Risk stratification in coronary artery disease : the role of (bio)markers and coronary CT-angiography

RISK STRATIFICATION IN CORONARY ARTERY DISEASE

THE ROLE OF (BIO)MARKERS AND CORONARY CT-ANGIOGRAPHY

ISBN: 978-90-8891-739-4

Cover design: Barry van Varik, Arbel Media, Echt
Printed by: Proefschriftmaken.nl || Uitgeverij BOXpress, ‘s Hertogenbosch
Published by: Uitgeverij BOXPress, ‘s Hertogenbosch

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PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de Universiteit Maastricht,
op gezag van de Rector Magnificus, Prof. Dr. L.L.G. Soete,
volgens het besluit van het College van Decanen,
in het openbaar te verdedigen,
op woensdag 20 november 2013 om 10.00 uur

door

Ivo Antonius Petronella Gerardus Joosen
geboren op 26 augustus 1981 te Nederweert

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Financial support by Stichting Hartsvrienden RESCAR for the publication of this thesis is gratefully acknowledged.

Additional financial support by Astellas Pharma BV, Bayer Healthcare BV, Guerbet BV, MSD BV and Stichting CIRC is similarly appreciated.

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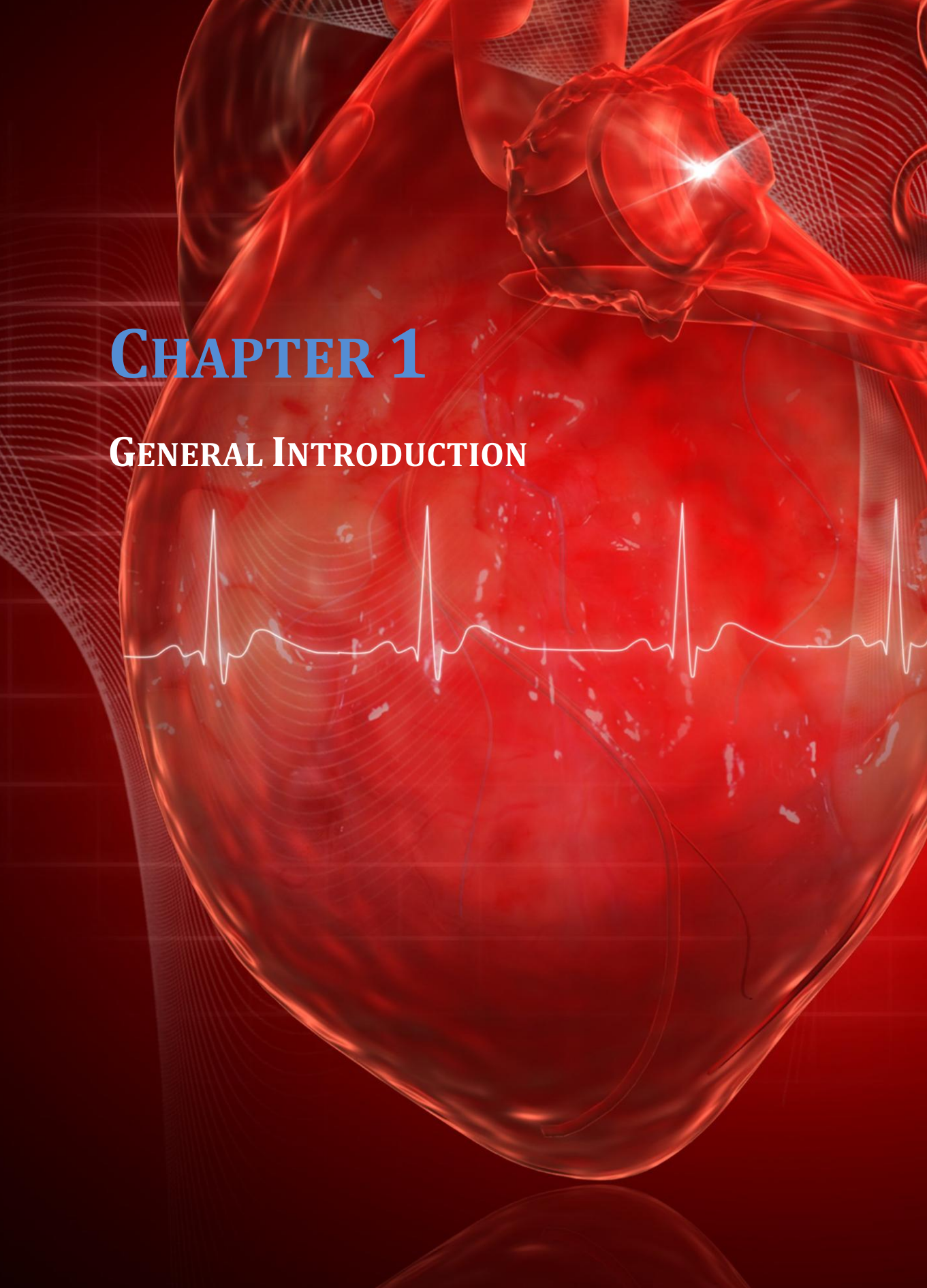


CHAPTER 1

GENERAL INTRODUCTION

MAGNITUDE OF CARDIOVASCULAR DISEASES

Cardiovascular disease (CVD), including ischemic heart disease and stroke, is still one of the major causes of mortality and morbidity in Western countries. Alarming is the expectation that the CVD burden will rise again after decades of decline. Due to the epidemic proportions of obesity and ageing, it is expected that the number of patients diagnosed with diabetes mellitus type II in The Netherlands is going to rise from the current 740.000 to 1.3 million in the year 2025, resulting in an increase of patients suffering from CVD [1]. In the USA, in 2009, a total of 787.931 people deceased due to CVD [2]. The proportion of mortality caused by CVD was 32.3% of total mortality. Moreover, each year an estimated 635.000 Americans will have a new myocardial infarction (MI), 280.000 Americans a recurrent MI and 150.000 Americans a silent first MI [2]. This is accompanied by high costs; the annual direct and indirect cost of CVD in the USA is estimated at $312.6 billion, which is 15% of total health expenditures, which is more than any other major diagnostic group [2].

Not only in Western countries, but also in developing countries, the prevalence of traditional risk factors for CVD has been increasing with consequent increases in cardiovascular event rates [3]. Therefore, CVD is a rising major global health problem.

ATHEROSCLEROSIS

Atherosclerosis, derived from the Greek athero (gruel-like) and sclerosis (hardening) is the main cause of coronary artery disease (CAD). Atherosclerosis is a multifactorial, chronic inflammatory disease in which immune mechanisms interact with metabolic risk factors to initiate, propagate and activate lesions in large and medium-sized arteries [4,5]. The atherosclerotic process already begins during adolescence. First, a fatty streak is formed, consisting of an accumulation of lipid-rich cells, macrophages and T-cells [6]. Fatty streaks can progress to atheromas, which are located in the intima layer of the artery (Figure 1) [7]. Atheromas consist of a core of lipids, connective tissue, debris and various inflammatory and immune cells like macrophages, T-cells and mast cells [8]. This core is surrounded by a protective, fibrous cap which forms the demarcation with the lumen of the artery.

Atheromas grow in the so-called 'shoulder region'. In this region, a lot of activated immune cells are present which secrete inflammatory cytokines that can weaken the fibrous cap. In this way, a stable plaque can convert into an unstable 'vulnerable' plaque. Rupture of a vulnerable plaque will also preferentially occur in the shoulder region of the plaque. As a consequence, prothrombotic material from inside the atheromatous plaque comes into contact with blood and forms an occluding thrombus, resulting in a MI (Figure 1) [9]. Atherosclerosis is not limited to the coronary arteries, but is a systemic disease, giving rise to acute and chronic vascular syndromes which can ultimately result in complications like MI, stroke and peripheral artery disease (PAD) [4].

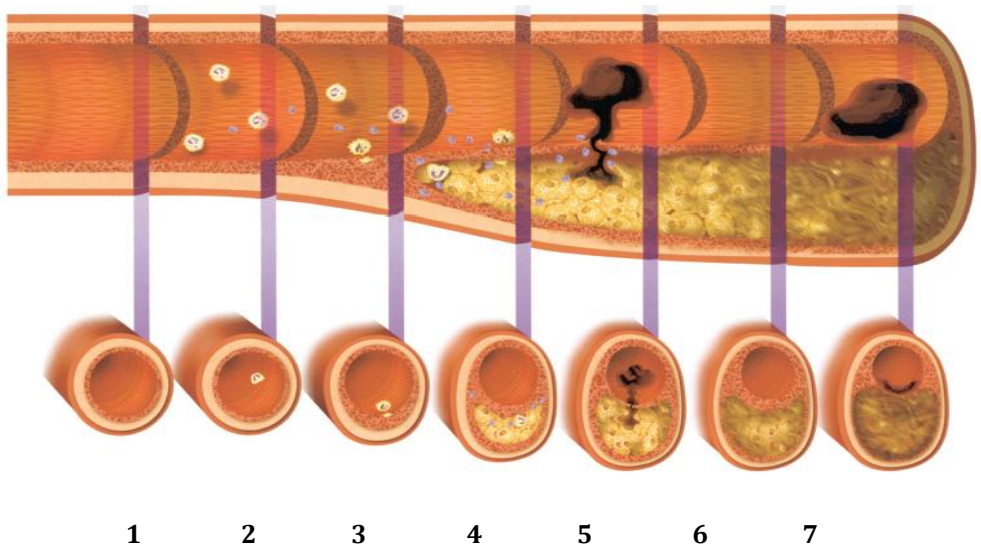


Figure 1. *Initiation, progression, and complication of human coronary atherosclerotic plaque. Top, Longitudinal section of an artery depicting "timeline" of human atherogenesis from normal artery (1) to atheroma that caused clinical manifestations by thrombosis or stenosis (5-7). Bottom, Corresponding cross sections of an artery during various stages of atheroma evolution. Reproduced with permission from: Libby P. Current concepts of the pathogenesis of the acute coronary syndromes. Circulation 2001;104:365–372 [7].*

DETECTION OF CORONARY ARTERY DISEASE AND VULNERABLE PLAQUES

There are various tools available in the search for patients with CAD and patients at high risk of a vulnerable plaque, ranging from basic office-based risk score algorithms to innovative invasive diagnostic imaging procedures like intravascular ultrasound (Figure 2) [10].

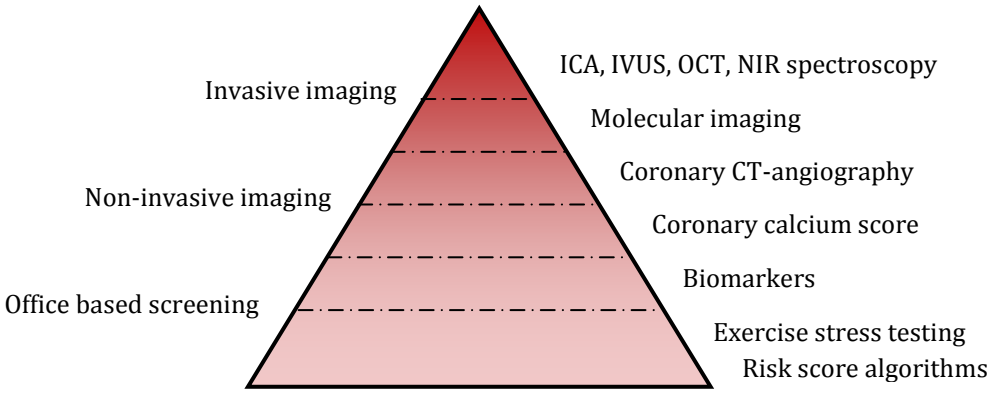


Figure 2. *The "vulnerable plaque pyramid", which shows a possible step-by-step method in order to detect a vulnerable plaque. Adapted and reproduced with permission from: Naghavi M, Libby P, Falk E, et al. From vulnerable plaque to vulnerable patient: a call for new definitions and risk assessment strategies: Part II. Circulation 2003;15:1772–1778 [10]. CT indicates computed tomography; ICA, invasive coronary angiography; IVUS, intravascular ultrasound; NIR, near infrared; OCT, optical coherence tomography.*

Risk Score Algorithms

There are several known traditional cardiovascular risk factors which are associated with the occurrence and progression of CAD, including male gender, age, diabetes mellitus, smoking, family history of premature atherosclerosis, obesity, hypertension, dyslipidemia and a sedentary lifestyle. These risk factors can be used in risk score algorithms in order to estimate the long term cardiovascular risk in patients. Frequently used risk score algorithms are the Framingham risk score (predicts 10-year risk of suffering any cardiovascular event), the PROCAM risk score (predicts 10-year risk of acute coronary events), the SCORE risk score (predicts 10-year risk of fatal cardiovascular disease) and the Diamond and Forrester score (predicts significant CAD on invasive coronary angiography) [11-14].

Partly because the use of risk score algorithms is cost- and time-efficient, this approach is preferable and recommended in clinical practice as a first step in cardiovascular risk assessment [15]. Detection of CAD in an early stage is useful, since the atherosclerotic process can be modified by lifestyle changes or pharmacological treatment, in order to prevent major adverse cardiac events (MACE) [16-18].

Exercise Stress Testing

Exercise stress testing, including exercise electrocardiography, exercise echocardiography and myocardial perfusion imaging, is a non-invasive, less expensive procedure. In guidelines it has been described in which scenarios exercise stress testing is recommended [19]. Exercise stress testing can be used for risk stratification beyond risk score algorithms. Moreover, it provides diagnostic and prognostic information [20-23]. However, diagnostic accuracy is variable and depends on age, gender, clinical patient characteristics and test modality [24]. Sensitivity and specificity rates for detection of CAD range from 68-88% (sensitivity) and from 73-84% (specificity) [25].

Biomarkers

In literature, several biomarkers are described which are related to CAD and the occurrence of MACE. C-reactive protein, a marker of systemic inflammation, is associated with cardiovascular events such as MI, coronary mortality and stroke in both men and women, with or without previous CVD [26-30]. These findings support the hypothesis that atherothrombosis is partly an inflammatory disease [31]. Experimental data indicate that blood coagulation proteins, including thrombin, are also involved in the pathophysiology of atherosclerosis. Beyond its central role in the process of thrombus formation, thrombin is described as a strong pro-inflammatory protein. It has the potential to activate different protease-activated receptors, resulting in pro-atherogenic and plaque destabilizing effects such as endothelial dysfunction, platelet activation, monocyte recruitment, oxidative stress and inflammation (Figure 3) [32,33]. However, evidence from clinical studies is lacking.

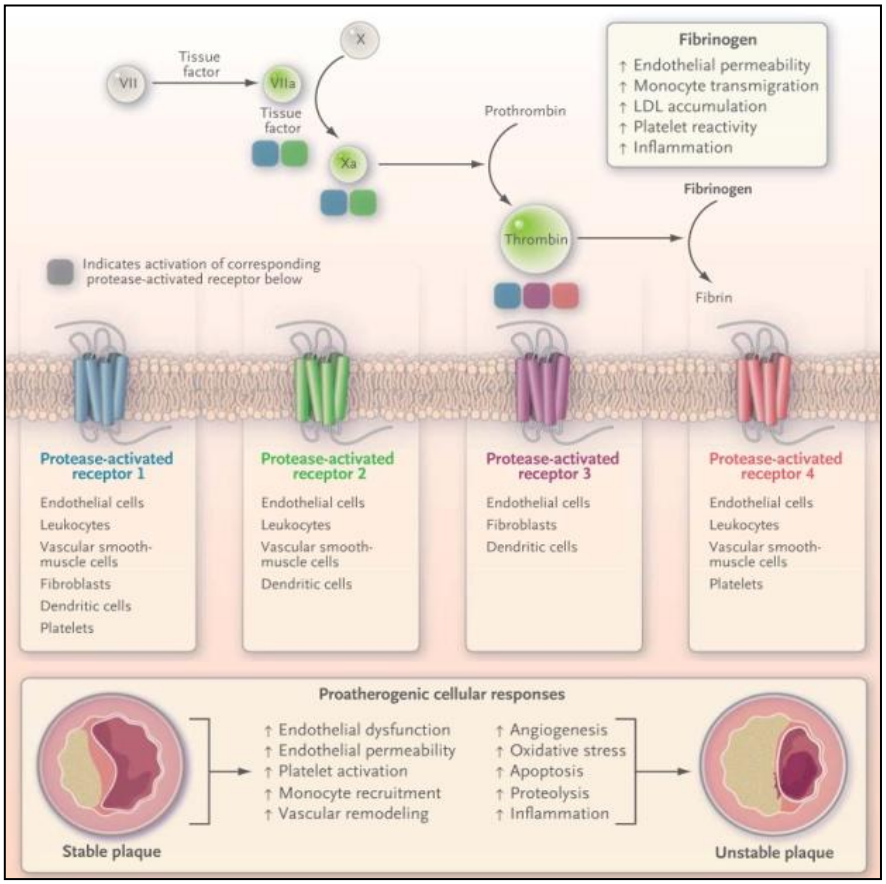


Figure 3. *Involvement of thrombin in the atherosclerotic process. Thrombin, factor Xa and tissue factor-factor VIIa complexes have the ability to activate protease-activated receptors, which results in a plethora of proatherogenic cellular responses. These proatherogenic actions are able to destabilize an atherosclerotic plaque. Reproduced with permission from: Borissoff JI, Spronk HM, ten Cate H. The hemostatic system as a modulator of atherosclerosis. N Engl J Med 2011;364:1746–1760 [32].*

Not only thrombin, but also neutrophils are involved in the atherosclerotic process [34,35]. Neutrophils are able to release extracellular traps (neutrophil extracellular traps [NETs]), which compose of chromatin threads, histones and granular proteins. NETs appear to be a form of innate immune response and were observed in cases of acute inflammation, in order to kill bacteria [36]. However, their role in the human atherosclerotic process remains unclear and needs further investigation.

Matrix Gla-protein (MGP), a vitamin K-dependent protein, is an inhibitor of calcification of arteries and cartilage [37]. Some studies already described the association between treatment with vitamin K-antagonists and increased calcification [38,39]. Furthermore, MGP is independently associated with mortality and CVD events, including MI, stroke, transient ischemic attack and heart failure, in outpatients with stable CAD [40]. These data support the hypothesis that calcification may contribute to the occurrence of MACE.

Another intriguing biomarker is the protein troponin. Cardiac troponin I and T are components of the contractile apparatus of myocardial cells and are expressed almost exclusively in the heart. They are released into the blood as a result of cardiomyocyte injury. Nowadays, cardiac troponin is the preferred biomarker to diagnose an acute MI [41]. Moreover, it can be used as a key diagnostic tool for decision making in patients presenting with chest pain. A few studies are published which focused on the prognostic value of hs-cTnT. However, these studies were performed in the elderly or in the general population, while less is known about the incremental value of hs-cTnT on top of other risk stratification tools in patients with stable chest pain [42-44].

Coronary Calcium Score

With the use of CT it is possible to visualize the presence and localization of calcium in the coronaries. The amount of calcium can be measured with software and expressed as total number of calcified lesions, calcium volume (mm^3), calcium mass (mg calcium hydroxyapatite) and Agatston score, which is the most widely used parameter to express the calcium score. An increased calcium score, which is associated with an increased risk of significant luminal stenosis, independently predicts MACE [45-47]. Moreover, it has incremental value on top of clinical risk stratification [48-50]. Despite advances in reconstruction techniques, the presence of coronary calcification leads to high levels of signal attenuation, causing blooming artifacts [51]. This leads to the effect of overestimating the calcium and thus potentially overestimating the degree of luminal stenosis. The higher the calcium score, the greater the risk of blooming artifacts, and the more difficult to reliable assess the CT-scan, possibly resulting in a non-diagnostic scan [52,53]. The choice whether to proceed with contrast enhanced CT-angiography in the presence of extensive calcification remains controversial [54].

Coronary Computed Tomographic Angiography

Coronary CT-angiography (CCTA) is a non-invasive, diagnostic imaging technique to visualize the coronary arteries. It provides valuable information regarding the presence, extent, degree and morphology of coronary plaques. The extent of CAD can be defined as the number of coronary segments with a plaque, irrespective of the degree of luminal stenosis. The degree of luminal stenosis can be classified as absent (no luminal stenosis), mild (<50% luminal stenosis), moderate (50-70% luminal stenosis) or severe (>70% luminal stenosis) [55]. Regarding plaque morphology, different plaques can be distinguished: calcified plaques (exclusively content with density >130 HU), non-calcified plaques (exclusively content with density <130 HU) and mixed plaques (characteristics of both calcified and non-calcified plaques) [55].

CCTA possesses reasonable diagnostic accuracy to detect obstructive CAD, but is also established to rule out obstructive CAD with a negative predictive value of 99% [56,57]. Moreover, CCTA has also proven prognostic value in predicting MACE, both in patients with acute chest pain and in outclinic patients with suspected CAD [58,59].

Recently, CCTA characteristics of vulnerable plaques are described, including large plaque volume, low plaque attenuation, large proportion of non-calcified plaque, spotty calcification and a high positive remodeling index (Figure 4) [60-63].

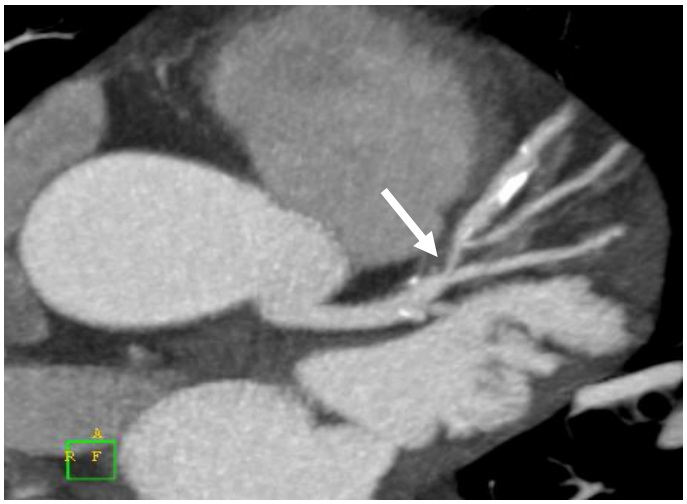


Figure 4. *Example of an axial coronary CT-image. The proximal part of the left anterior descending artery shows a large, non-calcified atherosclerotic plaque with positive outward remodeling (white arrow). These characteristics fits into the profile of a vulnerable plaque.*

Molecular Imaging

The field of molecular imaging has grown rapidly during the last decade and has brought new insights into cardiovascular disease. Molecular imaging focuses on the immunobiology behind the endothelium and can be helpful in identifying culprit atherosclerotic lesions [64,65]. An extensively studied concept is the possibility to image vascular inflammation by ^{18}FDG uptake with positron emission tomography (PET). However, not all clinical studies found a clear association between plaque vulnerability and ^{18}FDG uptake [66]. An alternative approach is PET/CT imaging using ^{18}F-NaF to depict calcification. ^{18}F-NaF is a promising marker of plaque biology, which has the potential to provide key insights into the role that calcification plays in the progression of atherosclerosis [67]. Moreover, molecular imaging remains expensive as well as time-consuming. In the future, once molecular imaging is successfully introduced in clinical practice, it has the potential to offer new therapeutic strategies in order to offer patients a personalized treatment [66].

Invasive Coronary Imaging Techniques

Invasive coronary angiography (ICA) is a widely used imaging technique, which is still considered as the reference standard for detecting obstructive CAD. However, one of the major disadvantages is that ICA only provides little information regarding characteristics of plaque vulnerability. The technique is limited or even inappropriate in its ability to visualize the vessel wall, fibrous cap thickness and plaque composition as well as plaque volume [68]. Degree of coronary artery stenosis is unreliable as a single parameter for plaque vulnerability, which is confirmed by a study in patients with a non-Q-wave MI. In more than 35% of these patients, ICA was not able to identify the culprit lesion [69].

In the past decade, complimentary intravascular imaging techniques have been developed which can be performed during ICA, including intravascular ultrasound (IVUS) and optical coherence tomography (OCT). IVUS uses ultrasound technology, while OCT uses depth-resolved back-reflection of infrared light in order to accurately visualize plaque characteristics as well as the vessel wall [63]. IVUS allow identification of four different tissue types including dense calcium, fibrous tissue, fibrofatty tissue and necrotic core [70].

Positive vascular remodeling, a feature which is associated with culprit and ruptured coronary plaques, is also visible with IVUS [71,72]. OCT offers a high resolution measurement of the fibrous cap thickness, which plays a critical role in plaque rupture as discussed earlier. Moreover, it is possible to image ruptures, thrombi, lipid cores and calcium. A major technical limitation of OCT is its lack of penetration, making it difficult to measure the true vessel size and plaque burden [63].

DETECTION OF CAD AND VULNERABLE PLAQUES IN DAILY CLINICAL PRACTICE

As previously discussed, it is expected that the number of patients suffering from CVD will rise in the near future. Therefore, improving risk profiling as presented in the vulnerable plaque pyramid is of paramount importance. Given the increasing burden of CVD and the ever rising health care expenditures, a focus on relatively low costs test, such as the development of novel serum biomarkers, is a desirable strategy.
In addition, development of a more precise diagnostic algorithm for risk profiling will help to decrease both cardiovascular risk and health care costs.

In daily clinical practice, it is not possible and not necessary for all patients to go through all 'phases' of the pyramid. This would even be undesirable since all tests have potential complications, especially the invasive procedures. Moreover, invasive techniques like molecular imaging, IVUS and OCT are not yet widespread available. In contrast, CCTA is available in an increasing number of hospitals. All procedures have their own strengths and weaknesses, and it is therefore crucial to require the most appropriate test and to extract as much information as possible from the chosen test.

AIM AND OUTLINE OF THIS THESIS

The aim of this thesis was to relate characteristics of CAD, as measured with CCTA, with (bio)markers, as a first step in order to improve risk stratification in patients with suspected CAD. Moreover, by using biomarkers that are not yet suitable for use in daily clinical practice, we tried to gain more insight into underlying mechanisms.

- Chapters 2 and 3 focus on clinical markers. Chapter 2 describes gender differences in clinical risk profile as well as gender differences in CAD. Chapter 3 studies the relationship between mild to moderate chronic kidney disease and CAD.
- Chapters 4-6 are reserved for more novel and specific biomarkers. Chapter 4 studies the incremental value of high-sensitivity cardiac troponin T as a risk stratification tool in patients with symptoms of chest discomfort suspected for CAD.

  Chapters 5 and 6 build a bridge between atherosclerosis, coagulation and immunity. Chapter 5 describes the association between in vivo thrombin formation and the presence and severity of coronary atherosclerosis. Chapter 6 describes the association between markers of neutrophil extracellular traps and coronary atherosclerosis.
- Chapter 7 demonstrates that vitamin K antagonists are able to accelerate the process of atherosclerotic calcification. Moreover, we show that vitamin K antagonists can induce vulnerable plaques in mice.

REFERENCES

1. Baan CA, Schoemaker CG, Jacobs-van der Bruggen MAM, et al. Diabetes tot 2025: Preventie en zorg in samenhang. *RIVM* 2009.
2. Go AS, Mozaffarian D, Roger Vl, et al. Heart disease and stroke statistics -- 2013 update: a report from the American Heart Association. *Circulation* 2013;127:6–245.
3. Celermajer DS, Chow CK, Marijon E, et al. Cardiovascular disease in the developing World: prevalences, patterns, and the potential of early disease detection. *J Am Coll Cardiol* 2012;14:1207–1216.
4. Ross R. Atherosclerosis -- an inflammatory disease. *N Eng J Med* 1999;340:115–126.
5. Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. *N Eng J Med* 2005;352:1685–1695.
6. Stary HC, Chandler AB, Glagov S, et al. A definition of initial, fatty streak, and intermediate lesions of atherosclerosis. *Circulation* 1994;89:2462–2478.
7. Libby P. Current concepts of the pathogenesis of the acute coronary syndromes. *Circulation* 2001;104:365–372.

8. Stary HC, Chandler AB, Dinsmore, RE, et al. A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis. *Circulation* 1994;92:1355–1374.
9. Davies MJ. Stability and instability: two faces of coronary atherosclerosis. *Circulation* 1996;94:2013–2020.
10. Naghavi M, Libby P, Falk E, et al. From vulnerable plaque to vulnerable patient: a call for new definitions and risk assessment strategies: Part II. *Circulation* 2003;15:1772–1778.
11. D'Agostino RB Sr, Vasan RS, Pencina MJ, et al. General cardiovascular risk profile for use in primary care: The Framingham Heart Study. *Circulation* 2008;117:743–753.
12. Assmann G, Cullen P, Schulte H. Simple scoring scheme for calculating the risk of acute coronary events based on the 10-year follow-up of the prospective cardiovascular Munster (PROCAM) study. *Circulation* 2002;105:310–315.
13. Conroy RM, Pyorala K, Fitzgerald AP, et al. Estimation of ten-year risk of fatal cardiovascular disease in Europe: The SCORE project. *Eur Heart J* 2003;24:987–1003.
14. Diamond GA, Forrester JS. Analysis of probability as an aid in the clinical diagnosis of coronary artery disease. *N Engl J Med* 1979;300:1350–1358.
15. Greenland P, Alpert JS, Beller GA, et al. 2010 ACCF/AHA guideline for assessment of cardiovascular risk in asymptomatic adults. *Circulation* 2010;122:584–636.
16. Bhatt DL, Fox KA, Hacke W, et al. Clopidogrel and aspirin versus aspirin alone for the prevention of atherothrombotic events. *N Engl J Med* 2006;16:1706–1717.
17. Smith SC Jr, Benjamin EJ, Bonow RO, et al. AHA/ACCF secondary prevention and risk reduction therapy for patients with coronary and other atherosclerotic vascular disease: 2011 update. *J Am Coll Cardiol* 2011;23:2432–2446.
18. Patel S, Adams MR. Prevention of cardiac disease: lifestyle modification or pharmacotherapy? *Intern Med J* 2008;3:199–203.
19. Gibbons RJ, Balady GJ, Bricker JT, et al. ACC/AHA 2002 guideline update for exercise testing: summary article. *J Am Coll Cardiol* 2002;40:1531–1540.
20. Sekhri N, Feder GS, Junghans C, et al. Incremental prognostic value of the exercise electrocardiogram in the initial assessment of patients with suspected angina: cohort study. *Br Med J* 2008;337:1272–1275.
21. Elhendy A, Mahoney DW, Burger KN, et al. Prognostic value of exercise echocardiography in patients with classic angina pectoris. *Am J Cardiol* 2004;94:559–563.

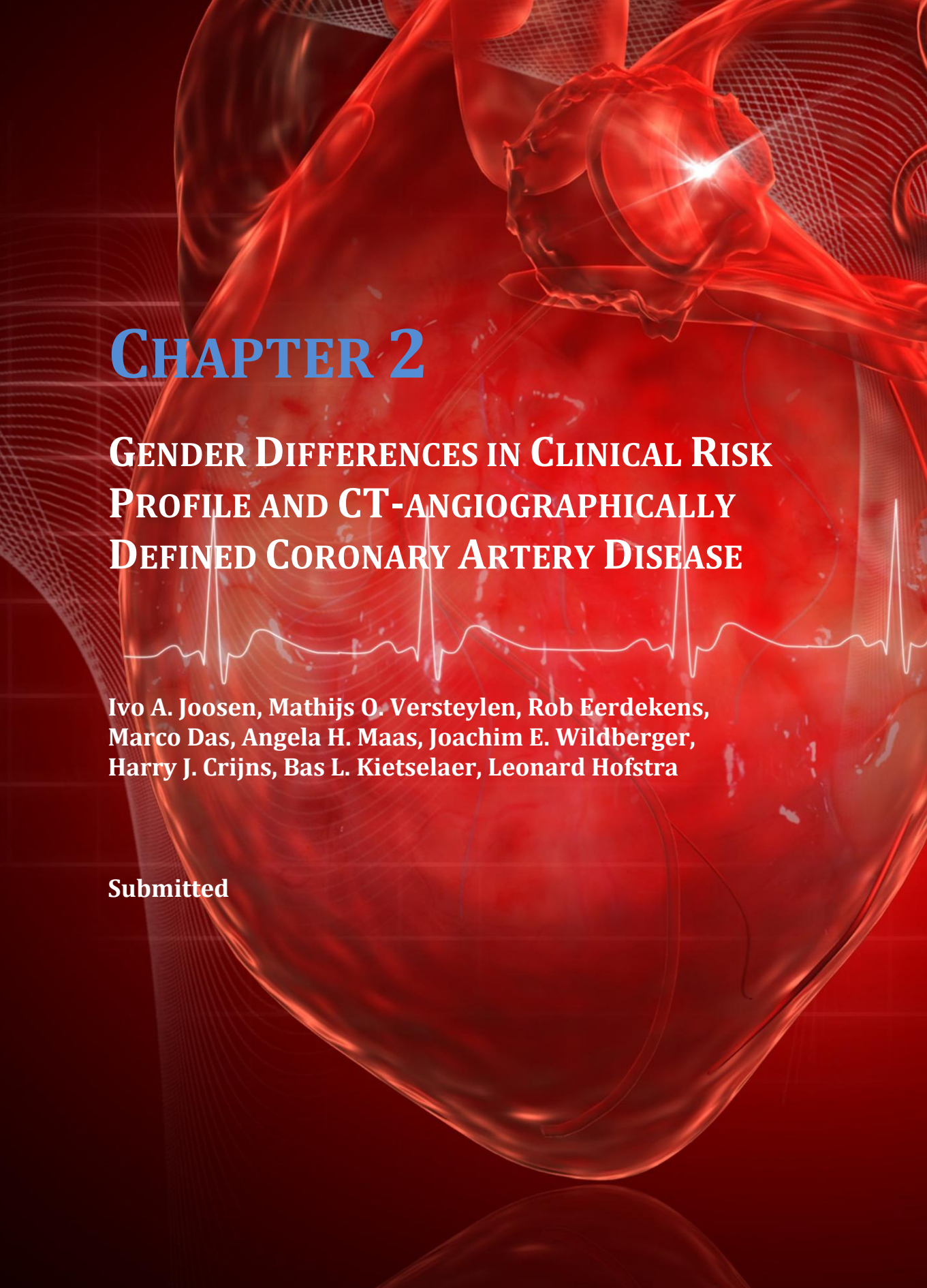
22. Groutars RG, Verzijlbergen JF, Zwinderman AH, et al. Incremental prognostic value of myocardial SPET with dual-isotope rest (201)TI/stress (99m)TC-tetrofosmin. *Eur J of Nucl Med Mol Imaging* 2002;29:46–52.
23. Elhendy A, Schinkel AF, van Domburg RT, et al. Risk stratification of patients with angina pectoris by stress 99mTc-tetrofosmin myocardial perfusion imaging. *J Nucl Med* 2005;46:2003–2008.
24. Banerjee A, Newman DR, Van den Bruel A, et al. Diagnostic accuracy of exercise stress testing for coronary artery disease: a systematic review and meta-analysis of prospective studies. *Int J Clin Pract* 2012;66:477–492.
25. Skinner JS, Smeeth L, Kendall JM, et al. NICE guidance. Chest pain of recent onset: assessment and diagnosis of recent onset chest pain or discomfort of suspected cardiac origin. *Heart* 2010;96:974–978.
26. Ridker PM, Cushman M, Stampfer MJ, et al. Inflammation, aspirin, and the risk of cardiovascular disease in apparently healthy men. *N Engl J Med* 1997;336:973–979.
27. Ridker PM, Burring JE, Shih J, et al. Prospective study of C-reactive protein and the risk of future cardiovascular events among apparently healthy women. *Circulation* 1998;98:731–733.
28. Danesh J, Wheeler JG, Hirschfield GM, et al. C-reactive protein and other circulating markers of inflammation in the prediction of coronary heart disease. *N Engl J Med* 2004;350:1387–1397.
29. Haverkate F, Thompson SG, Pyke SD, et al. Production of C-reactive protein and risk of coronary events in stable and unstable angina. *Lancet* 1997;349:462–466.
30. Retterstol L, Eikvar L, Bohn M, et al. C-reactive protein predicts death in patients with previous premature myocardial infarction – a 10 year follow-up study. *Atherosclerosis* 2002;160:433–440.
31. Ridker PM. C-reactive protein and the prediction of cardiovascular events among those at intermediate risk: moving an inflammatory hypothesis toward consensus. *J Am Coll Cardiol* 2007;49:2129–2138.
32. Borissoff JI, Spronk HM, ten Cate H. The hemostatic system as a modulator of atherosclerosis. *N Engl J Med* 2011;364:1746–1760.
33. Borissoff JI, Spronk HM, Heeneman S, et al. Is thrombin a key player in the 'coagulation-atherogenesis' maze? *Cardiovasc Res* 2009;82:392–403.

34. Drechsler M, Megens RT, van Zandvoort M, et al. Hyperlipidemia-triggered neutrophilia promotes early atherosclerosis. *Circulation* 2010;122:1837–1845.
35. Drechsler M, Döring Y, Megens RT, et al. Neutrophilic granulocytes – promiscuous accelerators of atherosclerosis. *Thromb Haemost* 2011;106:839–848.
36. Brinkmann V, Reichard U, Goosmann C, et al. Neutrophil extracellular trap kill bacteria. *Science* 2004;303:1532–1535.
37. Luo G, Ducy P, McKee MD, et al. Spontaneous calcification of arteries and cartilage in mice lacking matrix GLA protein. *Nature* 1997;386:78–81.
38. Weijs B, Blaauw Y, Rennenberg RJ, et al. Patients using vitamin K antagonists show increased levels of coronary calcification: an observational study in low-risk atrial fibrillation patients. *Eur Heart J* 2011;32:2555–2562.
39. Rennenberg RJ, van Varik BJ, Schurgers LJ, et al. Chronic coumarin treatment is associated with increased extracoronary arterial calcification in humans. *Blood* 2010;115:5121–5123.
40. Parker BD, Schurgers LJ, Brandenburg VM, et al. The associations of fibroblast growth factor 23 and uncarboxylated matrix Gla protein with mortality in coronary heart disease: the Heart and Soul study. *Ann Intern Med* 2010;152:640–648.
41. Thygesen K, Alpert JS, Jaffe AS, et al. Third universal definition of myocardial infarction. *J Am Coll Cardiol* 2012;60:1581–1598.
42. de Lemos JA, Drazner MH, Omland T, et al. Association of troponin T detected with a highly sensitive assay and cardiac structure and mortality risk in the general population. *JAMA* 2010;304:2503–2512.
43. deFilippi CR, de Lemos JA, Christenson RH, et al. Association of serial measures of cardiac troponin T using a sensitive assay with incident heart failure and cardiovascular mortality in older adults. *JAMA* 2010;304:2494–2502.
44. Otsuka T, Kawada T, Ibuki C, et al. Association between high sensitivity cardiac troponin T levels and the predicted cardiovascular risk in middle-aged men without overt cardiovascular disease. *Am Heart J* 2010;159:972–978.
45. Rumberger JA, Brundage BH, Rader DJ, et al. Electron beam computed tomographic coronary calcium scanning: a review and guidelines for use in asymptomatic persons. *Mayo Clin Proc* 1999;74:243–252.
46. Detrano R, Guerci AD, Carr JJ. Coronary calcium as a predictor of coronary events in four racial or ethnic groups. *N Engl J Med* 2008;358:1336–1345.

47. LaMonte MJ, FitzGerald SJ, Church TS, et al. Coronary artery calcium score and coronary heart disease events in a large cohort of asymptomatic men and women. *Am J Epidemiol* 2005;162:421–429.
48. Greenland P, LaBree L, Azen SP, et al. Coronary artery calcium score combined with Framingham score for risk prediction in asymptomatic individuals. *JAMA* 2004;2:210–215.
49. Hou ZH, Lu B, Gao Y, et al. Prognostic value of coronary CT angiography and calcium score for major adverse cardiac events in outpatients. *JACC Cardiovasc Imaging* 2012;5:990–999.
50. Erbel R, Möhlenkamp S, Moebus S, et al. Coronary risk stratification, discrimination, and reclassification improvement based on quantification of subclinical coronary atherosclerosis: the Heinz Nixdorf Recall study. *J Am Coll Cardiol* 2010;56:1397–1406.
51. Zhang S, Levin DC, Halpern EJ, et al. Accuracy of MDCT in assessing the degree of stenosis caused by calcified coronary artery plaques. *Am J Roentgenol* 2008;191:1676–1683.
52. Ong TK, Chin SP, Liew CK, et al. Accuracy of 64-row multidetector computed tomography in detecting coronary artery disease in 134 symptomatic patients: influence of calcification. *Am Heart J* 2006;151:1–6.
53. Achenbach S. Computed tomography coronary angiography. *J Am Coll Cardiol* 2006;48:1919–1928.
54. Abbara S, Arbab-Zadeh A, Callister TQ, et al. SCCT guidelines for performance of coronary computed tomographic angiography. *J Cardiovasc Comput Tomogr* 2009;3:190–204.
55. Raff GL, Abidov A, Achenbach S, et al. SCCT guidelines for the interpretation and reporting of coronary computed tomographic angiography. *J Cardiovasc Comput Tomogr* 2009;3:122–136.
56. Miller JM, Rochitte CE, Dewey M, et al. Diagnostic performance of coronary angiography by 64-row CT. *N Engl J Med* 2008;359:2324–2336.
57. Budoff MJ, Dowe D, Jollis JG, et al. Diagnostic performance of 64-multidetector row coronary computed tomographic angiography for evaluation of coronary artery stenosis in individuals without known coronary artery disease: results from the prospective multicenter ACCURACY trial. *J Am Coll Cardiol* 2008;52:1724–1732.
58. Schlett CL, Banerji D, Siegel E, et al. Prognostic value of CT angiography for major adverse cardiac events in patients with acute chest pain from the emergency department: 2-year outcomes of the ROMICAT trial. *JACC Cardiovasc Imaging* 2011;4:481–491.

