation, discrete data as frequencies and percentages. To compare the prevalence of abnormal ^{82}RbPET/CT by CAC category, Cochrane's Armitage test for trend was performed. To facilitate comparison with prior studies, a binary cut point of the CAC score (≥400) was used for survival analysis[22]. Patients were divided in four groups: considering both the presence or absence of ischemia and high (≥400)/low CAC score (<400). Cumulative event rates were computed for each category with the Kaplan-Meier method. Survival between groups was compared performing the Log-Rank test. To account for multiple testing post-hoc analysis with the Bonferroni correction was performed. An additional subanalysis without revascularization was performed. Cox-proportional hazard regression analysis was used to identify predictors for MACE. To appropriately account for heterogeneity among the study population, the analysis was adjusted for prespecified covariates, encompassing: age, sex, history of CAD and cardiovascular risk factors[23]. For all regression analyses, the proportional hazard assumption was tested with both a formal test and the Schoenfeld residual plots. No violation of the proportional hazard assumption was found.

To assess the potential clinical impact of the use of the use of this automated CAC score the net reclassification improvement (NRI) was calculated[24,25]. First internal validation was performed with bootstrapping to control for optimism and overfitting, additionally the NRI was calculated. For the computation of the NRI we censored follow up times at 4 years. Risk categories were defined as <5%; 5-7.5%; 7.5-20% and >20% as proposed by Greenland et al[5]. All hypotheses tests were two-sided with a critical significance level of <0.05. Statistical analysis was performed with R software (R software, version 3.5.1).

RESULTS

The complete study population of the MYOMARKER cohort consisted of 1265 patients. MPI was not performed in four patients (0.3%), no informed consent was obtained from 13 patients and 1 patient was lost to follow up. Another 500 patients were excluded because of a history of PCI/CABG. The remaining 747 patients are the subject of this report (figure 1). Among them a total of 195 (26.1%) showed an ischemic perfusion defect with SDS ≥2 on ^{82}RbPET/CT. The baseline characteristics for the complete cohort and stratified by MPI result are summarized in table 1. Mean age of the all patients was 67 (±10) and 50.5% were male. Most common risk factors were hypertension (62.1%) and hypercholesterolemia (50.9%). Patients with a ^{82}RbPET/CT positive for ischemia were more often male (45.7% vs. 64.1%, p value <0.001). No significant differences between both groups were seen regarding the known cardiovascular risk factors. Patients with ischemia more often had a history of coronary artery disease (16% vs 4%, p value 0.001) and more often used P2Y12 inhibitors (10.8% vs. 5.7%, p value 0.026) and beta blockade (54.1% vs. 42.5%, p value 0.007).

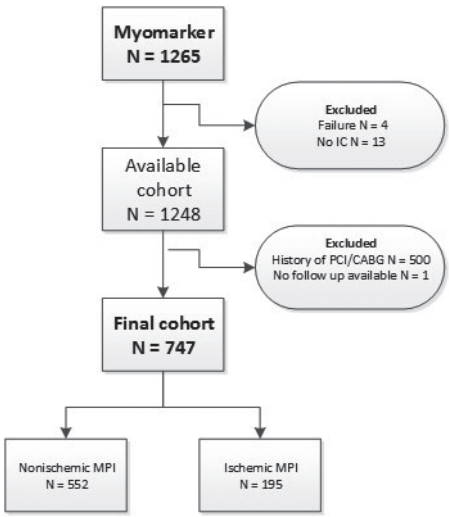

Figure 1. Flowchart study population

Table 1. Baseline characteristics

	Overall	Nonischemic	Ischemic	P value[1]
n	747	552	195	
Demographics				
Age, years	67±10	67±10	68±10	0.125
Male sex (%)	377 (50.5)	252 (45.7)	125 (64.1)	<0.001
BMI	27.6±5.2	27.3±5.0	28.3±5.8	0.020
Risk factors				
Current smoking	143 (19.1)	97 (17.8)	44 (22.7)	0.171
Diabetes Mellitus	140 (19.1)	97 (17.8)	42 (21.6)	0.289
Hypertension	464 (62.1)	340 (62.5)	121 (62.4)	1.000
Hypercholesterolemia	380 (50.9)	276 (50.7)	98 (50.5)	1.000
Family history CAD	180 (24.1)	129 (23.7)	49 (25.3)	0.738
Medical history				
Cardiovascular disease	567 (76.0)	413 (75.9)	148 (76.3)	0.996
Coronary artery disease	53 (7.1)	22 (4.0)	31 (16.0)	<0.001
Heart failure	37 (5.0)	24 (4.4)	13 (6.7)	0.288
Atrial fibrillation	119 (15.9)	84 (15.4)	34 (17.5)	0.571
Ischemic CVA	31 (4.1)	20 (3.7)	10 (5.2)	0.494
Drug therapy				
Aspirin	303 (40.6)	211 (38.8)	89 (45.9)	0.101
P2Y12-inhibitors	53 (7.1)	31 (5.7)	21 (10.8)	0.026
Anti-coagulants	140 (18.7)	97 (17.8)	42 (21.6)	0.289
Statin	350 (46.9)	253 (46.5)	92 (47.4)	0.892
ACE/AT-inhibitor	329 (44.0)	229 (42.1)	97 (50.0)	0.069
B-blocker	337 (45.1)	231 (42.5)	105 (54.1)	0.007

Values are shown as mean±SD or frequency with corresponding percentages. CAD = coronary artery disease, CVA = Cerebrovascular accident, AT = Angiontensine II, B-blocker = betablockade. [1]P values for comparison between nonischemic and ischemic ^{82}Rb PET/CT. Ischemia was defined as summed difference score ≥2.

CAC scores were successfully derived in 726 of the 747 patients. In 21 patients no valid scores could be obtained due to insufficient image quality. In total 126 patients had a CAC score of zero, and a ^{82}RbPET/CT positive for ischemia was found in 12% of these patients. The frequency of ^{82}RbPET/CT results positive for ischemia increased gradually with an increased CAC score (P value for trend <0.001, figure 2). An abnormal ^{82}RbPET/CT was seen in 38% of patients with a high CAC score (≥400).

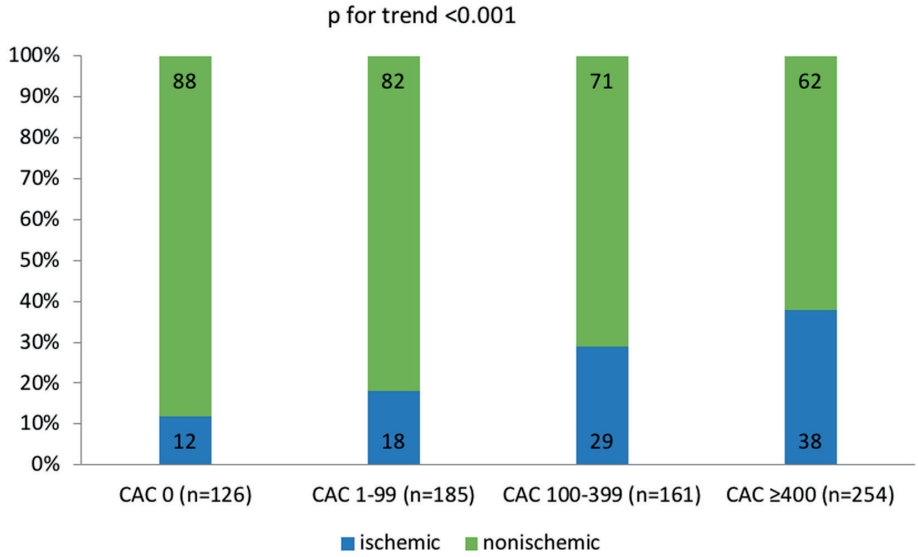

Figure 2 Prevalence of abnormal MPI results by CAC score. CAC=coronary artery calcium. MPI = myocardial perfusion imaging

Table 2 shows the results of the automatically derived CAC scores stratified by MACE separately for patients with and without ischemia on their ^{82}RbPET/CT. The occurrence of MACE was higher in patients with an ischemic ^{82}RbPET/CT (31%) result compared to patients without (10%). For both patients with and without ischemia, mean Agatston score was significantly higher in patients with a MACE (400±725 vs. 998±1110, P value <0.001 and 593±916 vs. 1443±1427, P value <0.001).

Median follow-up time was 3.62 (±0.82) years during which 115 patients reached the outcome MACE. Table 3 shows the occurrence of MACE and its components stratified by MPI result and CAC score. MACE consisted of 24 myocardial infarctions, 48 revascularizations and 43 all cause deaths.