59. Andreini D, Pontone G, Mushtaq S, et al. A long-term prognostic value of coronary CT angiography in suspected coronary artery disease. *JACC Cardiovasc Imaging* 2012;5:690–701.
60. Shmilovich H, Cheng VY, Tamarappoo BK, et al. Vulnerable plaque features on coronary CT angiography as markers of inducible regional myocardial hypoperfusion from severe coronary artery stenoses. *Atherosclerosis* 2011;219:588–595.
61. Pflederer T, Marwan M, Schepis T, et al. Characterization of culprit lesions in acute coronary syndromes using coronary dual-source CT angiography. *Atherosclerosis* 2010;211:437–444.
62. Motoyama S, Sarai M, Harigaya H, et al. Computed tomographic angiography characteristics of atherosclerotic plaques subsequently resulting in acute coronary syndrome. *J Am Coll Cardiol* 2009;54:49–57.
63. Fleg JL, Stone GW, Fayad ZA, et al. Detection of high-risk atherosclerotic plaque: report of the NHLBI Working Group on current status and future directions. *JACC Cardiovasc Imaging* 2012;5:941–955.
64. Leuschner F, Nahrendorf M. Molecular imaging of coronary atherosclerosis and myocardial infarction: considerations for the bench and perspectives for the clinic. *Circ Res* 2011;108:593–606.
65. Phinikaridou A, Andia ME, Shah AM, et al. Advances in molecular imaging of atherosclerosis and myocardial infarction: shedding new light on in-vivo cardiovascular biology. *Am J Physiol Heart Circ Physiol* 2012;303:1397–1410.
66. Kusters DH, Tegtmeier J, Schurgers LJ, et al. Molecular imaging to identify the vulnerable plaque -- from basic research to clinical practice. *Mol Imaging Biol* 2012;14:523–533.
67. Dweck MR, Joshi FR, Newby DE, et al. Noninvasive imaging in cardiovascular therapy: the promise of coronary arterial ^{18}F-sodium fluoride uptake as a marker of plaque biology. *Expert Rev Cardiovasc Ther* 2012;10:1075–1077.
68. Chan KH, Ng MKC. Is there a role for coronary angiography in the early detection of the vulnerable plaque? *Int J cardiol* 2013;164:262–266.
69. Kerensky RA, Wade M, Deedwania P, et al. Revisiting the culprit lesion in non-Q-wave myocardial infarction. Results from the VANQWISH trial angiographic core laboratory. *J Am Coll Cardiol* 2002;39:1456–1463.
70. Garcia-Garcia HM, Costa MA, Serruys PW. Imaging of coronary atherosclerosis: intravascular ultrasound. *Eur Heart J* 2010;31:2456–2469.

71. Kotani J, Mintz GS, Castagna MT, et al. Intravascular ultrasound analysis of infarct-related and non-infarct-related arteries in patients who presented with an acute myocardial infarction. *Circulation* 2003;107:2889–2893.
72. Maehara A, Mintz GS, Bui AB, et al. Morphologic and angiographic features of coronary plaque rupture detected by intravascular ultrasound. *J Am Coll Cardiol* 2002;40:904–910.



CHAPTER 2

GENDER DIFFERENCES IN CLINICAL RISK PROFILE AND CT-ANGIOGRAPHICALLY DEFINED CORONARY ARTERY DISEASE

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Submitted

BACKGROUND

To investigate gender differences in coronary CT-angiography (CCTA) defined coronary artery disease (CAD) as well as gender differences in the relationship between clinical risk profiling, extent and degree of CAD and short-term outcome.

METHODS

This retrospective study is approved by the Institutional Review Board and compliant to the Declaration of Helsinki. 1,042 patients (527 men, 515 women; mean age 56 years) with stable chest discomfort symptoms underwent CCTA to assess the extent (number of coronary segments with plaque) and degree (no, mild, moderate, severe) of CAD. Framingham risk score (FRS) was calculated in all patients. Binary logistic regression models were used to describe the relationship between gender and CAD. Presence of any plaque, presence of severe plaque, and different numbers of segments with plaque were used as dependent variables.

RESULTS

After adjustment for cardiovascular risk factors, men significantly more often harbored any plaque (OR: 2.6, 95%CI: 1.9-3.6), severe plaque (OR: 2.9, 95%CI: 1.7-4.9), 4-6 segments with plaque (OR: 3.3, 95%CI: 2.2-5.1) and >6 segments with plaque (OR: 4.0, 95%CI: 2.2-7.5), compared with women (all $P<0.001$). Although individual women had more risk factors, FRS was significantly lower in women as compared to men. A total of 28 patients suffered an event: 24 revascularizations, 2 acute coronary syndromes and 2 deaths.

CONCLUSION

Women had significantly lower FRS than men, regardless the extent and degree of CAD. This suggests that relative to men, the FRS underestimates the extent and degree of CAD in women as assessed with CCTA.

INTRODUCTION

Despite a significant decline in the incidence of cardiovascular diseases (CVD) in the last decades, ischemic heart disease and stroke are still major causes of morbidity and mortality. CVD is often regarded as a problem mostly seen in men. However, health statistics in Europe show that about 54% of all deaths in females are caused by CVD, compared to 43% of all deaths in males [1]. Despite these statistics, the risk of CVD is still underestimated in women. This has led the European Society of Cardiology to launch the 'Woman at Heart' program [2,3]. Similar programs have been initiated in the USA, such as the 'Go Red For Women' campaign [4]. These programs should improve risk stratification, diagnosis and therapy in women at risk for CVD. The importance of these programs is emphasized by studies showing that healthcare professionals often fail to identify cardiovascular risk factors in women, resulting in underdiagnosis and undertreatment [5-8]. As a consequence, women with CVD experience a worse quality of life [9,10] and have lower survival rates as compared with men [11,12].

Cardiovascular risk stratification algorithms, such as the Framingham risk score (FRS) are powerful tools to classify patients into risk groups [13]. The ability of FRS to predict for coronary artery disease (CAD) is similar or even better compared to other risk score algorithms like PROCAM and Diamond Forrester, in stable chest pain patients referred for coronary CT-angiography (CCTA) [14].

The aim of the present study was to investigate gender differences in CCTA defined CAD. Moreover, because the potential mismatch between clinical risk profiling and the extent and degree of CAD, we also investigate gender differences in the relationship between clinical risk profiling, extent and degree of CAD and short-term outcome.

METHODS

Study Population

We retrospectively studied 1,153 patients, referred from the cardiology outpatient department for CCTA because of stable chest discomfort, suspicious for CAD.

Scans were performed between June 2010 and July 2012, as part of the diagnostic work-up, using a second generation dual-source CT-scanner (Somatom Definition Flash, Siemens Medical Solutions, Forchheim, Germany). Included were patients who underwent coronary calcium scoring (CCS) as well as CCTA. Excluded were patients with a known history of CAD, patients with unavailable data regarding their cardiovascular risk profile and patients with an inconclusive scan (Figure 1).

This study is approved by the Institutional Review Board (IRB) and Ethics Committee. Written informed consent was waived because data were analyzed anonymously in accordance with IRB guidelines. The study complies with the ethical principles of the Declaration of Helsinki.

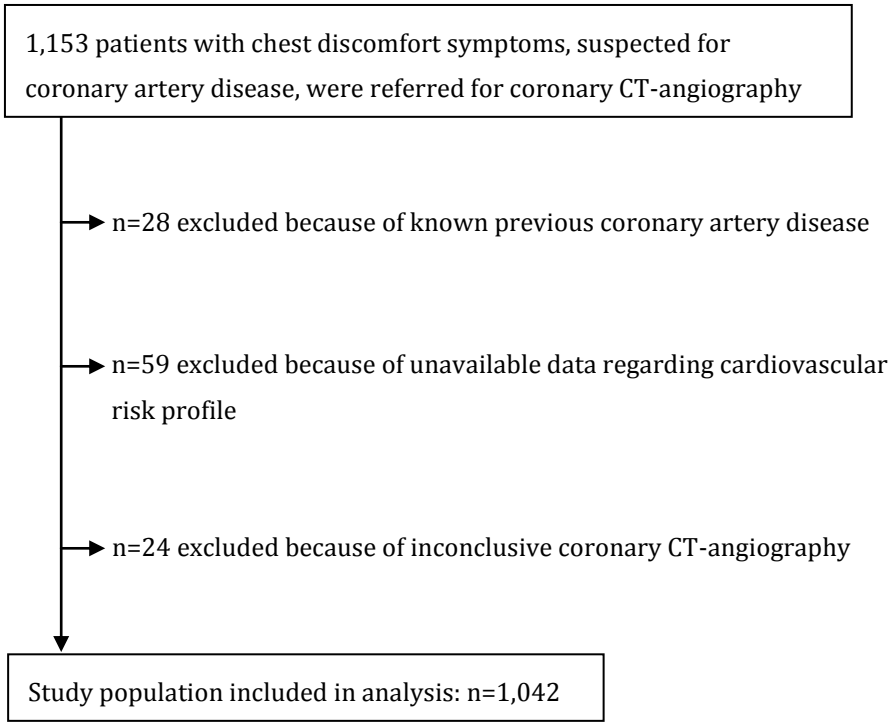


Figure 1. *Flowchart of the study design.*

Traditional Cardiovascular Risk Factors and Framingham Risk Score

Traditional cardiovascular risk factors were collected prior to the scan in order to calculate the FRS, which is an index of 10 year risk for adverse cardiovascular events.

Risk variables which are used for calculating the FRS are age, systolic blood pressure, smoking, diabetes mellitus, total cholesterol and high-density lipoprotein cholesterol [13]. Patients were classified as smoker if they were current smoker. A family history of premature CAD was defined as having a first-degree relative with a history of myocardial infarction or sudden cardiac death before the age of sixty [15]. Patients were classified as diabetic if diabetes mellitus was diagnosed according to the guidelines [16].

Coronary Calcium Score

A non-contrast-enhanced high pitch spiral scan was performed to determine the CCS, using the Agatston method [17]. Data acquisition parameters were: pitch 3.4, slice collimation 2 x 128 x 0.6 mm, gantry rotation time 280 ms, tube voltage 120 kV, tube current 100 mAs, slice thickness 3 mm/1.5 mm and dedicated reconstruction kernels for cardiac CT (B26f / I26f). CCS was calculated using dedicated software (Syngo Calcium Scoring, Siemens). An attenuation threshold of 130 Hounsfield units (HU) was used to identify calcifications in the main coronary branches. All individual calcifications were manually picked, summed and expressed as Agatston score.

Coronary CT-angiography

Adjacent to the CCS scan we performed CCTA. Data acquisition parameters for CCTA were: slice collimation 2 x 128 x 0.6 mm, gantry rotation time 280 ms and tube voltage 80-120 kV, depending on the individual patient size. Patients received 50 mg Metoprolol tartrate (AstraZeneca, Zoetermeer, The Netherlands) orally, two hours before CCTA. When indicated, an additional dose of 5-20 mg Metoprolol tartrate was administered intravenously on site, in an attempt to lower the heart rate <60 beats per minute (bpm). All patients received 0.8 mg Nitroglycerin spray (Pohl-Boskamp, Hohenlockstedt, Germany) sublingually just prior to CCTA. Heart rate and ECG were monitored prior and during CCTA. A 20 mL contrast test bolus was injected to assess the time to peak in the ascending aorta. CCTA was performed using 75–120 mL iopromide (Ultravist 300; Bayer Healthcare, Leverkusen, Germany), injected in the antecubital vein at a flow rate of 5.2–7.4 mL/s, directly followed by 40 mL intravenous saline at the same flow rate, using a dual-head power injector (Stellant, Bayer).

Amount of contrast agent as well as flow rate were dependent on individual patient characteristics.

Patients with a stable heart rate <60 bpm underwent a prospectively ECG-triggered high pitch spiral protocol ("Flash"). Patients with a stable heart rate between 60–90 bpm underwent a prospectively ECG-triggered protocol ("Adaptive sequence"). In patients with a heart rate >90 bpm or in case of arrhythmia, a retrospectively ECG-gated "Helical" low-pitch protocol with online dose modulation was performed.

All scans were independently analyzed by a cardiologist and radiologist, each with 4 years experience in CCTA assessment and blinded for patient details. In case of disagreement, consensus was reached by reviewing findings jointly. Dedicated software (Syngo CT 2010A, Siemens) was used to assess the source images. The coronary artery tree was analyzed for presence and severity of CAD, according to the 16-segment classification of the American Heart Association [18]. Plaques were defined as visible structures within or adjacent to the coronary artery lumen, which could be clearly distinguished from the vessel lumen and the surrounding pericardial tissue. The degree of CAD was visually estimated and classified as absent (no luminal stenosis), mild (1-50% luminal stenosis), moderate (50-70% luminal stenosis) or severe (>70% luminal stenosis) [19]. The extent of CAD was defined as the number of coronary segments with plaque, irrespective the degree of luminal stenosis.

Radiation Dose

The estimated effective radiation dose was calculated by multiplying the dose length product by a conversion factor of 0.014 mSv/(mGy x cm) for the chest region [20,21].

Follow-up and Outcome

Electronic patient records were monitored for 90-days outcome, including occurrence of revascularization procedures (percutaneous coronary intervention [PCI] or coronary artery bypass grafting [CABG]), acute coronary syndrome (ACS) and mortality. ACS was defined as typical angina pectoris, troponin T elevation (>0.01 µg/L), ST-segment elevation or depression of ≥ 1 mm, or two of these characteristics together with invasive angiographic confirmation of a culprit lesion [22].

Patients were seen by their cardiologist on a regular basis, and all hospital visits, including outpatient and emergency department visits, were recorded in the electronic patient system.

Statistical Analysis

Categorical baseline characteristics are expressed as absolute number and percentages, while continuous variables are expressed as means ± standard deviation (SD) or as median with interquartile range (IQR), for normal and non-parametric distributions, respectively. To test differences between men and women for statistical significance, we used the Pearson chi-square test for categorical variables and the Student t-test or Mann-Whitney test for continuous variables.

Odds ratios (OR) with corresponding 95% confidence intervals (CI) were used to quantify the association between gender and degree as well as extent of CAD, where women were used as reference category (OR=1.00). Three models were used to describe these associations: 1) an unadjusted model, 2) an age-adjusted model, and 3) a model adjusting for the FRS variables (age, diabetes mellitus, smoking, systolic blood pressure, total cholesterol and HDL-cholesterol) as well as other traditional cardiovascular risk factors (body mass index, family history of premature CAD and LDL-cholesterol). For these analyses we used binary logistic regression models with presence of any plaque, presence of severe plaque, 1-3 segments with plaque, 4-6 segments with plaque and >6 segments with plaque as dependent variables.

P values less than 0.05 were considered significant. Statistical analyses were performed using IBM SPSS software (version 19.0, SPSS Inc., Chicago, IL, USA).

RESULTS

Study Population

From 1,153 patients, 28 patients had a history of CAD, 59 patients had unavailable data regarding their risk profile and 24 patients had an inconclusive scan due to movement or breathing artefacts (Figure 1). Baseline characteristics of the remaining 1,042 patients (527 men; 515 women) are listed in Table 1.

Women were older and more likely to have diabetes, a family history of premature CAD and a higher systolic blood pressure as compared with men. The mean FRS was significantly lower in women compared to men (14.9 vs. 22.8; *P*<0.001).

Table 1. Baseline characteristics of the study population.

	Overall (n=1042)	**Men (n=527)**	**Women (n=515)**	***P* value**
Age (years)	56 ± 11	55 ± 11	57 ± 11	<0.001
Body Mass Index (kg/m^2)	27.2 ± 6.8	27.4 ± 8.1	26.9 ± 5.0	0.25
Diabetes mellitus	80 (7.7)	32 (6.1)	48 (9.3)	0.05
Current smoking	223 (21.4)	122 (23.1)	101 (19.6)	0.16
Family history of premature CAD	375 (36.0)	183 (34.7)	192 (37.3)	0.46
Systolic blood pressure (mmHg)	142 ± 20	141 ± 18	143 ± 22	0.03
Total cholesterol (mg/dL)	217 ± 46	209 ± 46	220 ± 46	<0.001
HDL-C (mg/dL)	54 ± 19	46 ± 16	58 ± 19	<0.001
LDL-C (mg/dL)	135 ± 43	135 ± 43	135 ± 39	0.46
Triglycerides (mg/dL)	159 ± 115	168 ± 115	151 ± 106	<0.01
Glucose (mg/dL)	106 ± 23	106 ± 27	105 ± 22	0.65
Framingham risk score	18.9 ± 13.8	22.8 ± 15.1	14.9 ± 11.0	<0.001
Coronary calcium score	3 (0-88)	11 (0-155)	0 (0-41)	<0.001

Values are presented as absolute number (percentage), or as mean ± standard deviation, except for calcium score, which is presented as median (interquartile range). CAD, coronary artery disease; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.

Radiation Dose

Estimated effective radiation dose for the CCS scan was 0.4 ± 0.2 mSv. Estimated effective radiation doses on top of the CCS scan were 1.2 ± 0.5 mSv for the "Flash" protocol (n=510), 3.4 ± 2.0 mSv for the "Adaptive sequence" protocol (n=465) and 11.0 ± 6.2 mSv for the "Helical" protocol (n=67).

Coronary Calcium Score, Extent and Degree of Coronary Artery Disease

Men had a significantly higher CCS as compared with women (*P*<0.001), Table 1. Coronary plaques, irrespective of the degree of luminal stenosis, were present in 346 (65.7%) men and 265 (51.5%) women. Men significantly more often harbored plaques with 50-70% luminal stenosis (16.3% vs. 11.1%; *P*=0.01) and plaques with >70% luminal stenosis (13.5 % vs. 4.9%; *P*<0.001) as compared with women. Moreover, both three vessel disease (23.3% vs. 9.1%; *P*<0.001) as well as left main vessel disease (17.1% vs. 11.8%; *P*=0.02) were significantly more often present in men. Regarding extent of CAD, a larger proportion of men had plaques in at least four coronary segments as compared with women (31.0% vs. 12.8%; *P*<0.001), Table 2.

Table 2. Extent and degree of coronary artery disease, according to gender.

	Men (n=527)	**Women (n=515)**	***P* value**
Presence of any plaque	346 (65.7)	265 (51.5)	<0.001
Maximal diameter luminal stenosis			
No plaque	181 (34.3)	250 (48.5)	<0.001
1-49%	189 (35.9)	183 (35.5)	0.91
50-70%	86 (16.3)	57 (11.1)	0.01
>70%	71 (13.5)	25 (4.9)	<0.001
Number of vessels with plaque			
None	181 (34.3)	250 (48.5)	<0.001
1-vessel	121 (23.0)	130 (25.2)	0.39
2-vessel	102 (19.4)	88 (17.1)	0.34
3-vessel	123 (23.3)	47 (9.1)	<0.001
Left main-vessel	90 (17.1)	61 (11.8)	0.02
Number of segments with plaque			
None	181 (34.3)	250 (48.5)	<0.001
1-3	183 (34.7)	199 (38.6)	0.19
4-6	112 (21.3)	50 (9.7)	<0.001
>6	51 (9.7)	16 (3.1)	<0.001

Values are presented as absolute number (percentage).

Adjustment for Traditional Cardiovascular Risk Factors

Table 3 shows the results of the binary logistic regression models, used to describe gender differences regarding presence of any and severe plaques. In both the unadjusted and the age-adjusted model (models 1 and 2), men had approximately 2-3 times greater odds of having any plaque as well as severe plaque, as compared with women. When adjusted for traditional cardiovascular risk factors (model 3), the odds ratios for men were 2.61 (95% CI 1.91-3.56) for the presence of any plaque and 2.89 (95% CI 1.71-4.88) for the presence of severe plaque (both $P<0.001$).

Table 3. Odds ratios of risk factors in relation to presence of any and severe plaque.

	Presence of any plaque			Presence of severe plaque		
	OR	**95% CI**	***P* value**	**OR**	**95% CI**	***P* value**
Model 1						
Male gender	1.80	1.41-2.31	<0.001	3.05	1.90-4.90	<0.001
Model 2						
Male gender	2.74	2.05-3.66	<0.001	3.62	2.23-5.87	<0.001
Age	1.11	1.09-1.13	<0.001	1.06	1.03-1.08	<0.001
Model 3						
Male gender	2.61	1.91-3.56	<0.001	2.89	1.71-4.88	<0.001
Age	1.12	1.10-1.13	<0.001	1.08	1.05-1.10	<0.001
Body mass index	1.01	0.98-1.03	0.58	1.00	0.98-1.03	0.83
Diabetes mellitus	1.52	0.83-2.76	0.18	0.85	0.34-2.15	0.73
Current smoking	1.74	1.21-2.51	<0.01	1.71	1.01-2.90	0.05
Family history	1.43	1.06-1.93	0.02	1.44	0.90-2.31	0.13
Systolic BP	1.01	1.01-1.02	<0.01	1.00	0.99-1.01	0.97
Total cholesterol	0.99	0.73-1.34	0.94	0.79	0.47-1.34	0.39
HDL-C	0.75	0.53-1.04	0.09	0.44	0.21-0.91	0.03
LDL-C	1.04	0.75-1.44	0.83	1.44	0.81-2.54	0.21

Female gender is used as reference category. Binary logistic regression analysis was performed using three models. Model 1 = unadjusted model; Model 2 = age-adjusted model; Model 3 = risk factor adjusted model. BP, blood pressure. HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.

Table 4 describes gender differences regarding extent of CAD in the same way. In all three models, men had 2.5-4 times greater odds of having 4-6 coronary segments as well as >6 coronary segments with a plaque, as compared to women (all *P*<0.001). Regarding the presence of 1-3 segments with plaque, odds ratios for men were in all models smaller than 1.00.

Table 4. Odds ratios of risk factors in relation to extent of CAD.

	1-3 segments with plaque			**4-6 segments with plaque**			**>6 segments with plaque**		
	OR	**95% CI**	***P***	**OR**	**95% CI**	***P***	**OR**	**95% CI**	***P***
Model 1									
Male gender	0.85	0.66-1.09	0.19	2.51	1.75-3.59	<0.001	3.34	1.88-5.94	<0.001
Model 2									
Male gender	0.91	0.71-1.18	0.48	3.53	2.39-5.20	<0.001	4.00	2.22-7.20	<0.001
Age	1.03	1.02-1.05	<0.001	1.10	1.08-1.12	<0.001	1.06	1.04-1.09	<0.001
Model 3									
Male gender	0.89	0.67-1.17	0.39	3.34	2.19-5.09	<0.001	4.01	2.15-7.49	<0.001
Age	1.03	1.02-1.05	<0.001	1.11	1.09-1.14	<0.001	1.05	1.02-1.09	<0.001
BMI	1.01	0.99-1.04	0.21	0.99	0.95-1.03	0.54	1.00	0.96-1.04	0.85
Diabetes	1.24	0.75-2.06	0.41	0.90	0.43-1.87	0.77	1.85	0.74-4.62	0.19
Smoking	1.08	0.77-1.50	0.67	1.57	0.97-2.52	0.06	2.15	1.14-4.05	0.02
Fam. history	1.21	0.92-1.59	0.17	1.93	1.30-2.85	<0.01	0.74	0.40-1.34	0.32
Systolic BP	1.00	1.00-1.01	0.60	1.01	1.00-1.02	0.20	1.01	1.00-1.03	0.04
Total-C	1.26	0.93-1.71	0.13	1.07	0.68-1.69	0.77	0.62	0.38-1.02	0.06
HDL-C	0.77	0.55-1.07	0.12	0.55	0.32-0.95	0.03	1.69	1.06-2.71	0.03
LDL-C	0.84	0.61-1.16	0.29	0.99	0.61-1.60	0.96	1.54	0.91-2.61	0.11

Female gender is used as reference category. Binary logistic regression analysis was performed using three models. Model 1 = unadjusted model; Model 2 = age-adjusted model; Model 3 = risk factor adjusted model. BMI, body mass index; BP, blood pressure; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; Total-C, total cholesterol.

Framingham Risk Score Versus Degree and Extent of Coronary Artery Disease

As shown in Figure 2, FRS is significantly higher in men as compared with women in all categories. Moreover, FRS in men shows a progressive increase as the degree of CAD becomes more severe (mean FRS 14.4% in absence of CAD, FRS 25.0% in mild CAD, FRS 29.3% in moderate CAD, and FRS 30.7% in severe CAD). An equal trend is visible in women for categories 'no CAD' (FRS 10.7%), 'mild CAD' (FRS 18.2%) and 'moderate CAD' (FRS 21.1%). However, women with severe CAD have a lower mean FRS (FRS 18.4%), probably explained by the small sample size in this subgroup. Figure 3 shows the relation between FRS and extent of CAD. As more segments show evidence of CAD, FRS increases in both men and women. Interestingly, patients with >6 affected coronary segments have, regardless of gender, a lower mean FRS as compared with patients with 4-6 affected segments, most likely due to the small number of patients with >6 affected segments.

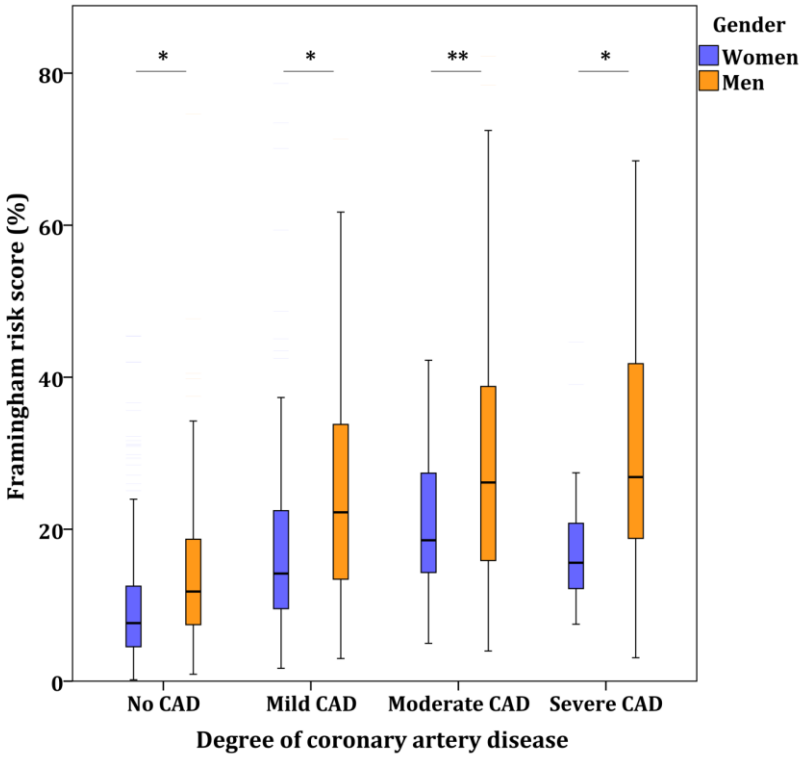


Figure 2. *Framingham risk score in relation to degree of coronary artery disease, according to gender. CAD = coronary artery disease; * P<0.001; ** P=0.002.*

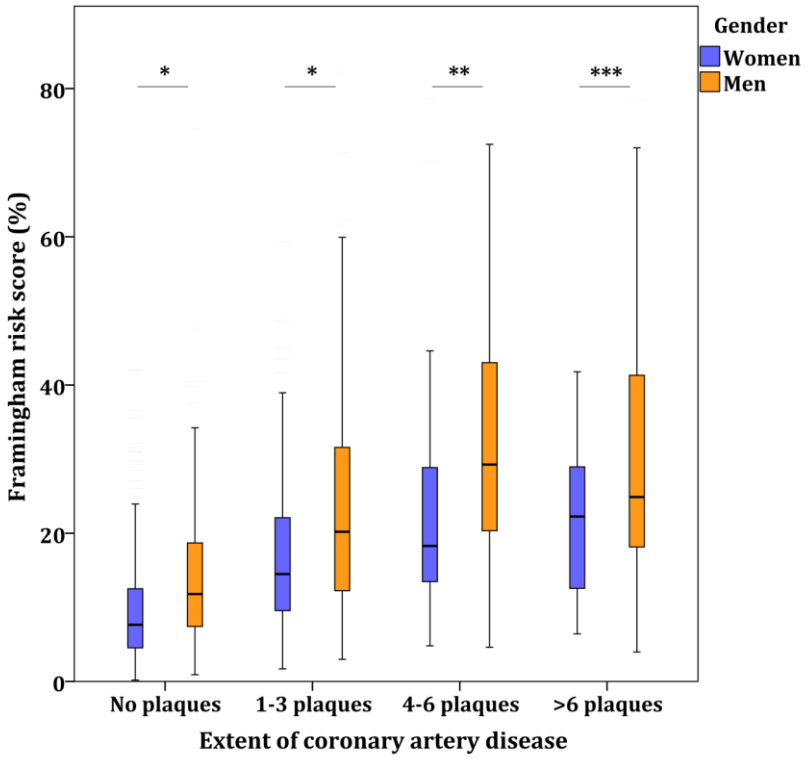


Figure 3: *Framingham risk score in relation to extent of coronary artery disease, according to gender. * P<0.001; ** P=0.001; *** P=0.104.*

Follow-up and Outcome

During the 90-days follow-up period, which was available for all patients, a total of 28 patients (2.7%) suffered an event. Coronary revascularization was performed in 24 patients: 19 PCI's (13 men, 6 women) and 5 CABG's (all men). Additionally, 2 men suffered an ACS, 1 man died (cardiovascular cause), while 1 woman died (non-cardiovascular cause).

DISCUSSION

Gender Differences in Coronary Artery Disease

Despite women in our study were having more traditional cardiovascular risk factors, they had a lesser extent and degree of CAD. This is consistent with previous CCTA studies, which observed that women were less likely to have obstructive CAD in symptomatic [23,24] and asymptomatic populations [25]. A possible explanation is that premenopausal women are relatively 'protected' from obstructive CAD due to favorable estrogen levels, while postmenopausal women are "catching up" in terms of developing CAD. On the other hand, several studies have shown that the prognosis of non-obstructive CAD in women is less benign than initially considered and even comparable with the more obstructive pattern of CAD in men [26,27]. Unfortunately, due to the short follow-up time we were not able to establish this theory in our cohort.

Gender Differences in Clinical Risk Profiling

In the past decades, several clinical risk profiling algorithms have been developed to estimate the long term cardiovascular risk in patients. These algorithms consist of traditional cardiovascular risk factors [13,28], more recently supplemented by biomarkers such as high-sensitivity C-reactive protein [29]. Risk factor analysis is an epidemiologic concept meant to be used in patient populations rather than in individuals. Nevertheless, it has proven to be a powerful tool in clinical practice. In addition to the fact that risk profiling is cost- and time-efficient, one of the major strengths is that several risk factors in one individual can be combined to calculate cardiovascular risk. When combined, risk factors increase the risk of cardiovascular events not in a linear but in an exponential way. Therefore, clinical risk profiling is preferable and recommended in clinical practice [30].

Although several studies have claimed to improve on the FRS algorithm, a recent review showed little evidence for any clinically relevant improvement beyond the FRS [31]. All risk factors used to calculate the FRS have a gender specific weighting factor. In the current study, women had a significant lower FRS compared to men, despite the fact that women had more individual risk factors, which is not an uncommon finding [32].

This implies that cardiovascular risk factors in women are not sufficiently translated into the FRS algorithm, resulting in much lower FRS. Our data showed that women, irrespective of the extent and degree of CAD, had significant lower FRS than men with corresponding extent and degree of CAD. This suggests that relative to men, the FRS underestimates extent and degree of CAD in women. This finding is supported by another study on the association between obstructive CAD and FRS. The authors found that a significant proportion of the patients categorized with a low (<10%) and intermediate (10–20%) FRS had obstructive CAD. Most women (93%) belonged to the low FRS category, which means that a significant proportion of them were having obstructive CAD [33]. However, underestimation of cardiovascular risk in women may not be a matter of inability of healthcare professionals to identify risk factors in women, but may be the result of underestimation of the weighting of risk factors in women by the FRS algorithm. This is supported by the multi-ethnic study of atherosclerosis. After excluding women with diabetes and those older than 79 years, 90% of women were classified as low risk, based on FRS [34]. This may contribute to the relative underuse of diagnostic and therapeutic care in women, resulting in worse outcomes. In the recent 2011 AHA prevention guidelines for women, it had therefore been proposed to introduce an additional category of "ideal cardiovascular health" to the FRS [35].

Our data also show that FRS in men increases as the degree of CAD becomes more severe. In our female patients however, this trend is only partially visible, which enhances the concept that there are gender differences in the pattern of CAD [36]. Based on the calculated FRS it is therefore almost impossible to predict the degree of CAD in women. In contrast, CCTA may provide incremental information beyond traditional cardiovascular risk factors, which may improve therapeutic decisions. This is strengthened by a study, in which the cardiac event rate was assessed after detection or exclusion of obstructive CAD by CCTA. This event rate was compared with the event rate as predicted by FRS. In patients without obstructive CAD, significantly fewer events occurred than predicted by FRS. Therefore, exclusion of obstructive CAD by CCTA identifies a patient population with an event risk lower than predicted by traditional risk factors [37].

Follow-up and Short-term Outcome

In the current study, almost 90% (1,024/1,153) of the initial patients who underwent CCTA were eligible for follow-up. These patients provide the basis for our general conclusions. The patient exclusion rate due to technical limitations of the scanning procedure was very low (24/1,153; 2%). This emphasizes that modern CT technology in combination with adequate medical preparation ensures robust CCTA assessment.

The vast majority of cardiovascular events in this study were revascularizations, driven by CCTA results. 'Hard' events, including ACS and cardiac death, were recorded in three male patients. Possibly, CAD remains silent for a longer time in women. Therefore, it is not inconceivable that women are catching up in developing adverse events in the long-term.

Study Limitations

Our study has several limitations that merit comment. First, because our population underwent CCTA recently, we were only able to report short-term outcome. However, although FRS is an index of 10 year risk of an adverse cardiovascular event, we believe that this risk finds its main origin in today's extent and degree of CAD. Therefore we felt justified to consider FRS in relation to the extent and degree of CAD. Second, although invasive coronary angiography is still the gold standard for assessment of CAD, CCTA is a well-established diagnostic imaging modality to evaluate extent and degree of CAD. Third, we had no available data regarding the menopausal state of our female patients and the use and duration of oral contraceptives or hormonal replacement therapy.

CONCLUSION

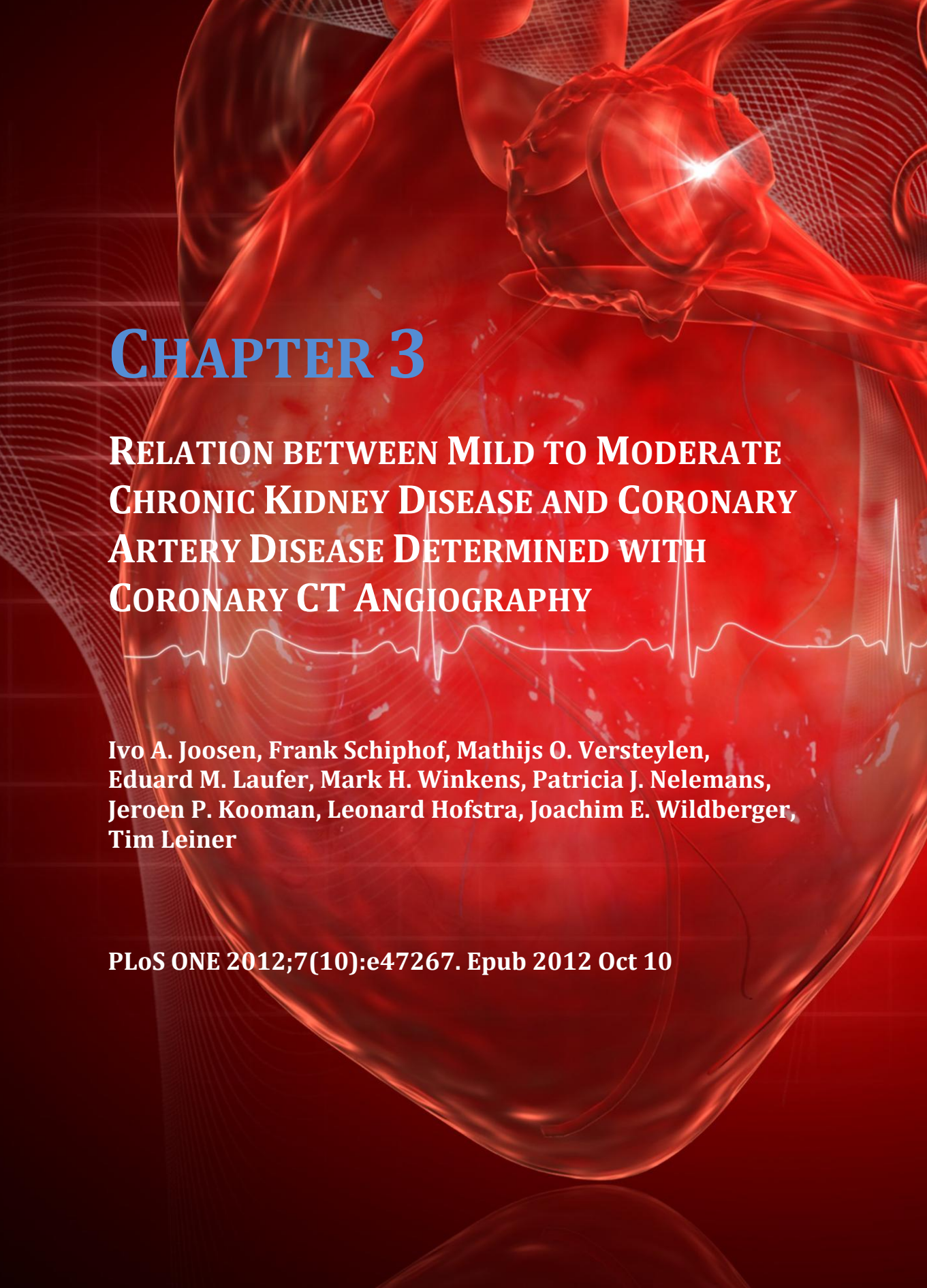
Men had a higher burden of CAD than women, as assessed with CCTA. Although individual women had more risk factors, FRS were significantly lower in women as compared with men, regardless extent and degree of CAD. This suggests that relative to men, the FRS underestimates the extent and degree of CAD in women.

REFERENCES

1. World Health Organization Statistical Information System 2009: www.who.int/whosis.
2. Stramba-Badiale M, Fox KM, Priori SG, et al. Cardiovascular diseases in women: a statement from the policy conference of the European Society of Cardiology. *Eur Heart J* 2006;27:994–1005.
3. Maas AH, van der Schouw YT, Regitz-Zagrosek V, et al. Red alert for women's heart: the urgent need for more research and knowledge on cardiovascular disease in women: proceedings of the workshop held in Brussels on gender differences in cardiovascular disease, 29 September 2010. *Eur Heart J* 2011;32:1362–1368.
4. Goldsmith J. The AHA 'goes red' for women. *Am J Nurs* 2004;104:25–26.
5. Weisz D, Gusmano MK, Rodwin VG. Gender and the treatment of heart disease in older persons in the United States, France, and England: a comparative, population-based view of a clinical phenomenon. *Gender Med* 2004;1:29–40.
6. Enriquez JR, Pratap P, Zbilut JP, et al. Women tolerate drug therapy for coronary artery disease as well as men do, but are treated less frequently with aspirin, β-blockers, or statins. *Gender Med* 2008;5:53–61.
7. Lansky AJ, Hochman JS, Ward PA, et al. Percutaneous coronary intervention and adjunctive pharmacotherapy in women: a statement for healthcare professionals from the American Heart Association. *Circulation* 2005;111:940–953.
8. Lahoz C, Mantilla T, Taboada M, et al. Gender differences in evidence-based pharmacological therapy for patients with stable coronary heart disease. *Int J Cardiol* 2009;133:336–340.
9. Norris CM, Ghali WA, Galbraith PD, et al. Women with coronary artery disease report worse health–related quality of life outcomes compared to men. *Health Qual Life Outcomes* 2004;2:21–31.
10. Pettersen KI, Reikvam A, Rollag A, et al. Understanding sex differences in health-related quality of life following myocardial infarction. *Int J Cardiol* 2008;130:449–456.
11. Gottlieb S, Harpaz D, Shotan A, et al. Sex differences in management and outcome after acute myocardial infarction in the 1990s. A prospective observational community-based study. *Circulation* 2000;102:2484–2490.
12. Marrugat J, Sala J, Masiá R, et al. Mortality differences between men and women following first myocardial infarction. *JAMA* 1998;280:1405–1409.