Table 2. CAC results according to MACE stratified by MPI result

	No MACE	**MACE**	P value
Total	632	115	
Nonischemic	**497**	**55**	
Agatston score	400±725	998±1110	<0.001
Categorical calciumscore			<0.001
0	108 (22.5)	3 (5.5)	
1-99	141 (29.3)	10 (18.2)	
100-399	103 (21.4)	12 (21.8)	
>400	129 (26.8)	30 (54.5)	
Ischemic	**135**	**60**	
Agatston score	593±916	1443±1427	<0.001
Categorical calciumscore			0.001
0	14 (10.8)	1 (1.7)	
1-99	28 (21.5)	6 (10.0)	
100-399	36 (27.7)	10 (16.7)	
>400	52 (40.0)	43 (71.7)	

Agatston score is shown as mean±SD, categorical calciumscores are shown as frequency (%). MACE comprises: nonfatal MI, revascularization and all-cause death. Ischemia was defined as SDS≥2

Table 3. MACE stratified by ischemia and CAC

	No ischemia/ low CAC *n=394*	**No ischemia/ high CAC** *n=159*	**Ischemia/low CAC** *n=99*	**Ischemia/high CAC** *n=95*
MACE	**24 (6.2%)**	**32 (20.2%)**	**17 (17.1%)**	**42 (44.2%)**
All cause death	16 (4.1%)	20 (12.6%)	4 (4.0%)	3 (3.2%)
Myocardial infarction	3 (0.8%)	6 (3.8%)	3 (3.0%)	12 (12.6%)
Revascularization	5 (1.3 %)	6 (3.8%)	10 (10.1%)	27 (28.4%)

MACE = Major cardiovascular event. Ischemia was defined as SDS≥2. CAC = Coronary artery calcium scores. Low = <400, high = ≥400.

Patients were divided in four groups according to their MPI result and CAC score for the survival analysis. Cumulative event rates differed significantly between the groups (P value Log-Rank test <0.001) (Figure 3). Post-hoc analysis showed, irrespective of the presence of ischemia, a significantly higher MACE rate in patients with high CAC scores compared to patients with low CAC scores (P value for both comparisons <0.001).

Cumulative event rates in patients without ischemia were 11.9% in patients with low CAC scores and 25.8% in patients with high CAC scores. The difference in cumulative event rates was even more pronounced in patients with ischemia: 23.4% in those with low CAC scores and 57.6% in patients with high CAC scores. Supplemental figure 1 shows the same survival analysis but only for the composite endpoint combining all cause death and myocardial infarction. It can be appreciated that the MACE rate was, irrespective of ischemia, significantly higher in patients with high CAC scores (P value patients without ischemia <0.001, P value patients with ischemia <0.01). These results are consistent with the findings in figure 3.

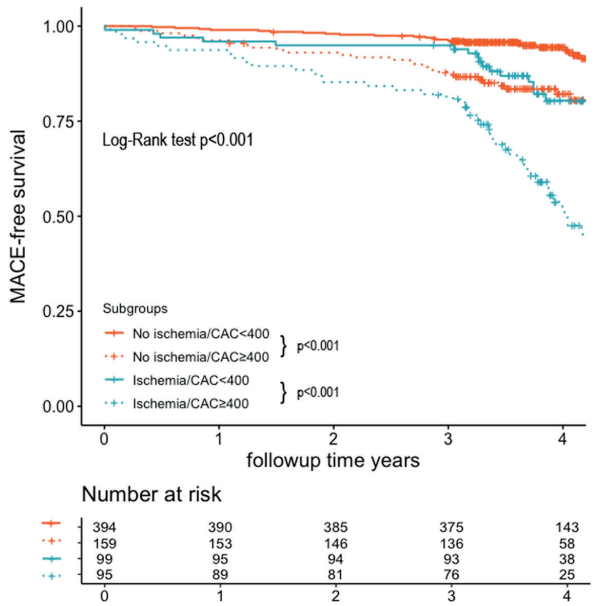

Figure 3 MACE free survival curves according to MPI result and CAC score.

Patients were divided in four groups according to both MPI results and CAC scores. P value for comparisons among subgroups were adjusted according to the Bonferroni-Holm correction.

To determine the association of the automated CAC score with MACE, univariable and multivariable cox regression analysis were performed with all preselected covariates (table 4). Age (HR 1.03 95%CI 1.01-1.05), male sex (HR 2.34 95%CI 1.57-3.48) and a history of CAD (HR 2.77 95%CI 1.69-4.53) were significant clinical predictors of MACE in the univariable analysis. Both established ischemia on ^{82}RbPET/CT (HR 3.19 95%CI 2.41-4.60) as well as high automated CAC scores (HR 3.49 95%CI 2.39-5.10) were significant univariable imaging predictors of MACE. After adjustment for all prespecified covariates both ischemia (HR 2.14 95%CI 1.45-3.17) and high automated CAC (HR 2.26 95%CI 1.48-3.46) remained independently associated with MACE.

Table 4. Unadjusted and adjusted Cox proportional regression analysis for MACE

	Univariable		Multivariable*	
Variable	HR (95% CI)	P value	HR (95% CI)	P value
Age	1.03 (1.01-1.05)	0.002	1.03 (1.00-1.05)	0.046
Male sex	2.34 (1.58-3.48)	<0.001	1.85 (1.21-2.81)	0.007
BMI	1.01 (0.98-1.05)	0.463	-	
Hypertension	1.40 (0.94-2.08)	0.095	-	
Hypercholesterolemia	0.95 (0.66-1.37)	0.780	-	
Diabetes Mellitus	1.17 (0.75-1.82)	0.498	-	
Smoking	1.30 (0.85-2.01)	0.228	-	
Family history CAD	1.16 (0.76-1.75)	0.494	-	
Known CAD	2.77 (1.69-4.53)	<0.001	-	
Ischemia	3.19 (2.21-4.60)	<0.001	2.14 (1.45-3.17)	<0.001
CAC≥400	3.49 (2.39-5.10)	<0.001	2.26 (1.48-3.46)	<0.001

CAD = Coronary Artery Disease, CAC = Coronary Artery Calcium, MACE comprises: nonfatal MI, revascularization and all-cause death, ischemia was defined as SDS≥2. *Adjusted for age, sex, history of CAD and cardiovascular risk factors

Addition of the automated CAC scores to a model with all predefined clinical predictors and ischemia yielded a total NRI of 0.13 (95% CI 0.022-0.245) which was significant. The NRI for patients with MACE was not significant 0.070 (95% CI -0.032-0.171) with a correctly reclassification of 19.1% compared to incorrect reclassification of 12.2%. For patients without MACE the NRI was 0.064 (0.018-0.109) In total 19.8% of patients were correctly reclassified compared to incorrect reclassification of 13.4%. Reclassification tables can be found in the supplemental materials.

DISCUSSION

In this study we showed that automated CAC scores are associated with MACE, irrespective of the MPI results and other prespecified confounders. Our results extend the existing knowledge regarding the combined use of CAC scoring and MPI by the use of an automated DL method that determines CAC from non-ECG-triggered CT images in one single imaging session. This DL method has recently been validated for a wide range of chest CT modalities (e.g. lung cancer screening CT, breast cancer CT and CT scans derived for radiotherapy planning)[12]. This method uses non-ECG-triggered CT images obtained for perfusion imaging, as a result no adaptations to the regular MPI are needed and no additional time is required to obtain ECG-triggered images. Most important advantage is that by using the non-ECG-triggered images the effective radiation dose remains the same. The aforementioned validation study showed both an excellent correlation between the DL derived CAC scores and manual CAC scores, and also correct classification of patients to their risk categories[12]. We therefore assume that implementation of this DL method to automatically determine CAC scores on ^{82}RbPET/CT images could have immediate clinical consequences.

In our cohort the prevalence of an abnormal ^{82}RbPET/CT result gradually increased by CAC score (P for trend <0.001). Since CAC is seen as marker to determine the extent of coronary sclerosis, this finding seems logical, as more coronary sclerosis translates into higher risk for future events. This finding is consistent with previous studies[8,9,26]. However, the extent of coronary sclerosis, e.g. a high CAC score is not the same as obstructive stenosis, e.g. stress-induced ischemia[27]. Normal ^{82}RbPET/CT results were found in 62% of the patients with high (≥400) CAC scores. Our results are in line with existing literature and confirm the poor correlation between CAC scores and flow-limiting obstructions[8,28,29]. In addition, a discrepancy between CAC scores and ^{82}RbPET/CT results was also found at the other end of the spectrum. Low CAC but abnormal ^{82}RbPET/CT was found in 12% of the patients. It has been suggested, and it is very likely that this is caused by noncalcified obstructive lesions[9]. Despite these discrepancies, CAC scores were independently associated with MACE in this study cohort.

Cox proportional hazard regression analysis showed that patients with high CAC scores had a 2.26 times higher risk of MACE compared to patients with a low CAC score. This association was found to be irrespective of known cardiovascular risk factors and the presence of ischemia on MPI. Several other studies show the same result, in both PET and SPECT perfusion imaging, as well as asymptomatic and symptomatic patients[8,9,29,30]. In contrast, Rozanski found that high CAC scores did not predict future MACE in patients with normal MPI results[31]. This might be due to the low risk profile of the study cohort and/

or short follow-up time in this cohort. After internal validation to correct for optimism and overfitting, the effect of the addition of the automated CAC score to a model with MPI data (and clinical covariates) on risk stratification was assessed. We found a significant overall NRI as a result of a significant NRI for patient without MACE. In total 19.8% of patients without MACE were correctly reclassified into a lower risk category with the addition of the automated CAC score. The greatest advantage of the reclassification was seen in the lower risk categories. This could be useful in clinical practice as in identifies patients that would not qualify for additional therapy and/or invasive CAG. The use of risk categories is however always arbitrary, a larger validation study is warranted to further elaborate this.

The results of our study strengthen the evidence of the complementary value of both functional and anatomical testing. With this DL method CAC scores can be obtained automatically without additional radiation exposure for the patient due to additional ECG-triggered CT scanning. The difference between the determination of the anatomical burden with CAC scores and functional testing could also be seen in the different components of MACE. Revascularization was mainly driven by ischemia on MPI (37 out of 48), and on the other hand 18 out of 24 patients suffering from a myocardial infarction showed high CAC scores. Previous studies have shown that the incremental predictive ability of a high CAC score can help identify patients with normal MPI PET/CT but obstructive CAD[9,32,33]. Also, in patients with ischemia there is a substantial difference in cumulative event rates between those with high and low CAC scores (57.6% vs. 23.4%). Our subanalysis without revascularization still revealed a significant difference in event rate in patients with ischemia between high and low CAC scores. Identification of patients with ischemia and high CAC scores might therefore lead to more aggressive treatment regimes, which potentially could reduce future events. Moreover, considering the significant NRI specifically for patients without MACE, it could also lead to more accurate identification of patients with a lower risk for future MACE. Our proposed combined assessment of a single imaging test could therefore improve clinical management by making a more accurate assessment of a patients' risk for future events than ischemia detection alone. This could lead to more efficient use of our healthcare system.

Although our results reflect regular care in a large hospital in the Netherlands it remains a single center, observational study. Considering its observational nature, it remains unclear if CAC score results would change clinical decision making. Future studies are needed for this. The proposed DL method is easily applicable, although only available with a purchased license. Lastly, the results of this study are only applicable to patients without a history of percutaneous intervention since our DL method is currently not able to distinguish between a coronary stent and calcium.

CONCLUSION

Automated CAC scores derived together with MPI in one single test are, irrespective of the presence of ischemia, associated with MACE in patients with suspected CAD. With the use of a DL algorithm CAC scores can be reliably obtained on non-ECG-triggered images. There is no additional radiation exposure for the patient and no manual scoring for the physician needed to obtain this automated CAC score. We therefore propose this single assessment DL method of calculating the CAC score to improve cardiovascular risk stratification in patients suspected of CAD undergoing MPI.

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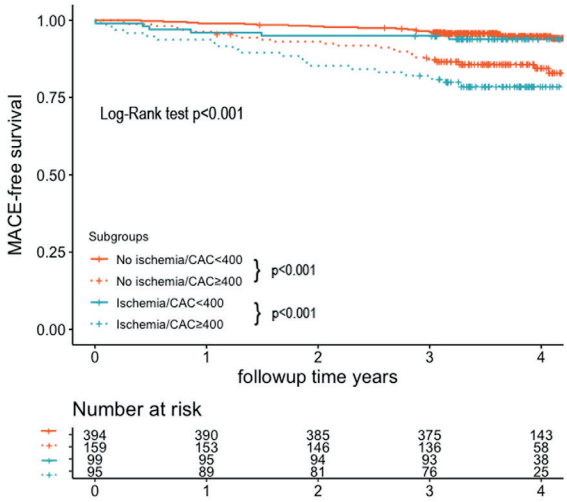
Supplemental table 1. Net reclassification Improvement for outcome MACE

Model consisting ischemia[1]	Model consisting of ischemia and CAC[1] <5%	5-7.5%	7.5-20%	>20%	Reclassified up, n(%)	Reclassified down, n(%)	NRI (95% CI)
MACE					22 (19.1)	14 (12.2)	0.070 (-0.032-0.171)
<5%	**4**	0	1	0			
5-7.5%%	2	**4**	8	0			
7.5-20%%	0	7	**43**	13			
>20%	0	0	5	**28**			
No MACE							
<5%	**128**	4	4	0	82 (13.4)	121 (19.8)	0.064 (0.018-0.109)
5-7.5%	44	**85**	48	0			
7.5-20%	0	61	**169**	26			
>20%	0	0	16	**26**			
Overall							0.133 (0.022-0.245)

[1]Both models consisted of general cardiovascular riskfactors, age, sex and history of coronary artery disease. CAC = Coronary Artery Calcium. Numbers in red indicate wrong reclassification, numbers in green indicate correct reclassification.



Supplemental figure 1. MACE free survival curves according to MPI result and CAC score

MACE included all cause death and myocardial infarction. Patients were divided in four groups according to both MPI results and CAC scores. P value for comparisons among subgroups were adjusted according to the Bonferroni-Holm correction.

PART III

Summary and discussion

CHAPTER 9

Summarizing discussion and future perspectives

SUMMARIZING DISCUSSION AND FUTURE PERSPECTIVES

Adequate risk stratification in patients complaining of chest pain is one of the major tasks for health care physicians. Not only will the physician determine if a patient has coronary artery disease (CAD) but also his or her risk for experiencing future cardiovascular events. Multiple risk scores, imaging techniques, and invasive measures exist to enable risk stratification. However, considering the costs for imaging techniques, the potential unnecessary exposure to radiation and the increased use in low-risk populations, there is an unmet need for a new gatekeeper with less impact on healthcare burden. The ultimate aim of this thesis was to investigate the role of extracellular vesicles (EVs) as potential gatekeeper in the risk stratification of patients suspected of CAD.

Part one: the diagnostic role of extracellular vesicles in coronary artery disease
Despite the existence of the aforementioned risk scores and guidelines to determine if referral to a cardiologist is necessary, ~80% of all referred patients do not show signs of clinically relevant CAD[1]. Lowering the number of unnecessary referrals will have immediate impact on healthcare burden for both patients and healthcare physicians. A widely available and inexpensive biomarker could serve an important role in this. During the last decade many different biomarkers have been proposed to diagnose patients with a chronic coronary syndrome (CCS)[2,3]. But until now, none of them reached sufficient accuracy to be implemented in clinical practice. A specific source for biomarkers gaining more and more attention are EVs, they are small bilayer membrane particles in the blood[4]. In several observational cohort studies EVs have shown to be associated with both the presence and extent of cardiovascular disease, and also with long term prognosis[4]. The diagnostic role for EVs in CAD is relatively unexplored. In **chapter 2,** we studied the associations between previously selected EV-derived proteins and the presence of stress induced myocardial ischemia. The analysis was performed in different EV subpopulations (with different densities), based on the hypothesis that the different populations provide additional information as a result of a more detailed cellular view. For this, we separated two subpopulations (called the HDL subpopulation and LDL subpopulation, respectively), as described in the study of Wang et al[5]. We showed in a cohort of 450 patients that in the EV-HDL subpopulation EV-Serpin C1, EV-CD14, EV-Serpin G1 and EV-Cystatin C were independently associated with CCS. In the LDL subpopulation, EV-CD14 and EV-Cystatin C were associated with CCS. Sex differences in diagnosis and prognosis of patients suspected for CCS are increasingly acknowledged and receive a lot of attention[6]. Therefore, a sex-stratified subgroup analysis was performed. This revealed that all associations found were

attributed to the associations in women. More specifically, none of the associations found in the complete cohort remained significant in men. Although this finding was interesting, these sex-specific results could only be interpreted as hypothesis generating, since the study was underpowered with only 46 women with the outcome. The exact mechanistic differences between men and women remain to be elucidated, however, one of the proposed ideas relies on the different role of inflammation between both sexes[7]. It is thought that inflammation, metabolic syndrome and obesity have a substantially larger impact on the progression of atherosclerotic disease in women compared to men[8]. The results found in our study contribute to this idea since the proteins that were associated with CCS in our sex-stratified subgroup analysis are all known for their role within the inflammatory cascade.