13. D'Agostino RB Sr, Vasan RS, Pencina MJ, et al. General cardiovascular risk profile for use in primary care: The Framingham Heart Study. *Circulation* 2008;117:743–753.
14. Versteylen MO, Joosen IA, Shaw LJ, et al. Comparison of Framingham, PROCAM, SCORE, and Diamond Forrester to predict coronary atherosclerosis and cardiovascular events. *J Nucl Cardiol* 2011;18:904–911.
15. Dutch Institute for Healthcare Improvement (CBO). Multidisciplinary guideline "Cardiovascular risk management 2006".
16. The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus. Report of the expert committee on the diagnosis and classification of diabetes mellitus. *Diabetes Care* 2003;26:5–20.
17. Agatston AS, Janowitz WR, Hildner FJ, et al. Quantification of coronary artery calcium using ultrafast computed tomography. *J Am Coll Cardiol* 1990;15:827–832.
18. Austen WG, Edwards JE, Frye RL, et al. A reporting system on patients evaluated for coronary artery disease. *Circulation* 1975;51: 5–40.
19. Raff GL, Abidov A, Achenbach S, et al. SCCT guidelines for the interpretation and reporting of coronary computed tomographic angiography. *J Cardiovasc Comput Tomogr* 2009;3:122–136.
20. Jessen KA, Shrimpton PC, Geleijns J, et al. Dosimetry for optimisation of patient protection in computed tomography. *Appl Radiat Isot* 1999;50:165–172.
21. Shrimpton PC. Assessment of patient dose in CT. http://www.msct.eu/PDF_FILES/Appendix%20paediatric%20CT%20Dosimetry.pdf
22. Braunwald E, Antman EM, Beasley JW, et al. ACC/AHA 2002 guideline update for the management of patients with unstable angina and non-ST-segment elevation myocardial infarction – summary article: *J Am Coll Cardiol* 2002;40:1366–1374.
23. Nasir K, Gopal A, Blankstein R, et al. Noninvasive assessment of gender differences in coronary plaque composition with multidetector computed tomographic angiography. *Am J Cardiol* 2010;105:453–458.
24. Yiu KH, de Graaf FR, Schuijf JD, et al. Age- and gender-specific differences in the prognostic value of CT coronary angiography. *Heart* 2012;98:232–237.
25. Blaha MJ, Nasir K, Rivera JJ, et al. Gender differences in coronary plaque composition by coronary computed tomography angiography. *Coron Artery Dis* 2009;20:506–512.
26. Gulati M, Cooper-DeHoff RM, McClure C, et al. Adverse cardiovascular outcomes in women with nonobstructive coronary artery disease. *Arch Intern Med* 2009;169:843–850.

27. Jespersen L, Hvelplund A, Abildstrøm SZ, et al. Stable angina with no obstructive coronary artery disease is associated with increased risks of major adverse cardiovascular events. *Eur Heart J* 2012;33:734–744.
28. Assmann G, Cullen P, Schulte H. Simples coring scheme for calculating the risk of acute coronary events based on the 10-year follow-up of the prospective cardiovascular Munster (PROCAM) study. *Circulation* 2002;105:310–315.
29. Ridker PM, Buring JE, Rifai N, et al. Development and validation of improved algorithms for the assessment of global cardiovascular risk in women. *J Am Coll Cardiol* 2007;297:611–619.
30. Greenland P, Alpert JS, Beller GA, et al. 2010 ACCF/AHA guideline for assessment of cardiovascular risk in asymptomatic adults. *Circulation* 2010;122:584–636.
31. Tzoulaki I, Liberopoulos G, Ioannidis JP. Assessment of claims of improved prediction beyond the Framingham risk score. *JAMA* 2009;302:2345–2352.
32. Ferrari R, Abergel H, Ford I, et al. Gender- and age-related differences in clinical presentation and management of outpatients with stable coronary artery disease. *Int J Cardiol* 2012;doi.10.1016/j.ijcard.2012.08.013 [Epub ahead of print].
33. Nair D, Carrigan TP, Curtin RJ, et al. Association of coronary atherosclerosis detected by multislice computed tomography and traditional risk-factor assessment. *Am J Cardiol* 2008;102:316–320.
34. Lakoski SG, Greenland P, Wong ND, et al. Coronary artery calcium scores and risk for cardiovascular events in women classified as "low risk" based on Framingham risk score: the multi-ethnic study of atherosclerosis (MESA). *Arch Intern Med* 2007;167:2437–2442.
35. Mosca l, Benjamin EJ, Berra K, et al. Effectiveness-based guidelines for the prevention of cardiovascular disease in women -- 2011 update. *Circulation* 2011;123:1243–62.
36. Shaw LJ, Bugiardini R, Merz CN. Women and ischemic heart disease: evolving knowledge. *J Am Coll Cardiol* 2009;54:1561–1575.
37. Hadamitzky M, Freissmuth B, Meyer T, et al. Prognostic value of coronary computed tomographic angiography for prediction of cardiac events in patients with suspected coronary artery disease. *JACC Cardiovasc Imaging* 2009;2:404–411.



CHAPTER 3

RELATION BETWEEN MILD TO MODERATE CHRONIC KIDNEY DISEASE AND CORONARY ARTERY DISEASE DETERMINED WITH CORONARY CT ANGIOGRAPHY

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PLoS ONE 2012;7(10):e47267. Epub 2012 Oct 10

BACKGROUND

The aim of this study was to investigate whether mild to moderate chronic kidney disease (CKD) is independently associated with coronary plaque burden beyond traditional cardiovascular risk factors.

METHODS

A total of 2,038 patients with symptoms of chest discomfort suspected for coronary artery disease (CAD) underwent coronary CT-angiography. We assessed traditional risk factors, coronary calcium score and coronary plaque characteristics. Patients were subdivided in three groups, based on their estimated glomerular filtration rate (eGFR): Normal renal function (eGFR ≥90 mL/min/1.73 m^2); mild CKD (eGFR 60-89 mL/min/1.73 m^2); and moderate CKD (eGFR 30-59 mL/min/1.73 m^2).

RESULTS

Coronary calcium score increased significantly with decreasing renal function (*P*<0.001). Coronary plaque prevalence was higher in patients with mild CKD (OR 1.83, 95%CI 1.52-2.21) and moderate CKD (OR 2.46, 95%CI 1.69-3.59), compared to patients with normal renal function (both *P*<0.001). Coronary plaques with >70% luminal stenosis were found significantly more often in patients with mild CKD (OR 1.67 (95%CI 1.16-2.40) and moderate CKD (OR2.36, 95%CI 1.35-4.13), compared to patients with normal renal function (both *P*<0.01). After adjustment for traditional cardiovascular risk factors, the association between renal function and the presence of any coronary plaque as well as the association between renal function and the presence of coronary plaques with >70% luminal stenosis becomes weaker and were no longer statistically significant.

CONCLUSION

Although decreasing renal function is associated with increasing extent and severity of CAD, mild to moderately CKD is not independently associated with coronary plaque burden after adjustment for traditional cardiovascular risk factors.

INTRODUCTION

Coronary artery disease (CAD) remains one of the leading causes of morbidity and mortality in developed countries. In 2007, a total of 406,351 people died due to CAD in the United States. Each year, an estimated 785,000 Americans will suffer a new coronary attack, and 470,000 will have a recurrent attack. It is estimated that an additional 195,000 silent first myocardial infarctions occur each year [1]. Chronic kidney disease (CKD) is also recognized as a major worldwide public health problem, as evidenced by an increasing incidence and prevalence of patients with kidney failure requiring renal replacement therapy, with poor outcomes and high costs [2]. Nearly 26 million people (13%) in the United States have CKD, and most are undiagnosed, while another 20 million Americans are at increased risk for CKD [3,4].

Several studies have found an increased prevalence of CAD, congestive heart failure and left ventricular hypertrophy in patients with end-stage CKD [5,6]. More recently, earlier stages of CKD have also been associated with a worse prognosis in patients with as well as without known CAD [7,8]. In order to further elucidate the interplay between renal function and symptomatic CAD, more detailed knowledge about coronary artery disease burden is desired in patients with different degrees of renal impairment.

Coronary computed tomographic angiography (CCTA) is a noninvasive diagnostic imaging tool, which provides precise information regarding the presence and extent of coronary calcium deposits as well as coronary plaque localization, degree of luminal stenosis and plaque morphology. Several studies already found an inverse association between renal function and coronary artery calcification, but it remains unclear if this association is independent from traditional cardiovascular risk factors [9-14].

Therefore, the aim of this retrospective cross-sectional study was to investigate whether mild to moderate chronic kidney disease is independently associated with coronary plaque burden beyond traditional cardiovascular risk factors.

METHODS

Ethics Statement

This study was approved by the Institutional Review Board (IRB) and Ethics Committee at the Maastricht University. Involved data were collected on a routine basis within the Maastricht Biomarker CT study. Analyses were carried out retrospectively. Informed consent was not obtained from individual patients because the data were analyzed anonymously in accordance with IRB guidelines. The study complies with the ethical principles of the Helsinki Declaration of 1964, revised by the World Medical Organization in Edinburgh in 2000.

Study Population

We studied 2,180 consecutive adult patients who were referred from the cardiology outpatient department for CCTA because of chest discomfort symptoms, suspected for CAD. All scans were performed in the Maastricht University Medical Center between 2008 and 2012 as part of the diagnostic work-up. Included were patients with a recently (not older than 1 month) measured creatinine, who underwent coronary calcium scoring (CCS) scan as well as CCTA. Excluded were patients with a known history of CAD, patients with missing data regarding their cardiac risk profile, patients with an inconclusive scan and patients with severely impaired renal function, defined as an estimated glomerular filtration rate (eGFR) ≤30 mL/min/1.73 m^2 (Figure 1).

Traditional Cardiovascular Risk Factors

Traditional cardiovascular risk factors were collected prior to the scan. Patients were classified as smoker if they were current smoker. A positive family history was defined as having a first-degree relative with a history of myocardial infarction or sudden cardiac death before the age of sixty. Patients were classified as diabetic if diabetes mellitus was diagnosed by a medical doctor according to the guidelines [15]. The body mass index (BMI) is defined as the individual's body mass (kg) divided by the square of the height (m^2).

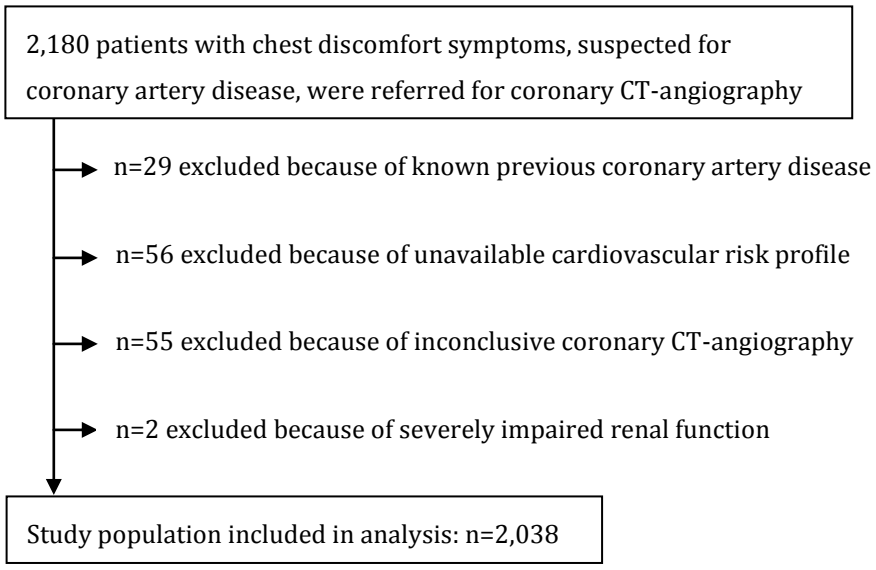


Figure 1. *Flowchart of the study design.*

Chronic Kidney Disease Categories

For all patients, the eGFR was calculated using the CKD-EPI Equation: eGFR = 141 x $\min(Scr/\kappa,1)^{\alpha}$ x $\max(Scr/\kappa,1)^{-1.209}$ x 0.993^{Age} x 1.018 [if female] x 1.159 [if black], where Scr is serum creatinine, κ is 0.7 for females and 0.9 for males, α is -0.329 for females and -0.411 for males, min indicates the minimum of Scr/κ or 1, and max indicates the maximum of Scr/κ or 1 [16]. The eGFR is expressed as mL/min/1.73 m^2. Patients were divided into three categories: normal renal function (eGFR ≥90 mL/min/1.73 m^2), mildly impaired renal function (eGFR 60-89 mL/min/1.73 m^2) and moderately impaired renal function (eGFR 30-59 mL/min/1.73 m^2) based on the National Kidney Foundation Kidney Disease Outcomes Quality Initiative practice guidelines for classification [17].

Coronary CT-angiography Protocol

Scans were performed using a 64–slice multidetector-row CT-scanner (Brilliance 64; Philips Healthcare, Best, The Netherlands, n=1,026) or a dual-source CT-scanner (Somatom Definition Flash, Siemens Medical Solutions, Forchheim, Germany, n=1,012).

Data acquisition parameters for the Brilliance 64 were a 64 x 0.625 mm slice collimation, a gantry rotation time of 420ms and a tube voltage of 80-120 kV, depending on the patient's height and weight. Data acquisition parameters for the Somatom Definition Flash were a 2 x 128 x 0.600 mm slice collimation, a gantry rotation time of 280 ms and a tube voltage of 80-120 kV. Patient preparation was identical for both CT-scanners. Patients received 50 mg Metoprolol tartrate (AstraZeneca, Zoetermeer, The Netherlands) orally, two hours before CCTA. When indicated, an additional dose of 5-20 mg Metoprolol tartrate was administered intravenously to lower the heart rate to <60 beats per minute (bpm). 0.8 mg Nitroglycerin spray (Pohl-Boskamp, Hohenlockstedt, Germany) was given sublingually just prior to CCTA. Heart rate and ECG were monitored during CCTA.

A non-enhanced scan was performed to determine the CCS using the Agatston method [18]. Subsequently, CCTA was performed using 75–120 mL of contrast agent (Xenetix 350; Guerbet, Roissy CdG Cedex, France or Ultravist 300; Bayer Pharma AG, Berlin, Germany), which was injected in the antecubital vein at a rate of 5.2–7.4 mL/s, directly followed by 40 mL intravenous saline (6.0 mL/s) using a dual-head power injector (Medrad Inc, Indianola, Pennsylvania, USA). Both the amount of contrast agent as well as the flow rate were dependent on individual patient characteristics.

Scan protocols were different for both CT-scanners. For the Brilliance 64, a prospectively gated "Step and shoot" protocol was used in all patients with a stable heart rate <65 bpm. In patients with a heart rate >65 bpm, we used a retrospectively gated "Helical" protocol with dose modulation to obtain the best image quality at minimal radiation dose [19,20]. For the Somatom Definition Flash, a prospectively gated high pitch spiral "Flash" protocol was used in patients with a stable heart rate <60 bpm. In patients with a stable heart rate between 60–90 bpm, we used a prospectively gated axial "Adaptive sequence" protocol. In patients with a heart rate >90 bpm or in case of an irregular heart rhythm, we used a retrospectively gated "Helical" protocol with dose modulation.

Coronary CT-angiography Analysis

Dedicated workstations (Philips Brilliance Workspace Portal and Siemens Syngo MultiModality Workplace) were used to assess the source images.

Scans were independently analyzed by a cardiologist and a radiologist, both experienced in the assessment of coronary CT-angiography and both blinded for patient details. In case of disagreement, consensus was reached by reviewing findings jointly.

CCS was expressed as the Agatston score using dedicated calcium scoring software with a threshold of 130 Hounsfield units (HU). The coronary artery tree was analyzed for the presence and severity of CAD, according to the classification of the American Heart Association [21]. Coronary plaques were defined as visible structures within or adjacent to the coronary artery lumen, which could be clearly distinguished from the vessel lumen and the surrounding pericardial tissue. Plaques were categorized as calcified (exclusively content >130 HU), non-calcified (exclusively content <130 HU) or mixed (characteristics of both calcified and non-calcified plaques). The degree of CAD was visually estimated and classified as absent (no luminal stenosis), mild (<50% luminal stenosis), moderate (50-70% luminal stenosis) or severe (>70% luminal stenosis) [22].

Statistical Analysis

Categorical baseline characteristics are expressed as percentages, while continuous variables are expressed as means (standard deviation; SD) or as median (interquartile range; IQR). To test differences between the CKD groups for statistical significance, we used analysis of variance (ANOVA).

Odds ratios (OR) with corresponding 95% confidence intervals (CI) were used to quantify the association between renal function and characteristics of coronary plaques, where patients with normal renal function (eGFR ≥90 mL/min/1.73 m^2) were used as reference category (OR=1.00). To adjust for traditional risk factors, multivariable logistic regression analysis was performed with presence of any plaque and presence of severe plaque as dependent variables. Included in the models were traditional cardiovascular risk factors as well as eGFR as categorical variable, where patients with normal renal function were used as reference category.

The smallest detectable OR (alpha = 5%, power = 80%) for the detection of any coronary plaque was 1.3 for patients with mild CKD and 1.6 for patients with moderate CKD (proportion exposed among controls = 50%).

For the detection of severe coronary plaque, the smallest detectable OR (alpha = 5%, power = 80%) was 1.6 for patients with mild CKD and 2.1 for patients with moderate CKD, respectively (proportion exposed among controls = 6%). A *P*-value <0.05 was considered significant. Statistical analyses were performed using SPSS software (version 19.0, SPSS Inc., Chicago, IL, USA).

RESULTS

Study Population

Patients with chest discomfort symptoms, suspected for CAD were studied. From 2,180 patients, 29 patients had a history of CAD, 56 patients had missing data concerning their risk profile, 55 patients had an inconclusive CCTA due to poor image quality because of movement and/or breathing artifacts and 2 patients suffered from severely impaired renal function (Figure 1). Baseline characteristics of the remaining 2,038 patients are listed in Table 1.

A total of 1,778 patients underwent a prospectively gated scan protocol (mean radiation dose 3.2 mSv), whereas 260 patients underwent a retrospectively gated scan protocol (mean radiation dose 11.1 mSv).

Among the 2,038 patients, 745/2,038 (36.6%) had normal renal function, 1,138/2,038 (55.8%) had mildly impaired renal function, and 155/2,038 (7.6%) had moderately impaired renal function. Compared to patients with normal renal function, patients with mild or moderately impaired renal function were older, had a higher mean body mass index and a higher mean systolic blood pressure. On the other hand, they were less likely to have a positive family history and they were less often male and smoker, Table 1.

Coronary Calcium Score and Coronary CT-angiography

The mean CCS increased significantly with decreasing renal function. The mean CCS was 94 in patients with normal renal function, 103in patients with mildly impaired renal function and 148 in patients with moderately impaired renal function (all *P*<0.01), (Figure 2).

Table 1. Baseline characteristics of the study population, stratified by eGFR.

		eGFR (mL/min/1.73m²)			
	All	**≥90**	**60-89**	**30-59**	
Baseline characteristics	**(n=2,038)**	**(n=745)**	**(n=1,138)**	**(n=155)**	***P* value**
Age (years)	56 ± 11	50 ± 10	59 ± 9	66 ± 9	<0.001
Body Mass Index (kg/m²)	26.8 ± 4.5	26.5 ± 4.7	26.8 ± 4.2	27.7 ± 4.8	0.01
Male gender	51.5	56.1	51.0	32.9	<0.001
Diabetes mellitus	7.5	7.5	6.6	13.5	<0.01
Smoking	22.3	28.6	19.1	15.5	<0.001
Positive family history	37.0	41.1	36.1	23.9	<0.001
Systolic BP (mmHg)	142 ± 19	138 ± 18	143 ± 19	149 ± 21	<0.001
Total cholesterol (mg/dL)	213 ± 46	209 ± 43	213 ± 43	201 ± 50	<0.01
HDL-C (mg/dL)	50 ± 19	50 ± 19	50 ± 19	50 ± 19	0.71
LDL-C (mg/dL)	131 ± 39	131 ± 39	135 ± 39	124 ± 46	<0.01
Triglycerides (mg/dL)*	159 ± 106	159 ± 106	159 ± 106	151 ± 80	0.92
Glucose (mg/dL)**	105 ± 34	106 ± 27	105 ± 27	108 ± 36	0.50
Coronary calcium score	4 (0-95)	0 (0-40)	10 (0-115)	38 (0-220)	<0.001
Presence of any plaque	59.6	49.8	64.5	71.0	<0.001
Presence of severe plaque	8.4	5.9	9.5	12.9	<0.01

Values are presented as percentage, or as mean ± standard deviation, except for calcium score, which is presented as median (interquartile range). BP, blood pressure; eGFR, estimated glomerular filtration rate; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol. * Measured in n= 1,786. ** Measured in n= 1,568.

Coronary plaques were found in 1,215 of 2,038 patients (59.6%). Patients with mildly (OR 1.83, 95%CI 1.52-2.21) or moderately (OR 2.46, 95%CI 1.69-3.59) impaired renal function significantly more often harbored coronary plaques compared to patients with normal renal function (both *P*<0.001). This observation extended to the presence of severe coronary plaques (luminal stenosis >70%), which were also found significantly more often in patients with mildly (OR 1.67, 95%CI 1.16-2.40) or moderately (OR 2.36, 95% CI 1.35-4.13) impaired renal function compared to patients with normal renal function (both *P*<0.01), Table 2.

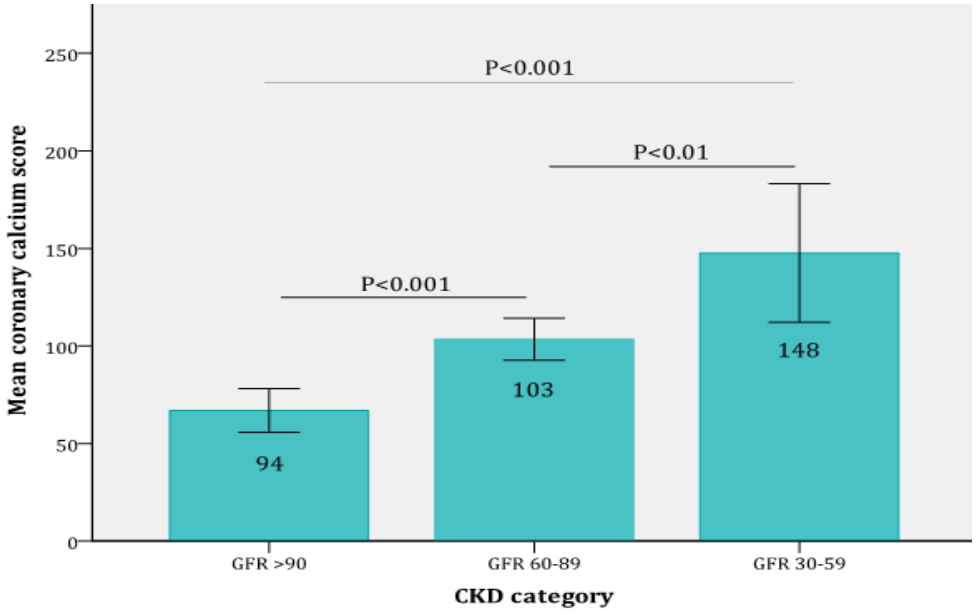


Figure 2. *Mean coronary calcium score in the different CKD-categories. The graph shows that the mean calcium score increased significantly with decreasing renal function.*

Table 2. Presence of any and severe coronary plaque in patients, stratified by eGFR.

(n=2,038)		**eGFR ≥90**	**eGFR 60-89**	**eGFR 30-59**
Any plaque	**OR (95% CI)**	1.00 (reference)	1.83 (1.52-2.21)	2.46 (1.69-3.59)
	***P* value**	---	<0.001	<0.001
Severe plaque	**OR (95% CI)**	1.00 (reference)	1.67 (1.16-2.40)	2.36 (1.35-4.13)
	***P* value**	---	<0.01	<0.01

Odds ratios for the presence of any and severe coronary plaques, stratified by eGFR. Patients with normal renal function (eGFR ≥90 mL/min/1.73m^2) were used as reference category (OR=1.00).

As expected, patients with mild (OR 1.70, 95%CI 1.28-2.26, *P*<0.001) or moderately (OR 1.68, 95%CI 1.01-2.79, *P*=0.05) impaired renal function had significantly higher proportions of calcified plaques, while there were no significant differences regarding the presence of mixed and non-calcified plaques, Table 3.

Table 3. Plaque morphology in patients, stratified by eGFR.

(n=1,215)		eGFR ≥90	eGFR 60-89	eGFR 30-59
Calcified plaque	**OR (95% CI)**	1.00 (reference)	1.70 (1.28-2.26)	1.68 (1.01-2.79)
	***P* value**	---	<0.001	0.05
Mixed plaque	**OR (95% CI)**	1.00 (reference)	1.00 (0.78-1.28)	1.02 (0.66-1.56)
	***P* value**	---	0.98	0.94
Non-calcified plaque	**OR (95% CI)**	1.00 (reference)	0.91 (0.70-1.19)	0.90 (0.57-1.43)
	***P* value**	---	0.48	0.66

Odds ratios for plaque morphology, stratified by eGFR. Patients with normal renal function (eGFR ≥90 mL/min/1.73m^2) were used as reference category (OR=1.00).

Correction for Traditional Cardiovascular Risk Factors

The results of multivariable logistic regression analysis are presented in Table 4. Age, male gender, smoking, diabetes mellitus, systolic blood pressure and positive family history are independent risk factors for the presence of any coronary plaque. Age, male gender and smoking remain the only independent risk factors for the presence of severe coronary plaques.

After adjustment for traditional cardiovascular risk factors, the association between impaired renal function (expressed by eGFR as categorical variable where patients with normal renal function were used as reference category) and the presence of any coronary plaque as well as the association between impaired renal function and the presence of severe coronary plaque becomes weaker and were no longer statistically significant.

Table 4. Multivariable logistic regression of risk factors for the presence of any and severe coronary plaque.

	Presence of any plaque			Presence of severe plaque		
Characteristic	**OR**	**95% CI**	***P* value**	**OR**	**95% CI**	***P* value**
Age	1.11	1.09-1.12	<0.001	1.06	1.04-1.08	<0.001
Gender (male = 1)	2.85	2.30-3.52	<0.001	3.18	2.22-4.57	<0.001
Smoking (yes = 1)	1.94	1.51-2.50	<0.001	1.84	1.27-2.66	0.001
Diabetes mellitus (yes = 1)	1.81	1.18-2.78	<0.01	0.64	0.32-1.29	0.21
Systolic blood pressure	1.01	1.01-1.02	<0.001	1.00	0.99-1.01	0.48
Fam. history (positive = 1)	1.37	1.11-1.69	<0.01	1.38	0.98-1.94	0.07
Total cholesterol	0.98	0.89-1.07	0.58	1.00	0.87-1.15	1.00
Normal renal function	1.00	(reference)	---	1.00	(reference)	---
Mild CKD	0.92	0.73-1.15	0.45	1.21	0.81-1.80	0.36
Moderate CKD	0.75	0.48-1.18	0.21	1.52	0.80-2.90	0.21

Multivariable logistic regression analysis was performed with known traditional cardiovascular risk factors as well as the CKD categories. Patients with normal renal function (eGFR ≥90 mL/min/1.73m^2) were used as reference category (OR=1.00). CKD, chronic kidney disease.

DISCUSSION

Our results demonstrate that patients with mild or moderately impaired renal function have a higher coronary plaque burden including a higher prevalence of severe coronary plaques compared to patients with normal renal function. Moreover, coronary plaques in patients with impaired renal function exhibit an increased degree of calcification. However, after adjustment for traditional cardiovascular risk factors, mild to moderately impaired renal function is not an independent risk factor for the presence of any coronary plaque nor for the presence of severe plaques. This means that variations in coronary plaque burden in this study can be explained by variations in traditional cardiovascular risk factors.

Our study was motivated by the paucity of data regarding the association between mild to moderately impaired renal function and CAD. Several studies investigated the association between CKD and coronary artery calcification [13-18].

Although all studies found that impaired renal function was associated with an increasing coronary calcium score, it still remains unclear whether this association is independent from traditional cardiovascular risk factors. Some of these studies found an independent association [13-15], while other studies did not [16-18]. CCS is a marker for atherosclerotic plaque burden and has been shown to be a predictor for the occurrence of myocardial infarction and cardiovascular death [23]. On the other hand, in case of a CCS of zero, it is still possible to have a so called 'vulnerable' plaque, which may rupture and cause an acute coronary event [24]. By means of contrast enhanced CT-angiography, it is possible to visualize these non-calcified plaques. Therefore, we were also able to focus on plaque morphology and degree of luminal stenosis, instead of only using CCS as determinant of CAD. However, in our study, non-calcified plaques were not found more often in patients with an impaired renal function compared to patients with a normal renal function.

At present, a limited number of reports have been published regarding the relationship between impaired renal function and coronary plaque morphology. Cho et al. studied 4,297 asymptomatic subjects undergoing CCTA as part of a general health evaluation. They found that subjects with early CKD (eGFR ≥45 mL/min/1.73 m^2) had significantly higher prevalence of (obstructive) CAD and CCS >100 compared to subjects without CKD. However, after adjustment for proteinuria and other traditional risk factors, there was no significant association between a decrease in eGFR and the risk of (obstructive) CAD or CCS >100 [25]. Although these findings are in line with our results, the main difference is that our population consists of symptomatic patients with suspected CAD, whereas Cho et al. studied asymptomatic subjects. Also other studies did not found an independent correlation between CKD and coronary plaque burden [26] or did not adjust for traditional cardiovascular risk factors [27].

The search for novel markers that better predict cardiovascular events in patients with impaired renal function is of considerable clinical interest as it may lead to improved strategies to prevent major adverse cardiovascular events (MACE). Recently, Clase et al. examined the contribution of eGFR and urinary albumin-creatinine ratio beyond traditional cardiovascular risk factors in a large cohort of patients with high cardiovascular risk. They conclude that eGFR as well as the urinary albumin-creatinine ratio add only little to traditional cardiovascular risk factors.

However, in contrast to our study, their study outcomes were all-cause mortality and long-term dialysis [28]. It would be of interest to investigate the possible additional contribution of renal impairment over traditional cardiovascular risk factors in the prediction of MACE instead of all-cause mortality and long-term dialysis. Because of the relatively short follow-up time, we did not yet focus on the cardiovascular event rate in our population.

Question remains what precise mechanism can explain the relationship between CKD and MACE. It is well known that traditional risk factors, such as age, hyperlipidemia, smoking and hypertension are associated with the development of both CAD and CKD. However, non-traditional risk factors like albuminuria, proteinuria, homocysteinemia and elevated levels of uric acid are also established factors for the progression of renal disease. Other factors which are suggested in the literature to contribute to this mechanism are anemia, oxidative stress, derangements in calcium-phosphate homeostasis, inflammation and conditions promoting coagulation, which are all associated with accelerated atherosclerosis and endothelial dysfunction [29-34].

This study is among the first coronary CT-angiography studies in which various degrees of CKD were compared with coronary plaque characteristics in patients with symptoms of chest discomfort, suspected for CAD. In order to calculate the eGFR, we used the CKD-EPI equation instead of other equations like the Cockcroft-Gault equation or the Modification of Diet in Renal Disease (MDRD) equation since the CKD-EPI equation intends to be more generalizable across various clinical settings [16].

Study Limitations

Our study has several limitations that merit comment. First, the eGFR was based on a single creatinine measurement. We did not take information into account regarding the course of renal function over time. This may have influenced the results since the serum creatinine concentration depends on various. However, we only used creatinine values less than 1 month old. Second, we did not have information regarding proteinuria and albuminuria because we did not collect urine samples. Third, although CCTA is a well-established imaging technique for detection of coronary plaques, the functional relevance of the plaques remains unsubstantiated.

Fourth, this study was performed in outpatient department patients presenting with symptoms of chest discomfort. Results in the general population might have been different. Finally, we were not able yet to investigate patient outcomes because of the relatively short follow-up time.

CONCLUSION

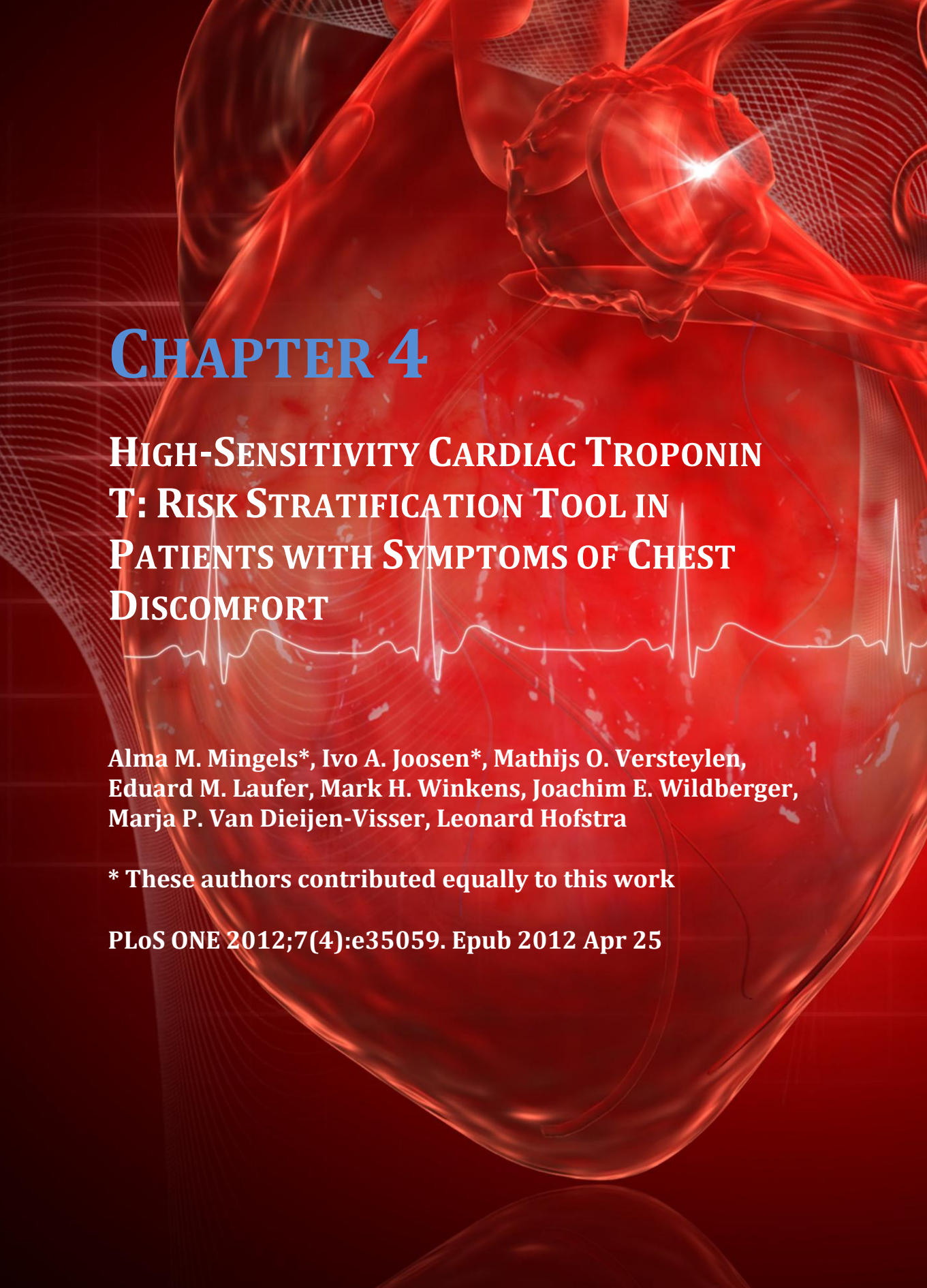
Although decreasing renal function is associated with increased extent and severity of coronary artery disease in patients with symptoms of chest discomfort, mild to moderate CKD is not independently associated with coronary plaque burden after adjustment for traditional cardiovascular risk factors.

REFERENCES

1. Roger VL, Go AS, Lloyd-Jones DM, et al. Heart disease and stroke statistics -- 2011 update: a report from the American Heart Association. *Circulation* 2011;123:18–209.
2. Eknoyan G, Lameire N, Barsoum R, et al. The burden of kidney disease: improving global outcomes. *Kidney Int* 2004;66:1310–1314.
3. Coresh J, Selvin E, Stevens LA, et al. Prevalence of chronic kidney disease in the United States. *JAMA* 2007;298:2038–2047.
4. Centers for Disease Control and Prevention (CDC). Hospitalization discharge diagnoses for kidney disease: United States, 1980-2005. *MMWR Morb Mortal Wkly Rep* 2008;57:309–312.
5. Becker BN, Himmelfarb J, Henrich WL, et al. Reassessing the cardiac risk profile in chronic hemodialysis patients: a hypothesis on the role of oxidant stress and other non-traditional cardiac risk factors. *J Am Soc Nephrol* 1997;8:475–486.
6. Venkatesan J, Henrich WL. Anemia, hypertension, and myocardial dysfunction in end-stage renal disease. *Semin Nephrol* 1997;17:257–269.
7. Anavekar NS, McMurray JJ, Velazquez EJ, et al. Relation between renal dysfunction and cardiovascular outcomes after myocardial infarction. *N Engl J Med* 2004;351:1285–1295.
8. Go AS, Chertow GM, Fan D, et al. Chronic kidney disease and the risks of death, cardiovascular events, and hospitalization. *N Engl J Med* 2004;351:1296–1305.

9. Fox CS, Larson MG, Keyes MJ, et al. Kidney function is inversely associated with coronary artery calcification in men and women free of cardiovascular disease: The Framingham Heart Study. *Kidney Int* 2004;66:2017–2021.
10. Budoff MJ, Rader DJ, Reilly MP, et al. Relationship of estimated GFR and coronary artery calcification in the CRIC (Chronic Renal Insufficiency Cohort) Study. *Am J Kidney Dis* 2011;4: 519–526.
11. Roy SK, Cespedes A, Li D, et al. Mild and moderate pre-dialysis chronic kidney disease is associated with increased coronary artery calcium. *Vasc Health Risk Man* 2011;7:719–724.
12. Kramer H, Toto R, Peshock R, et al. Association between chronic kidney disease and coronary artery calcification: The Dallas Heart Study. *J Am Soc Nephrol* 2005;16:507–513.
13. Ix JH, Katz R, Kestenbaum B, et al. Association of mild to moderate kidney dysfunction and coronary calcification. *J Am Soc Nephrol* 2008;19:579–585.
14. Parikh NI, Hwang SJ, Larson MG, et al. Indexes of kidney function and coronary artery and abdominal aortic calcium (from the Framingham Offspring Study). *Am J Cardiol* 2008;102: 440–443.
15. The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus. Report of the expert committee on the diagnosis and classification of diabetes mellitus. *Diabetes Care* 2003;26:5–20.
16. Levey AS, Stevens LA, Schmid CH, et al. A new equation to estimate glomerular filtration rate. *Ann Intern Med* 2009;150:604–612.
17. National Kidney Foundation. K/DOQI clinical practice guidelines for chronic kidney disease: evaluation, classification, and stratification. *Am J Kidney Dis* 2002;39:1–266.
18. Agatston AS, Janowitz WR, Hildner FJ, et al. Quantification of coronary artery calcium using ultrafast computed tomography. *J Am Coll Cardiol* 1990;15:827–832.
19. Hausleiter J, Meyer T, Hadamitzky M, et al. Radiation dose estimates from cardiac multislice computed tomography in daily practice: impact of different scanning protocols on effective dose estimates. *Circulation* 2006;113:1305–1310.
20. Borissoff JI, Joosen IA, Versteylen MO, et al. Accelerated in vivo thrombin formation independently predicts the presence and severity of CT-angiographic coronary atherosclerosis. *JACC Cardiovasc Imaging* 2012;5:1201–1210.
21. Austen WG, Edwards JE, Frye RL, et al. A reporting system on patients evaluated for coronary artery disease. *Circulation* 1975;51:5–40.