Another entity of CAD, often considered as less stable, is unstable angina (UA). The incidence of UA has markedly decreased after the development of high-sensitive cardiac troponin assays, but patients suffering from UA remain an important group of patients[9]. We investigated the associations of EV-derived proteins with the diagnosis of UA in **chapter 3**. We studied whether the aforementioned EV-proteins were associated with UA, indicating a potential use as biomarker. Only EV-Cystatin C in the TEX-subpopulation was found as independent predictor for the presence of UA. The protein Cystatin C is an inhibitor of proteases, serving an important role in the inflammatory cascade[10]. Cystatin C has been described widely in the cardiovascular field for its association with renal dysfunction but also for the close relation with coronary artery disease in general[11–13]. Although a clear association between Cystatin C and atherosclerotic disease seems to exist, it remains questionable if Cystatin C would be specific enough to serve as a biomarker for UA[10].

In **chapter 4** we review the role of EVs and biomarkers in diagnosing CCS. Most important steps to make towards clinical implementation are: 1. Optimization of isolation and quantification techniques with automated methods, 2. The use of internal standards during isolation, and 3. To analyze large cohorts. In this chapter, we describe the significant heterogeneity within the population of CAD, which raises the question if the same proteins should be used for all patients. Large cohorts are warranted to enable subanalyses to study potential differences among different patient characteristics. In **chapter 5** we performed a large cohort study in 1034 patient from the ongoing prospective Basel VIII cohort study. This study was designed to further elaborate on the early detection of functional relevant CAD (fCAD). We found Cystatin C in the LDL subpopulation to be an independent predictor of fCAD. After addition of EV-derived proteins to a model with a clinical judgement (CJ) score a significant increase was found. However, the accuracy (measured with area under

the curve (AUC)) of the combined model was only moderate with an AUC of 0.68. We were not able to replicate the sex-based differences from **chapter** 2 in this large unselected cohort. An interesting subgroup analysis based on the type of stress induction (physiological versus pharmacological) revealed a significantly increased diagnostic accuracy among patients undergoing physiological stress from 0.73 (model with CJ + outcome of exercise test) to 0.75 (model with CJ, exercise results + EV-protein data). This still does not suffice for clinical implementation but it is clearly better compared to the diagnostic accuracy in the entire cohort (AUC 0.68). Not much is understood about the pathophysiological difference on myocardial cell level between pharmacological and physical stress, but it is thought that pharmacological stress enables identification of flow disabilities rather than actual myocardial ischemia[14]. Previous studies have shown differences between pharmacological stress and physical stress in both physiological response of the left ventricle as well as biomarker release[14,15]. Considering the easy availability and low costs of physical exercise testing, our findings encourage to further investigate the potential use of EVs as adjunct to physical exercise testing. This might potentially enable physicians to better stratify a patients risk of having fCAD and eventually reduce the number of unnecessary referrals and subsequently reduce healthcare burden.

Part two: the bone-heart axis

Risk stratification is also important to assign patients to their appropriate risk category concerning future cardiovascular events, i.e. assessing prognosis rather than diagnosis. Currently one of the strongest prognostic predictors available is the coronary artery calcium (CAC) score. CAC score measures relate to the extent of vascular calcification (VC), and are considered the best anatomical measure of atherosclerotic disease burden[16]. VC shows similarities with the process of bone formation and degradation[17]. A key-regulator of bone formation and degradation is osteoprotegerin (OPG) but OPG is also implicated as a regulator of VC[18]. Existing literature regarding the exact role of OPG in VC remains unclear. In mice studies high levels of plasma OPG were associated with less VC whereas in humans high levels of plasma OPG seems to be associated with future cardiovascular events[19–21]. EVs derived from vascular smooth muscle cells (VSMCs) are thought to play a key role in VC. Previous studies indicate that OPG is transported by these VSMC-derived EVs, since they were found near calcium deposits in the vascular wall[22]. Moreover, OPG transported by VSMC-derived EVs seems to lower the amount of calcification in the vessel wall/plaque. The role of OPG derived from (VSMC or circulating) EVs might help to understand the different association found between mice and humans. However, measuring OPG in VSMC-derived EVs is not suitable for clinical practice. We therefore investigated in **chapter 6** if OPG derived from circulating EVs rather than directly

from plasma or VSMC-derived EVs might help to further unravel the role of OPG in VC. Our hypothesis was that circulating EV-derived OPG, in contrast to plasma levels of OPG and in line with the experimental studies, would show a negative association with the CAC score. We measured OPG levels in 750 patients suspected of CCS in plasma and two EV-subpopulations (LDL and TEX). Our main finding was that both levels of plasma OPG as well as EV-derived OPG were independently associated with CAC scores as surrogate of VC. For both plasma and EV-derived OPG this association was positive. This was in contrast to our hypothesis of an inverse association in EVs compared to plasma. Our results suggest OPG levels in plasma and EVs both mirror the same process more than actively regulating it. Additionally, circulating EVs seems to have a different role in the process of VC compared to VSMC derived EVs.

Combined assessment of coronary calcium and myocardial perfusion

Although, CAC is considered as the best anatomical measure of atherosclerotic disease burden, studies have shown that the CAC score does not correspond well with luminal degree of stenosis[23]. Thus, the diagnostic accuracy of coronary CT derived CAC score to diagnose significant CAD is limited. Although the role of CT angiography is currently rapidly developing, also in the intermediate risk patients, it remains an anatomical assessment without functional information[24]. An upcoming imaging technology to overcome this is FFR_{CT}. Several large studies have shown sensitivities and specificities of this technique ranging from 76%-89% and 61%-86%[25]. However, in up to 30% of patients, image quality is insufficient which makes it impossible to measure FFR_{CT}. Despite adequate image quality, the results are also highly dependent on adequate use of betablockade and nitroglycerin during CT[26]. All factors that complicate a wide applicability of the technique. Another novel technique studied in coronary CT, is perfusion (CTP). Diagnostic sensitivity and specificity of CTP range between 83%-92% and 82%-90% at respectively segment and patient level[25]. Maybe the most important remark is the amount of radiation needed for this technique, approximately 15mSv is used compared to 3.7mSv which is used for MPI with a ^{82}Rb-PET/CT-scanner, which was used in this thesis. MPI with PET/CT is a widely accepted non-invasive test for the presence of significant CAD[27]. It seems obvious that a combination of both an anatomical measure (CAC) and an actual flow measure would provide additional information. Several studies investigated and confirmed this idea[28-30]. However, CAC scores are in principle determined on an ECG-gated CT scan, whereas MPI often uses non-gated low-dose CT images to plan perfusion measurements. Combination of both modalities therefore often involves additional radiation exposure and additional manual work. As a result, this approach is often used only in an experimental set up. Different studies showed the determination of CAC scores on CT images acquired for a different purpose is

feasible, as long as acquisition parameters are sufficient, with at least 120kVp or adaptation of the threshold value to determine CAC[31]. In our center, a deep learning method was trained to determine CAC scores automatically from CT images acquired for other purposes than primary CAC scoring. External validation of this method showed excellent results for both accuracy and risk stratification in different types of CT scans (e.g. pulmonary cancer screening, non-ECG gated perfusion scans and scans for radiotherapeutic planning). In **chapter 7** we used this automated CAC score, determined on the low-dose attenuation correction CT images acquired during MPI (120kVp, 35mA), in combination with perfusion data. We investigated if this combined assessment could improve the detection of significant CAD. In this chapter we showed that addition of CAC scores increased the AUC to detect significant CAC with 4% (AUC from 0.87 to 0.91). In addition to this, the prognostic value of this combined assessment was studied in **chapter 8**. Both the presence of ischemia as well as a high CAC score (>400) are independent predictors of future MACE in patients suspected of CAD. Considering the easy availability of this automated method and amount of radiation needed, this is a promising alternative for advanced CT-scanning with FFR_{CT} and CTP as gatekeeper for invasive CAD.

FUTURE PERSPECTIVES

Diagnostic strategy

Considering our ageing population, the unmet need for certainty in risk stratification, and growing healthcare costs, gatekeepers for patient referral will become increasingly important. This thesis presents two different strategies for this; the use of biomarkers (EVs) and the use of machine learning. The results on EVs presented in this thesis are a first step to discover their potential but there are major remaining steps to make. Combining information of different sources to improve risk stratification, for example with the use of machine learning might also serve an important role in the near future. In this paragraph I will discuss future perspectives from my point of view, based on the knowledge gained during my PhD.

One of the persistent limitations of EV-derived biomarker research is the design of the studies. Most studies are observational, often retrospective studies, with relatively small numbers. Some of the experiments in EV studies are performed using blood of patients being part of a randomized clinical trial (RCT), but there are no RCTs primarily developed to investigate the clinical impact of EVs. This is somewhat logical, considering the existing evidence. The absence of high through-put techniques hampers the analysis of large confirmatory trials needed for clinical implementation.

Currently, ultracentrifugation is the reference standard for the isolation of EVs[32]. This is however; time-consuming, labor intensive, and requires a large amount of blood and several manual steps that all influence reproducibility and quantification[33]. To enable the analysis of large cohorts, future studies should focus on automated methods to isolate and quantify the EVs combined with robust platforms for accurate protein quantification. Additional to isolation, the implementation of an internal standard is a very important step to take[34].

Common problem with biomarker studies in general is their limited ability to add incremental information on top of existing clinical models in the prediction of a patients risk[35]. Potential solution for this, is the use of multimarker models[36,37]. Multimarker models are thought to combine information of different pathways and thereby providing complementary information. However, considering the astonishing power of clinical variables one should think on how to use this in the development of new (multimarker) biomarkers. For example, a study performed by Ouellete et al. showed marked differences in the clinical profile with varying degree of CAD[38]. Another study investigated sex difference in biomarker profiling, showing that nearly 85% of biomarker profiles differed between men and women[39]. Also, subgroup analysis of trials performed in EVs showed differences among patient groups with different characteristics[40,41]. It is therefore questionable if one single biomarker model could fit this heterogeneous study population. Considering the ability to look at a cellular level with EVs, different stages of the atherosclerotic disease could be accompanied by a different EV-protein signature, mirrored in different clinical characteristics. Another solution to reduce the number of unnecessary referrals for MPI is to use biomarkers to improve the diagnostic accuracy of easily available information, such as for example an exercise test, laboratory results or echocardiographic measures. Finally, we are instinctively focused to develop strategies to detect disease, but for the purpose of reducing the healthcare burden it might be interesting to focus on safely excluding patients without the disease. EVs might be useful for this since they tend to reflect processes on cellular level and thereby detect more accurate sign of chronic diseases such as atherosclerosis rather than the outcome (ACS, or other cardiovascular complications).

Prognostic strategy

Considering risk stratification for future events, the results of the ISCHEMIA trial placed identification of patients with non-ACS lesions in a different perspective, since optimal medical treatment seems to be as safe as invasive treatment considering future events[42]. Key-player in future events is the vulnerability of plaques. Identification of vulnerability could improve a patient-tailored approach with regards

to revascularization and preventive medical strategies. A relatively novel method to assess plaque vulnerability is near-infrared spectroscopy (NIRS). Recent results from the PROSPECT-II trial are encouraging to perform a large, sufficiently powered RCT to assess the impact of using NIRS on MACE[43]. Despite the promising results, it is important to realize NIRS requires invasive measurements with advanced catheters. For this, preselection of patients that would or would not qualify for NIRS would be very welcome. Evaluation of blood from patients undergoing NIRS would enable future researchers to study biological processes involved in plaque vulnerability and potentially identify (EV)biomarkers to identify either patients qualifying for NIRS or even more advanced, qualifying for intensified treatment.

Our future healthcare systems should focus more on sustainability and efficiency, by using our healthcare facilities in the best way. We should focus on collaborations between studies to improve the efficiency, for example by performing biomarker discoveries in patients participating in a large RCT as in the proposed NIRS study. This could help to unravel biological pathways in different stages of atherosclerosis and plaque vulnerability. Biomarker studies are not intended to replace but add to clinical, diagnostic or prognostic imaging modalities, neither are imaging modalities intended to replace invasive strategies. Figure 1 represents the presumed role for EVs in clinical practice from point of view. It can be appreciated their presumed role will mainly be to improve risk stratification and improve preselection for patients who require a referral to a cardiologist. The use of an EV-based risk stratification method on top of existing risk scores and basic testing could than result in less unnecessary referrals, shorter waiting time and thereby lower healthcare burden for both the patients and healthcare physicians. To enable this, it is my belief we should redirect our focus to the exclusion of stable coronary artery disease rather than confirming it.

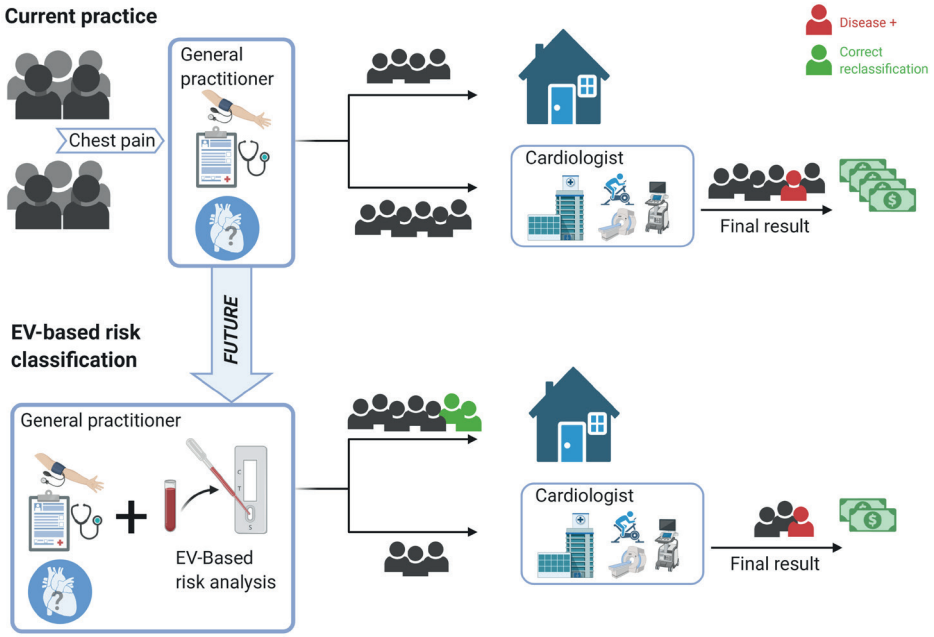


Figure 1. potential role of EVs to improve the diagnostic strategy of patients with suspected coronary artery disease.

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CHAPTER 10

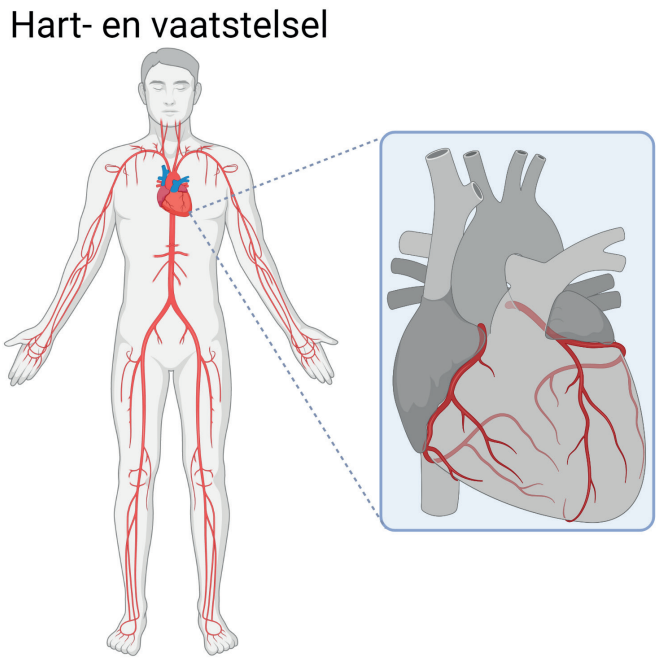
Summary in Dutch

een samenvatting in het Nederlands

SUMMARY IN DUTCH
EEN SAMENVATTING IN HET NEDERLANDS

Hart- en vaatziekten

Jaarlijks overlijden er nog altijd te veel mensen aan de gevolgen van hart- en vaatziekten. Samen met kanker is het de meest voorkomende oorzaak van overlijden in Nederland. Hart- en vaatziekten is een verzamelnaam voor verschillende ziektebeelden, waaronder: aandoeningen van onze bloedvaten zoals (slag)aderverkalking, een verwijding van de bloedvaten, maar ook aandoeningen van het hart zelf zoals hartfalen of een ritmestoornis. Figuur 1 toont een overzicht van het hart- en vaatstelsel en een detailplaatje waarin de ligging van de bloedvaten van het hart zichtbaar zijn.