22. Raff GL, Abidov A, Achenbach S, et al. SCCT guidelines for the interpretation and reporting of coronary computed tomographic angiography. *J Cardiovasc Comput Tomogr* 2009;3:122–136.
23. Budoff MJ, Gul KM. Expert review on coronary calcium. *Vasc Health Risk Manag* 2008;2: 315–324.
24. Morita H, Fujimoto S, Kondo T, et al. Prevalence of computed tomographic angiography-verified high-risk plaques and significant luminal stenosis in patients with zero coronary calcium score. *Int J Cardiol* 2012;158:272–278.
25. Cho I, Min HS, Chun EJ, et al. Coronary atherosclerosis detected by coronary CT angiography in asymptomatic subjects with early chronic kidney disease. *Atherosclerosis* 2010;208:406–411.
26. Kono K, Fujii H, Miyoshi N, et al. Coronary plaque morphology using virtual histology-intravascular ultrasound analysis in hemodialysis patients. *Ther Apher Dial* 2011;15:44–50.
27. Ogita M, Funayama H, Nakamura T, et al. Plaque characterization of non-culprit lesions by virtual histology intravascular ultrasound in diabetic patients: impact of renal function. *J Cardiol* 2009;54:59–65.
28. Clase CM, Gao P, Tobe SW, et al. Estimated glomerular filtration rate and albuminuria as predictors of outcomes in patients with high cardiovascular risk: a cohort study. *Ann Intern Med* 2011;154:310–318.
29. Luft FC. Renal disease as a risk factor for cardiovascular disease. *Basic Res Cardiol* 2000;95: 72–76.
30. Foley RN, Parfrey PS, Sarnak MJ. Epidemiology of cardiovascular disease in chronic renal disease. *J Am Soc Nephrol* 1998;9:16–23.
31. McCullough PA. Cardiorenal risk: an important clinical intersection. *Rev Cardiovasc Med* 2002;3:71–76.
32. Kasiske BL. Hyperlipidemia in patients with chronic renal disease. *Am J Kidney Dis* 1998;32: 142–156.
33. Bostom AG, Selhub J. Homocysteine and arteriosclerosis: subclinical and clinical disease associations. *Circulation* 1999;99:2361–2363.
34. Rabelink TJ, Zwaginga JJ, Koomans HA, et al. Thrombosis and hemostasis in renal disease. *Kidney Int* 1994;46:287–296.



CHAPTER 4

HIGH-SENSITIVITY CARDIAC TROPONIN T: RISK STRATIFICATION TOOL IN PATIENTS WITH SYMPTOMS OF CHEST DISCOMFORT

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PLoS ONE 2012;7(4):e35059. Epub 2012 Apr 25

BACKGROUND

Recent studies demonstrated the association between increased concentrations of high-sensitivity cardiac troponin T (hs-cTnT) and the incidence of myocardial infarction, heart failure, and mortality in elderly and the general population. The value of hs-cTnT in symptomatic outclinic patients remains unclear. Aim of this study was to investigate the prognostic value of hs-cTnT as a biomarker in patients with symptoms of chest discomfort suspected for coronary artery disease and to assess its additional value in combination with other risk stratification tools in predicting cardiac events.

METHODS

We studied 1,088 patients (follow-up 2.2 ± 0.8 years) with chest discomfort who underwent coronary calcium scoring and coronary CT-angiography. Traditional cardiovascular risk factors and concentrations of hs-cTnT, N-terminal pro-brain-type natriuretic peptide (NT-proBNP) and high-sensitivity C-reactive protein (hsCRP) were assessed. Study endpoint was the occurrence of late coronary revascularization (>90 days), acute coronary syndrome, and cardiac mortality.

RESULTS

Hs-cTnT was a significant predictor for the composite endpoint (highest quartile [Q4]>6.7 ng/L, HR 3.55; 95%CI 1.88-6.70; *P*<0.001). Survival analysis showed that hs-cTnT had significant predictive value on top of risk stratification tools (Chi-square change *P*<0.01). In patients with hs-cTnT in Q4 versus <Q4, a 2- to 3-fold increase in cardiovascular risk was noticed, either when corrected for high or low Framingham risk score, coronary calcium scoring, or CT-angiography assessment (HR 3.11; 2.73; 2.47; respectively; all *P*<0.01). This was not the case for hsCRP and NT-proBNP.

CONCLUSION

Hs-cTnT is a useful prognostic biomarker in patients with suspected CAD and an independent predictor for cardiac events when corrected for cardiovascular risk profiling, calcium score and CT-angiography results.

INTRODUCTION

Identification of patients at risk for acute cardiovascular events remains a challenge. One promising avenue to improve the identification of these patients is the use of serum biomarkers, which could provide a relatively easy and cost-effective step in risk stratification. Several biomarkers have been evaluated with respect to their incremental diagnostic and prognostic value [1,2]. Elevated concentrations of high-sensitivity C-reactive protein (hsCRP), an inflammatory biomarker, are associated with future cardiovascular events, which supports the hypothesis that atherothrombosis is partly an inflammatory disease [3]. Elevated concentrations of N-terminal pro-B-type natriuretic peptide (NT-proBNP), the inactive fragment from BNP which is secreted by the cardiomyocytes in response to ventricular wall stretch, have also been associated with an increased risk of death and cardiovascular events [4]. However, none of these biomarkers have achieved widespread acceptance in daily practise as a risk stratification tool for the detection of coronary artery disease (CAD).

With the development of more accurate high-sensitivity cardiac troponin (hs-cTn) assays, new possibilities become available to improve risk stratification [5-7]. Recently, we demonstrated the association between hs-cTnT and CAD, as determined by coronary computed tomographic angiography (CCTA), in patients with stable chest pain [8]. We found that even mild CAD is associated with quantifiable circulating levels of hs-cTnT, which was confirmed by others [9]. This could be the result of episodes of cardiac ischemia due to a mismatch between metabolic demand and supply. An alternative mechanism could be that cardiac troponin T release is the result of dislodgement of small localized thrombi, causing micro-injury in small coronary vessels. Recently, a number of studies were published, which focused on the prognostic value of hs-cTnT. Most of these studies were performed in the elderly or the general population [10-13]. Less is known about the possible incremental value of hs-cTnT on top of existing risk stratification tools in patients visiting the cardiology outpatient department because of symptoms of chest discomfort suspected for CAD.

In the present study, we investigated the prognostic value of hs-cTnT in symptomatic patients with suspected CAD, and assessed its additional value in combination with other risk stratification tools in predicting cardiac events. As a comparison, we also studied hsCRP and NT-proBNP.

METHODS

Ethics Statement

This study complies with the Declaration of Helsinki and all patients gave written informed consent. The study was approved by the Institutional Review Board and Ethics Committee at the Maastricht University.

Study Population

We studied 1,114 patients with symptoms of chest discomfort who were referred from the cardiology outpatient department for CCTA because of suspected CAD, according to the appropriateness criteria for cardiac computed tomography [14]. All scans were performed in our university medical center between 2007 and 2009. Part of this population was studied previously [8]. Included were patients with a recent history of chest discomfort symptoms in the presence of additional cardiovascular risk factors and/or inconclusive diagnostic test results, resulting in a population with an intermediate pretest probability of CAD. Excluded were eight patients with missing data regarding their cardiovascular risk profile and eighteen patients with a history of proven CAD, acute myocardial infarction (AMI), percutaneous coronary intervention (PCI) or coronary artery bypass grafting (CABG). The remaining 1,088 patients were subject of this study (Figure 1). Patients who were referred from the emergency department for CCTA because of acute chest pain, suspect for an acute coronary syndrome (ACS), were not included in this study. In general, patients with an allergy to iodinated contrast agent, pregnant patients and patients with an impaired renal function (defined as an estimated glomerular filtration rate <45 mL/min/1.73m^2) were ineligible for CCTA.

Cardiovascular Risk Factors

Cardiovascular risk factors were prospectively gathered in order to calculate the Framingham risk score (FRS). The FRS is used to estimate the 10-year risk of suffering a myocardial infarction or cardiovascular death, based on age, gender, diabetes mellitus, smoking, systolic blood pressure, total cholesterol, and high-density lipoprotein (HDL) [15].

Patients were classified as smokers if they had smoked in the 12 weeks before CCTA. A positive family history was defined as having a first-degree relative with a history of myocardial infarction or sudden cardiac death before the age of sixty.

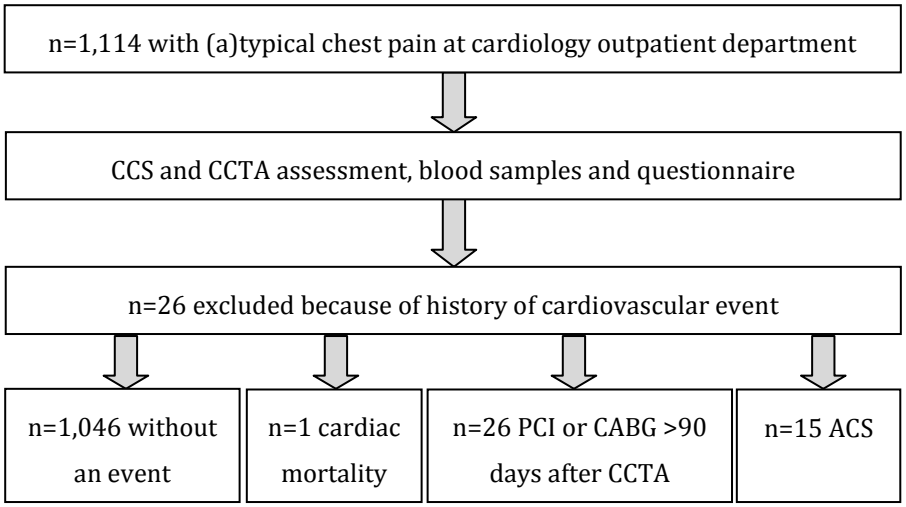


Figure 1. *Flowchart of the study design. * Early revascularizations within 90 days after CCTA were censored at the time of PCI or CABG.*

Echocardiography Acquisition

Echocardiography (Sonos 5500, Hewlett Packard, Palo Alto, CA, USA) was performed in 612 patients, using Xcelera software (Philips Healthcare, Best, the Netherlands). The left ventricular ejection fraction (LVEF) was assessed using 2D echo images. To assess the presence of left ventricular hypertrophy (LVH), we used three parameters: interventricular septum end-diastolic wall thickness (IVSEDWT), posterior wall end-diastolic wall thickness (PWEDWT) and left ventricular mass (LVMASS). Measurement of the IVSEDWT and PWEDWT were performed in the parasternal long axis.

Coronary CT-angiography Acquisition

CT-scans were performed in all patients using a 64-slice multidetector-row CT-scanner (Brilliance 64, Philips Healthcare) with a 64 x 0.625-mm slice collimation, a gantry rotation time of 420 ms and a tube voltage of 80-120 kV.

Patients received 50 mg metoprolol tartrate orally, two hours before CCTA, to lower the heart rate (HR). When the HR was >65 beats per minute (bpm), 5-20 mg metoprolol tartrate (AstraZeneca, Zoetermeer, the Netherlands) was administered intravenously. All patients received 0.8 mg nitroglycerin spray (Pohl-Boskamp, Hohenlockstedt, Germany). HR and ECG were continuously monitored.

A non-enhanced scan was performed to determine the CCS using the Agatston method [16]. Subsequently, CCTA was performed using 85-110 mL contrast agent (Xenetix 350, Guerbet, Roissy CdG Cedex, France), which was injected in the antecubital vein with a flow rate of 6.0 mL/s, directly followed by 40 mL saline (6.0 mL/s) using a dual-head power injector. In patients with a stable HR <65 bpm, a prospective ECG-gated 'step and shoot' protocol was used (radiation dose 3.5 ± 1.2 mSv). In patients with a HR >65 bpm, a retrospective ECG-gated 'helical' protocol with dose modulation was used (radiation dose 11.8 ± 3.6 mSv).

Coronary Plaque Assessment

All scans were independently analyzed by two cardiologists, both with level III expertise in coronary CT-angiography and blinded for patient details, using source images in Cardiac Comprehensive Analysis software (Philips Healthcare). In case of disagreement, consensus was reached by reviewing findings jointly.

CCS was expressed as Agatston score using calcium scoring software (Philips Healthcare) with a threshold of 130 Hounsfield units. The coronary tree was analyzed for the presence and severity of CAD, according to the 16-segment classification of the American Heart Association [17]. The extent of CAD was classified as absent, mild (<50% luminal stenosis), moderate (50-70% luminal stenosis) or severe (≥70% luminal stenosis), according to the guidelines of the Society of Cardiovascular Computed Tomography [18].

Biomarker Measurement

Samples were collected just before the scan, processed within two hours, and stored at -80°C until analysis. Total cholesterol, HDL and triglycerides concentrations were measured using the Synchron LX20 (Beckman Coulter Inc., Brea, CA, USA).

Low-density lipoprotein (LDL) was calculated using the Friedewald equation [19] except for subjects with triglycerides >400 mg/dL and total cholesterol <50 mg/dL, in which case LDL was determined on the Cobas Mira Plus (Roche Diagnostics, Basel, Switzerland). HsCRP was measured on the BN ProSpec using the CardioPhase hsCRP assay (Siemens Diagnostics, Deerfield, IL, USA). Hs-cTnT (high sensitivity fifth generation cTnT assay) and NT-proBNP were measured on the Elecsys 2010 (Roche Diagnostics).

Study Endpoint and Follow-up

The composite study endpoint was the occurrence of revascularization (PCI/CABG) >90 days after CCTA, cardiac mortality and ACS, including myocardial infarction and unstable angina requiring hospitalization. ACS was defined as typical angina pectoris, troponin T elevation (>0.01 µg/L) and ST-segment elevation/depression of ≥1 mm, or at least two of these characteristics together with invasive angiographic confirmation of a culprit lesion [20]. So, this means that patients with only troponin T elevation did not meet the criteria for an ACS. We censored follow-up when revascularization was performed within 90 days and after occurrence of the study endpoint. Patients were seen by their cardiologist on a regular basis, and all hospital visits, both outpatient department visits as well as emergency room visits, were recorded in the electronic patient records. Additionally, the national mortality records were checked. None of the attending clinicians had access to the results of the hs-cTnT, hsCRP and NT-proBNP measurements.

Statistical Analysis

To test for differences in baseline patient characteristics, we used the Pearson χ^2 test for discrete variables and the *t*-test for continuous variables. Logistic regression and survival analysis were used to study prediction of the composite endpoint of late revascularization procedures, ACS, and cardiac mortality. Confounding was considered for baseline characteristics that differed significantly between the event group and non-event group. For Kaplan-Meier analysis, categories of independent variables were compared using the log-rank test. Cox proportional hazard regression was validated for proportionality using log-minus-log and for time dependency.

It was used to evaluate the additive value of the cardiac biomarkers, based on the Chi-square change (-2 log likelihood ratio) and whether biomarkers remained significant predictors. Biomarker concentrations less than the limit of detection were set equal to the limit of detection. The threshold for statistical significance was $P<0.05$, two-sided unless stated otherwise. All data were analyzed using SPSS Statistics 18.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

The baseline characteristics of the 1,088 patients who met the inclusion and exclusion criteria are presented in Table 1. Follow-up information was available for all patients (mean follow-up time 2.2 ± 0.8 years). As a result of CCTA, 50 patients underwent (early) revascularization within 90 days (35x PCI, 15x CABG) and these were censored at the time of revascularization. Final survival analysis included a total of 42 patients who suffered a cardiac event: 26 patients underwent (late) revascularization >90 days after CCTA (20x PCI and 6x CABG), 15 patients suffered an ACS (6x AMI and 9x unstable angina requiring hospitalization), and one patient died due to heart failure. The overall cardiac event rate was 4%.

Table 1 shows that patients who suffered a cardiac event consisted of significantly more smokers, had higher systolic blood pressure and FRS, higher hs-cTnT concentrations, lower LVEF, higher CCS, and more severe lesions on CCTA.

Prognostic Value of hs-cTnT

Logistic regression revealed that 1-unit increase in hs-cTnT concentration resulted in a significant increase in cardiac risk (HR 1.04, 95% CI 1.01-1.06, $P=0.004$), in contrast to NT-proBNP (HR 1.00, 95% CI 1.00-1.00, $P=0.645$) and hsCRP (HR 1.00, 95% CI 0.98-1.03, $P=0.712$). Comparable results were obtained when biomarker concentrations were corrected for age and gender.

Kaplan-Meier analysis shows that hs-cTnT and NT-proBNP concentrations were both significant predictors for the occurrence of cardiac events ($P<0.001$ and $P=0.009$, respectively), in contrast to hsCRP ($P=0.355$).

Table 1. Baseline characteristics of the study population.

Baseline characteristics	All Participants (n=1,088)	No Event (n=1,046)	Cardiac Event (n=42)	*P* value
Age, years	56 ± 11	56 ± 11	59 ± 11	0.067
Male gender	53.8	53.6	57.1	0.655
Systolic BP, mmHg	142 ± 19	141 ± 19	149 ± 16	0.010
Smoking	26.5	25.6	50.0	0.001
Diabetes mellitus	8.4	8.3	12.5	0.347
Positive family history	39.7	39.2	51.2	0.123
Framingham risk score	16.7 (9.3-27.2)	16.4 (9.2-26.4)	25.1 (14.6-48.1)	<0.001
Total cholesterol, mg/dL	206.1 ± 46.3	205.7 ± 46.2	215.7 ± 47.1	0.179
LDL cholesterol, mg/dL	127.6 ± 41.4	127.3 ± 41.5	134.0 ± 40.5	0.303
HDL cholesterol, mg/dL	50.4 ± 29.9	50.5 ± 30.3	47.9 ± 16.0	0.581
Triglycerides, mg/dL	153.2 ± 102.6	152.6 ± 102.9	166.5 ± 93.3	0.391
hsCRP, mg/dL	0.14 (0.07-0.31)	0.14 (0.07-0.31)	0.16 (0.10-0.48)	0.079
hs-cTnT, pg/mL	4.1 (<3.0-6.7)	4.0 (<3.0-6.6)	6.8 (<3.0-10.3)	0.015
NT-proBNP, pg/mL	75.5 (34.3-153.2)	74.2 (33.4-155.4)	92.3 (55.2-136.6)	0.218
LVEF, % †	60.3 ± 7.8	60.4 ± 7.7	57.2 ± 9.4	0.048
IVSEDWT, mm †	8.9 ± 1.7	8.9 ± 1.7	9.5 ± 1.7	0.103
PWEDWT, mm †	8.7 ± 1.1	8.7 ± 1.1	9.3 ± 1.4	0.060
LVMASS, gram †	185 ± 55	185 ± 54	198 ± 72	0.252
Coronary calcium score	7 (0-122)	6 (0-110)	252 (8-644)	<0.001
CCTA luminal stenosis, %				<0.001
No CAD	36.8	37.9	9.5	
Mild CAD (<50%)	38.1	39.0	16.7	
Moderate CAD (50-70%)	14.5	14.0	28.6	
Severe CAD (>70%)	10.5	9.2	45.2	

Values are presented as %, as mean ± SD or as median (interquartile range). † n=612 underwent echocardiography. hsCRP, high-sensitive CRP; hs-cTnT, high-sensitivity cardiac troponin T; IVSEDWT, interventricular septum end-diastolic wall thickness; LVEF, left ventricular ejection fraction; LVMASS, left ventricular mass; NT-proBNP, N-terminal pro-brain-type natriuretic peptide; PWEDWT, posterior wall end-diastolic wall thickness.

Moreover, Cox regression reveals that hs-cTnT was the only significant biomarker predicting for cardiac events, either when testing the biomarker concentrations as a continuous variable (Table 2: Model 1, 4, and 7, respectively) or when present in the highest quartile Q4 (Table 2: Model 2, 5, and 8, respectively).

Table 2. Cox regression analysis of cardiac biomarkers for the composite endpoint.

Model	Cardiac biomarker	Chi-square	*P*	HR	95% CI	*P*
1	hs-cTnT	28.93	<0.001	1.03	1.01-1.04	<0.001
2	hs-cTnT in Q4 (>6.7 ng/L) *	17.30	<0.001	3.55	1.88-6.70	<0.001
3	hs-cTnT > URL (14 ng/L) *,†	1.08	0.299	1.85	0.57-6.02	0.307
4	NT-proBNP	0.84	0.359	1.00	1.00-1.00	0.380
5	NT-proBNP in Q4 (>18 pmol/L) *	0.72	0.396	0.70	0.31-1.60	0.399
6	NT-proBNP > URL (36 pmol/L) *,†	0.47	0.492	0.66	0.20-2.16	0.495
7	hsCRP	0.11	0.742	1.00	0.98-1.02	0.746
8	hsCRP in Q4 (>3.1 mg/L) *	0.86	0.355	1.38	0.70-2.73	0.357
9	hsCRP > URL (3 mg/L) *,†	0.61	0.436	1.31	0.66-2.60	0.437

* Dichotomous variable (yes or no); † URL = upper reference limit (used for diagnosis). hsCRP, high-sensitive C-reactive protein; hs-cTnT, high-sensitivity cardiac troponin T; NT-proBNP, N-terminal pro-brain-type natriuretic peptide; Q4, fourth quartile.

Additional Value of hs-cTnT on Top of FRS

Clinical risk profiling using FRS predicted significantly for the occurrence of cardiac events (Table 1, *P*<0.001). This was confirmed using logistic and Cox regression for FRS as a continuous variable or when categorized as follows: <5% (low risk), 5-20% (intermediate risk), >20% (high risk) (all *P*<0.05). Kaplan-Meier analysis confirmed this relation, as shown in Figure 2A (*P*=0.018). Noticeably, almost no cardiac events were observed in patients with FRS <5%. When regarding time to event using Cox regression, hs-cTnT concentrations were predictive on top of FRS (Table 3, without versus with hs-cTnT). This was true when hs-cTnT was added to the individual parameters of the FRS (Model 1: HR hs-cTnT 1.02, *P*=0.007 and Chi-square change 5.23, *P*=0.022) or marginally significant when added to the complete FRS algorithm (Model 2: HR hs-cTnT 1.02, *P*=0.018 and Chi-square change 3.58, *P*=0.058).

To further illustrate, we noticed a 3-fold increase in cardiac risk in patients with hs-cTnT concentrations in Q4 as compared to <Q4, independent from high or low FRS (cut-off 20%) (Model 3: HR hs-cTnT 3.11, *P*=0.001 and Chi-square change 10.56, *P*=0.001). In patients with FRS <20%, the cardiac event rate increased from 2.1% to 5.9% when hs-cTnT concentrations were in Q4 compared to <Q4. In patients with FRS >20%, the cardiac event rate increased from 3.6% to 10.6%, respectively. This is also illustrated using Kaplan-Meier analysis in Figure 2B (*P*=0.001). In contrast, no significant additional value was found for NT-proBNP and hsCRP.

Table 3. Cox regression analysis of Framingham risk profiling for the composite endpoint of cardiac events.

	Without hs-cTnT			**With hs-cTnT**		
	HR	**95% CI**	***P* value**	**HR**	**95% CI**	***P* value**
Model 1						
Age	1.03	1.00-1.07	0.074	1.02	0.98-1.06	0.332
Male gender *	1.26	0.61-2.63	0.531	1.12	0.53-2.36	0.770
Total cholesterol	1.11	0.84-1.47	0.468	1.16	0.87-1.54	0.308
HDL cholesterol	0.92	0.45-1.88	0.812	0.92	0.44-1.88	0.811
Systolic blood pressure	1.02	1.00-1.03	0.071	1.02	1.00-1.04	0.030
Smoking *	3.73	1.83-7.60	<0.001	3.34	1.62-6.92	0.001
Diabetes mellitus *	1.32	0.44-3.94	0.618	0.95	0.27-3.35	0.939
hs-cTnT	-	-	-	1.02	1.01-1.04	0.007
Model 2						
Framingham	1.03	1.02-1.04	<0.001	1.03	1.01-1.04	<0.001
hs-cTnT	-	-	-	1.02	1.00-1.03	0.018
Model 3						
Framingham >20% *	2.33	1.19-4.55	0.013	1.79	0.89-3.57	0.101
hs-cTnT in Q4 *	-	-	-	3.11	1.58-6.11	0.001

* Dichotomous variable (yes or no). HDL, high-density lipoprotein; hs-cTnT, high-sensitivity cardiac troponin T; Q4, fourth quartile.

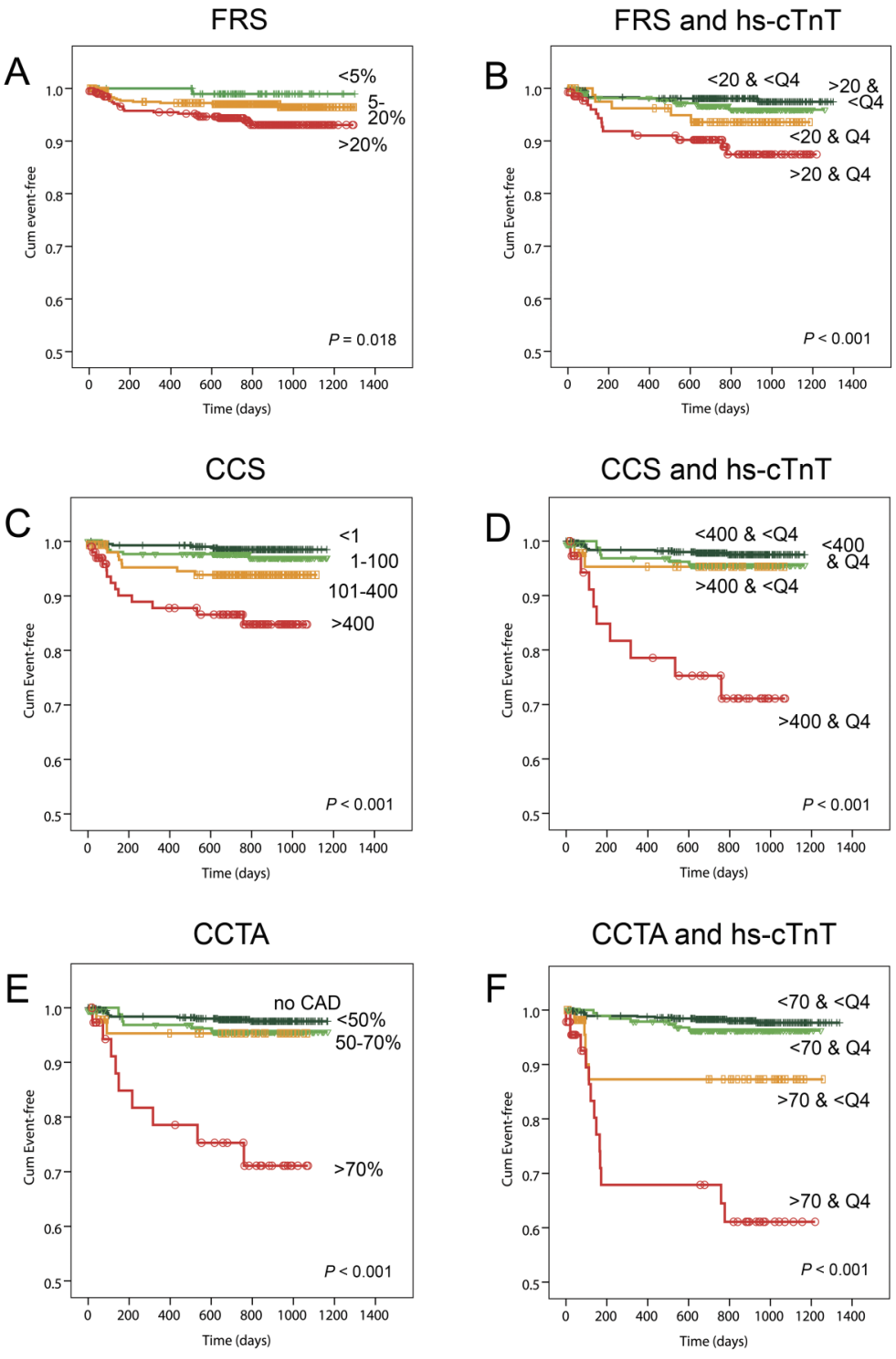

FRS
A
<5%
5-20%
>20%
Cum event-free
P = 0.018
Time (days)
FRS and hs-cTnT
B
<20 & <Q4
>20 & <Q4
<20 & Q4
>20 & Q4
Cum Event-free
P < 0.001
Time (days)
CCS
C
<1
1-100
101-400
>400
Cum Event-free
P < 0.001
Time (days)
CCS and hs-cTnT
D
<400 & <Q4
<400 & Q4
>400 & <Q4
>400 & Q4
Cum Event-free
P < 0.001
Time (days)
CCTA
E
no CAD
<50%
50-70%
>70%
Cum Event-free
P < 0.001
Time (days)
CCTA and hs-cTnT
F
<70 & <Q4
<70 & Q4
>70 & <Q4
>70 & Q4
Cum Event-free
P < 0.001
Time (days)

Figure 2 (previous page). *Kaplan-Meier analysis illustrating improved classification by including hs-cTnT to current risk stratification tools. Figures on the left shows Kaplan-Meier curves for FRS (A), CCS (C) and CCTA assessment (E). Figures on the right shows Kaplan-Meier curves when hs-cTnT was added to FRS (B), CCS (D) and CCTA assessment (F). Q4 = fourth quartile of hs-cTnT concentrations.*

Finally, Table 1 shows that LVEF was significantly lower in the cardiac event group as compared to the non-event group (*P*=0.048). Cox regression confirmed LVEF as a significant predictor (HR 0.96, 95% CI 0.93-1.00, *P*=0.049). Nevertheless, the predictive value of hs-cTnT remained significant when corrected for LVEF (HR hs-cTnT 1.02, 95% CI 1.01-1.04, *P*=0.002 and HR LVEF 0.97, 95% CI 0.93-1.01, *P*=0.124). When LVEF was added to risk profiling as presented in Table 4, smoking (Model without hs-cTnT) or smoking and hs-cTnT (Model with hs-cTnT) remained the only significant predictors.

Additional Value of hs-cTnT on Top of CCS and CCTA

Kaplan-Meier analysis shows an apparent gradient of adverse survival for more severe CAD (Figure 2C and 2E). Furthermore, hs-cTnT concentrations (median, IQR) were significantly higher in mild (4.2 pg/mL, <3.0-7.3), moderate (4.7 pg/mL, <3.0-7.3), and severe CAD (6.5 pg/mL, 3.6-9.4) as compared to patients without CAD (3.3 pg/mL, <3.0-5.3), all *P*<0.001. A similar trend was found for CCS (*P*<0.001). These data show that increasing concentrations of hs-cTnT were associated with the severity of CAD, which is in line with our previous results in part of this population [8].

Table 4. Cox regression analysis of risk profiling and coronary plaque assessment including LVEF for the composite endpoint of cardiac events.

	Without hs-cTnT			With hs-cTnT		
	HR	**95% CI**	***P* value**	**HR**	**95% CI**	***P* value**
Model FRS						
Age	1.03	0.98-1.08	0.249	1.01	0.96-1.06	0.747
Male gender *	0.82	0.31-2.18	0.697	0.68	0.25-1.90	0.464
Total cholesterol	1.22	0.86-1.74	0.259	1.32	0.92-1.91	0.135
HDL cholesterol	0.59	0.14-2.53	0.475	0.63	0.14-2.78	0.540
Systolic blood pressure	1.02	1.00-1.04	0.104	1.02	1.00-1.05	0.051
Smoking *	6.85	2.57-18.2	<0.001	5.94	2.19-16.1	<0.001
Diabetes mellitus *	2.06	0.58-7.38	0.265	1.35	0.29-6.27	0.700
LVEF	0.97	0.93-1.02	0.262	0.97	0.93-1.02	0.267
hs-cTnT	-	-	-	1.02	1.00-1.04	0.041
Model CCS						
CCS	1.00	1.00-1.00	0.012	1.00	1.00-1.00	0.057
LVEF	0.98	0.93-1.02	0.337	0.99	0.94-1.04	0.653
hs-cTnT	-	-	-	1.02	1.00-1.04	0.022
Model CCTA						
CCTA luminal stenosis, %			<0.001			<0.001
No CAD = reference	1.00			1.00		
Mild CAD (<50%)	0.45	0.04-4.91	0.509	0.44	0.04-4.84	0.501
Moderate CAD (50-70%)	7.07	1.36-36.7	0.020	7.26	1.40-37.7	0.018
Severe CAD (>70%)	29.2	6.58-129	<0.001	27.3	6.10-122	<0.001
LVEF	0.98	0.94-1.02	0.313	0.99	0.95-1.03	0.570
hs-cTnT	-	-	-	1.01	1.00-1.02	0.183

* Dichotomous variable (yes or no). HDL, high-density lipoprotein; hs-cTnT, high-sensitivity cardiac troponin T; LVEF, left ventricular ejection fraction; Q4, fourth quartile.

Table 5. Cox regression analysis of coronary plaque assessment for the composite endpoint of cardiac events.

Models	Without hs-cTnT			With hs-cTnT		
	HR	**95% CI**	***P* value**	**HR**	**95% CI**	***P* value**
Model 1 (CCS)						
CCS	1.00	1.00-1.00	<0.001	1.00	1.00-1.00	<0.001
hs-cTnT	-	-	-	1.02	1.01-1.04	0.006
Model 2 (CCS)						
CCS >400 *	5.68	2.72-11.86	<0.001	4.53	2.13-9.64	<0.001
hs-cTnT in Q4 *	-	-	-	2.73	1.32-5.62	0.007
Model 3 (CCTA)						
CCTA luminal stenosis, %			<0.001			<0.001
No CAD = reference	1.00			1.00		
Mild CAD (<50%)	1.47	0.42-5.22	0.549	1.44	0.41-5.11	0.571
Moderate CAD (50-70%)	7.25	2.27-23.11	0.001	7.09	2.22-22.62	0.001
Severe CAD (>70%)	23.98	8.10-70.96	<0.001	21.76	7.27-65.10	<0.001
hs-cTnT	-	-	-	1.01	1.00-1.03	0.028
Model 4 (CCTA)						
Severe CAD (>70%) *	11.33	5.98-21.47	<0.001	9.23	4.79-17.82	<0.001
hs-cTnT in Q4 *	-	-	-	2.47	1.29-4.77	0.007

* Dichotomous variable (yes or no). CCS, coronary calcium score; CCTA, coronary CT-angiography; hs-cTnT, high-sensitivity cardiac troponin T; CAD, coronary artery disease; Q4, fourth quartile.

Cox regression in Table 5 shows the additional value of hs-cTnT on top of CAD assessment with CCS and CCTA. One unit increase in hs-cTnT resulted in a minor increase in cardiac risk (Model 1 CCS: HR hs-cTnT 1.02, *P*=0.006 and Chi-square change 4.23, *P*=0.040; Model 3 CCTA: HR hs-cTnT 1.01, *P*=0.028 and Chi-square change 3.04, *P*=0.081). To illustrate, a 2- to 3-fold increase in cardiac risk was noticed in patients with hs-cTnT concentrations in Q4, independent from high or low CCS (cut-off Agatston score 400) or luminal stenosis on CCTA (cut-off 70%) (Model 2 CCS: HR hs-cTnT 2.73, *P*=0.007 and Chi-square change 7.20, *P*=0.007; Model 4 CCTA: HR hs-cTnT 2.47, *P*=0.007 and Chi-square change 7.24, *P*=0.007.

In patients with high CCS (Agatston score >400, n=85), the cardiac event rates increased from 4.3% to 24% when hs-cTnT concentrations were in Q4 as compared to <Q4. In patients with a CCTA lesion of >70% luminal stenosis (n=103), the cardiac event rates were 8.8% and 28% when hs-cTnT concentrations were in <Q4 and Q4, respectively. This is also illustrated using Kaplan-Meier analysis in Figure 2D and 2F for CCS and CCTA assessment, respectively (both *P*=0.001). Again, no significant additional value was found for NT-proBNP and hsCRP.

DISCUSSION

Our study shows that in patients with symptoms of chest discomfort suspected for CAD, hs-cTnT was a significant predictor for the composite endpoint of late revascularizations, ACS and cardiac mortality. Over three times as much cardiac events were found in patients with hs-cTnT concentrations in the fourth quartile (cut-off 6.7 ng/L, HR 3.55, *P*<0.001) as compared to patients with hs-cTnT concentrations in the lowest three quartiles. Moreover, survival analysis showed that hs-cTnT significantly contributed to the identification of a subgroup of patients with higher risk for cardiac events. When using traditional risk factors, smoking (HR 3.34, *P*=0.001), hs-cTnT (HR 1.02, *P*=0.007), and systolic blood pressure (HR 1.02, *P*=0.030) remained the only significant predictors. Hs-cTnT remained significantly predictive independent from FRS (HR 1.02-3.11, dependent whether variables were continuous or categorized). In addition, hs-cTnT improved classification on top of the extent of CAD as assessed with CCS and CCTA. To illustrate, a 2- to 3-fold increase in cardiac risk was noticed in patients with hs-cTnT concentrations in the highest quartile, independent from high or low CCS (cut-off Agatston score 400) or luminal stenosis on CCTA (cut-off 70%) (HR 2.73 and 2.47, both *P*=0.007).

In a previous study, Reichlin et al showed that the positive predictive value of hs-cTnT in diagnosing acute myocardial infarction was only 19% (cut-off 2 pg/mL, limit of detection) or 50% (cut-off 14 pg/mL, 99th percentile of healthy reference population), while the negative predictive value was nearly perfect (99-100%, dependent on cut-off) [5]. This indicates that it is of great importance to exclude false positives before widespread introduction of hs-cTn as a risk factor.

On the other hand, the present study as well as other studies has shown the adverse outcome of elevated hs-cTn on cardiovascular events [10-12,21,22]. The reference change value for hs-cTnT concentrations, that is based on biological variations in healthy individuals and analytical variations, was 58% and around 95% for the short-term (4 hours) and long-term (8 weeks), respectively [23]. There are no results reported on optimal delta cut-offs considering a longer follow-up period of years apart from the study of deFilippi et al, who recently showed that for an increase in hs-cTnT concentrations >50% over two to three years, the risk for heart failure and cardiovascular death were 1.7 and 1.8-fold, respectively [11].

Question remains what the underlying pathophysiological mechanisms of elevated hs-cTnT concentrations in these patients are. Korosoglou and colleagues concluded that the presence of non-calcified coronary plaques may result in continuous leakage of troponins, possibly due to repetitive micro-embolization of atherosclerotic debris [9]. In our study, we observed a stepwise increase in hs-cTnT concentrations with increasing atherosclerotic plaque burden which supports this explanation. Alternative explanations for troponin leakage which have been supposed are demand ischemia, myocardial ischemia (for example due to coronary vasospasm), direct myocardial damage, chronic renal insufficiency, or myocardial strain because of volume or pressure overload [24]. Other possible causes of elevated hs-cTnT concentrations could be chest trauma, strenuous exercise, pericarditis, myocarditis and cardiac amyloidosis. However, in the present study we could exclude chest trauma and strenuous exercise as causes. Furthermore, ECG findings, CCTA and echocardiography did not reveal convincing evidence for alternative diagnoses like pericarditis, myocarditis or amyloidosis. Since concentrations of NT-proBNP were not increased in our patients, we felt we could exclude digestive heart failure as cause of the increased hs-cTnT concentrations.

Recently, two papers were published which feed the thought that the identification of patients at risk for a cardiovascular event may soon become easier and more accurate using hs-cTnT [10,11]. DeFilippi et al performed serial measures of hs-cTnT in community-dwelling older adults [11]. They found a significant association between baseline hs-cTnT concentrations, changes in hs-cTnT concentrations and the development of heart failure and cardiovascular death.