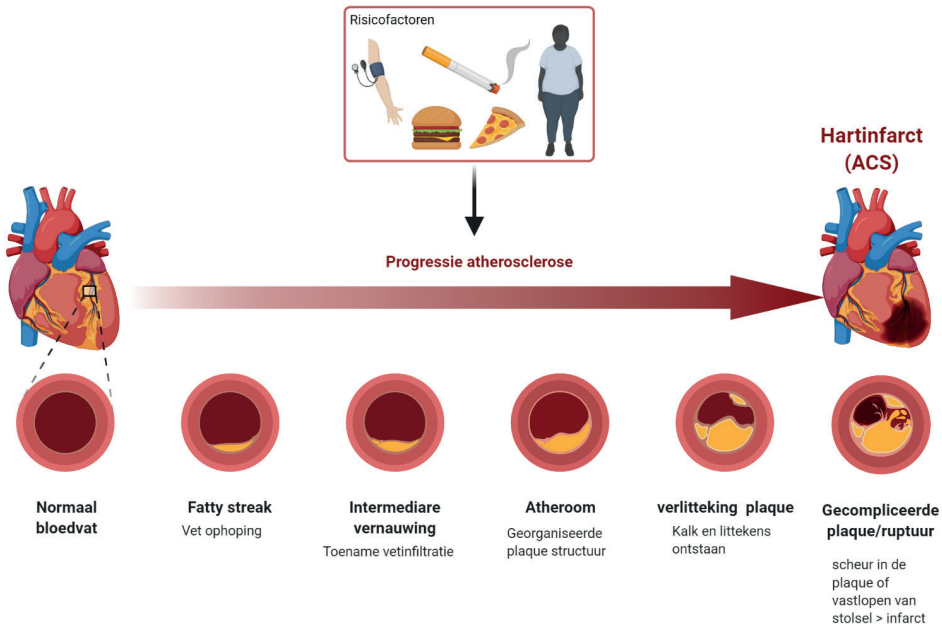


Figuur 1. Hart en vaatstelsel, met een detail van het hart en de bloedvoorziening. Created with BioRender.com

Atherosclerose

Het belangrijkste en meest voorkomende onderliggende ziektebeeld dat hart- en vaatziekten kan veroorzaken is slagaderverkalking, ook wel atherosclerose genoemd (Figuur 2). Atherosclerose is een verouderingsproces van de bloedvaten dat al kort na de geboorte begint. Er is jarenlang sprake van een continue opbouw en afbraak van

cholesterol en uiteindelijk ook vaatkalk in de vaatwanden. Bepaalde leefstijlgewoonten zoals roken, vet eten, suikerziekte, een hoge bloeddruk en ook familiaire belasting beïnvloeden de snelheid en ernst van atherosclerose. Dit proces kan in ieder bloedvat in het lichaam optreden en ter plaatse zorgen voor een vernauwing. Omdat de bloedvaten onder andere verantwoordelijk zijn voor het vervoeren van zuurstof zal de hoeveelheid zuurstof die naar de organen of weefsels gaat afnemen als de vernauwing toeneemt. Het uiteindelijke gevolg hiervan is een tekort aan zuurstof, dit noemen we "ischemie". Naast alle organen in het lichaam wordt ook het hart van bloed, en dus zuurstof, voorzien. De drie bloedvaten die hier verantwoordelijk voor zijn noemen we de kransslagaders of coronairen (zie ook figuur 1). Atherosclerose in de coronairen staat bekend als coronairlijden en is het onderwerp van dit proefschrift.



Figuur 2. Verschillende stadia van atherosclerose. Created with BioRender.com

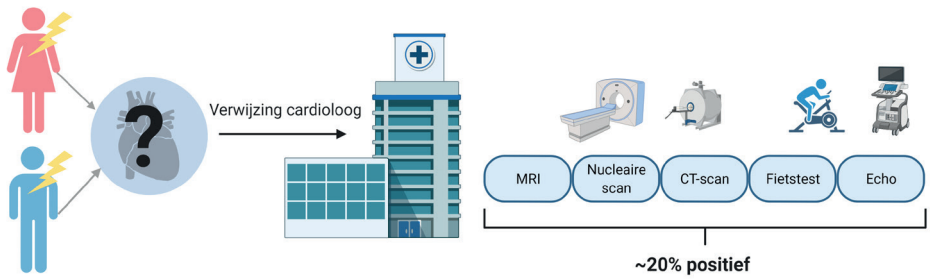
Coronairlijden

Op het moment dat >~70% van het bloedvat wordt afgesloten door de ophoping van vaatkalk spreken we van een ernstige vernauwing die kan leiden tot ischemie en klachten (figuur 2 – stadium: atheroom/verlittekening plaque). De meest bekende zijn; pijn op de borst met of zonder uitstraling naar de armen/kaken, misselijkheid en soms ook een kortademig gevoel. Meestal treden de klachten in het begin vaak alleen op bij inspanning, dit is het moment dat het hart de grootste zuurstofbehoefte heeft.

In dit geval spreken we van stabiel coronairlijden, ook wel een chronisch coronair syndroom (CCS). Dit proces van opbouw en afbraak van vaatkalk is vaak jarenlang stabiel maar kan plotseling verergeren als bijvoorbeeld een bloedpropje zorgt voor een acute afsluiting van het bloedvat. In dit geval spreek je van een hartinfarct, ook wel bekend als een acuut coronair syndroom (ACS). Hierbij ontstaan als gevolg van de volledige afsluiting duidelijke afwijkingen op het hartfilmpje. Daarnaast kunnen we in het bloed snel schade van de hartcellen terugvinden, het belangrijkste stofje dat wordt gemeten bij een verdenking op hartschade is Troponine. Hoe langer het vat afgesloten is, hoe hoger het Troponine in het bloed zal worden. Dit komt omdat Troponine vrijkomt in de bloedbaan na het afsterven van de hartcellen. Het is dan ook van belang om dit afgesloten bloedvat zo snel mogelijk weer open te maken om de doorbloeding en zuurstofvoorziening van het hart te herstellen.

Diagnose hart- en vaatziekten – huidige stand van zaken

De eerste stap voor de huisarts als een patiënt komt met "pijn op de borst" is een risico inschatting maken hoe groot de kans is dat deze klachten door het hart worden veroorzaakt. We weten namelijk dat er vele oorzaken van pijn op de borst kunnen zijn, anders dan door een probleem met het hart. Het is daarom de kunst om patiënten met pijn op de borst als gevolg van atherosclerose (CCS) adequaat te identificeren. Dit is belangrijk omdat patiënten met CCS een verhoogd risico hebben op cardiovasculaire problemen in de toekomst. Naast leefstijl adviezen moeten ze daarom vaak preventief medicatie gebruiken of zelfs een ingreep ondergaan om de vernauwing op te heffen. Kortom, het tijdig en adequaat identificeren van patiënten met hart- en vaatziekten, of een verhoogd risico hierop is uitermate belangrijk. Op dit moment wordt ongeveer 30% van de patiënten met pijn op de borst door de huisarts verwezen naar de cardioloog voor aanvullende diagnostiek (figuur 3).



Figuur 3. Huidige diagnostische proces pijn op de borst. Created with BioRender.com

De meest gebruikte aanvullende onderzoeken zijn een inspanningstest (fietstest) waarbij er continu een hartfilmpje wordt gemaakt, een nucleaire scan van het hart of een MRI. Bij de laatste twee wordt er gebruik gemaakt van medicatie om het hart op te jagen om op deze manier inspanning na te bootsen. Afwijkingen op het hartfilmpje tijdens de fietstest of een afwijkende doorbloeding op de scan vormen de doorslag om te bepalen of een patiënt wel of geen CCS heeft. Naast de hiervoor genoemde diagnostische mogelijkheden bestaat er ook nog een coronair angiogram, hierbij wordt via een bloedvat de doorbloeding van de coronairen bekeken. Dit is de gouden standaard om te bepalen of er sprake is van CCS maar omdat dit een invasief onderzoek is zijn we terughoudend met het gebruik als eerste diagnosticum. Om deze reden vind je deze ook niet terug in figuur 3.

Prognose inschatting hart- en vaatziekten – huidige stand van zaken

Naast het inschatten of een patiënt op dit moment klachten heeft die direct het gevolg zijn van atherosclerose (ACS/CCS) is het belangrijk vast te stellen wat het risico is op toekomstige problemen. Uit onderzoek is gebleken dat de totale hoeveelheid kalk in de bloedvaten hier een van de beste voorspellers voor is. Met behulp van een geavanceerde CT-scan kan een totale "kalkscore" berekend worden. Hoe hoger deze score, hoe groter de kans dat je in de toekomst symptomatisch coronairlijden krijgt (prognose). Aan de andere kant maakt de afwezigheid van kalk in de coronairen de aanwezigheid van coronairlijden (diagnose) zeer onwaarschijnlijk. Het helpt dus bij het inschatten van het risico van een patiënt op het hebben of krijgen van coronairlijden.

Risico inschatting met behulp van biomarkers

Op dit moment is het zo dat slechts ~20% van de patiënten die één, of meer van deze aanvullende onderzoeken ondergaat uiteindelijk coronairlijden blijkt te hebben (figuur 3). Dit betekent dat er ten onrechte patiënten aan belastende onderzoeken worden blootgesteld en veel onnodig werk wordt verricht. Het zou dus helpen als er een betere inschatting gemaakt kan worden bij wie deze aanvullende onderzoeken juist wel of niet nodig zijn, bijvoorbeeld met het gebruik van signaalstoffen. Deze signaalstoffen, ook wel biomarkers genoemd, kunnen helpen om deze risico inschatting te verbeteren. Een biomarker kan bijvoorbeeld een eiwit of een andere bouwstof zijn die meetbaar is in het bloed van een patiënt. Een specifieke groep signaalstoffen is afkomstig uit extracellulaire vesicles (EVs). EVs zijn kleine blaasjes die door alle cellen in ons lichaam gemaakt kunnen worden. Ze worden gezien als de vuilniszakken van ons lichaam, omdat ze afbraakproducten van ons lichaam opruimen. We weten uit onderzoek dat de hoeveelheid en samenstelling van de EVs waardevolle informatie bevat over processen die plaatsvinden in het lichaam. Interessant aan deze bron van signaalstoffen is dat het bij chronische ziekten zoals atherosclerose subtiele

aanwijzingen van het lichaam lijkt te kunnen detecteren. Het probleem met chronische ziekten en signalen meten in bloed is vaak dat de concentraties te laag zijn voor de detectiegrens. Het gebruik van EVs zorgt mogelijk voor een meer gedetailleerde weergave dan in het gewone bloed mogelijk is in de vroeg fase van een chronische ziekte zoals bijvoorbeeld atherosclerose. De focus van dit proefschrift is om nader onderzoek te verrichten hoe deze EVs in te zetten zijn om de risicostratificatie in patiënten met (een verdenking) coronairlijden te optimaliseren.

Deel 1 – de rol van extracellulaire vesicle eiwitten bij het diagnosticeren van coronairlijden

In deel 1 van dit proefschrift onderzoeken we de rol van een aantal geselecteerde eiwitten afkomstig uit EVs. We hebben daarbij gekeken hoe deze EV-eiwitten kunnen helpen met het identificeren van coronairlijden. De onderzochte eiwitten zijn gebaseerd op eerdere studies. In **hoofdstuk 2** van dit proefschrift hebben we 6 verschillende eiwitten (Cystatin C, CD14, Serpin G1, Serpin C1, Serpin F2 en Plasminogeen) onderzocht als mogelijke biomarkers om CCS te detecteren in een groep van 450 patiënten met pijn op de borst klachten. **Hoofdstuk 2** laat zien dat een deel van deze eiwitten inderdaad geassocieerd zijn met het hebben van CCS. Er is steeds meer bewijs dat hart- en vaatziekten zich anders ontwikkelen en presenteren voor mannen en vrouwen. We hebben daarom in dit hoofdstuk ook gekeken of de associaties die gevonden werden verschillend waren voor mannen en vrouwen. Hierbij hebben we een bijzondere bevinding gedaan. Het bleek zo te zijn dat alle gevonden associaties wel standhielden voor de vrouwen maar niet binnen de groep mannen. Hierbij is het belangrijk om aan te geven dat de groep vrouwen waarin dit getoetst kon worden klein was en dat er verder onderzoek nodig is om definitieve conclusies te trekken. Wel roept het de vraag op of we de zoektocht naar een biomarker niet voor mannen en vrouwen apart zouden moeten afleggen.

Naast ACS en CCS is er nog een groep die hier een beetje tussenin valt, de "instabiele" patiënten (IAP). Deze patiënten hebben hevige, vaak acuut ontstane klachten maar geen afwijkend hartfilmpje en in het bloed geen aanwijzingen voor schade (een laag Troponine). Ondanks dit lage Troponine en normale hartfilmpje blijkt toch een deel van deze patiënten een dreigend hartinfarct te hebben. In **hoofdstuk 3** bestuderen we de 6 eerdergenoemde eiwitten en de aanwezigheid van een dreigend hartinfarct (IAP) in deze groep. We vinden in deze studie 1 eiwit, Cystatin C, dat geassocieerd is met een dreigend hartinfarct. We weten uit eerder onderzoek dat er een relatie bestaat tussen Cystatin C met vele uitingen van hart- en vaatziekten. Het is dus niet verassend dat Cystatin geassocieerd is met een dreigend hartinfarct, dit is immers ook een uiting van hart- en vaatziekten.

Maar omdat Cystatin C bij zoveel verschillende processen betrokken is, lijkt het niet waarschijnlijk dat dit eiwit specifiek genoeg is om dreigende hartinfarcten op te sporen. De vraag is dus of dit in de dagelijkse praktijk echt bruikbaar zal zijn. In **hoofdstuk 4** geven we een overzicht van de (on)mogelijkheden van het gebruik van EV-eiwitten als biomarkers in coronairlijden. We vatten de bestaande literatuur kort samen en bespreken een aantal klinische en technologische aspecten die het potentiele succes van deze biomarkerbron beïnvloeden. Verder gaan we in op de heterogeniteit van de groep patiënten met hart- en vaatziekten, waarbij we oproepen na te denken over de vraag of we niet op zoek moeten gaan naar biomarkers voor specifieke gedefinieerde subgroepen in plaats van voor de groep als geheel. **Hoofdstuk 5** toont de resultaten van de grootste EV-eiwit studie tot op heden verricht. Op basis van de resultaten uit **hoofdstuk 2 en 3** hebben we ervoor gekozen 4 van de 6 eiwitten te bestuderen (Cystatin C, CD14, Serpin G1 en Serpin C1). In ruim 1000 patiënten laten we zien dat EV-Cystatin C onafhankelijk geassocieerd is met CCS. Het toevoegen van EV-eiwitten aan een model met alleen klinische gegevens zorgt voor een significant beter voorspelmodel voor het vaststellen van CCS. Om te onderzoeken of er sprake is van coronairlijden ondergaan de patiënten in **hoofdstuk 2 en 5** een zogeheten stresstest. Hierbij kan de stress worden geïnduceerd met behulp van medicatie of door een echte inspanningstest. In **hoofdstuk 5** hebben we ook onderzocht of de rol en waarde van EV-eiwitten verschilt bij de verschillende manieren van stress. Hoewel er dus een significante verbetering van de voorspelling wordt gezien is de accuraatheid op dit moment niet voldoende om deze test in te zetten in de dagelijkse praktijk. De resultaten in de groep patiënten die de fysieke inspanning hebben ondergaan zijn echter wel veelbelovend. Het verrichten van een fysieke inspanningstest, vaak een fietstest, is namelijk relatief goedkoop en gemakkelijk. De waarde van de fietstest op zichzelf is duidelijk minder dan de eerdergenoemde scans, als we dit kunnen optimaliseren door dit te combineren met EVs zou dit veel onnodige scans en dus kosten, werk en straling kunnen voorkomen.

Deel 2 – de bot-hart-as

Het tweede deel van dit proefschrift heet de bot-hart-as. Deze naam is gebaseerd op een onderzoeker die ontdekte dat mensen met veel kalk in de bloedvaten vaak juist weinig kalk in de botten van het skelet hebben. Dit laatstgenoemde klinische fenomeen staat bekend als "botontkalking", en heeft als gevolg een grotere kans op spontante breuken in het skelet. Het proces van vaatverkalking en botontkalking wordt gedreven door verschillende eiwitten, maar een van de belangrijkste regulatoren hierin is osteoprotegerine (OPG). De precieze rol van dit eiwit hebben we nog niet ontdekt. Dierstudies die zijn gedaan naar dit eiwit laten zien dat grote hoeveelheden OPG in

het bloed van muizen zorgt voor minder vaatverkalking, hetgeen suggereert dat OPG een beschermende rol heeft. Studies in mensen laten echter het tegenovergestelde zien, namelijk dat grote hoeveelheden OPG in het bloed geassocieerd zijn met een hogere kans op hart- en vaatziekten. Uit experimentele studies weten we ook dat EVs afkomstig uit vaatwandcellen verantwoordelijk zijn voor het afleveren van de kalk. In de studies die in mensen zijn gedaan is alleen gekeken naar OPG gemeten in het plasma. Het zou interessant zijn de rol van OPG in EVs te bestuderen. De EVs die wij bestuderen zijn circulerende EVs, wat ook weer iets anders is dan de EVs afkomstig uit vaatwandcellen. In **hoofdstuk 6** onderzoeken we de associatie tussen vaatkalk en OPG gemeten in EVs en plasma in 750 patiënten met pijn op de borst klachten. Het meten van vaatkalk is een lastig proces, maar zoals gezegd is een kalkscore, bepaald met behulp van een CT-scan, een goede maat voor vaatkalk. Voor alle 750 patiënten is daarom deze kalkscore bepaald. Het resultaat van **hoofdstuk 6** laat zien dat de relatie tussen OPG en de kalkscore positief geassocieerd is. Dit geldt voor zowel OPG gemeten in het plasma als in de EVs. Het lijkt er dus op dat ze beide hetzelfde proces weergeven in plaats van verschillende processen te weerspiegelen. Welke relatie EVs, OPG en vaatverkalking onderling precies hebben moet in toekomstige studies verder worden uitgezocht.