De Lemos et al found an association between increased hs-cTnT and structural heart disease, especially left ventricular hypertrophy, and subsequent risk for all-cause mortality [10]. However, the study by deFilippi was focusing on elderly with a mean age above 70 years, while in the study of de Lemos the vast majority of the population (77%) consisted of patients with FRS <10%. These characteristics are not typical for the patients presenting at the cardiology outpatient department. Therefore, it is not clear from those studies to what extent hs-cTnT would be of incremental value in patients presenting with symptoms of chest discomfort at the cardiology outpatient department. Moreover, the published studies focused on left ventricular hypertrophy and heart failure, respectively. It is not inconceivable that the main cause of the elevated hs-cTnT is the presence of atherosclerosis, because it is known that the majority of patients with heart failure have underlying coronary atherosclerotic disease [25]. Moreover, hypertension is an important risk factor for atherosclerosis and also the major determinant of left ventricular hypertrophy. In this study, we show that although the predictive value of LVEF for events was significant, this did not seem to confound our results. In previous work, we demonstrated that even mild CAD is associated with increased concentrations of hs-cTnT and we suggested that hs-cTnT may become a potential serum biomarker to improve the identification of patients at risk for developing cardiovascular events [8]. There is increasing evidence that ACS may be predominantly caused by such mild stenoses [26]. On the other hand, it is known that the extent of CAD provides important prognostic information in both asymptomatic and symptomatic patients. Both high CCS and ≥50% luminal stenosis on CCTA deprive prognosis significantly [27]. In this study we show that measuring hs-cTnT provides additional value on these already strong prognostic parameters. In our opinion, these findings strengthen the hypothesis that hs-cTnT is a prognostic clinical biomarker. In addition, we provide new insights into the use of hs-cTnT, which can help the physician to better identify the patient at risk of a cardiovascular event.

Study Limitations

This study has several limitations that merit comment. First, the follow-up period is relatively short and therefore we found relatively few events. However, the event rate which we found is comparable to other previously published large CCTA-trials.

Second, clinicians were not blinded for CCTA findings. Therefore, early revascularizations (within 90 days) were censored for survival analysis. However, the knowledge of the CCTA findings could still bias the clinician's behaviour after the 90 day time period. It would be interesting to blind clinicians for CCTA results, but since CCTA is part of the diagnostic work-up in our university medical center, this would be unethical. On the other hand, none of the clinicians had access to the results of the hs-cTnT, hsCRP and NT-proBNP measurements. Third, all patients were of Western European descent. It remains uncertain whether our results can be generalized to other populations. Fourth, we performed a single hs-cTnT measurement and it remains unclear in which manner hs-cTnT varies in time. Fifth, despite the fact that invasive coronary angiography is still the golden standard for coronary artery stenosis, we are convinced that the use of CCTA gives an adequate reflection of the extent and severity of plaque burden in our study population.

CONCLUSION

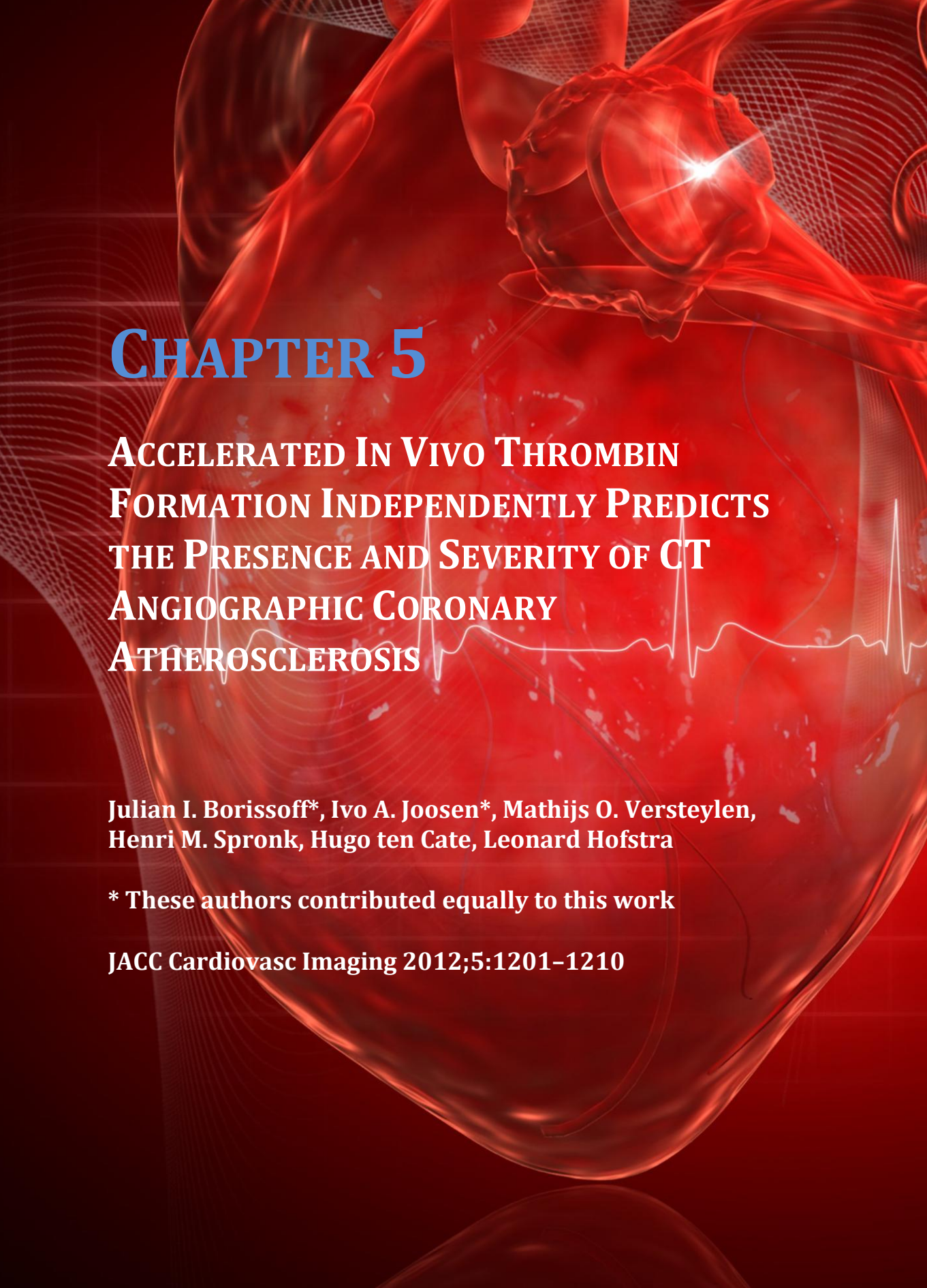
Hs-cTnT is a useful prognostic biomarker in patients with symptoms of chest discomfort suspected for CAD. Hs-cTnT is associated with the extent of CAD, assessed by CCS and CCTA, and is a significant predictor for the occurrence of a future cardiac event (late revascularization, ACS, and cardiac mortality). Even better performance was obtained when hs-cTnT concentrations were combined with Framingham risk profiling. Finally, hs-cTnT also provided additional value to the assessment of CAD by coronary computed tomographic angiography.

REFERENCES

1. Kaptoge S, Di Angelantonio E, Lowe G, et al. C-reactive protein concentration and risk of coronary heart disease, stroke, and mortality: an individual participant meta-analysis. *Lancet* 2010;375:132–140.
2. Wang TJ, Gona P, Larson MG, et al. Multiple biomarkers for the prediction of first major cardiovascular events and death. *N Engl J Med* 2006;355:2631–2639.

3. Ridker PM. C-reactive protein and the prediction of cardiovascular events among those at intermediate risk: moving an inflammatory hypothesis toward consensus. *J Am Coll Cardiol* 2007;49:2129–2138.
4. Wang TJ, Larson MG, Levy D, et al. Plasma natriuretic peptide levels and the risk of cardiovascular events and death. *N Engl J Med* 2004;350:655–663.
5. Reichlin T, Hochholzer W, Bassetti S, et al. Early diagnosis of myocardial infarction with sensitive cardiac troponin assays. *N Engl J Med* 2009;361:858–867.
6. Keller T, Zeller T, Peetz D, et al. Sensitive troponin I assay in early diagnosis of acute myocardial infarction. *N Engl J Med* 2009;361:868–877.
7. Lindahl B, Venge P, James S. The new high-sensitivity cardiac troponin T assay improves risk assessment in acute coronary syndromes. *Am Heart J* 2010;160:224–229.
8. Laufer EM, Mingels AM, Winkens MH, et al. The extent of coronary atherosclerosis is associated with increasing circulating levels of high sensitive cardiac troponin T. *Arterioscler Thromb Vasc Biol* 2010;30:1269–1275.
9. Korosoglou G, Lehrke S, Mueller D, et al. Determinants of troponin release in patients with stable coronary artery disease: insights from CT angiography characteristics of atherosclerotic plaque. *Heart* 2011;97:823–831.
10. de Lemos JA, Drazner MH, Omland T, et al. Association of troponin T detected with a highly sensitive assay and cardiac structure and mortality risk in the general population. *JAMA* 2010;304:2503–2512.
11. deFilippi CR, de Lemos JA, Christenson RH, et al. Association of serial measures of cardiac troponin T using a sensitive assay with incident heart failure and cardiovascular mortality in older adults. *JAMA* 2010;304:2494–2502.
12. Otsuka T, Kawada T, Ibuki C, et al. Association between high-sensitivity cardiac troponin T levels and the predicted cardiovascular risk in middle-aged men without overt cardiovascular disease. *Am Heart J* 2010;159:972–978.
13. Alehagen U, Dahlstrom U, Rehfeld JF, et al. Prognostic assessment of elderly patients with symptoms of heart failure by combining high-sensitivity troponin T and N-terminal pro B-type natriuretic peptide measurements. *Clin Chem* 2010;56:1718–1724.
14. ACCF/ACR/SCCT/SCMR/ASNC/NASCI/SCAI/SIR 2006 appropriateness criteria for cardiac computed tomography and cardiac magnetic resonance imaging. *J Am Coll Cardiol* 2006;48:1475–1497.

15. D'Agostino RB Sr, Vasan RS, Pencina MJ, et al. General cardiovascular risk profile for use in primary care: the Framingham Heart Study. *Circulation* 2008;117:743–753.
16. Agatston AS, Janowitz WR, Hildner FJ, et al. Quantification of coronary artery calcium using ultrafast computed tomography. *J Am Coll Cardiol* 1990;15:827–832.
17. Austen WG, Edwards JE, Frye RL, et al. A reporting system on patients evaluated for coronary artery disease. Report of the Ad Hoc Committee for Grading of Coronary Artery Disease, Council on Cardiovascular Surgery, American Heart Association. *Circulation* 1975;51:5–40.
18. Raff GL, Abidov A, Achenbach S, et al. SCCT guidelines for the interpretation and reporting of coronary computed tomographic angiography. *J Cardiovasc Comput Tomogr* 2009;3:122–136.
19. Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin Chem* 1972;18:499–502.
20. Braunwald E, Antman EM, Beasley JW, et al. ACC/AHA 2002 guideline update for the management of patients with unstable angina and non-ST-segment elevation myocardial infarction. *J Am Coll Cardiol* 2002;40:1366–1374.
21. Omland T, de Lemos JA, Sabatine MS, et al. A sensitive cardiac troponin T assay in stable coronary artery disease. *N Engl J Med* 2009;361:2538–2547.
22. Kavsak PA, Wang X, Ko DT, et al. Short- and long-term risk stratification using a next-generation, high-sensitivity research cardiac troponin I (hs-cTnI) assay in an emergency department chest pain population. *Clin Chem* 2009;55:1809–1815.
23. Vasile VC, Saenger AK, Kroning JM, et al. Biological and analytical variability of a novel high-sensitivity cardiac troponin T assay. *Clin Chem* 2010;56:1086–1090.
24. Thygesen K, Alpert JS, White HD. Universal definition of myocardial infarction. *J Am Coll Cardiol* 2007;50:2173–2195.
25. Fox KF, Cowie MR, Wood DA, et al. Coronary artery disease as the cause of incident heart failure in the population. *Eur Heart J* 2001;22:228–236.
26. Virmani R, Burke AP, Farb A, et al. Pathology of the vulnerable plaque. *J Am Coll Cardiol* 2006;47:13–18.
27. Hadamitzky M, Freissmuth B, Meyer T, et al. Prognostic value of coronary computed tomographic angiography for prediction of cardiac events in patients with suspected coronary artery disease. *JACC Cardiovasc Imaging* 2009;2:404–411.



CHAPTER 5

ACCELERATED IN VIVO THROMBIN FORMATION INDEPENDENTLY PREDICTS THE PRESENCE AND SEVERITY OF CT ANGIOGRAPHIC CORONARY ATHEROSCLEROSIS

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JACC Cardiovasc Imaging 2012;5:1201–1210

BACKGROUND

Besides its pivotal role in thrombus formation, experimental data indicate that thrombin also plays a role in the pathophysiology of atherosclerosis progression and vascular calcification. Nevertheless, the clinical evidence remains limited. The objective of this study was to investigate the relationship between thrombin generation and the presence and severity of coronary artery disease (CAD).

METHODS

Using 64-slice coronary CT-angiography, we assessed the presence and characteristics of CAD in patients (n=295; median age: 58 years) with stable chest pain. Coronary artery calcification (CAC) was graded as absent (Agatston 0), mild (Agatston 1-100), moderate (Agatston 101-400) and severe (Agatston >400). Calibrated automated thrombography was used to assess endogenous thrombin potential in plasma in vitro. Thrombin-antithrombin complexes (TATc) levels were measured as a marker for thrombin formation in vivo.

RESULTS

TATc levels were substantially higher in patients with CAD vs. patients without CAD (p=0.004). Significant positive correlations were observed between steadily increasing TATc levels and the severity of CAD (r=0.225, *P*<0.001). In multinomial logistic regression models, after adjusting for established risk factors, TATc levels predicted the degree of CAC: mild (OR:1.56, *P*=0.006); moderate (OR:1.56, *P*=0.007); and severe (OR:1.67, *P*=0.002). Trends were comparable between the groups when stratified according to the degree of coronary luminal stenosis.

CONCLUSION

This study provides novel clinical evidence indicating a positive independent association between enhanced in vivo thrombin generation and the presence and severity of coronary atherosclerosis, which may suggest that thrombin plays a role in atherogenesis.

INTRODUCTION

Atherosclerosis is a multifactorial chronic inflammatory vascular disorder [1,2]. Given the abundant experimental evidence showing extensive interactions between the hemostatic, immune and inflammation systems, we have proposed a role for the clotting proteins in modulating atherosclerosis progression and atherosclerotic plaque phenotype [3]. In particular, thrombin, which is the most central coagulation protein, is also recognized as a strong pro-inflammatory mediator. Endowed with a potent cell signaling capacity, thrombin can induce an array of pro-atherogenic and plaque-destabilizing effects such as inflammation, vascular smooth muscle cell migration and proliferation, leukocyte chemotaxis, proteolysis, apoptosis, angiogenesis, etc. [3,4]. Recently, we demonstrated that thrombin, as well as other coagulation proteins, are widely expressed and functionally active throughout distinct compartments of the arterial vessel wall [5], supporting an active cell-based coagulation network within human atherosclerotic plaques. G-protein-coupled protease-activated receptors (PARs), which are selectively cleaved by thrombin, are also abundantly distributed in the vasculature under normal conditions and overexpressed in atherosclerotic lesions [6]. Experimental animal studies have clearly indicated that variations in the clotting activity affect the progression and thrombogenicity of atherosclerotic plaques [3].

Antithrombotic therapy is a cornerstone in the management and prevention of atherothrombosis in patients [2]. Experimental data demonstrate that direct thrombin inhibition substantially attenuates atherosclerosis development in ApoE-null mice [7] and protects against severe plaque progression in prothrombotic mice [8]. However, the role of blood coagulation proteins in atherogenesis, in particular thrombin, has not been adequately addressed in previously conducted clinical research. Cardiac computed tomographic angiography (CCTA) is a well-established non-invasive imaging modality, which has high diagnostic accuracy for detection and characterization of coronary plaques [9,10].

Using CCTA, we investigated the association between thrombin formation in plasma and the presence and severity of coronary atherosclerosis in patients with suspected coronary artery disease (CAD).

METHODS

Study Population

We studied 295 adult patients who were referred from the cardiology outpatient department for CCTA because of stable chest pain, suspected for CAD. Scans were performed in our university medical center between January 2008 and June 2010 as part of the diagnostic work-up in these patients. Included were patients with a recent history of (a)typical chest pain, who underwent a coronary calcium score scan as well as CCTA. Excluded were patients with acute chest pain suspected for an acute coronary syndrome (ACS), patients with a history of acute myocardial infarction (AMI), percutaneous coronary intervention and/or coronary artery bypass grafting surgery, patients with missing data regarding their cardiac risk profile, patients with an inconclusive CT-scan and patients currently on anti-coagulation therapy (oral vitamin K antagonist/selective anticoagulants or low-molecular weight heparins). In vitro hemolysis of blood samples was also an exclusion criterion. We calculated the Framingham risk score (FRS) in all patients to estimate the 10-year risk of suffering a myocardial infarction or cardiovascular death [11]. The Institutional Review Board and Ethics Committee at the Maastricht University Medical Center approved the study, and all patients gave written informed consent.

CCTA Protocol

Scans were performed using a 64–slice CT-scanner (Brilliance 64; Philips Healthcare, Best, The Netherlands) with a 64 x 0.625 mm slice collimation, a gantry rotation time of 420 ms and a tube voltage of 80-120 kV. Tube current varied from 150-210 mAs for the prospectively gated “Step and shoot” protocol and from 600-1000 mAs for the retrospectively gated “Helical” protocol. Patients received 50 mg Metoprolol tartrate orally, two hours before CCTA. When indicated, an additional dose of 5–20 mg Metoprolol tartrate (AstraZeneca, Zoetermeer, The Netherlands) was administered intravenously to lower the heart rate <65 beats per minute (bpm). 0,8 mg Nitroglycerin spray (Pohl-Boskamp, Hohenlockstedt, Germany) was given sublingually just prior to CCTA. Heart rate and ECG were monitored during CCTA.

A non-enhanced scan was performed to determine the amount of coronary artery calcification (CAC), using the Agatston method [12]. Subsequently, CCTA was performed using 85–110 mL of contrast agent (Xenetix 350; Guerbet, Roissy CdG Cedex, France), which was injected in the antecubital vein at a rate of 6.0 mL/s, directly followed by 40 mL intravenous saline (6.0 mL/s) using a dual-head power injector. A prospectively gated "Step and shoot" protocol was used in all patients with a stable heart rate <65 bpm. In patients with an irregular heart rate or a stable heart rate >65 bpm, we used a retrospectively gated "Helical" protocol with dose modulation to obtain the best image quality at minimal radiation dose [13,14].

CCTA Analysis

All scans were independently analyzed by two cardiologists with level III expertise in coronary CT-angiography, blinded for patient details, using source images in the Cardiac Comprehensive Analysis software (Philips Healthcare). In case of disagreement, consensus was reached by reviewing findings jointly.

CAC was expressed as the Agatston score using calcium scoring software (Philips Healthcare) with a threshold of 130 Hounsfield units (HU). The coronary artery tree was analyzed for the presence and severity of CAD, according to the 16-segment classification of the American Heart Association [15]. Coronary plaques were defined as visible structures within or adjacent to the coronary artery lumen, which could be clearly distinguished from the vessel lumen and the surrounding pericardial tissue. Plaques were categorized as calcified (exclusively content with density >130 HU), non-calcified (exclusively content with density <130 HU) or mixed (characteristics of both calcified and non-calcified plaques). The degree of CAD was classified as absent (no luminal stenosis), mild (<50% luminal stenosis), moderate (50-70% luminal stenosis) or severe (>70% luminal stenosis) [16]. The degree of CAC was classified as absent (Agatston score 0), mild (Agatston score 1-100), moderate (Agatston score 100-400) or severe (Agatston score >400) [17].

Blood Samples and Laboratory Measurements

Blood samples were taken just before the scan, processed within 2 hours and plasma was stored at -80°C until analysis.

Continuous thrombin generation in clotting platelet-poor plasma was monitored in vitro by using the Calibrated Automated Thrombography (CAT) method (Thrombinoscope B.V., Maastricht, The Netherlands) [18]. The reaction was triggered by adding 5pM tissue factor (PPP Reagent, Thrombinoscope B.V., Maastricht, The Netherlands) in the presence of 4 μM phospholipids and 16 mM added $CalCl_2$ (in duplicate). Endogenous thrombin potential (ETP) was analyzed (corresponds to the area under the curve). ETP values were normalized based on platelet-poor normal pooled plasma (NPP) obtained from healthy volunteers, the latter used as a reference [19]. Data are expressed as % of NPP [20]. In addition, using a commercially available micro-enzyme immunoassay kit (Enzygnost® TAT Micro, Siemens Healthcare Diagnostics, Deerfield, IL, USA)–we established thrombin-antithrombin complexes (TATc) levels in all patients (in duplicate) as a highly specific marker for thrombin formation in vivo.

Statistical Analysis

Statistical analyses were performed using IBM SPSS Statistics 19.0.0 (SPSS Inc., Chicago, IL, USA). Categorical variables are presented as numbers (percentages), whereas continuous data are expressed as mean ± SD (standard deviation), unless otherwise indicated. TATc plasma levels were normalized by natural logarithm transformation. We used the score plus 1 to also include patients with a TATc plasma level below 1 ng/mL. Demographic differences between patients with or without CAD were tested either using a Student's t-test or Mann-Whitney U test, depending on the distribution characteristics of the data. Pearson's chi-square test (χ^2) was used to compare proportions (binary or categorical), whereas continuous variables were analysed via one-way analysis of variance test (ANOVA), including Bonferroni correction. Correlations are presented as Pearson or Spearman's coefficients according to the observed distribution.

Multivariate analyses were conducted using binary/multinomial logistic regression, computed in a multiple main effects or forward stepwise manner, including variables with $P<0.05$. χ^2 and odds ratios with 95% CI were calculated to determine which variables demonstrated significant independent associations with atherosclerotic plaque presence, degree of luminal stenosis and CAC.

Receiver operating characteristic (ROC) analysis was carried out to evaluate the potential of using TATc and Framingham risk score (separately or in combination) for determining the presence or absence of CAD. Areas under the ROC curve (AUROC) were compared using the Hanley and McNeil's method. We performed the net reclassification index to evaluate the incremental effect of adding TATc to the Framingham risk score for predicting the presence of coronary plaques. A 2-sided *P* value <0.05 was considered statistically significant.

RESULTS

Study Population Characteristics

The study population consisted of 295 individuals [182 males (61.7%) and 113 females (38.3%)] with a median age of 57 years (min-max: 30-87). A total of 226 patients underwent a "Step and shoot" scan (mean radiation dose 3.6 mSv), whereas 69 patients underwent a "Helical" scan (mean radiation dose 11.6 mSv). CAD was detected in 205 (69.5%) patients. The prevalence of absent, mild, moderate and severe CAD was 30.5%, 22.7%, 26.4% and 20.3%, respectively. Compared to the non-CAD group, patients with CAD were predominantly male (65.4%) and older, showed increased systolic blood pressure and had lower LDL plasma concentrations. However, the prevalence of statin use in the CAD group was significantly higher compared to the non-CAD group [100 (48.8%) vs. 22 (24.4%)]. Baseline characteristics are presented in Table 1.

Increased In Vivo Thrombin Formation Independently Reflects Presence of CAD

As depicted in Figure 1A, among the population with CAD (n=205), the average baseline (lg 10 transformed) TATc levels were significantly higher compared to the group without CAD (mean 0.41; 95% CI 0.38–0.45 vs. mean 0.32; 95% CI 0.29–0.36, *P*=0.001). Multivariate logistic regression analysis showed that higher TATc levels (OR: 1.47, 95% CI 1.10–1.97, *P*=0.010), in addition to other established risk factors such as male gender (OR: 3.36; 95% CI 1.75–6.45, *P*<0.001), age (OR: 1.09; 95% CI 1.05–1.12, *P*<0.001) and smoking (OR: 2.17; 95% CI 1.09–4.33, *P*<0.001), were all independently associated with the presence of CAD (data not shown).

Table 1. Baseline characteristics of subjects stratified into groups per presence of CAD.

	All patients	Patients without CAD	Patients with CAD	
Characteristics	**(n = 295)**	**(n = 90)**	**(n = 205)**	***P***
Age, years	57 (30–87)	54 (30–76)	59 (36–87)	<0.001
Male	182 (61.7)	48 (53.3)	134 (65.4)	0.053
Body Mass Index, kg/m^2	26.4 (24.2–29.4)	26.6 (23.8–29.7)	26.4 (24.3–29.3)	0.620
Systolic BP, mmHg	143 (130–156)	139 (127–153)	145 (132–157)	0.023
Diastolic BP, mmHg	80 (73–87)	80 (71–86)	81 (74–87)	0.615
Smoking	71 (24.1)	18 (20.0)	53 (25.9)	0.304
Diabetes mellitus	26 (8.8)	6 (6.7)	20 (9.8)	0.505
Positive family history	126 (42.7)	36 (40.0)	90 (43.9)	0.609
Lipid-lowering therapy	122 (41.4)	22 (24.4)	100 (48.8)	<0.005
Anticoagulation therapy	0 (0)	0 (0)	0 (0)	---
Framingham Risk Score	18.9 (12.3–30.0)	14.1 (9.8–21.4)	21.6 (13.4–33.1)	<0.001
Total cholesterol, mg/dL	213 (174–244)	217 (192–240)	205 (170–247)	0.077
LDL, mg/dL	128 (101–166)	135 (116–163)	124 (97–166)	0.043
HDL, mg/dL	46 (35–58)	46 (35–57)	44 (37–58)	0.980
Triglycerides, mg/dL	134 (90–208)	146 (91–231)	130 (89–202)	0.180
Coronary lesions, n	2 (0–5)	0 (0–0)	4 (2–7)	<0.001
Coronary calcium score	27 (0–214)	0 (0–0)	117 (23–355)	<0.001

Values are median (interquartile range), or n (%). BP, blood pressure; CAD, coronary artery disease; HDL, high-density lipoprotein; LDL, low-density lipoprotein; n, number.

AUROC for coronary plaque presence, calculated by using the Framingham risk score, was 0.663 (95% CI 0.61–0.72; *P*<0.001). Addition of TATc as a marker to the FRS improved the predictive value, resulting in a significant increase of the AUROC to 0.676 (95% CI 0.62–0.73; *P*=0.048 – difference between areas). The net reclassification index to assess the incremental value of TATc over the Framingham risk score in predicting the presence of coronary plaques was 3.1%, which was not significant (p=0.68).

CAT measurement, which was carried out to assess the potential to generate thrombin in vitro, resulted in ETP values, which are almost the same in the non-CAD group and the CAD group (Figure 1B).

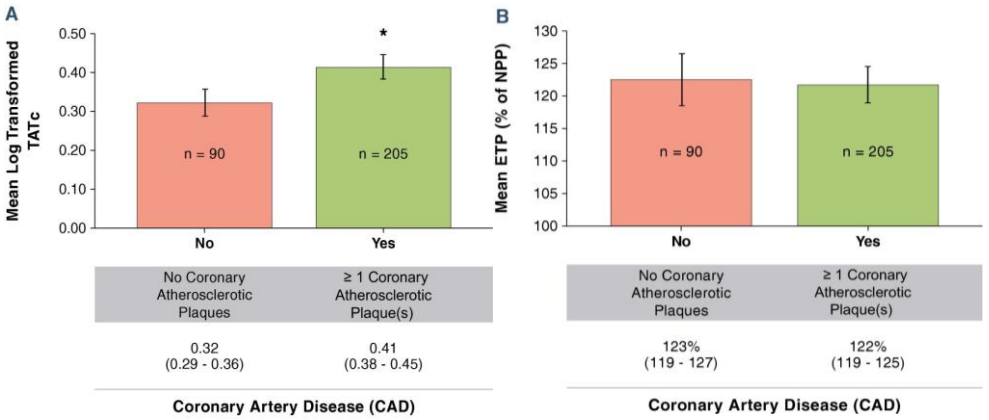

Figure 1. *In vivo/in vitro thrombin formation and the presence of CAD. Panel A shows that patients with CAD exhibit significantly higher in vivo thrombin generation compared to patients without CAD (P=0.001). Bars represent average lg 10 transformed TATc levels, whereas data distribution is presented in mean (95% CI) in the table below the bars. Panel B shows that patients with CAD show comparable thrombin generation potential compared to patients without CAD (P<0.004). Bars represent average ETP levels, whereas data distribution is presented in mean (95% CI) in the table below the bars. NPP = normal pool plasma.*

TATc as Determinant of CAC and Luminal Stenosis

In the entire study population, TATc levels showed a significant positive association with the degree of CAC (Agatston score, r=0.209, *P*<0.001), as presented in Figure 2A. In contrast, no significant relationship was noted between TATc concentrations and the number of non-calcified plaques (r=0.052, *P*=0.376). Multivariate logistic regression analyses, using multiple main effects and forward stepwise techniques, identified higher TATc formation as an independent risk factor for developing CAC (Table 2). Compared to a reference group, consisting of all patients without any coronary calcifications, the odds ratios associated with CAC burden were as follows: mild CAC (OR: 1.60, 95% CI 1.18–2.16, *P*<0.005); moderate CAC (OR: 1.58, 95% CI 1.16–2.15, *P*<0.005); and severe CAC (OR: 1.71, 95% CI 1.26–2.33, *P*<0.005).

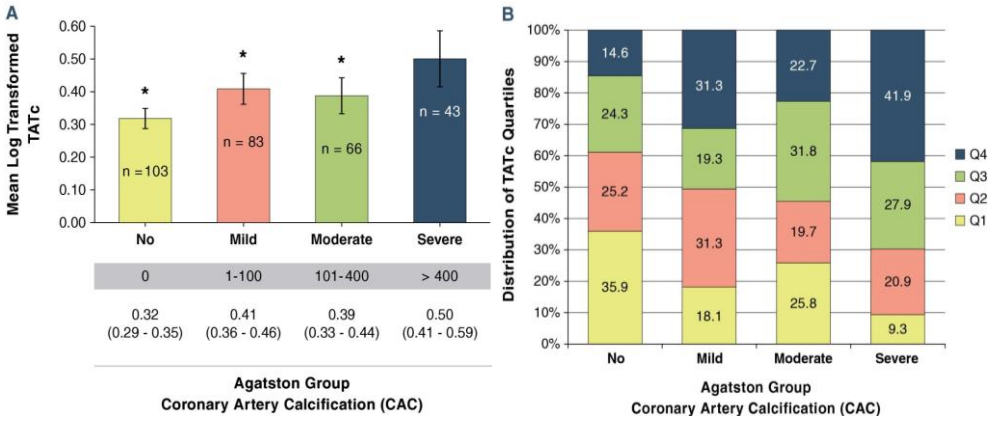

Figure 2. *TATc as an independent predictor of CAC. Panel A: Association between increasing TATc and severity of CAC. Bars represent lg 10 transformed TATc levels, presented as mean (95% CI) in the table below, stratified per CAC group. * Statistical significance at P<0.05 when compared with the severe CAC group. Panel B: Distribution of TATc quartiles between CAC score groups.*

As shown in Figure 2B, we also found a significant difference in the distribution of the TATc quartiles (Q1-Q4) between the different CAC groups (*P*=0.002). While in the no CAC group, 35.9% of the patients had TATc values in the lowest quartile (Q1) and only 14.6% of them had values in the highest quartile (Q4), the distribution of the TATc quartiles in the severe CAC group was 9.3% and 41.9% in Q1 and Q4, respectively.

There was a strong positive association between Agatston score and severity of CAD (r=0.712, *P*<0.001). Nevertheless, we also tested the relationship between TATc formation and the degree of luminal stenosis by performing multivariate logistic regression analyses with degree of luminal stenosis as a dependent variable. Similarly, TATc concentrations accurately identified worsening atherosclerosis. The odds ratios associated with mild, moderate and severe CAD are depicted in Table 3.

Table 2. Multivariate models of factors associated with odds of CAC.

	Mild CAC		Moderate CAC		Severe CAC	
	Agatston 1–100		Agatston 100–400		Agatston >400	
	OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*
Model 1: "Multinomial Logistic Regression: Main Effects Model"						
No CAC, Agatston 0	1.00 (reference)		1.00 (reference)		1.00 (reference)	
Age	1.05 (1.01–1.09)	0.021	1.12 (1.07–1.17)	<0.005	1.15 (1.09–1.22)	<0.005
Gender (male = 0)	2.61 (1.22–5.57)	0.014	3.46 (1.50–8.01)	0.004	6.09 (2.17–17.07)	<0.005
Smoking (yes = 1)	0.71 (0.32–1.53)	0.377	0.51 (0.21–1.22)	0.132	0.34 (0.12–0.95)	0.039
Diabetes (yes = 1)	1.04 (0.30–3.61)	0.951	0.71 (0.19–2.61)	0.603	0.50 (0.12–2.17)	0.355
Fam. history (yes = 1)	0.60 (0.31–1.16)	0.127	0.42 (0.20–0.88)	0.021	0.41 (0.16–1.01)	0.053
BMI	0.99 (0.91–1.08)	0.788	1.02 (0.93–1.12)	0.687	0.94 (0.83–1.06)	0.329
Total Chol	0.88 (0.66–1.15)	0.346	0.96 (0.71–1.29)	0.769	0.84 (0.59–1.21)	0.354
Systolic BP	1.01 (0.99–1.04)	0.219	1.02 (0.99–1.04)	0.130	1.03 (1.00–1.06)	0.051
Diastolic BP	1.00 (0.96–1.03)	0.911	1.00 (0.96–1.04)	0.973	0.99 (0.95–1.04)	0.752
TATc	1.56 (1.14–2.15)	0.006	1.56 (1.13–2.15)	0.007	1.67 (1.21–2.31)	0.002
Model 2: "Multinomial Logistic Regression: Forward Stepwise Model"						
No CAC, Agatston 0	1.00 (reference)		1.00 (reference)		1.00 (reference)	
Age	1.05 (1.01–1.09)	0.010	1.12 (1.07–1.17)	<0.005	1.16 (1.10–1.21)	<0.005
Gender (male = 0)	2.45 (1.25–4.81)	0.009	3.14 (1.49–6.61)	<0.005	4.86 (1.98–11.95)	<0.005
Fam. history (yes = 1)	0.59 (0.31–1.11)	0.104	0.40 (0.20–0.82)	0.012	0.40 (0.17–0.93)	0.034
TATc	1.60 (1.18–2.16)	<0.005	1.58 (1.16–2.15)	<0.005	1.71 (1.26–2.33)	<0.005

BMI, body mass index; BP, blood pressure; CAC, coronary artery calcification; TATc, thrombin-antithrombin complex; Total chol, total cholesterol.

Table 3. Multinomial logistic regression models for CAD severity as the dependent variable.

	Mild CAD		Moderate CAD		Severe CAD	
	Stenosis <50%		Stenosis 50–70%		Stenosis >70%	
	OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*
Model 1: "Multinomial Logistic Regression: Main Effects Model"						
No CAD	1.00 (reference)		1.00 (reference)		1.00 (reference)	
Age	1.08 (1.03–1.12)	<0.005	1.09 (1.04–1.13)	<0.005	1.10 (1.05–1.15)	<0.005
Gender (male = 0)	2.50 (1.12–5.55)	0.025	3.02 (1.37–6.69)	0.006	5.70 (2.33–13.94)	<0.005
Smoking (yes = 1)	0.62 (0.26–1.47)	0.276	0.54 (0.23–1.28)	0.161	0.28 (0.12–0.67)	0.004
Diabetes (yes = 1)	0.69 (0.20–2.36)	0.557	0.82 (0.23–2.89)	0.756	0.98 (0.23–4.28)	0.980
Fam. history (yes = 1)	0.60 (0.30–1.22)	0.160	0.62 (0.31–1.25)	0.177	0.63 (0.30–1.34)	0.226
BMI	0.98 (0.90–1.08)	0.704	1.04 (0.95–1.14)	0.356	1.00 (0.90–1.10)	0.944
Total Chol	0.77 (0.57–1.04)	0.093	0.82 (0.61–1.11)	0.198	0.97 (0.72–1.32)	0.861
Systolic BP	1.01 (0.98–1.03)	0.524	1.02 (0.99–1.04)	0.207	1.02 (0.99–1.04)	0.232
Diastolic BP	1.00 (0.96–1.04)	0.939	1.00 (0.97–1.04)	0.870	1.01 (0.97–1.05)	0.745
TATc	1.37 (0.99–1.88)	0.056	1.57 (1.16–2.14)	0.004	1.47 (1.07–2.02)	0.017
Model 2: "Multinomial Logistic Regression: Forward Stepwise Model"						
No CAD	1.00 (reference)		1.00 (reference)		1.00 (reference)	
Age	1.08 (1.03–1.12)	<0.005	1.09 (1.05–1.13)	<0.005	1.09 (1.05–1.14)	<0.005
Gender (male = 0)	2.46 (1.20–5.08)	0.014	3.12 (1.52–6.41)	0.002	5.35 (2.38–12.03)	<0.005
Smoking (yes = 1)	0.73 (0.32–1.65)	0.443	0.65 (0.29–1.46)	0.296	0.31 (0.14–0.69)	0.004
TATc	1.39 (1.02–1.89)	0.036	1.59 (1.18–2.14)	0.002	1.50 (1.11–2.04)	0.009

BMI, body mass index; BP, blood pressure; CAD, coronary artery disease; TATc = thrombin-antithrombin complex; Total chol, total cholesterol.

Furthermore, we found a U-shaped relationship between the potential to generate thrombin in vitro and the extent of CAD (Figure 3). Within the group of patients with detected CAD, ETP did not correlate to the extent of CAC (r=-0.036, *P*=0.604), whereas it was significantly associated with the degree of CAD (r=0.271, *P*<0.001). In a multivariate logistic regression analysis, when compared to mild CAD, the odds ratios associated with moderate and severe CAD were as follows: moderate (OR: 1.02, 95% CI 1.00–1.04, *P*=0.056) and severe (OR: 1.04, 95% CI 1.02–1.06, *P*<0.001).

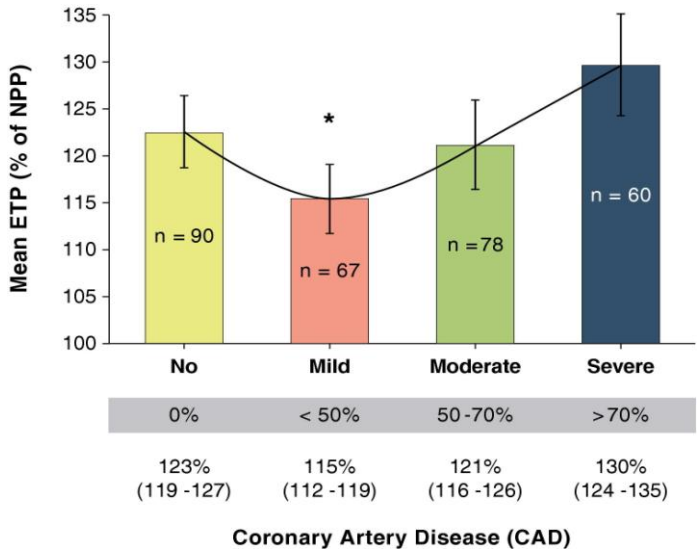

Figure 3: *U-shaped relationship between ETP and CAD. Bars represent endogenous thrombin potential (ETP) values (% of normal pool plasma [NPP]), presented as mean (95% confidence interval), stratified according to degree of luminal stenosis. * Statistical significance at the P<0.05 level when compared with the severe coronary artery disease group.*

DISCUSSION

Major Findings

The present study examines the relationship between thrombin formation and CAD. In a cohort of 295 patients with suspected CAD, we established baseline TATc concentrations in plasma and used CCTA imaging to assess the presence and severity of coronary atherosclerotic plaques.

We found several novel findings of potential clinical relevance. The primary observation of this study is that higher TATc levels, the latter considered a sensitive marker of thrombin formation in vivo, are independently related to the presence and severity of CAD. Net reclassification index analysis showed that incorporation of TATc as an additional test did not significantly improves the cardiovascular risk stratification capacity of the Framingham risk score. On the other hand, the amount of CAC and degree of luminal stenosis were consistently higher with increasing thrombin generation, indicating that TATc measurement is useful to detect even mild-grade coronary artery calcification or stenosis. In daily practice, TATc concentrations may therefore contribute to predict which patients are more likely to have CAD. However, as a single biomarker it seems not to have enough power to be a substitute for other diagnostic imaging tools like CCTA, magnetic resonance imaging, ultrasound, nuclear imaging tests or invasive coronary angiography.

The detrimental role of thrombin in atherothrombosis is not a matter of dispute [21]. Numerous previously published reports have documented increased rates of thrombin synthesis and blood coagulation activation upon the onset of major adverse cardiovascular events [22-25]. However, besides being linked to blood thrombogenicity and determining the magnitude of thrombus formation upon atherosclerotic plaque rupture, thrombin activity per se may be also relevant to the pathophysiology of atherosclerosis progression [3,4]. Several research groups have attempted to study the relationship between hypercoagulability and atherosclerosis progression by assessing ankle brachial pressure index (ABPI) in patients with peripheral artery disease [26-28] or evaluating other markers of subclinical atherosclerosis such as carotid intima-media thickness (cIMT) [29,30]. Nevertheless, these studies do not provide sufficient insight into this problem due to the very limited potential of ABPI and cIMT techniques to evaluate CAD.

To our knowledge, this is the first study to precisely examine the relationship between thrombin generation and the angiographic presence, severity and calcification of coronary artery plaques by using CCTA in a population with suspected, but not previously established CAD.

Enhanced In Vivo Thrombin Generation During Atherogenesis: Potential Clinical Implications

Given the capacity of thrombin to modulate pro-atherogenic actions related to plaque destabilization, it becomes important to define what the clinical implications of these findings may be. Previously, we have demonstrated that early atherosclerotic lesions exert an enhanced pro-coagulant state in comparison to stable advanced ones [5]. This phenomenon was partially explained by the increased activity of many key coagulation proteins (incl. thrombin), but also by the ability of different vascular cell types to synthesize coagulation factors at a local level. There is abundant histopathological and experimental evidence to demonstrate that thrombosis occurs long before an atherosclerotic plaque ruptures, named as subclinical or "buried" thrombosis [8,31,32]. The latter is also considered a potential trigger of plaque vulnerability [33-35].

Furthermore, blood coagulation is an important component of the host-defence system to fight tissue injury and infection [36]. Despite that the exact mechanisms of the enhanced TATc formation in blood during atherosclerosis progression remain unclear to date, one may speculate that inflammation and coagulation operate in a perpetual mode to repair worsening atherosclerosis vascular damage. While oral vitamin K-antagonists and antiplatelet therapy remain the cornerstone in primary and secondary prevention therapy against atherothrombosis and reduce cardiovascular mortality by ~ 30%, numerous clinical trials have failed to account a clear atheroprotective effect [3]. In contrast, a few experimental studies have indicated that administration of direct thrombin inhibitors in atherosclerotic mice substantially inhibits plaque volume and results in plaque stability [7,8]. Given the improved safety profile that these novel therapeutic agents show [37,38], it becomes important to further investigate the effects of selective thrombin inhibition on plaque volume and phenotype determination in patients.

Although the CAT method is meant to determine the potential to generate thrombin in plasma in vitro, the U-shaped association between ETP and CAD, which we demonstrate, may also be of physiological relevance. We have previously reported that early atherosclerotic lesions show an increased thrombin generation potential in comparison to stable advanced lesions [5].

Since the absence of angiographic CAD does not exclude early-stage morphological changes of the arterial vessel wall, one may assume that the increased thrombin-forming capacity in the non-CAD group may be due to first signs of atherosclerotic alterations. Thrombin is a central enzyme in the coagulation-inflammation axis and represents a potential therapeutic target via which atherosclerosis might be modulated. Moreover, thrombin is well-known for its dual-faceted character in both hemostasis and cell signaling [4]. At very low concentrations, thrombin can mediate atheroprotective effects such as endothelial barrier protection, reduction in apoptosis and trans-endothelial migration of leukocytes, and promote atheroprotective IL-10 synthesis [39-41]. Some of those actions are dependent on the occupancy of endothelial protein C receptor by its natural ligand protein C/activated protein C. Hence, the net effect of specific long-term thrombin inhibition remains hard to predict.

Other Findings

The role of CAC in inducing plaque vulnerability remains controversial. Clinical evidence shows that CAC is associated with coronary vasomotor dysfunction and reduced myocardial perfusion, even in the absence of luminal stenosis [42]. Novel concepts of plaque vulnerability propose that atherosclerotic plaque hypoxia may induce angiogenesis, intraplaque hemorrhage and increased risk for rupture [43,44]. Besides serving as a precise indicator of the presence and severity of coronary plaque burden [45], the Agatston score is considered a better predictor of cardiovascular outcomes than the Framingham risk score [46]. In the current study, we present new evidence indicating higher thrombin generation as an independent determinant of CAC. Despite that the clinical significance of these findings remains to be further investigated, a recent ex vivo human study reports that aortic valve calcification can be induced through increased thrombin generation [47].

Study Limitations

Our study has several limitations. First, we performed a single-center study in which all patients were of Western European descent. Second, the patient number was relatively small, which limits the options for analyzing the relevance of TATc for predicting follow-up events.

Moreover, because the relatively healthy population, we did not found a high rate of (hard) cardiovascular events, especially not in short term, which is in line with other CT-studies. Third, while we screened for angiographic CAD, these findings may reflect other existing atherosclerosis settings (carotid or peripheral artery lesions), which we did not assess in this study. Therefore, the association between thrombin formation and atherosclerosis in other vascular beds remain open. Fourth, the purpose of this study was to investigate the association of thrombin formation and CAD and was not meant to unravel this complex causal relationship.

CONCLUSION

Thrombin formation is a useful tool in determining the presence and severity of coronary artery disease, but more importantly, may be also involved in the pathophysiology of vascular calcification and atherosclerosis progression.

REFERENCES

1. Ross R. Atherosclerosis - an inflammatory disease. *N Engl J Med* 1999;340:115–126.
2. Holmes DR, Jr., Kereiakes DJ, Kleiman NS, et al. Combining antiplatelet and anticoagulant therapies. *J Am Coll Cardiol* 2009;54:95–109.
3. Borissoff JI, Spronk HM, ten Cate H. The hemostatic system as a modulator of atherosclerosis. *N Engl J Med* 2011;364:1746–1760.
4. Borissoff JI, Spronk HM, Heeneman S, et al. Is thrombin a key player in the 'coagulation-atherogenesis' maze? *Cardiovasc Res* 2009;82:392–403.
5. Borissoff JI, Heeneman S, Kilinc E, et al. Early atherosclerosis exhibits an enhanced procoagulant state. *Circulation* 2010;122:821–830.
6. Nelken NA, Soifer SJ, O'Keefe J, et al. Thrombin receptor expression in normal and atherosclerotic human arteries. *J Clin Invest* 1992;90:1614–1621.
7. Bea F, Kreuzer J, Preusch M, et al. Melagatran reduces advanced atherosclerotic lesion size and may promote plaque stability in apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol* 2006;26:2787–2792.

8. Borissoff JI, Loubele ST, Leenders P, et al. Direct thrombin inhibition by dabigatran protects against severe atherosclerosis progression in prothrombotic mice. *Circulation* 2010;122:A19384.
9. Budoff MJ, Dowe D, Jollis JG, et al. Diagnostic performance of 64-multidetector row coronary computed tomographic angiography for evaluation of coronary artery stenosis in individuals without known coronary artery disease: results from the prospective multicenter ACCURACY trial. *J Am Coll Cardiol* 2008;52:1724–1732.
10. Voros S, Rinehart S, Qian Z, et al. Coronary atherosclerosis imaging by coronary CT angiography: current status, correlation with intravascular interrogation and meta-analysis. *JACC Cardiovasc Imaging* 2011;4:537–548.
11. D'Agostino RB, Sr., Vasan RS, Pencina MJ, et al. General cardiovascular risk profile for use in primary care: the Framingham Heart Study. *Circulation* 2008;117:743–753.
12. Agatston AS, Janowitz WR, Hildner FJ, et al. Quantification of coronary artery calcium using ultrafast computed tomography. *J Am Coll Cardiol* 1990;15:827–832.
13. Hausleiter J, Meyer T, Hadamitzky M, et al. Radiation dose estimates from cardiac multislice computed tomography in daily practice: impact of different scanning protocols on effective dose estimates. *Circulation* 2006;113:1305–1310.
14. Laufer EM, Mingels AM, Winkens MH, et al. The extent of coronary atherosclerosis is associated with increasing circulating levels of high sensitive cardiac troponin T. *Arterioscler Thromb Vasc Biol* 2010;30:1269–1275.
15. Austen WG, Edwards JE, Frye RL, et al. A reporting system on patients evaluated for coronary artery disease. Report of the Ad Hoc Committee for Grading of Coronary Artery Disease, Council on Cardiovascular Surgery, American Heart Association. *Circulation* 1975;51:5–40.
16. Raff GL, Abidov A, Achenbach S, et al. SCCT guidelines for the interpretation and reporting of coronary computed tomographic angiography. *J Cardiovasc Comput Tomogr* 2009;3:122–136.
17. Pletcher MJ, Tice JA, Pignone M, et al. Using the coronary artery calcium score to predict coronary heart disease events: a systematic review and meta-analysis. *Arch Intern Med* 2004;164:1285–1292.
18. Dielis AW, Castoldi E, Spronk HM, et al. Coagulation factors and the protein C system as determinants of thrombin generation in a normal population. *J Thromb Haemost* 2008;6:125–131.

19. Smid M, Dielis AW, Winkens M, et al. Thrombin generation in patients with a first acute myocardial infarction. *J Thromb Haemost* 2011;9:450–456.
20. Spronk HM, Dielis AW, De Smedt E, et al. Assessment of thrombin generation II: Validation of the calibrated automated thrombogram in platelet-poor plasma in a clinical laboratory. *Thromb Haemost* 2008;100:362–364.
21. Furie B, Furie BC. Mechanisms of thrombus formation. *N Engl J Med* 2008;359:938–949.
22. Danesh J, Lewington S, Thompson SG, et al. Plasma fibrinogen level and the risk of major cardiovascular diseases and nonvascular mortality: an individual participant meta-analysis. *JAMA* 2005;294:1799–1809.
23. Di Tullio MR, Homma S, Jin Z, et al. Aortic atherosclerosis, hypercoagulability, and stroke the APRIS study. *J Am Coll Cardiol* 2008;52:855–861.
24. Henareh L, Jogestrand T, Agewall S. Prothrombin fragment 1 + 2 is associated with intima media thickness of the carotid artery in patients with myocardial infarction. *Thromb Res* 2009;124:526–530.
25. Undas A, Szuldrzynski K, Brummel-Ziedins KE, et al. Systemic blood coagulation activation in acute coronary syndromes. *Blood* 2009;113:2070–2078.
26. van der Bom JG, Bots ML, van Vliet HH, et al. Antithrombin and atherosclerosis in the Rotterdam Study. *Arterioscler Thromb Vasc Biol* 1996;16:864–867.
27. McDermott MM, Green D, Greenland P, et al. Relation of levels of hemostatic factors and inflammatory markers to the ankle brachial index. *Am J Cardiol* 2003;92:194–199.
28. Tzoulaki I, Murray GD, Price JF, et al. Hemostatic factors, inflammatory markers, and progressive peripheral atherosclerosis: the Edinburgh Artery Study. *Am J Epidemiol* 2006;163:334–341.
29. Paramo JA, Orbe J, Beloqui O, et al. Prothrombin fragment 1+2 is associated with carotid intima-media thickness in subjects free of clinical cardiovascular disease. *Stroke* 2004;35:1085–1089.
30. Bernhard H, Wipfler P, Leschnik B, et al. Relationship between thrombin generation and carotid intima-media thickness. *Hamostaseologie* 2010;30:168–171.
31. Rittersma SZ, van der Wal AC, Koch KT, et al. Plaque instability frequently occurs days or weeks before occlusive coronary thrombosis: a pathological thrombectomy study in primary percutaneous coronary intervention. *Circulation* 2005;111:1160–1165.

32. Kramer MC, Rittersma SZ, de Winter RJ, et al. Relationship of thrombus healing to underlying plaque morphology in sudden coronary death. *J Am Coll Cardiol* 2010;55:122–132.
33. Mann J, Davies MJ. Mechanisms of progression in native coronary artery disease: role of healed plaque disruption. *Heart* 1999;82:265–268.
34. Burke AP, Kolodgie FD, Farb A, et al. Healed plaque ruptures and sudden coronary death: evidence that subclinical rupture has a role in plaque progression. *Circulation* 2001;103:934–940.
35. Finn AV, Nakano M, Narula J, et al. Concept of vulnerable/unstable plaque. *Arterioscler Thromb Vasc Biol* 2010;30:1282–1292.
36. Degen JL, Bugge TH, Goguen JD. Fibrin and fibrinolysis in infection and host defense. *J Thromb Haemost* 2007;5:24–31.
37. Schulman S, Kearon C, Kakkar AK, et al. Dabigatran versus warfarin in the treatment of acute venous thromboembolism. *N Engl J Med* 2009;361:2342–2352.
38. Connolly SJ, Ezekowitz MD, Yusuf S, et al. Dabigatran versus warfarin in patients with atrial fibrillation. *N Engl J Med* 2009;361:1139–1151.
39. Naldini A, Bernini C, Pucci A, et al. Thrombin-mediated IL-10 up-regulation involves protease-activated receptor (PAR)-1 expression in human mononuclear leukocytes. *J Leukoc Biol* 2005;78:736–744.
40. Bae JS, Kim YU, Park MK, et al. Concentration dependent dual effect of thrombin in endothelial cells via Par-1 and Pi3 Kinase. *J Cell Physiol* 2009;219:744–751.
41. Bae JS, Rezaie AR. Thrombin inhibits nuclear factor kappaB and RhoA pathways in cytokine-stimulated vascular endothelial cells when EPCR is occupied by protein C. *Thromb Haemost* 2009;101:513–520.
42. Wang L, Jerosch-Herold M, Jacobs DR Jr., et al. Coronary artery calcification and myocardial perfusion in asymptomatic adults: the MESA (Multi-Ethnic Study of Atherosclerosis). *J Am Coll Cardiol* 2006;48:1018–1026.
43. Sluimer JC, Gasc JM, van Wanroij JL, et al. Hypoxia, hypoxia-inducible transcription factor, and macrophages in human atherosclerotic plaques are correlated with intraplaque angiogenesis. *J Am Coll Cardiol* 2008;51:1258–1265.
44. Sluimer JC, Daemen MJ. Novel concepts in atherogenesis: angiogenesis and hypoxia in atherosclerosis. *J Pathol* 2009;218:7–29.

45. McEvoy JW, Blaha MJ, Defilippis AP, et al. Coronary artery calcium progression: an important clinical measurement? *J Am Coll Cardiol* 2010;56:1613–1622.
46. Alexopoulos N, Raggi P. Calcification in atherosclerosis. *Nat Rev Cardiol* 2009;6:681–688.
47. Breyne J, Juthier F, Corseaux D, et al. Atherosclerotic-like process in aortic stenosis: activation of the tissue factor-thrombin pathway and potential role through osteopontin alteration. *Atherosclerosis* 2010;213:369–376.

CHAPTER 6

ELEVATED LEVELS OF CIRCULATING DNA AND CHROMATIN ARE INDEPENDENTLY ASSOCIATED WITH SEVERE CORONARY ATHEROSCLEROSIS AND A PROTHROMBOTIC STATE

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Arterioscler Thromb Vasc Biol 2013;33:2032–2040

BACKGROUND

Aberrant neutrophil activation occurs during the advanced stages of atherosclerosis. Once primed, neutrophils can undergo apoptosis or release neutrophil extracellular traps (NETs). This extracellular DNA exerts potent pro-inflammatory, pro-thrombotic and cytotoxic properties. The goal of this study was to examine the relationships among extracellular DNA formation, coronary atherosclerosis, and the presence of a prothrombotic state.

METHODS

In a prospective, observational cohort of 282 individuals with suspected coronary artery disease (CAD), we examined the severity, extent, and phenotype of coronary atherosclerosis using coronary CT-angiography. Double-stranded DNA, nucleosomes, citrullinated histone H4 and myeloperoxidase-DNA complexes, considered in vivo markers of cell death and NETosis, respectively, were established. We further measured plasma markers of coagulation activation and inflammation.

RESULTS

Plasma double-stranded DNA, nucleosomes and myeloperoxidase-DNA complexes were positively associated with thrombin generation and significantly elevated in patients with severe coronary atherosclerosis or extremely calcified coronary arteries. Multinomial regression analysis, adjusted for confounding factors, identified high plasma nucleosome levels as an independent risk factor of severe coronary stenosis (odds ratio: 2.14, 95% confidence interval: 1.26-3.63; *P*=0.005). Markers of neutrophil extracellular traps, such as myeloperoxidase-DNA complexes, predicted the number of atherosclerotic coronary vessels and the occurrence of major adverse cardiac events.

CONCLUSION

Markers of cell death and NET formation are independently associated with CAD, prothrombotic state, and occurrence of adverse cardiac events. These biomarkers potentially aid in the prediction of cardiovascular risk in patients with chest pain.

INTRODUCTION

Atherosclerotic plaque disruption and subsequent intraluminal thrombus formation are the pathological hallmark of both acute coronary syndrome (ACS), including myocardial infarction (MI), and ischemic stroke. Discharge of plaque or thrombotic debris from unstable or ulcerated lesions into the circulation is considered the inciting cause of arterial thromboembolic complications. Despite all modern advances in pharmacological and interventional therapy, atherothrombosis remains one of the most significant clinical and burdens worldwide [1].

During the progression of atherosclerosis, circulating cells and cellular constituents of the vessel wall become more prone to DNA damage. As a result of inadequate DNA repair capacity, processes such as cellular senescence, necrosis and apoptosis could prevail, thus inducing extracellular DNA and nucleosome (chromatin fragments/histone-DNA complexes) release [2]. There is also a distinct cell death pathway, named extracellular trap formation (ETosis), via which neutrophils and other cell types, such as monocytes, mast cells, and eosinophils, can dismantle and expel nuclear or mitochondrial DNA [3]. This process serves as host defense against infection, wherein highly decondensed chromatin threads, carrying both histones and granule proteins, are cast out, providing an extracellular scaffold to trap and kill microbial pathogens [4]. There is growing evidence showing the relationship among different infective pathogens, atherosclerosis progression, and atherothrombosis [5]. In addition to the well-established anti-bacterial properties, pioneer experimental studies have documented that excessive generation of circulating DNA, nucleosomes and histones can be deleterious in several disease settings (eg, sepsis, pulmonary inflammation, thrombocytopenia, venous thrombosis, cancer) [6-11]. Given their potent cytotoxic and pro-thrombotic effects, extracellular DNA may establish a new interface between inflammation and coagulation [12].

There are different imaging techniques to assess the presence and severity of coronary atherosclerosis. Coronary computed tomography angiography (CCTA) has evolved as a widely available, highly accurate noninvasive diagnostic imaging tool for the assessment of coronary artery disease (CAD) [13,14].

The primary aim of this study was to determine the associations between circulating levels of markers of cell death and NETosis and the severity, extent, and phenotype of CCTA-assessed coronary atherosclerosis in individuals with suspected CAD. We further sought to examine the relationship between extracellular DNA and nucleosomes released in plasma, the presence of a prothrombotic state, and the occurrence of major adverse cardiac events during follow-up.

METHODS

Study Population

We studied 282 adult patients who were referred from the cardiology outpatient department for CCTA because of chest discomfort symptoms, suspected for CAD. All patients were referred according to the AHA 2010 Appropriate Use Criteria for Cardiac Computed Tomography [15]. Scans were performed in the Maastricht University Medical Center between September 2010 and February 2012 as part of the diagnostic work-up. Excluded were patients with an acute onset of chest pain suspected for an ACS, patients with a known history of CAD, patients with unavailable data regarding their cardiac risk profile and patients with an inconclusive CT-scan. A history of systemic inflammatory disease, myocarditis, pericarditis, active infection or cancer were also exclusion criteria.

The Institutional Review Boards and Ethics Committees at Maastricht University Medical Center and Boston Children's Hospital approved the study, and all patients and healthy volunteers gave written informed consent. The study was performed in compliance with the ethical standards laid down in the 1975 Declaration of Helsinki.

Cardiovascular Risk Profile

In all patients, traditional cardiovascular risk factors were collected prior to the scan. Patients were classified as smokers if they were current smoker. A family history of premature CAD was defined as having a first-degree relative with a history of MI or sudden cardiac death before the age of sixty. Patients were classified as diabetic or hypertensive if diagnosed according to current guidelines [16,17].

Coronary CT-angiography Acquistion

CCTA was performed using a dual-source CT scanner (Somatom Definition Flash, Siemens Medical Solutions, Germany). Scan parameters were: slice collimation 2x128x0.6 mm, gantry rotation time 280 ms, tube voltage 80-120 kV and temporal resolution 75 ms. Patients took 50 mg Metoprolol tartrate (AstraZeneca, The Netherlands) orally, 2 hours before CT, unless contra-indicated. An additional dose of 5-20 mg Metoprolol tartrate was administered intravenously just before the scan when the heart rate was >60 beats per minute (bpm). All patients received 0.8 mg nitroglycerin (Pohl-Boskamp, Germany) sublingually just prior to CT.

First, a native scan was performed to calculate the calcium score by the Agatston method. Subsequently, a 20 mL contrast test bolus was injected to assess the time to peak in the ascending aorta. CT-angiography was performed by administrating 80-100 mL of contrast agent (Ultravist 300, Bayer Pharma AG, Germany) in the antecubital vein, at a flow rate of 5.4-7.6 mL/s, followed by a 60 mL saline flush (6.0 mL/s) using a dual-head power injector (Medrad Inc, PA, USA). The amount of contrast agent, as well as the flow rate, were dependent on individual patient characteristics. In patients with a stable heart rate <60 bpm, we used a prospectively ECG-gated high pitch spiral protocol. In patients with a stable heart rate between 60-90 bpm, a prospectively ECG-gated sequential axial protocol was used. In patients with higher or irregular heart rates, we used a retrospectively ECG-gated helical protocol with dose modulation.

Coronary CT-angiography Analysis

All scans were independently analyzed by a cardiologist and a radiologist, both experienced in reading CCTA's and blinded to clinical information. In case of disagreement, consensus was reached by discussion. The coronary calcium score (CCS) was expressed as the Agatston score using dedicated calcium scoring software with a threshold of 130 Hounsfield Units (HU). The coronary artery tree was analyzed for the presence and severity of CAD according to the 16 coronary segments model of the American Heart Association [18], using the source images on the provided software (Syngo, Siemens Medical Solutions, Germany). The degree of stenosis was visually defined as absent (no luminal stenosis), mild (1-50% luminal stenosis), moderate (50-70% luminal stenosis) or severe (>70% luminal stenosis) [19].

In addition, an involvement score was calculated by counting all diseased vessel segments (irrespective of degree of stenosis), with a score ranging from 0-16 [20]. Plaques were categorized as calcified (exclusively content with density >130 HU), non-calcified (exclusively content with density <130 HU), or mixed (characteristics of both calcified and non-calcified plaques).

Blood Samples, Laboratory Measurements and Platelet-poor Normal Pooled Plasma Preparation

Venous blood was collected in 3.2% citrate (w/v) prior to the exercise ECG testing and scan, processed within one hour and samples were stored at -80°C until analysis. Double-stranded DNA was established by using Sytox® Green nucleic acid stain (S7020, Invitrogen, CA, USA), lambda DNA standard (Quant-iT™ PicoGreen® dsDNA Assay Kit, Invitrogen, CA, USA) and a microplate fluorometer (Fluoroskan Ascent, Thermo Fisher Scientific Inc., MA, USA) at 485 nm excitation and 538 nm emission [21]. Circulating mono- and oligo-nucleosome levels were quantified with an ELISA kit, following the manufacturer's instructions (Cell Death Detection ELISA^{PLUS,} Cat. No.11774425001, Roche Diagnostics, IN, USA). Citrullinated histone H4 was identified using an in-house capture ELISA method. Briefly, plasma samples were added together with a monoclonal mouse anti-histone biotinylated antibody (Component 1) to a streptavidin-coated plate (Component 9, Cell Death Detection ELISA^{PLUS}, Clone H11-4, Roche Diagnostics). Rabbit polyclonal anti-histone H4 (citrulline 3) (ab81797; Abcam Inc., MA, USA) antibody was used as a secondary step. Detection was accomplished via incubation with peroxidase-labeled tertiary antibody. Myeloperoxidase-DNA (MPO-DNA) complexes and von Willebrand factor (VWF) levels were established as previously published [22,23]. Commercially available human ELISA kits were used for measuring thrombin-antithrombin complexes (ab108907; Abcam Inc., MA, USA) as a sensitive marker of in vivo thrombin formation, and PMN Elastase-α1-PI complexes (ab119553; Abcam Inc., MA, USA), soluble (s)CD163 (Human CD163 Quantikine ELISA Kit DC1630, R&D Systems, MN, USA) and CXCL4/platelet factor 4 levels (Human ELISA Kit DY795, R&D Systems, MN, USA) as markers of neutrophil, monocyte and platelet activation, respectively.

Both platelet-poor normal poor plasma (NPP) and plasma obtained from healthy volunteers (separate group, n=10) were used as a reference for all measured markers. NPP was prepared at the departments of Hematology and Clinical Chemistry of the Maastricht University Medical Center, The Netherlands, by pooling plasma from 85 healthy volunteers, not using any medication. Venous blood from healthy volunteers was collected in 3.2% (w/v) citrate using a 21-gauge needle (Becton, Dickinson and Company, UK) or a Winged Infusion Set (Becton, Dickinson and Company, UK) through venapuncture. The first 10 ml of venous blood were discarded. Platelet-poor plasma was prepared by two centrifugation steps: the first at 2,000x g for 15 minutes (min) and the second at 11,000x g for 10 min. Plasma aliquots were snap-frozen in liquid nitrogen and stored at –80°C until use. Data are expressed as a fold change over NPP (nucleosomes, MPO-DNA complexes, citrullinated histone H4, and VWF) or as concentration (ng/ml).

Study Endpoint and Follow-up

The composite study endpoint was the occurrence of MACE including revascularization >60 days following CCTA (percutaneous coronary intervention [PCI] or coronary artery bypass grafting [CABG]), ACS and cardiac mortality. ACC/AHA guidelines were applied as clinical criteria for the diagnosis of ACS (MI or unstable angina requiring hospitalization) [24]. Follow-up was censored in case when revascularization was performed within 60 days of CCTA or following the occurrence of MACE, as these events are primarily CCTA-driven. Patients were regularly examined by their cardiologist. All outpatient visits, emergency room visits and hospital admissions were accordingly recorded in the electronic patient records. National mortality records were also checked.

Statistical Analysis

Continuous variables are expressed as median (interquartile range [IQR]), and categorical data as numbers (percentages), unless otherwise indicated. Based on the distribution characteristics of the data, non-normally distributed continuous variables were transformed by using natural logarithm of score plus 1, to account for patients with levels less than 1, and to provide improved normality before further analyses.

Student's t-test, Mann-Whitney U test or one-way analysis of variance (ANOVA) test including Bonferroni correction were used as appropriate. Pearson's chi-square test (χ^2) was utilized to detect differences among categorical data (binary or ordinal). Pearson or Spearman's correlation coefficients were established depending on the observed data distribution. Multinomial logistic regression analyses were performed to identify the factors that independently determine ordinal variables such as the degree of luminal stenosis/atherosclerotic plaque severity. Multiple linear regression models were created to describe the impact of multiple predictors on a single continuous dependent variable. Accordingly, standardized coefficients β were calculated as measures of association. Pearson's chi-square test (χ^2) and odds ratio (OR) with 95% confidence interval (CI) were used to calculate the association between dichotomized parameters (via a median split – high versus low levels) and outcomes. Standardized coefficient β indicates the change of the dependent variable (in SD) that is induced by 1 SD change in the determinant. Receiver operating characteristic (ROC) analysis was performed to establish the potential of the tested markers to predict MACE and their incremental value when compared to CCTA. Areas under the ROC curve (AUROC) were compared using the Hanley and McNeil's method. Statistical analyses were performed using IBM SPSS Statistics 20.0.0 (SPSS Inc., IL, USA) and GraphPad Prism 6.0a (GraphPad Software Inc., CA, USA).

RESULTS

Clinical Characteristics

We studied 282 patients (183 males; 64.9%) with chest discomfort symptoms, suspected for CAD. The median age of the study population was 60 years (min-max 34-83 years). A total of 245 patients underwent both coronary calcium score scan and CCTA. In the remaining 37 patients, CCTA was waived because of an extremely high CCS. Baseline characteristics are presented in Table 1. The prevalence of absent, mild, moderate and severe CAD was 18.1%, 26.6%, 26.2% and 16.0%, respectively. The 37 patients (13.1%) who did not undergo CCTA are considered as a separate category and are labeled as "Extremely Calcified" in all Tables and Figures.

Table 1. Baseline characteristics of the study population, stratified by severity of CAD.

Characteristics	All patients (n=282)	No CAD (n=51)	Mild CAD (n=75)	Moderate CAD (n=74)	Severe CAD (n=45)	Extremely calcified* (n=37)	*P*
Age, median (min-max)	60 (34-83)	55 (36-76)	60 (34–78)	61 (43–83)	60 (37–79)	64 (42–78)	0.004
Male gender	183 (64.9)	31 (60.8)	40 (53.3)	46 (62.2)	37 (82.2)	29 (78.4)	0.017
BMI, kg/m^2	27 (25–29)	27 (25–29)	26 (24–29)	26 (24–29)	27 (26–29)	27 (24–30)	0.595
Creatinine, μmol/l	79 (70–88)	80 (70–88)	78 (70–88)	80 (72–90)	82 (70–91)	76 (65–88)	0.558
Smoking	70 (24.8)	9 (17.6)	17 (22.7)	18 (24.3)	14 (31.1)	12 (32.4)	0.482
Diabetes mellitus	29 (10.3)	5 (9.8)	9 (12.0)	4 (5.4)	4 (8.9)	7 (18.9)	0.565
Family history of premature CAD	105 (37.2)	16 (31.4)	35 (46.7)	27 (36.5)	15 (33.3)	12 (32.4)	0.280
VKA therapy	40 (14.2)	3 (5.9)	14 (18.7)	7 (9.5)	10 (22.2)	6 (16.2)	0.046
Aspirin therapy	93 (33.0)	15 (29.4)	24 (32.0)	21 (28.4)	19 (42.2)	14 (37.8)	0.432
Lipid-lowering therapy	101 (35.8)	11 (21.6)	30 (40.0)	26 (35.1)	20 (44.4)	14 (37.8)	0.087
Antihypertensive therapy	66 (23.4)	5 (9.8)	19 (25.3)	19 (25.7)	12 (26.7)	11 (29.7)	0.114
Involvement score	2 (0–5)	0 (0–0)	2 (1–4)	5 (2–6)	5 (3–7)	---	<0.001
Calcified plaques	1 (0–3)	0 (0–0)	1 (0–2)	2 (1–4)	2 (1–4)	---	<0.001
Mixed plaques	1 (0–2)	0 (0–0)	0 (0–1)	1 (1–2)	2 (0–2)	---	<0.001
Non-calcified plaques	0 (0–1)	0 (0–0)	0 (0–0)	0 (0–1)	0 (0–1)	---	<0.001
Coronary calcium score	88 (1–356)	0 (0–0)	44 (5–102)	168 (63–355)	197 (46–501)	1211 (948–2295)	<0.001

Categorical variables are presented as numbers (percentages). Continuous data are expressed as median (interquartile range), unless otherwise indicated. BMI, body mass index; CAD, coronary artery disease; CCTA, coronary CT-angiography; VKA, vitamin K antagonists. * Patients with no CT-angiographically confirmed coronary atherosclerotic plaques as a result of severe coronary calcification.

Increased Levels of Circulating dsDNA, Nucleosomes and MPO-DNA Complexes in Patients With Severe Coronary Atherosclerosis

Figure 1A shows individual double-stranded (dsDNA) levels in patients according to the presence and severity of CAD. Extreme coronary artery calcification (CAC) is shown as a separate group of patients. The release of extracellular dsDNA into the circulation was significantly greater in patients with severe CAD (69.59 ng/ml (41.25–87.75); *P*=0.003) or abundant CAC (79.37 ng/ml (57.97–92.60); *P*<0.001) compared with individuals with no angiographically detected CAD (50.09 ng/ml (13.91–63.92)). Whereas the median fold change in free plasma nucleosomes over NPP was 1.32 (1.05–2.09) in the no CAD group, there was a significant fold increase in nucleosome release into the circulation in both the severe CAD (2.02 [1.55–3.51]; *P*<0.001) and extremely calcified groups (2.36 [1.60–3.41]; *P*<0.001; Figure 1B). A similar significant increase was observed with respect to plasma levels of MPO-DNA, VWF and TAT complexes (Figures 1D and 2D and 2E). Circulating levels of polymorphonuclear (PMN) elastase-α1-proteinase inhibitor (PI) complexes were significantly higher in the extremely calcified patient group (64.21 ng/ml [20.79–91.04]) compared with the other groups (*P*<0.05 for all comparisons; Figure 2A). Citrullinated histone H4, CXCL4/platelet factor 4, and sCD163 levels in plasma did not significantly differ among the groups (Figures 1C and 2B and 2C). There was a positive association between the severity of luminal stenosis, assessed by CCTA, and several plasma parameters, including dsDNA (Spearman's rho=0.316; *P*<0.001), nucleosomes levels (Spearman's rho=0.271; *P*<0.001), MPO-DNA (Spearman's rho=0.215; *P*=0.001), TAT complexes (Spearman's rho=0.216; *P*=0.001), and VWF (Spearman's rho=0.186; *P*=0.003; data not shown). To evaluate the strength of associations between all tested variables and CAD severity, we performed multinomial logistic regression, in which a main effects model was implemented (Table 2). Increasing TAT formation robustly predicted all levels of severity of coronary artery stenosis: mild CAD (OR: 1.36; 95% CI 1.09–1.68; *P*=0.005), moderate CAD (OR: 1.31; 95% CI 1.06–1.62; *P*=0.013) and severe CAD (OR: 1.33; 95% CI 1.06–1.68; *P*=0.015), respectively. In addition to established risk factors, such as age and male gender, nucleosome (OR: 2.14; 95% CI 1.26–3.63; *P*=0.005) and VWF (OR: 7.31; 95% CI 1.33–40.07; *P*=0.022) levels independently predicted the presence of severe coronary stenosis, (Table 2).

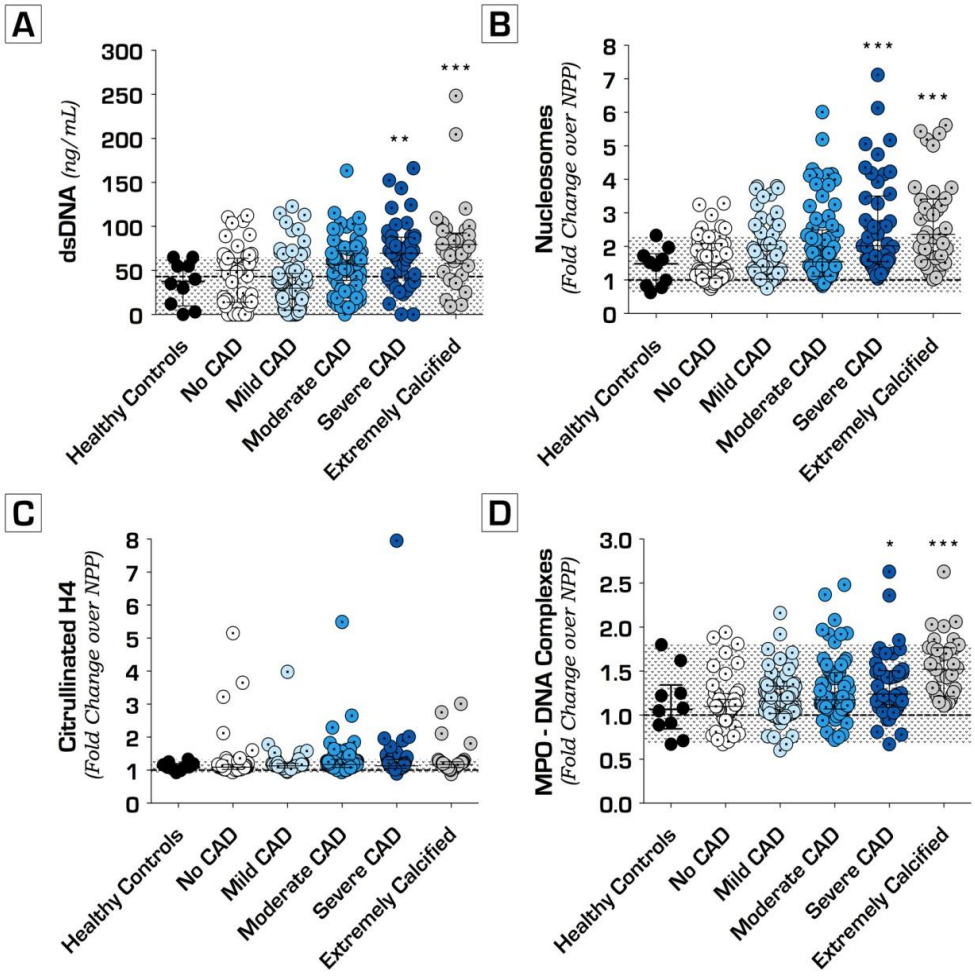


Figure 1. *Circulating DNA, nucleosome fragments and markers of neutrophil extracellular traps according to the presence and severity of coronary artery disease (CAD). Patients were divided in categories, based on the severity of CAD, as assessed with coronary computed tomographic angiography (CCTA). Patients who did not undergo CCTA because of a high calcium score were considered as a separate category (Extremely Calcified). A total of four markers were measured in all patients (panels A-D). Shaded area demonstrates the range of the measured markers in plasma from healthy controls (n=10). Normal pooled plasma (from n=85 healthy volunteers) is indicated by a horizontal dotted line. * P<0.05; ** P<0.01; *** P<0.001. dsDNA indicates double stranded DNA.*

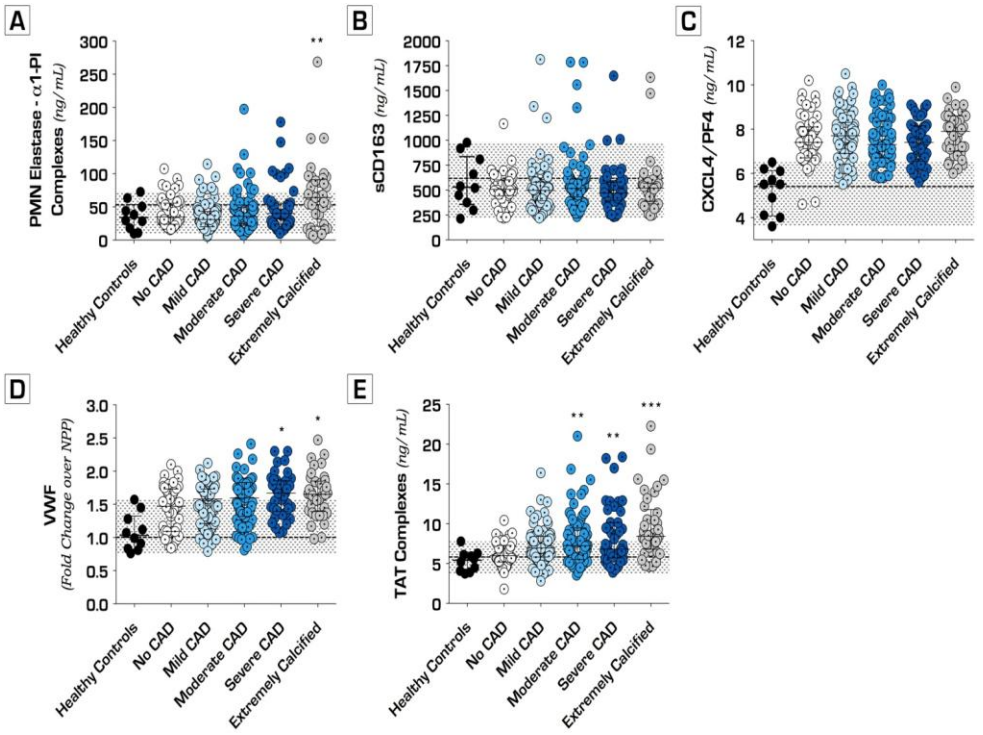


Figure 2. *Plasma levels of markers of leukocyte, platelet, endothelial and coagulation activation according to the presence and severity of coronary artery disease (CAD). Patients were divided in categories, based on the severity of CAD, as assessed with coronary computed tomographic angiography (CCTA). Patients who did not undergo CCTA because of a high calcium score were considered as a separate category (Extremely Calcified). A total of five markers were measured in all patients (panels A-E). Shaded area demonstrates the range of the measured markers in plasma from healthy controls (n=10). Normal pooled plasma (from n=85 healthy volunteers) is indicated by a horizontal dotted line.* P<0.05; ** P<0.01; *** P<0.001. MPO, myeloperoxidase; PMN, polymorphonuclear; α1-PI, alpha 1-proteinase inhibitor; PF4, platelet factor 4; TAT, thrombin-antithrombin; VWF, von Willebrand factor.*

Table 2. Multinomial logistic regression models for CAD severity as dependent variable.