Perfusiescans die de doorbloeding van het hart kunnen meten zijn het meest geschikt om te kijken of de patiënt klachten heeft door een lokaal zuurstofgebrek als gevolg van een vernauwing. Naast het vaststellen van de oorzaak van de klachten is de tweede taak om tegelijk een inschatting te maken van de kans op toekomstige problemen. Hiervoor is de kalkscore de best bestudeerde maat. Het klinkt logisch, maar het combineren van onderzoeken waarbij zowel de perfusie als de kalkscore bepaald kan worden levert aanvullende informatie. Toch wordt er vaak gekozen voor óf een scan gefocust op de doorbloeding (perfusie) van het hart, of een scan om de kalk score (anatomie) te bepalen. Als onderdeel van de nucleaire (perfusie) scan wordt voor de anatomische oriëntatie altijd een CT-scan gemaakt, maar deze is van een lagere kwaliteit dan bij de kalkscore berekening, andersom zijn de CT-scan beelden zoals ze voor de kalkscore gemaakt worden niet bruikbaar om de perfusie in het hart te meten. In het laatste deel van dit proefschrift gebruiken we een zelflerend computermodel om op de standaard (en dus lagere kwaliteit) CT-scan beelden die voor de perfusie scan worden gemaakt kalkscores te bepalen. Deze methode is verder ontwikkelt en inmiddels gevalideerd en breed inzetbaar. Hierdoor kunnen we nu extra voorspellende informatie halen uit scans die met een ander (diagnostisch) doel gemaakt zijn. In **hoofdstuk 7** laten we zien dat de automatische calciumscore in combinatie met de resultaten van de nucleaire perfusiescan zorgt voor 4% (87% naar 91%) verbetering van de risico inschatting of een patiënt CCS heeft. **Hoofdstuk 8** laat zien dat ook de

prognose van een patiënt met pijn op de borst beter kan worden ingeschat met behulp van deze gecombineerde methode. Het gebruik van deze gecombineerde methode zorgt ervoor dat de patiënt geen aanvullende scans hoeft te ondergaan, en dus geen extra radioactieve straling ontvangt. Daarnaast kost het de patiënt en de dokter minder tijd wat uiteindelijk zorgt voor minder zorgkosten.

In het laatste en afsluitende **hoofdstuk 9** proberen we alle bevinding samen te vatten en in perspectief te plaatsen. Er zijn nog grote stappen te zetten voordat EVs inzetbaar zijn in de praktijk. Een van de belangrijkste punten hiervoor is het verbeteren van de technieken die worden gebruikt. Met de steeds doorgroeiende zorgvraag is het daarnaast belangrijk dat we meer gaan focussen op het efficiënt en duurzaam gebruiken van de zorg en onze onderzoeksprojecten hierop aanpassen. Op dit moment doen veel onderzoeksgroepen allemaal individueel onderzoek om binnen hun niche bestaande hypothesen of technieken nog verder te verbeteren. Neem als voorbeeld een grote gerandomiseerde studie waarbij in duizenden patiënten een nieuw medicijn wordt getest. Als we van al deze patiënten bloed zouden kunnen afnemen kunnen de experimentele onderzoekers veel informatie verzamelen over de (patho)fysiologie van verschillende ziektebeelden. Door op deze manier samen te werken kunnen we sneller tot gevraagde antwoorden komen. Daarnaast is het belangrijk om ons te realiseren dat het doel van bijvoorbeeld biomarkers niet is om geavanceerde beeldvormende technieken te vervangen, maar om het onterechte gebruik ervan te beperken. Met andere woorden, we zijn geen concurrenten maar partners, met allemaal hetzelfde doel voor ogen: het verbeteren van de patiëntenzorg. Door op deze manier samen te gaan werken, kunnen we een start maken om ons zorgstelsel te ontlasten en onnodige kosten verminderen. Hierdoor blijft uiteindelijk meer geld en tijd over voor geavanceerde zorg voor de juiste patiënten.

APPENDIX

LIST OF PUBLICATIONS

Dekker M, Waissi F, Timmerman N, Silvis MJM, Timmers L, de Kleijn DPV. Extracellular vesicles in diagnosing chronic coronary syndromes—the bumpy road to clinical implementation. *Vol. 21, International Journal of Molecular Sciences. MDPI AG; 2020. p. 1–19.*

Dekker M, Waissi F, van Bennekom J, Silvis MJM, Timmerman N, Bank IEM, et al. Plasma extracellular vesicle proteins are associated with stress-induced myocardial ischemia in women presenting with chest pain. *Scientific Reports. 2020 Dec 1;10(1).*

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Timmerman N, Waissi F, **Dekker M**, Q.Y. van de Pol, J. van Bennekom, A. Schoneveld, M.J.M. klein Avink, R.J. de Winter, G. Pasterkamp, G.J. de Borst, D.P.V. de Kleijn "Protein levels in extracellular vesicles predict high-risk patients for secondary cardiovascular events" *In press European Journal of vascular & endovascular surgery*

Dekker M, Waissi F, Bank IEM, Lessmann N, Išgum I, Velthuis BK, et al. Automated calcium scores collected during myocardial perfusion imaging improve identification of obstructive coronary artery disease. *IJC Heart & Vasculature 2020 Feb;26:100434.*

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Zivelonghi C, Verheye S, Timmers L, van Kuijk JP, Giannini F, **Dekker M**, et al. Efficacy of Coronary Sinus Reducer in Patients With Non-revascularized Chronic Total Occlusions. *American Journal of Cardiology. 2020;126.*

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Silvis MJM, **Dekker M**, Zivelonghi C, Agostoni P, Stella PR, Doevendans PA, et al. The Coronary Sinus Reducer; 5-year Dutch experience. *Netherlands Heart Journal. 2021;29(4).*

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Silvis MJM, Fiolet ATL, Opstal TJS, **Dekker M** et al. Extracellular vesicle NLRP3 protein concentrations identify the effect of colchicine on the inflammasome in chronic coronary disease: a LoDoCo2 substudy. *Accepted atherosclerosis, in press*

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Verwer MC, Waissi F, Mekke JM, **Dekker M** et al. High Lipoprotein(a) is associated with Major Adverse Limb Events after femoral artery endarterectomy. *Atherosclerosis. 2021.11.019*

SUBMITTED/UNDER REVIEW

Waissi F, **Dekker M,** Timmerman N et al. Tenascin-C expression is associated with unstable carotid atherosclerotic plaques. *Revisions nature scientific reports*

Timmerman N, Waissi F, **Dekker M** et al. Ceramides and phospholipids are associated with high risk of major cardiovascular events after carotid endarterectomy. *Under review Atherosclerosis*

Waissi F, Timmerman N , **Dekker M** et al. High tenascin-C plasma extracellular vesicles levels are associated with an increased risk of major adverse cardiovascular events in carotid endarterectomy patient. *Under review ATVB*

IN PREPARATION

Dekker M, Schäfer I, Waissi F. Extracellular vesicles derived proteins improve the detection of functional relevant coronary artery disease in adjunct to clinical judgement. *In preparation*

F. Waissi, N.Timmerman, **M. Dekker**, J. Kroon, G.J. de Borst, D.P.V. de Kleijn "Combined plasma Extracellular Vesicle biomarkers improve risk stratification for major adverse cardiovascular events after carotid endarterectomy" *Submission is postponed until other biomarker studies are accepted for publication.*

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ABOUT THE AUTHOR

Mirthe Dekker was born on September 29th, 1991 in Biddinghuizen, the Netherlands, as the first daughter of Ron and Carla. After 3 years of full attention of both parents her sister Beryl was born. She graduated from the Christian College Nassau Veluwe in Harderwijk in 2010. She moved to Groningen and started the study "Human Movement Sciences", after realizing how important direct patient care was for her she decided to apply for medical school which she started in 2011, luckily also in Groningen. On invitation she applied for the honours college programme of the Rijks University of Groningen. In 2015 she finished both bachelors. During her study she participated as treasurer in the board of student association Parafrid. During her internships in Almelo her interest in cardiology was confirmed. After obtaining her medical degree, she moved to Utrecht to start as a resident not-in-training under supervision of dr. J.H. Kirkels. After 5 months she started as a PhD student under supervision of prof. dr. D.P.V. de Kleijn and dr. L. Timmers. During her position as a PhD she completed the post-graduate master Epidemiology. Her work was rewarded internationally with a young investigator award at the ESC conference of preventive cardiology. During her PhD she started a valuable collaboration with prof. dr. C. Mueller from the University Hospital in Basel which will be continued after finishing this PhD. In December 2020 she started as a resident in training cardiology under supervision of dr. G.T.J. Sieswerda at the University Medical Centre Utrecht. She is currently doing her general training in internal medicine at the Meander Medical Centre in Amersfoort under supervision of dr. R. Fijnheer and dr. J.M.M.B Otten. After that she will proceed with her dedicated cardiology training. Mirthe aspires to become a passionate and outstanding cardiologist while maintaining time for the other important things in life; friends family and cycling, together with her beloved Geert.