	Multinomial logistic regression: Main effects model					
	Mild CAD Stenosis: 1–50 %		**Moderate CAD Stenosis: 50–70 %**		**Severe CAD Stenosis: >70%**	
Variable	**OR (95% CI)**	***P***	**OR (95% CI)**	***P***	**OR (95% CI)**	***P***
Reference, no CAD (stenosis 0%)	1.0 (Reference)		1.0 (Reference)		1.0 (Reference)	
Age	1.04 (0.99–1.10)	0.097	1.09 (1.03–1.14)	0.002	1.07 (1.01–1.14)	0.030
Gender (male = 1)	1.07 (0.37–3.05)	0.904	1.49 (0.51–4.38)	0.464	5.85 (1.47–23.34)	0.012
Body Mass Index	0.94 (0.84–1.05)	0.250	0.99 (0.89–1.11)	0.885	1.06 (0.93–1.20)	0.393
Creatinine	1.00 (0.97–1.04)	0.826	1.00 (0.97–1.04)	0.817	0.99 (0.95–1.03)	0.614
Smoking (yes = 1)	1.72 (0.61–4.89)	0.306	1.82 (0.61–5.38)	0.280	2.69 (0.78–9.24)	0.116
Family history of CAD (yes = 1)	2.44 (1.02–5.82)	0.045	1.89 (0.76–4.65)	0.168	1.58 (0.52–4.84)	0.420
Diabetes mellitus (yes = 1)	1.13 (0.28–4.65)	0.862	0.44 (0.09–2.11)	0.306	0.82 (0.15–4.60)	0.823
dsDNA	0.99 (0.97–1.00)	0.058	1.01 (0.99–1.02)	0.269	1.01 (1.00–1.03)	0.094
Nucleosomes	1.02 (0.62–1.68)	0.933	1.41 (0.88–2.27)	0.153	2.14 (1.26–3.63)	0.005
Citrullinated histone H4	0.74 (0.33–1.66)	0.469	0.85 (0.44–1.66)	0.638	1.04 (0.50–2.14)	0.925
MPO–DNA complexes	1.04 (0.20–5.37)	0.962	1.40 (0.28–7.00)	0.682	1.74 (0.28–10.78)	0.554
TAT complexes	1.36 (1.09–1.68)	0.005	1.31 (1.06–1.62)	0.013	1.33 (1.06–1.68)	0.015
VWF	1.44 (0.39–5.32)	0.582	1.78 (0.48–6.67)	0.390	7.31 (1.33–40.07)	0.022
PMN elastase–α1–PI complexes	0.99 (0.97–1.00)	0.146	0.99 (0.97–1.01)	0.207	0.99 (0.97–1.01)	0.229
sCD163	1.00 (1.00–1.00)	0.581	1.00 (1.00–1.00)	0.695	1.00 (1.00–1.00)	0.399
CXCL4/PF4	1.11 (0.76–1.62)	0.577	0.95 (0.64–1.42)	0.808	0.77 (0.47–1.27)	0.312
VKA therapy (yes = 1)	2.59 (0.59–11.31)	0.205	0.93 (0.19–4.54)	0.933	3.62 (0.67–19.56)	0.135
Lipid-lowering therapy (yes = 1)	2.34 (0.87–6.30)	0.092	1.43 (0.52–3.92)	0.485	1.54 (0.48–4.92)	0.470
Antihypertensive therapy (yes = 1)	1.90 (0.59–6.15)	0.282	2.24 (0.68–7.33)	0.184	2.84 (0.75–10.80)	0.126
Aspirin therapy (yes = 1)	1.02 (0.41–2.51)	0.973	0.72 (0.28–1.85)	0.494	1.93 (0.60–6.18)	0.266

dsDNA, double stranded DNA; MPO, myeloperoxidase; PF4, platelet factor 4; α1–PI, α1–proteinase inhibitor; PMN, polymorphonuclear; VWF, von Willebrand factor; VKA, vitamin K antagonists.

Associations Between Markers of Cell Death and NETosis and the Extent and Phenotype of Coronary Atherosclerosis

In all patients who underwent CCTA, we were able to assess the number of coronary artery segments affected by atherosclerosis, the degree of luminal stenosis and characteristics with respect to plaque morphology. In this population, we found a significant positive association between the number of diseased coronary artery segments and plasma dsDNA (Spearman's rho=0.242; *P*<0.001), nucleosomes (Spearman's rho=0.219; *P*=0.001), MPO-DNA (Spearman's rho=0.337; *P*<0.001), TAT complexes (Spearman's rho=0.330; *P*<0.001), and VWF levels (Spearman's rho=0.155; *P*=0.015; data not shown). Using a multiple linear regression model (Table 3) and adjusting for various confounding factors, we examined the independent relationships between candidate determinants and the extent of coronary atherosclerosis in all patients who underwent CCTA. Standardized regression coefficients β are reported in Table 3. The involvement score (number of affected coronary segments) was mainly determined by male gender, statin use, elevated plasma nucleosomes (β=0.140; *P*=0.026), MPO-DNA (β=0.134; *P*=0.041) and TAT levels (β=0.164; *P*=0.016). Age, family history of premature CAD, and dsDNA remained of borderline statistical significance. With respect to phenotype of coronary plaques, nucleosomes (β=0.152; *P*=0.022) and TAT complexes (β=0.151; *P*=0.036), but also age, male gender, and family history of premature CAD, were found independently associated with the number of calcified plaques, quantified by CCTA (Table 3). We also observed an independent relationship among circulating dsDNA (β=0.201; *P*=0.005), TAT complexes (β=0.176; *P*=0.015), CXCL4/platelet factor 4 (β=0.128; *P*=0.042), male gender, and the number of mixed plaques. Free plasma dsDNA (β=0.224; *P*=0.003), MPO-DNA complexes (β=0.150; *P*=0.040) and VWF (β=0.152; *P*=0.022) were further associated with a non-calcified coronary atherosclerotic plaque phenotype.

Table 3. Multiple linear regression models for the extent and CAD phenotype as dependent variables.

	Number of segments with CAD		Number of calcified lesions		Number of mixed lesions		Number of non-calcified lesions	
Determinants	**β**	***P***	**β**	***P***	**β**	***P***	**β**	***P***
Age	0.128	0.052	0.154	0.027	0.103	0.138	-0.124	0.089
Gender (Male = 1)	0.220	0.002	0.196	0.009	0.163	0.030	0.014	0.856
Body Mass Index	0.060	0.312	0.064	0.309	0.004	0.948	0.045	0.497
Creatinine	-0.015	0.827	-0.007	0.920	0.001	0.989	-0.040	0.602
Smoking (Yes = 1)	0.068	0.258	0.049	0.436	0.054	0.395	0.028	0.670
Family history of CAD (Yes = 1)	0.101	0.097	0.128	0.045	0.015	0.812	-0.002	0.979
Diabetes mellitus (Yes = 1)	-0.037	0.525	-0.025	0.686	-0.008	0.894	-0.059	0.362
dsDNA	0.123	0.065	-0.033	0.644	0.201	0.005	0.224	0.003
Nucleosomes	0.140	0.026	0.152	0.022	0.046	0.487	0.030	0.665
Citrullinated histone H4	-0.080	0.181	-0.092	0.147	-0.051	0.423	0.041	0.542
MPO–DNA complexes	0.134	0.041	0.109	0.117	0.036	0.602	0.150	0.040
TAT complexes	0.164	0.016	0.151	0.036	0.176	0.015	-0.101	0.182
VWF	0.088	0.141	0.007	0.915	0.098	0.122	0.152	0.022
PMN elastase–α1-PI complexes	0.051	0.425	0.061	0.369	0.032	0.636	-0.031	0.660
sCD163	-0.065	0.274	-0.015	0.805	-0.065	0.303	-0.095	0.149
CXCL4/PF4	0.005	0.938	-0.078	0.208	0.128	0.042	0.018	0.782
VKA therapy (Yes = 1)	0.069	0.263	0.102	0.121	-0.021	0.751	0.016	0.816
Lipid-lowering therapy (Yes = 1)	0.145	0.021	0.094	0.156	0.111	0.095	0.102	0.142
Antihypertensive therapy (Yes = 1)	0.020	0.736	0.029	0.646	0.010	0.876	-0.022	0.741
Aspirin therapy (Yes = 1)	-0.007	0.910	-0.018	0.786	-0.002	0.977	0.027	0.694

Standardized coefficient β indicates the change of the dependent variable (in SD) that is induced by 1 SD change in the determinant. Abbreviations see Table 2.

Extracellular DNA Is an Independent Determinant of a Pronounced Prothrombotic State

Because extracellular DNA, nucleosomes and neutrophil extracellular traps (NETs) have been functionally implicated in a number of prothrombotic mechanisms both in vitro and in vivo [3,12,25], we investigated the associations between extracellular dsDNA and TAT and VWF levels, which are considered well-established prothrombotic markers in various clinical conditions, including atherosclerosis and atherothrombosis [26]. In the entire study population, significant positive correlations were observed between TAT formation and dsDNA (Spearman's rho=0.367; $P<0.001$), nucleosomes (Spearman's rho=0.195; $P=0.001$), citrullinated histone H4 (Spearman's rho=0.231; $P<0.001$), MPO-DNA complexes (Spearman's rho=0.322; $P<0.001$), PMN elastase-α1-PI complexes (Spearman's rho=0.274; $P<0.001$) and VWF (Spearman's rho=0.127; $P=0.033$; data not shown). In a multiple linear regression model, after accounting for various confounding factors (Table 4), dsDNA (β=0.306; $P<0.001$), MPO-DNA (β=0.230; $P<0.001$) and PMN elastase-α1-PI complexes (β=0.177; $P=0.003$) independently predicted thrombin generation.

Plasma levels of VWF showed significant positive associations with dsDNA (Spearman's rho=0.171; $P=0.004$), nucleosomes (Spearman's rho=0.132; $P=0.026$) and citrullinated histone H4 levels (Spearman's rho=0.322; $P<0.001$). As shown in Table 4, VWF levels were independently determined by both dsDNA (β=0.146; $P=0.037$) and CXCL4/platelet factor 4 (β=0.200; $P=0.001$) levels in plasma. Overall, there seems to be a strong relationship among extracellular DNA generation (dsDNA), markers of NETosis (MPO-DNA complexes; citrullinated histone H4) and the presence of a prothrombotic state, as determined by the increase in TAT and VWF levels (Fig 3).

High Baseline Levels of Circulating DNA, Nucleosomes and Markers of NETs Are Significantly Associated With the Occurrence of Major Adverse Cardiac Events

During a median total follow-up of 545 days (interquartile range 446–666), 27 (9.7%) patients suffered from MACEs (11 PCIs, 10 CABGs, 4 ACS, 2 cardiac deaths). MACEs occurred more frequently in men (92.6%), with a median age of 59 years (min-max: 42-76), who were overweight (66.7%) and who were less often diabetic (14.8%).

Table 4. Multiple linear regression models: prothrombotic markers as dependent variables.

	TAT complexes		Von Willebrand Factor	
Determinants	**β**	***P* value**	**β**	***P* value**
Age	0.050	0.408	-0.005	0.937
Gender (Male = 1)	-0.008	0.902	0.056	0.432
Body Mass Index	0.020	0.713	-0.064	0.296
Creatinine	-0.088	0.154	-0.032	0.654
Smoking (Yes = 1)	-0.027	0.628	0.052	0.409
Family history of CAD (Yes = 1)	0.029	0.592	-0.057	0.358
Diabetes mellitus (Yes = 1)	-0.022	0.685	0.025	0.686
dsDNA	0.306	<0.001	0.146	0.037
Nucleosomes	0.041	0.486	0.056	0.398
Citrullinated histone H4	0.037	0.499	0.084	0.174
MPO–DNA complexes	0.230	<0.001	0.032	0.634
TAT complexes	---		-0.027	0.699
Von Willebrand Factor	-0.021	0.699	---	
PMN elastase–α1-PI complexes	0.177	0.003	0.018	0.788
sCD163	0.069	0.197	0.083	0.176
CXCL4/PF4	0.050	0.351	0.200	0.001
VKA therapy (Yes = 1)	0.001	0.992	0.094	0.150
Lipid-lowering therapy (Yes = 1)	-0.041	0.463	-0.039	0.546
Antihypertensive therapy (Yes = 1)	0.056	0.317	0.073	0.246
Aspirin therapy (Yes = 1)	0.032	0.573	0.045	0.495

Standardized coefficient β indicates the change of the dependent variable (in SD) that is induced by 1 SD change in the determinant. --- = The parameter was excluded from the model. Abbreviations, see Table 2.

Two patients died because of non-cardiac reasons and were excluded from the outcome analyses (Table 5). Significantly elevated baseline levels of circulating dsDNA (*P*=0.0016), nucleosomes (*P*=0.0013), MPO-DNA (*P*=0.0169), TAT (*P*=0.0042) and PMN elastase-α1-PI complexes (*P*=0.0011) were observed in the group who suffered a MACE compared with the event-free group (Figure 4).

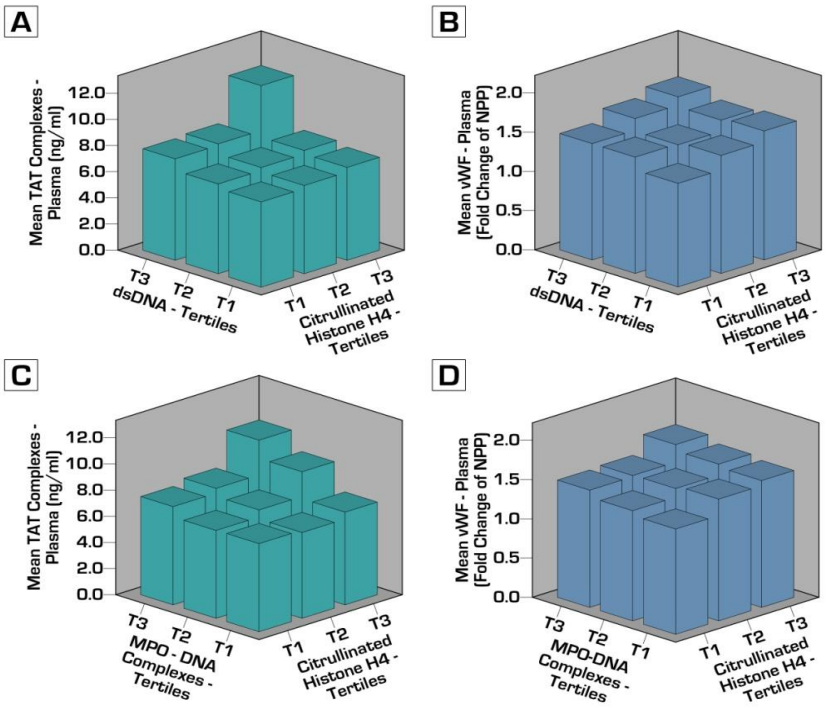


Figure 3. *Relationship among extracellular DNA generation, NETosis markers, and a prothrombotic state. Panels A and B show the relationship among levels of double-stranded DNA (dsDNA), citrullinated histone H4, and mean thrombin-antithrombin (TAT) levels (panel A) or mean von Willebrand factor (VWF) levels (panel B), respectively. Panels C and D show the relationship among levels of myeloperoxidase (MPO)-DNA, citrullinated histone H4, and mean TAT levels (panel C) or mean VWF levels (panel D). Elevated levels of both extracellular DNA generation (dsDNA) and NETosis markers (MPO-DNA complexes; citrullinated histone H4) are associated with the presence of a prothrombotic state, defined by increased TAT and VWF levels. NPP indicates normal pooled plasma; and T indicates tertile.*

To gain further insight into the predictive value of low versus high levels of the tested markers, we dichotomized all continuous predictor variables into two groups by using a median split. High baseline values (≥ total group median) of dsDNA (OR: 3.12; 95% CI 1.27–7.63; *P*=0.013), nucleosomes (OR: 2.59; 95% CI 1.09–6.14; *P*=0.030), MPO-DNA (OR: 3.53; 95% CI 1.38–9.03; *P*=0.009), TAT (OR: 2.59; 95% CI 1.09–6.14; *P*=0.030) and PMN elastase-α1-PI complexes (OR: 3.22; 95% CI 1.31–7.88; *P*=0.011) were significantly associated with the occurrence of MACEs (Table 6).

Table 5. Major adverse events.

Major adverse cardiac events	Patients
Percutaneous coronary intervention (PCI)	11
Coronary artery bypass grafting (CABG)	10
Acute coronary syndrome (ACS)	4
Cardiac death	2

Major adverse non-cardiac events	
Cerebrovascular death	1
Death due to cancer	1

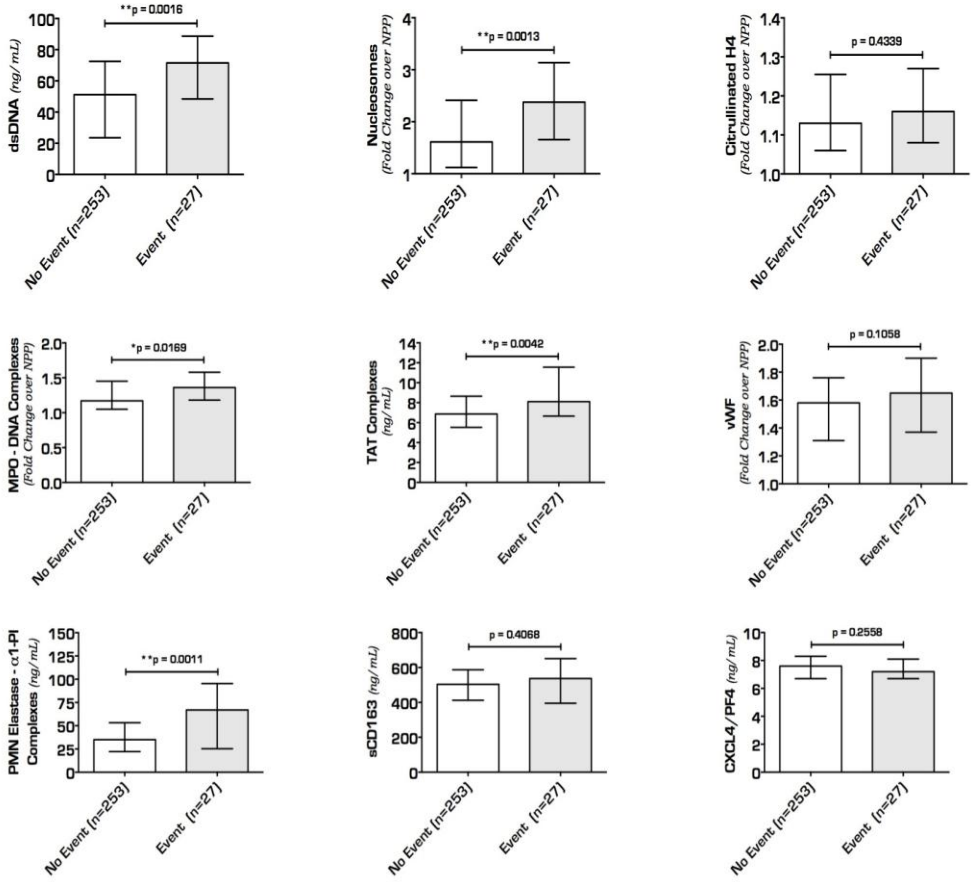


Figure 4 (previous page). *All tested plasma markers according to the occurrence of MACEs. dsDNA, double stranded DNA; MPO, myeloperoxidase; NPP, normal pooled plasma; PF4, platelet factor 4; α1-PI, alpha 1-proteinase inhibitor; PMN, polymorphonuclear; TAT, thrombin–antithrombin; VWF, von Willebrand factor. * P<0.05; ** P<0.01; *** P<0.001.*

Table 6. Dichotomized plasma markers (high versus low values).

Predictors	**Relative risk OR (95% CI)**	***P* value**
dsDNA	3.12 (1.27-7.63)	0.013
Nucleosomes	2.59 (1.09-6.14)	0.030
Citrullinated H4	1.51 (0.67-3.43)	0.324
MPO-DNA complexes	3.53 (1.38-9.03)	0.009
TAT complexes	2.59 (1.09-6.14)	0.030
VWF	1.66 (0.73-3.77)	0.225
PMN elastase–α1–PI complexes	3.22 (1.31-7.88)	0.011
sCD163	1.49 (0.67-3.34)	0.333
CXCL4/PF4	0.51 (0.23-1.17)	0.111

High values ≥ total group median, while low values < total group median. Abbreviations, see Table 2.

In addition, we investigated CAD characteristics in all 245 patients that underwent CCTA. Patients developing MACE had a significantly higher involvement score: 5.7 vs. 2.8, *P*<0.001. These patients showed significantly more mixed plaques (1.9±1.4 versus 0.9±1.2; *P*=0.002), as well as more calcified plaques (3.2±2.5 versus 1.6±2.0; *P*=0.003). As expected, ROC analysis indicated that CT score (stenosis >70%) is very useful to discriminate between patients with or without MACEs (AUROC: 0.83; 95% CI 0.71–0.95; *P*<0.001). In comparison, measurement of dsDNA, nucleosomes, MPO-DNA, TAT and PMN elastase-α1-PI complexes also showed the potential to significantly predict MACE during follow-up (AUROC: 0.76; 95% CI 0.68–0.89; *P*<0.001). There was no significant difference between the predictive capacity of CCTA and the aforementioned plasma biomarkers (difference between AUROCs, 0.04; *P*=0.571).

However, the addition of these biomarkers to CT score (stenosis >70%) improved its predictive value, whereas the difference between the AUROCs as compared to CT score alone indicated a trend toward statistical significance (AUROC, 0.92; 95% CI 0.87–0.96; *P*<0.001; difference between AUROCs, 0.09; *P*=0.086).

Table 7. One-way analysis of variance (ANOVA) and multiple comparisons.

Dependent variable	**sCD163 – Tertiles**		***P* value**
dsDNA	T1	T2	1.000
		T3	0.157
Nucleosomes	T1	T2	1.000
		T3	1.000
Citrullinated histone H4	T1	T2	1.000
		T3	0.148
MPO–DNA complexes	T1	T2	1.000
		T3	1.000

Dependent variable	**PMN Elastase – Tertiles**		***P* value**
dsDNA	T1	T2	1.000
		T3	<0.001
Nucleosomes	T1	T2	0.222
		T3	0.002
Citrullinated histone H4	T1	T2	1.000
		T3	0.214
MPO–DNA complexes	T1	T2	1.000
		T3	<0.001

Statistical significance at the *P*<0.05 level (post-hoc Bonferroni multiple comparison test). dsDNA, double stranded DNA; MPO, myeloperoxidase; T, tertile.

Both neutrophil and monocyte hyperactivation have been demonstrated to play a key role in the initiation of prothrombotic responses [27].

To provide a better understanding on the potential origin of extracellular DNA traps, we performed additional analyses by stratifying all cell death and NETs markers by tertiles of either plasma PMN elastase-α1-PI complexes or sCD163, considered sensitive markers of neutrophil and monocyte activation, respectively. Although extracellular DNA might originate from different cell types, here we focused on neutrophils and monocytes because of their significant role in the pathogenesis of atherosclerosis, their involvement in ETosis, but also due to the fact that neutrophils are the most predominant white blood cell type in circulation. Our data suggests that the increase in plasma nucleosomes and MPO-DNA complexes is strictly specific to neutrophil activation and thus might be considered potential markers of NET formation (Table 7). Platelet activation, as reflected by CXCL4/platelet factor 4 levels, showed a weak positive correlation with citrullinated histone H4 only (Spearman's rho=0.138; *P*=0.020). Significant positive associations were observed between neutrophil activation marker PMN elastase-α1-PI complexes and dsDNA (Spearman's rho=0.257; *P*<0.001), nucleosomes (Spearman's rho=0.209; *P*<0.001) and MPO-DNA complexes (Spearman's rho=0.240; *P*<0.001).

DISCUSSION

The current cross-sectional observational prospective clinical study is the first to examine the relationship among plasma markers of extracellular DNA, circulating nucleosomal fragments, NET formation and the severity, extent, and phenotype of CCTA-defined coronary atherosclerosis in individuals with suspected CAD.

The principal finding in this study is the independent association between increased levels of circulating markers of cell death and NETosis and the severity and extent of CAD in patients with chest discomfort symptoms. Furthermore, we provide new data of potential clinical relevance, which demonstrate an independent relationship between extracellular DNA generation and the presence of a prothrombotic state in patients with CAD. Increased baseline levels of circulating dsDNA, nucleosomes and markers of NETosis were also significantly associated with the occurrence of MACEs during follow-up. Importantly, these biomarkers could potentially aid in the prediction of MACE in patients with chest discomfort.

Endothelial injury or dysfunction, driven by distinct hemodynamic, oxidative, biochemical and proinflammatory insults (eg, smoking, perturbed lipid metabolism, or hypertension), precedes the onset of atherosclerosis [28]. In response to tissue injury, the host defense system promotes wound healing by triggering a variety of inflammatory and hemostatic reactions, designed to restore the homeostatic equilibrium [29]. A state of persistent activation and cross talk between inflammation and coagulation can result either in exacerbation of tissue injury (eg, atherosclerotic plaque progression) or thrombosis [30,31]. In fact, chronic inflammation and hypercoagulability are considered integral mechanisms in the pathogenesis of both atherosclerosis and thrombosis [30,32]. A complex network of cellular and molecular interactions, bridging innate and adaptive immunity in atherogenesis, orchestrate all proinflammatory, fibroproliferative and prothrombotic changes in the arterial vessel wall [33,34]. Neutrophils are the most abundant white blood cell type responsible for the early response to tissue injury. Neutrophils migrate to the site of tissue damage and extrude decondensed chromatin threads (NETs), consisting of nuclear histones and azurophilic granule proteins, such as MPO and PMN elastase [4]. Histone degradation and citrullination, driven by PMN elastase and peptidylarginine deiminase 4, respectively, are key processes, which comprise the cornerstone of chromatin decondensation and subsequent NET formation [3,35].

Histological studies have reported the presence of NETs within the luminal portion of human atherosclerotic vessels, but also in coronary thrombosuction specimens obtained from patients after acute myocardial infarction [36,37]. There are various potential pathways via which extracellular DNA traps might induce either initiation or exacerbation of atherosclerosis. Extracellular DNA represents a link between the innate and adaptive immune systems and may aggravate atherosclerosis through activation of T lymphocytes and antigen-presenting cells [38,39]. The chronic inflammatory atherosclerotic environment can induce neutrophil priming, resulting in enhanced neutrophil activation and MPO-dependent respiratory burst [40]. Elevated MPO levels independently predict endothelial dysfunction, the risk of CAD and ACS in patients [41]. Here, we demonstrate an independent association among increased MPO-DNA levels, a marker of NETosis, and the extent of CAD and presence of a hypercoagulable state.

Neutrophils can undergo apoptosis during inflammation. Macrophages play a crucial role in the clearance of apoptotic neutrophils, thus resulting in resolution of inflammation. This process is also known as efferocytosis. In patients with ACS, delayed neutrophil apoptosis is a commonly observed phenomenon [42,43]. Inflammation can be exacerbated as a result of overloaded efferocytosis [44]. Interestingly, extracellular histones (H3 and H4) significantly impair the clearance of apoptotic neutrophils by macrophages, and activated protein C, known to cleave histones, restores the efferocytotic capacity of macrophages [6,45]. Hence, extracellular DNA trap formation may have deleterious effects by aggravating chronic inflammation during atherosclerosis progression. There are several clinical studies demonstrating a significant increase in circulating deoxyribonuclease I (DNase I) levels during acute coronary events [46,47]. Since DNase I is an endonuclease, which selectively cleaves DNA and contributes to extracellular chromatin degradation, this may be considered additional indirect evidence, suggesting a role for extracellular DNA in the pathogenesis of myocardial infarction [48]. Recent experimental studies show that administration of DNase I prevents thrombus formation in mouse models of deep vein thrombosis [8,49].

Extracellular DNA and histones also exert powerful prothrombotic effects in vitro and in vivo [3]. Nucleosomes and histones can promote thrombin formation through the activation of either extrinsic or intrinsic coagulation pathways and through platelet activation [7,12,49-51].

In addition, excess of extracellular histones can affect the function of the anticoagulant protein C pathway by inhibiting protein C activation, thus resulting in enhanced thrombin formation [52]. PMN elastase, which is an integral component of NETs, cleaves tissue factor pathway inhibitor and promotes thrombin generation in a factor Xa-dependent manner [53]. To our knowledge, this is the first clinical study to demonstrate an independent association among increased extracellular DNA generation, TAT formation, and VWF release in cardiac patients. We show that steadily increasing TAT levels are also independently linked to an increased degree of coronary artery stenosis and plaque extent, also comparable to our previous findings [54]. Thrombin is a key molecule that is important to hemostasis, and also to atherogenesis [30,55,56]. Hence, one can postulate that circulating DNA, chromatin, and possibly NETs might exacerbate atherosclerosis via coagulation activation.

Our data demonstrated that increased levels of plasma nucleosomes, which indicate ongoing chromatin decondensation, predicted the number of calcified plaques and were not associated with any other plaque phenotype. However, it seems that other cell death markers, such as dsDNA, were not phenotype specific. None of the markers more specific to NET formation (citrullinated histone H4 and MPO-DNA complexes) were independently associated with an increased risk of any type of CAD. MPO-DNA complexes seemed a useful tool only in patients with confirmed CAD, as they predicted the extent and number of non-calcified lesions [57,58].

Not all studied patients underwent CCTA. In 37 patients the coronary calcium score scan already revealed an extremely high calcium score. In those patients, CCTA was waived because of the very high a priori chance for a nondiagnostic test result. Predominantly as a result of blooming artifacts, reliable estimation of the severity of the coronary plaque becomes greatly impaired. Furthermore, it is known that blooming can lead to overestimation of the severity of CAD [59]. We know at least that these patients have calcified plaques, but it is conceivable and even plausible that they also have mixed and non-calcified plaques. However, we were not able to prove this because CCTA was waived. Despite the fact that we can not exactly say to what extent these patients have obstructive CAD, it is known that a high calcium score is associated with an increased risk for severe stenosis and cardiovascular events. In fact, it is even considered to be a better predictor than the Framingham risk score [60]. Instead of excluding these patients, we therefore considered them as a separate high-risk group.

Our data indicate that measurement of biomarkers, such as dsDNA, nucleosomes, MPO-DNA, TAT and PMN elastase-α1-PI complexes, may be useful for the evaluation of patients with chest discomfort. Although the addition of these five biomarkers to CT score did not significantly improve risk stratification, we observed a trend in increasing the prediction capacity of traditional CCTA. However, one should consider that CT score provides significant prognostic information, thus it might be difficult to establish an incremental value for any plasma biomarker measured. Larger studies with a longer follow-up are needed to better assess the sensitivity and specificity of all tested biomarkers to identify vulnerable plaques in patients with coronary atherosclerosis, as well as to study their potential to predict the occurrence of MACEs.

It also remains of interest to further test whether levels of the different markers predict adverse outcomes within a single CAD phenotype, as determined by CCTA. Because some of these tests (eg, dsDNA) are inexpensive and technically simple to determine, a broader use might be considered even before CCTA, if they prove useful as diagnostic and prognostic tools in patients suspected of having CAD.

The relatively short-term follow-up may be considered a limitation, which resulted in smaller numbers of recorded composite end points. Although not established in this cohort of patients, it remains of interest to also study the relationship among extracellular DNA and chromatin release, NETosis, and neutrophil counts. High neutrophil counts are considered a potent inflammatory marker for risk stratification in patients with coronary atherosclerosis. Although we found independent associations between elevated markers of cell death and NETosis and the occurrence of MACEs, it is too early yet to use these markers in daily clinical practice. Longitudinal prospective studies will unravel their prognostic power, whereas mechanistic studies are needed to establish whether there is a causal relationship between NET formation and atherosclerotic plaque progression.

CONCLUSION

Our report provides evidence demonstrating that elevated levels of markers of extracellular DNA, chromatin, and NETosis are independently associated with the severity, extent and phenotype of coronary atherosclerosis and with the occurrence of MACEs. Our data reveal potential clinical application of these biomarkers to predict cardiovascular risk in patients. Further experimental and clinical studies are necessary to explore the involvements of NETs in the pathogenesis of atherosclerosis and atherothrombosis.

REFERENCES

1. Roger VL, Go AS, Lloyd-Jones DM, et al. Executive summary: Heart disease and stroke statistics--2012 update: A report from the american heart association. *Circulation* 2012;125:188–197.

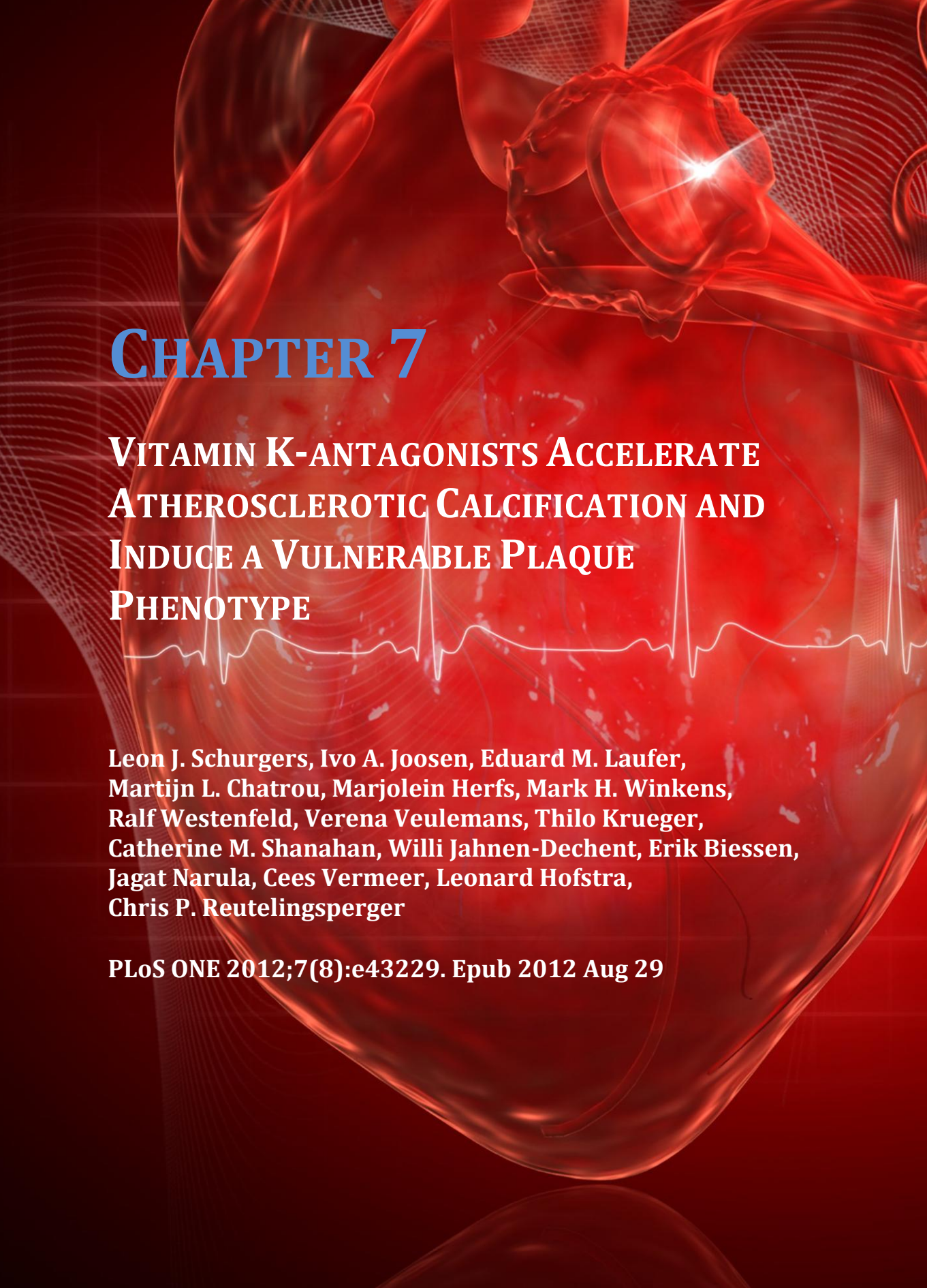
2. Mahmoudi M, Mercer J, Bennett M. DNA damage and repair in atherosclerosis. *Cardiovasc Res* 2006;71:259–268.
3. Fuchs TA, Brill A, Wagner DD. Neutrophil extracellular trap (net) impact on deep vein thrombosis. *Arterioscler Thromb Vasc Biol* 2012;32:1777–1783.
4. Brinkmann V, Reichard U, Goosmann C, et al. Neutrophil extracellular traps kill bacteria. *Science* 2004;303:1532–1535.
5. Tufano A, Di Capua M, Coppola A, et al. The infectious burden in atherothrombosis. *Semin Thromb Hemost* 2012;38:515–523.
6. Xu J, Zhang X, Pelayo R, et al. Extracellular histones are major mediators of death in sepsis. *Nature Medicine* 2009;15:1318–1321.
7. Fuchs TA, Bhandari AA, Wagner DD. Histones induce rapid and profound thrombocytopenia in mice. *Blood* 2011;118:3708–3714.
8. Brill A, Fuchs TA, Savchenko AS, et al. Neutrophil extracellular traps promote deep vein thrombosis in mice. *J Thromb Haemost* 2012;10:136–144.
9. Thomas GM, Carbo C, Curtis BR, et al. Extracellular DNA traps are associated with the pathogenesis of trali in humans and mice. *Blood* 2012;119:6335–6343.
10. Demers M, Krause DS, Schatzberg D, et al. Cancers predispose neutrophils to release extracellular DNA traps that contribute to cancer-associated thrombosis. *Proc Natl Acad Sci USA* 2012;109:13076–13081.
11. van Montfoort ML, Stephan F, Lauw MN, et al. Circulating nucleosomes and neutrophil activation as risk factors for deep vein thrombosis. *Arterioscler Thromb Vasc Biol* 2013;33:147–151.
12. Massberg S, Grahl L, von Bruehl ML, et al. Reciprocal coupling of coagulation and innate immunity via neutrophil serine proteases. *Nature Medicine* 2010;16:887–896.
13. Min JK, Shaw LJ, Berman DS. The present state of coronary computed tomography angiography a process in evolution. *J Am Coll Cardiol* 2010;55:957–965.
14. Hulten EA, Carbonaro S, Petrillo SP, et al. Prognostic value of cardiac computed tomography angiography: A systematic review and meta-analysis. *J Am Coll Cardiol* 2011;57:1237–1247.
15. Taylor AJ, Cerqueira M, Hodgson JM, et al. ACCF/SCCT/ACR/AHA/ASE/ASNC/NASCI/ SCAI/SCMR 2010 appropriate use criteria for cardiac computed tomography. *J Am Coll Cardiol* 2010;56:1864–1894.

16. ECDCDM. Report of the expert committee on the diagnosis and classification of diabetes mellitus. *Diabetes care* 2003;26:5–20.
17. The seventh report of the joint national committee on prevention, detection, evaluation, and treatment of high blood pressure. *Bethesda (MD)*; 2004.
18. Austen WG, Edwards JE, Frye RL, et al. A reporting system on patients evaluated for coronary artery disease. *Circulation* 1975;51:5–40.
19. Raff GL, Abidov A, Achenbach S, et al. SCCT guidelines for the interpretation and reporting of coronary computed tomographic angiography. *J Cardiovasc Comput Tomogr* 2009;3:122–136.
20. Min JK, Shaw LJ, Devereux RB, et al. Prognostic value of multidetector coronary computed tomographic angiography for prediction of all-cause mortality. *J Am Coll Cardiol* 2007;50:1161–1170.
21. Fuchs TA, Kremer Hovinga JA, Schatzberg D, et al. Circulating DNA and myeloperoxidase indicate disease activity in patients with thrombotic microangiopathies. *Blood* 2012;120:1157–1164.
22. Kessenbrock K, Krumbholz M, Schonermarck U, et al. Netting neutrophils in autoimmune small-vessel vasculitis. *Nature Medicine* 2009;15:623–625.
23. Andersson HM, Siegerink B, Luken BM, et al. High vwf, low adamts13, and oral contraceptives increase the risk of ischemic stroke and myocardial infarction in young women. *Blood* 2012;119:1555–1560.
24. Braunwald E, Antman EM, Beasley JW, et al. ACC/AHA 2002 guideline update for the management of patients with unstable angina and non-st-segment elevation myocardial infarction--summary article. *J Am Coll Cardiol* 2002;40:1366–1374.
25. Borissoff JI, ten Cate H. From neutrophil extracellular traps release to thrombosis: An overshooting host-defense mechanism? *J Thromb Haemost* 2011;9:1791–1794.
26. Lowe G. Can haemostatic factors predict atherothrombosis? *Intern Emerg Med* 2011;6:497–501.
27. Gawaz M. The evolving science of atherothrombotic disease. *Eur Heart J Supplements* 2008;10:I4–I7.
28. Nabel EG, Braunwald E. A tale of coronary artery disease and myocardial infarction. *N Engl J Med* 2012;366:54–63.
29. Delvaeye M, Conway EM. Coagulation and innate immune responses: Can we view them separately? *Blood* 2009;114:2367–2374.

30. Borissoff JI, Spronk HM, ten Cate H. The hemostatic system as a modulator of atherosclerosis. *N Engl J Med* 2011;364:1746–1760.
31. Engelmann B, Massberg S. Thrombosis as an intravascular effector of innate immunity. *Nat Rev Immunol* 2013;13:34–45.
32. Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. *N Engl J Med* 2005;352:1685–1695.
33. Weber C, Noels H. Atherosclerosis: Current pathogenesis and therapeutic options. *Nature Medicine* 2011;17:1410–1422.
34. Borissoff JI, Heeneman S, Kilinc E, et al. Early atherosclerosis exhibits an enhanced procoagulant state. *Circulation* 2010;122:821–830.
35. Martinod K, Demers M, Fuchs TA, et al. Neutrophil histone modification by peptidylarginine deiminase 4 is critical for deep vein thrombosis in mice. *Proc Natl Acad Sci USA* 2013;110:8674–8679.
36. Megens RT, Vijayan S, Lievens D, et al. Presence of luminal neutrophil extracellular traps in atherosclerosis. *Thromb Haemost* 2012;107:597–598.
37. deBoer OJ, Li X, Teeling P, et al. Neutrophils, neutrophil extracellular traps and interleukin-17 associate with the organisation of thrombi in acute myocardial infarction. *Thromb Haemost* 2013;109:290–297.
38. Tillack K, Breiden P, Martin R, et al. T lymphocyte priming by neutrophil extracellular traps links innate and adaptive immune responses. *J Immunol* 2012;188:3150–3159.
39. Doring Y, Manthey HD, Drechsler M, et al. Auto-antigenic protein-DNA complexes stimulate plasmacytoid dendritic cells to promote atherosclerosis. *Circulation* 2012;125:1673–1683.
40. Soehnlein O. Multiple roles for neutrophils in atherosclerosis. *Circ Res* 2012;110:875–888.
41. Vita JA, Brennan ML, Gokce N, et al. Serum myeloperoxidase levels independently predict endothelial dysfunction in humans. *Circulation* 2004;110:1134–1139.
42. Garlichs CD, Eskafi S, Cicha I, et al. Delay of neutrophil apoptosis in acute coronary syndromes. *J Leukoc Biol* 2004;75:828–835.
43. Biasucci LM, Liuzzo G, Giubilato S, et al. Delayed neutrophil apoptosis in patients with unstable angina: Relation to c-reactive protein and recurrence of instability. *Eur Heart J* 2009;30:2220–2225.
44. Soehnlein O, Lindbom L. Phagocyte partnership during the onset and resolution of inflammation. *Nat Rev Immunol* 2010;10:427–439.

45. Friggeri A, Banerjee S, Xie N, et al. Extracellular histones inhibit efferocytosis. *Mol Med*. 2012;18:825–833.
46. Kawai Y, Yoshida M, Arakawa K, et al. Diagnostic use of serum deoxyribonuclease i activity as a novel early-phase marker in acute myocardial infarction. *Circulation* 2004;109:2398–2400.
47. Morikawa N, Kawai Y, Arakawa K, et al. Serum deoxyribonuclease i activity can be used as a novel marker of transient myocardial ischaemia: Results in vasospastic angina pectoris induced by provocation test. *Eur Heart J* 2007;28:2992–2997.
48. Kumamoto T, Kawai Y, Arakawa K, et al. Association of gln222arg polymorphism in the deoxyribonuclease i (dnase i) gene with myocardial infarction in japanese patients. *Eur Heart J* 2006;27:2081–2087.
49. von Bruhl ML, Stark K, Steinhart A, et al. Monocytes, neutrophils, and platelets cooperate to initiate and propagate venous thrombosis in mice in vivo. *J Exp Med* 2012;209:819–835.
50. Semeraro F, Ammollo CT, Morrissey JH, et al. Extracellular histones promote thrombin generation through platelet-dependent mechanisms: Involvement of platelet tlr2 and tlr4. *Blood* 2011;118:1952–1961.
51. Fuchs TA, Brill A, Duerschmied D, et al. Extracellular DNA traps promote thrombosis. *Proc Natl Acad Sci USA* 2010;107:15880–15885.
52. Ammollo CT, Semeraro F, Xu J, et al. Extracellular histones increase plasma thrombin generation by impairing thrombomodulin-dependent protein c activation. *J Thromb Haemost* 2011;9:1795–1803.
53. Higuchi DA, Wun TC, Likert KM, et al. The effect of leukocyte elastase on tissue factor pathway inhibitor. *Blood* 1992;79:1712–1719.
54. Borissoff JI, Joosen IA, Versteylen MO, et al. Accelerated in vivo thrombin formation independently predicts the presence and severity of ct angiographic coronary atherosclerosis. *JACC Cardiovasc Imaging* 2012;5:1201–1210.
55. Borissoff JI, Spronk HM, Heeneman S, et al. Is thrombin a key player in the "coagulation-atherogenesis" maze? *Cardiovasc Res* 2009;82:392–403.
56. Borissoff JI, Otten JJ, Heeneman S, et al. Genetic and pharmacological modifications of thrombin formation in apolipoprotein e-deficient mice determine atherosclerosis severity and atherothrombosis onset in a neutrophil-dependent manner. *PloS One* 2013;8:e55784.

57. Motoyama S, Sarai M, Harigaya H, et al. Computed tomographic angiography characteristics of atherosclerotic plaques subsequently resulting in acute coronary syndrome. *J Am Coll Cardiol* 2009;54:49–57.
58. Versteylen MO, Kietselaer BL, Dagnelie PC, et al. Additive value of semi-automated quantification of coronary artery disease using cardiac CT-angiography to predict for future acute coronary syndrome. *J Am Coll Cardiol* 2013;61:2296–2305.
59. Zhang S, Levin DC, Halpern EJ, et al. Accuracy of mdct in assessing the degree of stenosis caused by calcified coronary artery plaques. *Am J Roentgenol* 2008;191:1676–1683.
60. Alexopoulos N, Raggi P. Calcification in atherosclerosis. *Nat Rev Cardiol* 2009;6:681–688.



CHAPTER 7

VITAMIN K-ANTAGONISTS ACCELERATE ATHEROSCLEROTIC CALCIFICATION AND INDUCE A VULNERABLE PLAQUE PHENOTYPE

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PLoS ONE 2012;7(8):e43229. Epub 2012 Aug 29

BACKGROUND

Vitamin K-antagonists (VKA) are treatment of choice and standard care for patients with venous thrombosis and thromboembolic risk. In experimental animal models as well as humans, VKA have been shown to promote medial elastocalcinosis. As vascular calcification is considered an independent risk factor for plaque instability, we here investigated the effect of VKA on coronary calcification in patients and on calcification of atherosclerotic plaques in ApoE$^{-/-}$ mice.

METHODS

A total of 266 patients (133 VKA users and 133 gender and Framingham risk score matched non-VKA users) underwent 64-slice MDCT to assess the degree of coronary artery disease (CAD). ApoE$^{-/-}$ mice (10 weeks) received a Western type diet (WTD) for 12 weeks, after which mice were fed a WTD supplemented with vitamin K_1 (VK_1, 1.5 mg/g) or vitamin K_1 and warfarin (VK_1&W; 1.5 mg/g & 3.0 mg/g) for 1 or 4 weeks, after which mice were sacrificed.

RESULTS

VKA-users developed significantly more calcified coronary plaques as compared to non-VKA users. Warfarin significantly increased frequency and extent of vascular calcification in ApoE$^{-/-}$ mice. Also, plaque calcification comprised microcalcification of the intimal layer. Furthermore, warfarin treatment decreased plaque expression of calcification regulatory protein carboxylated matrix Gla-protein, increased apoptosis and, surprisingly outward plaque remodeling, without affecting overall plaque burden.

CONCLUSION

VKA use is associated with coronary artery plaque calcification in patients with suspected CAD and causes changes in plaque morphology with features of plaque vulnerability in ApoE$^{-/-}$ mice. Our findings underscore the need for alternative anticoagulants that do not interfere with the vitamin K cycle.

INTRODUCTION

Vitamin K antagonists (VKA) are the most frequently prescribed drugs to control blood coagulation of patients with thrombosis and patients at risk of thromboembolic events. VKA block the vitamin K epoxide reductase complex that drives conversion of certain glutamate residues of vitamin K-dependent coagulation factors into γ-carboxyglutamic acid (Gla)-residues [1]. VKA therapy may have undesired side-effects in addition to risk of bleeding because a number of proteins outside the coagulation system also require γ-glutamylcarboxylation to become biologically active [2].

Matrix Gla-protein (MGP) is a vitamin K-dependent protein not related to blood coagulation but also affected by VKA [3]. Animal models showed that MGP is a strong inhibitor of calcification of arterial vessel wall and cartilage [4]. In arteries, MGP acts as a local inhibitor of media calcification [5,6]. Its inhibitory mechanism is still not fully understood but involves inhibition of bone morphogenetic protein 2 and 4 (BMP-2 and -4) [7,8], suppression of osteochondrogenic transdifferentiation of vascular smooth muscle cells [9] and direct inhibition of calcium-crystal growth [10,11]; in all cases MGP requires vitamin K-dependent γ-carboxylation [10]. Concordantly, clinical studies and case reports revealed that VKA treatment is associated with arterial calcification and upregulation of uncarboxylated MGP (ucMGP) [12-15].

MGP expression is increased in human atherosclerotic lesions [16] and vascular smooth muscle cells (VSMCs) are predominantly involved in intimal calcification [17]. Overexpression of MGP in the apoE$^{-/-}$ mouse model of atherosclerosis reduced both intimal and medial calcification of atherosclerotic plaques whereas gene deletion of MGP in apoE$^{-/-}$ mice accelerated intimal calcification of plaques [18]. BMP-2 transgenic apoE$^{-/-}$ mice displayed increased calcification of intima of atheromatous lesions, suggesting a key role for MGP in suppressing BMP-2 induced vascular calcification [19]. Since intimal calcification of atherosclerotic plaques is considered a risk factor for plaque rupture [20,21] we were interested in effects of VKA on atherosclerotic intima calcification. The aim of this study was to investigate the effects of VKA on calcification of coronary atherosclerotic lesions in patients, using coronary CT-angiography (CCTA). CCTA allows quantifying calcification of vascular tissue but is insufficient to distinguish between medial and intimal calcification. Therefore, we investigated effects of VKA on calcification of atherosclerotic plaque in apoE$^{-/-}$ mice.

METHODS

Study Population

Between January 2008 and April 2010, a total of 1,973 patients underwent a coronary calcium score scan as well as coronary computed tomographic angiography (CCTA) in our medical center. All patients were referred from the cardiology outpatient department because of cardiac symptoms suspected for CAD. CCTA was performed as part of the diagnostic work-up in these patients. Included were patients with complete data regarding their cardiac risk profile in order to calculate the Framingham Risk Score (FRS). The FRS is used to estimate the 10-year risk of cardiovascular disease and takes the following risk factors into account: age, gender, diabetes mellitus, smoking status, systolic blood pressure (treated as well as untreated), total cholesterol and high-density lipoprotein (HDL). Excluded were patients presenting to the emergency department because of acute chest pain. Patients with a known history of CAD, especially a history of myocardial infarction or coronary revascularization, were also excluded from this analysis. Patients were asked if they are using VKA. The duration of VKA use was verified in medical records. The VKA users (n=133) were divided into tertiles, based on duration of VKA use (Table 1). Each VKA user was individually matched with a non-VKA user, based on equal FRS (Table 2). This study complies with the guidelines for good clinical practice and was performed in accordance with the Declaration of Helsinki.

Risk Factor Assessment

Cardiac risk factors were prospectively collected by the referring cardiologists. Patients were classified as diabetics if they were treated with hypoglycemic medication or in case of a fasting plasma glucose ≥ 6.7 mmol/L (≥ 121 mg/dL). Patients were classified as smoker if they were current smoker. A positive family history was defined as having a first degree with a history of myocardial infarction or sudden cardiac death before the age of sixty. Total cholesterol, HDL, triglycerides and glucose were measured with the Synchron LX20 (Beckman Coulter, Brea, CA, USA). LDL was calculated using the Friedewald equation.

CCTA Acquisition

Scans were performed using a 64-slice MDCT-scanner (Brilliance 64, Philips Healthcare, Best, the Netherlands) with a 64x0.625-mm slice collimation, a gantry rotation time of 420 ms and a tube voltage of 80 to 120 kV, depending on the patient's height and weight. Patients received 50 mg Metoprolol tartrate orally, two hours before CCTA. When indicated, an additional dose of 5-20 mg Selokeen (AstraZeneca, Zoetermeer, the Netherlands) was given intravenously to lower the heart rate <65 beats per minute (bpm). All patients received 0,8 mg nitroglycerin spray (Pohl-Boskamp, Hohenlockstedt, Germany) sublingually just prior to CCTA to achieve optimal vasodilatation. Heart rate and ECG were monitored during the scan.

A non-enhanced scan was performed to determine coronary calcium score using the Agatston method [22]. Subsequently, CCTA was performed using 85–110 mL of contrast agent (Xenetix 350; Guerbet, Roissy CdG Cedex, France), which was injected in the antecubital vein at a flow rate of 6.0 mL/s, directly followed by 40 mL intravenous saline (6.0 mL/s) using a dual-head power injector. The most appropriate scan protocol was chosen based on the patient's heart rate. In patients with a stable heart rate <65 bpm, a prospective ECG-gated 'step and shoot' protocol was used. In patients with a heart rate >65 bpm, a retrospective ECG-gated 'helical' protocol with dose modulation was used.

Coronary Plaque Assessment

All scans were independently analyzed by two experienced imagers (IJ, EL), blinded for patient details, using source images in the Cardiac Comprehensive Analysis software (Philips Healthcare, Best, The Netherlands). In case of disagreement, consensus was reached by reviewing findings jointly.

The coronary calcium score was expressed as the Agatston score using dedicated calcium-scoring software (Philips Healthcare, Best, the Netherlands). All consecutive pixels (minimal total area of 1 mm^2) with a threshold of 130 Hounsfield Units (HU) within the coronary arteries are considered as coronary calcification. The software automatically detects these coronary calcifications. The Agatston score for each calcification is calculated by multiplying the area of the lesion (mm^2) by a weighting factor, which depends on the maximum density of the pixels.

The total Agatston score is calculated by summing all individual calcifications [23]. The coronary artery tree was analyzed for the presence and severity of coronary artery disease, according to the 16-segment classification of the American Heart Association. Coronary plaques were defined as visible structures within or adjacent to the coronary artery lumen, which could be clearly distinguished from the vessel lumen and the surrounding pericardial tissue. Plaques were categorized as calcified (exclusively content with density >130 HU), non-calcified (exclusively content with density <130 HU) or mixed (characteristics of both calcified and non-calcified plaques). The degree of stenosis was classified as absent (no luminal stenosis), mild (<50% luminal stenosis), moderate (50-70% luminal stenosis) or severe (>70% luminal stenosis).

Follow-up

Follow-up was performed in all patients regarding the occurrence of coronary revascularization procedures, cardiac mortality and acute coronary syndromes (ACS), including myocardial infarction and unstable angina requiring hospitalization. ACS was defined as typical angina pectoris, troponin T elevation (>0.01 μg/L) and ST-segment elevation/depression of ≥1 mm, or at least two of these characteristics together with invasive angiographic confirmation of a culprit lesion [24]. Patients were seen by their cardiologist on a regular basis, and all hospital visits, both outpatient department visits as well as emergency room visits, were recorded in the electronic patient records. Additionally, the national mortality records were checked.

Animals & Diet

Both male and female apoE$^{-/-}$ mice were purchased from the Maastricht University. Mice were aged 10 weeks when entering the study and all animals were housed in normal cages with free access to water and the provided foods. Irradiated (0.9Mrad) vitamin K-deficient WTD (0.25% cholesterol and 15% cocoa butter) was from Arie Blok, Woerden, the Netherlands. Vitamin K_1, dissolved in corn oil, was added to the vitamin K-deficient food in the required amounts. The VKA warfarin was added directly to the food. The Experimental Animal Experimental Committee of the Maastricht University approved all described animal protocols.

The VKA/vitamin K_1 model is based on high doses of VKA administered to mice with concomitant administration of vitamin K_1 to overcome the antagonism of VKA in liver but not in extrahepatic tissues such as the vasculature [25]. Thus the effects of VKA on extrahepatic tissues can be studied without the animals suffering from major bleedings. To induce atherosclerosis, mice (n=40) received a WTD containing vitamin K_1 (1.5 mg/g food) for three months. After 12 weeks of treatment, 8 mice were sacrificed to monitor baseline atherosclerosis (t = 0; baseline group). The remaining mice were divided into two groups of 16 mice. The first group continued the WTD + vitamin K_1 (1.5 mg/g food) (VK_1 group), the second group received the WTD + vitamin K_1 (1.5 mg/g food) + warfarin (3.0 mg/g food) (VK_1&W group). These two diets were continued for another week and 8 mice from each group were sacrificed. The remaining mice continued the VK_1 and VK_1&W diet and were sacrificed 4 weeks after the start of the experimental diets.

Experimental Animal Procedures

Thirty minutes prior to sacrificing the mice, annexin A5-biotin [26] was injected via the tail vein (16 μg/gram body weight). After 30 minutes, mice were anesthetized with 4% isoflurane and kept anesthetized using isoflurane 1.5–2.5%. Blood was collected in 105 mM trisodium citrate via the portal vein and plasma aliquots were frozen at –80°C. Before collecting all required tissues, the vasculature was perfused with a sterile vasodilating saline solution (150 mM saline, 2.5 mM $CaCl_2$, and 100 pM sodium nitroprusside in HEPES, pH 7.3) via the portal vein. The aortic arch, thoracic and abdominal aorta (bifurcation till one cm above), and right and left carotid artery were dissected, transferred to a physiological salt solution in a silicon-coated Petri dish, and adipose and connective tissue were carefully removed. The abdominal aorta was frozen in liquid nitrogen for assessment of the calcium content. The aortic arch and right carotid artery were fixed in 4% (v/v) HEPES buffered formaldehyde containing 2.5 mM calcium and 150 mM saline and transferred after one hour to 1% (v/v) HEPES-buffered formaldehyde containing 2.5 mM calcium and 150 mM saline and kept at 4°C for 24 hours. Vascular tissue was next transferred to 70% ethanol before use for embedding and subsequent immunohistochemistry.

The thoracic aorta was snap frozen in liquid nitrogen and stored at –80°C for alkaline phosphatase (ALK) analysis. The left carotid artery was snap frozen in liquid nitrogen and stored at –80°C for mRNA analysis.

Analysis of Vascular Lesions in ApoE$^{-/-}$ Mice

Cryosections and paraffin sections, 4 μm thick, were cut from the abdominal aorta starting (bifurcation of iliaca till one cm higher) and aortic arch. Cryosections were stained with hematoxylin/eosin (HE) for histochemical analysis. Masson's trichrome stain and von Kossa stain were performed. Quantitative analysis of lesions was performed using ImageJ histomorphometry software on at least 4 sections from each mouse.

Antibodies And Chemicals

Parallel sections were stained with monoclonal rat anti- mouse Mac3 (1:30, BD Pharmingen, #S2031-30), aSMactin (1:200, Abcam, #AB-15734), BMP2 (1:50, Santa Cruz, #sc-6895), cleaved caspase-3 (1:100, Cell signaling tech, #9661S), Collagen type2 (1:100, Developmental Studies Hybridoma Bank, #II-II6B3), MGP (against various epitopes, Vascular Products BV, Maastricht, the Netherlands), designated as anti-cMGP (1:250, recognizing carboxylated MGP; cMGP), and anti-ucMGP (1:250, recognizing uncarboxylated MGP; ucMGP), respectively.

Biotinylation of mono- and polyclonal antibodies, and subsequent streptavidin-HRP labeling were performed using the Dako-Kit System-HRP (LSAB2, #K0673). Antibodies were visualized with a Nova-RED substrate (Vector #SK-4800, Vector Laboratories, Inc). In case of annexin A5-biotin, staining was performed using streptavidin-AP. Visualization was done using AP-vector red or AP-vector blue. Sections were counterstained with hematoxylin (Klinipath, #4085-9002) and mounted with imsol (Klinipath, #7961) and entellan (Merck #7961). In negative controls, incubation with primary antibody was omitted.

Vitamin K_1 and warfarin were purchased from Sigma (Saint Louis, USA). All chemicals were of analytical grade or better.

Biochemical And Immunohistochemical Measurements of ApoE$^{-/-}$ Tissues

Tissue calcium was determined after lyophilization and expressed per mg dry weight; the freeze-dried tissues were extracted with a tenfold excess (v/w) of 10% formic acid (overnight at 4°C) and calcium concentrations were measured using atomic absorption-spectrometry (AAS, Department of Clinical Chemistry, University Hospital Maastricht, The Netherlands). For the ALK assay, part of the vessel was homogenized in ice-cold TBS supplemented with protein inhibitor cocktail using a Polytron-type homogenizer. ALK activity was measured colorimetrically at 405 nm using a p-nitrophenyl phosphate substrate (Sigma). The total ALK level per vessel part was normalized to the protein content for comparisons. Immunohistochemistry was performed after embedding the vascular tissues in paraffin and subsequent sectioning (4 μm thick). Each seventh section was used for calcium detection by von Kossa staining. Each antibody staining was performed in one batch. Annexin A5-biotin was stained using streptavidin-AP, after blocking for endogenous peroxidase. Appropriate negative controls were used for antibody and annexin A5 staining. The relative extent of staining was measured using a microscope coupled to a computerized morphometry system (quantimed 570, Leica, the Netherlands). Quantification was expressed as area in mm^2 or as percentage staining of the total plaque area. To reliably compare staining of different antibodies both microscope and camera adjustments were kept constant [6,27]. Calcium, phosphate, cholesterol, and triglycerides were measured in plasma. Lipids were measured at the end of the experiments by standard enzymatic techniques.

RNA Isolation And Quantitative Reverse Transcription Real-time PCR

Messenger RNA was extracted using RNeasy Mini Kit for kidney and lung tissues and RNeasy Fibrous Tissue Kit with proteinase K digestion before RNA extraction for aorta to maximize mRNA yield. mRNA concentration and 260nm/280nm ratio were measured by Nanodrop 1000 (Thermo Scientific, USA). Integrity and amount of mRNA were analyzed by capillary electrophoresis (Agilent Bioanalyzer 2100; Agilent Technologies, Germany). Reverse transcription and real-time PCR were performed using the TaqMan Gene Expression Master Mix (PE, Applied Biosystems, USA) and the 7500 Fast Real Time PCR System according to the manufacturer's instructions.

The following temperature profile was used: 2 min at 50°C, 10 min at 95°C, and 40 cycles at 95°C following 60°C. All primers (TaqMan Gene Expression Assays) were from Applied Biosystems, including MGP, SM22-α, RunX2, VEGF, BMP-2, BMP-4, MMP-2 and MMP-9. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as housekeeping target. The expression of each target gene was normalized to GAPDH. For calculating relative expression levels the ΔCt (cycle threshold) method was used.

Statistical Analysis

Statistical analyses were performed using SPSS software (version 17.0, SPSS Inc., Chicago, IL, USA). Categorical baseline characteristics are expressed as numbers (percentages) while continuous variables are expressed as means ± SD. To test differences between patients in the tertiles for statistical significance, we used the Pearson chi-square test for categorical variables and analysis of variance (ANOVA) for continuous variables. Values in animals are expressed as mean ± SD. The difference between two groups was determined by Kruskal Wallis test. Differences for multiple comparisons were determined by ANOVA with Bonferroni correction. Differences were considered to be significant at $P<0.05$.

RESULTS

Coronary Calcification in Patients

133 VKA users and 133 individually age, gender and FRS matched non-VKA users were included in this study. Of the 133 VKA users, 52 patients had no plaque and of the 133 non-VKA 41 patients had no plaque at time of screening. VKA users were divided in tertiles based on duration of VKA use. The mean duration of VKA use is 2.5 ± 1.5 months in the first tertile (T1), 18.7 ± 8.8 months in the second tertile (T2) and 86.4 ± 47.1 months in the third tertile (T3). The categorization of the VKA users into tertiles distributed the non-VKA users also in three groups because each non-VKA user was individually matched with a VKA user. Tables 1 and 2 summarize the baseline characteristics of the tertiles of non-VKA users and VKA users, respectively.

Table 1. Baseline characteristics of patients on VKA treatment.

Variable	1st Tertile (n=44)	2nd Tertile (n=44)	3rd Tertile (n=45)	*P* value
Male gender	29 (65.9)	27 (61.4)	35 (77.8)	0.231
Age (years)	57.6 ± 10.8	59.7 ± 10.1	63.6 ± 8.5	0.016
Smoking	7 (15.9)	7 (15.9)	3 (6.7)	0.325
Diabetes mellitus	3 (6.8)	1 (2.3)	3 (6.7)	0.560
Positive family history	10 (22.7)	11 (25.0)	10 (22.2)	0.948
Systolic BP (mmHg)	140 ± 16	142 ± 21	140 ± 18	0.861
Total cholesterol (mmol/L)	5.5 ± 1.1	5.3 ± 1.2	5.2 ± 1.1	0.294
LDL-cholesterol (mmol/L)	3.5 ± 1.0	3.3 ± 1.2	3.3 ± 1.0	0.537
HDL-cholesterol (mmol/L)	1.3 ± 0.5	1.3 ± 0.5	1.2 ± 0.4	0.349
Triglycerides (mmol/L)	1.7 ± 1.3	1.7 ± 1.0	1.8 ± 1.3	0.981
Framingham Risk Score	21.6 ± 15.6	21.4 ± 13.9	29.8 ± 17.2	0.017
Agatston score	79.6 ± 159.8	142.4 ± 306.0	252.5 ± 399.3	0.029

Continuous variables are presented as mean ± SD. Categorical variables are expressed as number (%).
BP, blood pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

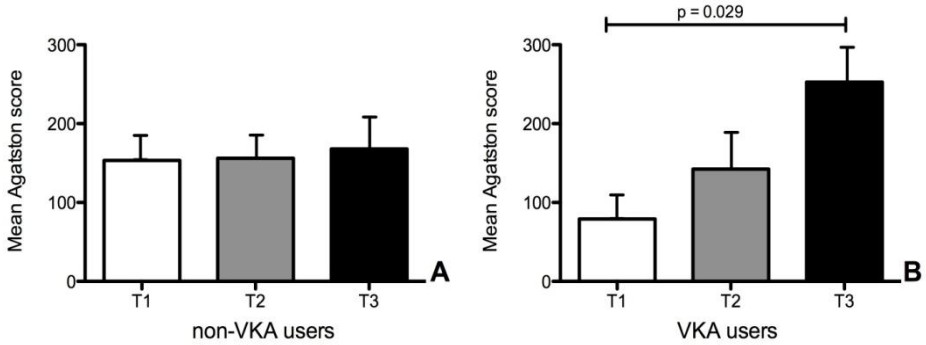
Coronary calcification was quantified as the Agatston score. There were no significant differences between the mean Agatston score in the tertiles of non-VKA users (p=0.965; Figure 1A). On the other hand, the mean Agatston score increased significantly in VKA users, as the duration of VKA use increased (p=0.029; Figure 1B). Next, we analyzed all coronary segments in each patient to assess plaque morphology and the degree of luminal stenosis. Plaques were categorized as calcified, mixed or non-calcified plaque. Plaque morphology did not differ significantly between the three tertiles of non-VKA users (Figure 1C). In contrast, the fraction of calcified coronary plaques increased significantly with prolonged VKA use. Fifty percent of the plaques in the 1st tertile of VKA users were calcified, compared to 61.5% in the 2nd tertile and 68.5% in the 3rd tertile (p<0.01; Figure 1D). Follow-up information was available for all patients (mean follow-up time 2.9 ± 0.6 years).

Table 2. Baseline characteristics of patients not on VKA treatment.

Variable	1st Tertile (n=44)	2nd Tertile (n=44)	3rd Tertile (n=45)	*P* value
Male gender	29 (65.9)	27 (61.4)	35 (77.8)	0.231
Age (years)	57.6 ± 8.9	59.8 ± 9.4	59.2 ± 9.5	0.536
Smoking	11 (25.0)	7 (15.9)	9 (20.0)	0.569
Diabetes mellitus	5 (11.4)	1 (2.3)	4 (8.9)	0.247
Positive family history	23 (52.3)	17 (38.6)	16 (35.6)	0.238
Systolic BP (mmHg)	140 ± 17	146 ± 19	145 ± 20	0.354
Total cholesterol (mmol/L)	5.3 ± 1.1	5.2 ± 1.1	5.5 ± 1.4	0.581
LDL-cholesterol (mmol/L)	3.3 ± 1.0	3.3 ± 1.0	3.5 ± 1.3	0.470
HDL-cholesterol (mmol/L)	1.3 ± 0.4	1.3 ± 0.4	1.1 ± 0.3	0.111
Triglycerides (mmol/L)	1.7 ± 0.8	1.5 ± 0.8	2.1 ± 1.3	0.022
Framingham Risk Score	21.6 ± 15.6	22.2 ± 14.1	28.7 ± 17.2	0.065
Agatston score	153.4 ± 261.4	156.0 ± 285.1	167.8 ± 263.0	0.965

Continuous variables are presented as mean ± SD. Categorical variables are expressed as number (%).
BP, blood pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

In the group of VKA users, 2 patients underwent coronary revascularization, 1 patient suffered an acute coronary syndrome and 2 patients died as a result of coronary artery disease. In the group of non-VKA users, 5 patients underwent coronary revascularization, no patients suffered an acute coronary syndrome and 1 patient died as a result of coronary artery disease.



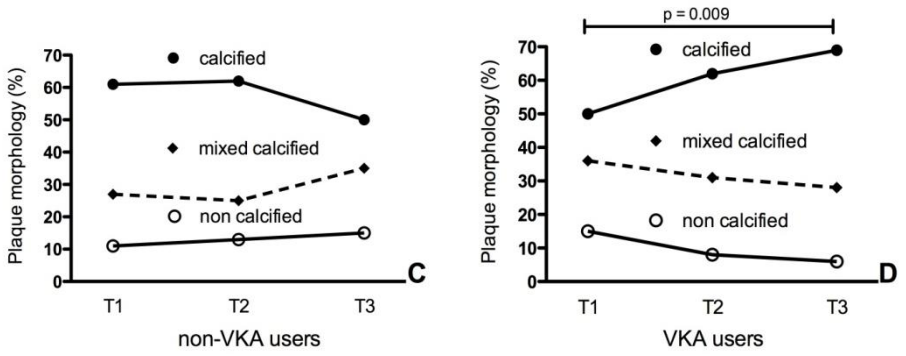


Figure 1 (partly previous page). *Duration of VKA treatment significantly increases the amount of coronary calcification and number of calcified plaques. Panel B shows a significant increase in Agatston score in VKA users (P=0.029), as patients use VKA for a longer time. This increase is not visible in matched patients not on VKA (panel A; P=0.965). In patients using VKA, a significant trend (P=0.009) was seen towards a higher percentage of calcified plaques in those patients treated longest with VKA (panel D). In contrast, this was not the case for the non-VKA users (panel C). T indicates tertile.*

We also checked other medication use between VKA and non-VKA patients. VKA users were using Verapamil significantly more often compared to non VKA users (P<0.005). This suggests that the difference in Agatston score between the VKA users and non VKA users may be even larger, since Verapamil is an inhibitor of calcification [28]. There were no significant differences in other frequent used drugs such as beta blockers, aspirin and statins between VKA and non VKA users.

In conclusion, these results indicate that VKA use is associated with an increase of atherosclerotic plaque calcification. Size and location of calcium deposits are important determinants for plaque stability. Unfortunately, due to the limited spatial resolution of MDCT and the presence of blooming artifacts, it is not possible to assess if these calcium deposits are localized in the intima or the media of the arterial wall. Therefore we performed a more detailed study of effect of VKA on plaque calcification in an established model of atherosclerosis, the apoE$^{-/-}$ mice.

Effect of Warfarin on Atherosclerotic Burden of apoE$^{-/-}$ Mice

40 apoE$^{-/-}$ mice entered the experimental scheme at the age of 10 weeks. Vitamin K_1 (VK_1) and warfarin (most prescribed VKA) supplementation to the Western type diet (WTD) did neither change plasma cholesterol, calcium and phosphate levels nor body weight as compared with WTD supplemented with VK_1 only demonstrating that warfarin was well tolerated (Table 3). Vitamin K1 was co-administered because warfarin alone would cause internal bleeding of mice. Vitamin K1 counteracts warfarin's antagonistic activity in liver but not in extrahepatic tissue [25]. We quantified intimal plaque area in the aortic arch by histomorphometry of hematoxylin/eosin (HE) and Masson's trichrome stained sections. Both H/E and Masson's trichrome staining showed that dietary supplementation of warfarin did not change plaque expansion during the 4 weeks treatment regimen (Figure 2A) and neither number nor size distribution of plaques were affected. Additionally, collagen content of the plaques did not differ significantly.

Table 3. Characteristics of apoE$^{-/-}$ mice at baseline, and after one and four weeks of treatment.

	Baseline	Control		Warfarin	
Variable	**3 month WTD**	**1 week**	**4 weeks**	**1 week**	**4 weeks**
Cholesterol	11.0 ± 0.3	11.0 ± 0.4	10.9 ± 0.5	11.1 ± 0.5	11.0 ± 0.3
Calcium	2.29 ± 0.04	2.28 ± 0.05	2.29 ± 0.03	2.27 ± 0.03	2.27 ± 0.03
Phosphate	1.94 ± 0.04	1.93 ± 0.04	1.95 ± 0.04	1.94 ± 0.03	1.94 ± 0.02
Weight male	21.7 ± 0.6	21.3 ± 1.2	23.7± 0.6	22.3 ± 0.6	23.0 ± 1.0
Weight female	31.7± 0.6	31.7 ± 1.5	32.3 ± 2.3	29.7 ± 1.2	31.3 ± 2.3

WTD, Western type diet.

Effect of Warfarin on Plaque Calcification

We quantified vascular calcification of the thoracic aorta using two different techniques. Atomic absorption spectrometry (AAS; Department of Clinical Chemistry, University Hospital Maastricht, The Netherlands) revealed that calcium levels were already elevated at one week post-baseline and increased further 4 weeks post-baseline if warfarin was added to the WTD (Figure 2B).

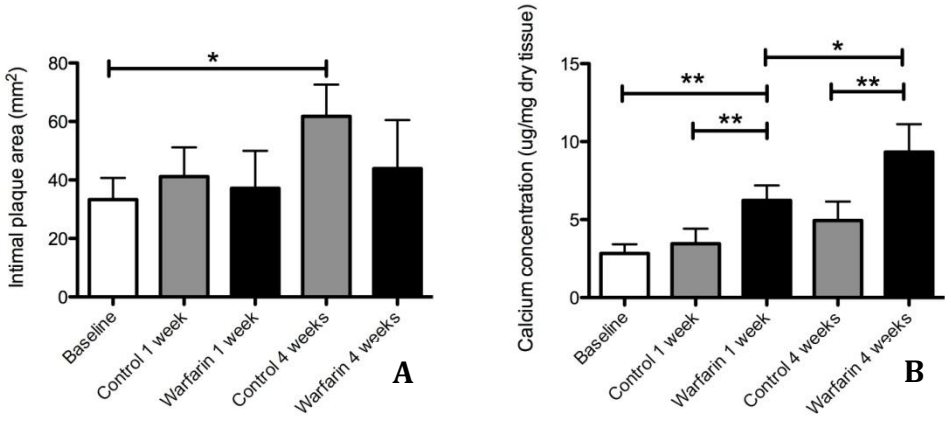


Figure 2. *Warfarin treatment of apoE$^{-/-}$ mice on Western type diet (WTD) does not influence plaque size but increases vascular calcification. ApoE$^{-/-}$ mice developed atherosclerotic lesions when maintained on WTD for 12 weeks. From baseline mice were fed with WTD plus vitamin K or WTD plus vitamin K and warfarin for the duration of one week or four weeks. Growth of intimal area was not significantly affected by warfarin (A). Vascular calcium was determined by AAS and revealed significant increase in calcium at 1 and 4 weeks warfarin treatment (B). * P<0.05, ** P<0.01.*

Whereas AAS measures overall calcium of tissue, von Kossa staining allows pinpointing the actual location and morphology of intra-plaque calcium deposits. Von Kossa analysis of serial sections showed that calcium deposits were mainly confined to intimal layers of atherosclerotic lesions. No medial calcification was observed at baseline but warfarin induced significant calcification of the medial layer of the plaque (Figure 3A,C). In all cases intimal calcification was more prevalent than medial calcification (Figure 3B,D). Warfarin treatment significantly increased number of plaques with intimal calcification and also increased calcification nodule area (Figure 3B). We were also able to discriminate between microcalcifications (<2 μm) and macrocalcification (>50 μm) (Figure 3E,F). Macrocalcification was often accompanied by microcalcification (Figure 3F). Microcalcifications were also observed in absence of macrocalcification (Figure 3E). We conclude that warfarin accelerates both medial and intimal calcification of atherosclerotic plaque.

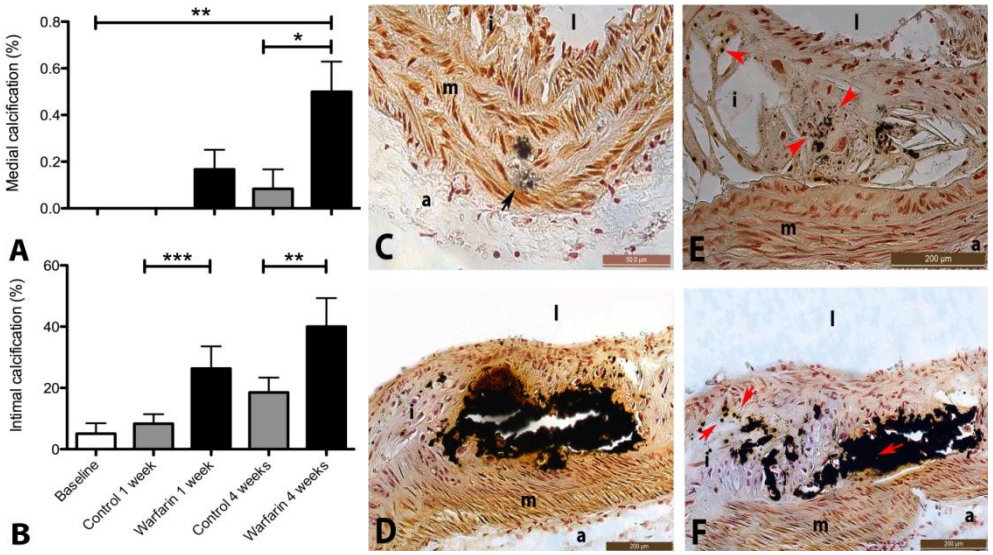


Figure 3. *Warfarin treatment rapidly increases medial and intimal plaque calcification in apoE$^{-/-}$ mice. Von Kossa stained calcified plaques were scored for medial (A,C) and intimal plaque calcification (B,D). In addition calcification was categorized as microcalcification (E, arrow heads) and macrocalcification (F, arrows). Microcalcifications occur either alone or in conjunction with macrocalcification. * P<0.05, ** P<0.01, *** P<0.001. a, adventitia; i, intima; l, lumen; m, media.*

Effect of Warfarin on Plaque Phenotype

Histochemistry of calcified plaques in the 4 weeks warfarin treated animals revealed abundant presence of chondrocyte like cells in close proximity to macro calcium deposits (Figure 4A). The presence of bone-associated proteins in these plaques was confirmed by collagen type-II staining (Figure 4B, C) as well as alkaline phosphatase measurement (Figure 5A) and staining (Figure 5B,C). Of note, warfarin treatment (4 weeks) did not only augment calcification but also significantly increased the presence of both chondrocytic markers.

As carboxylated MGP was previously shown to inhibit chondrogenesis via inactivation of BMP2 [7], we stained aortic plaques for the presence of carboxylated versus uncarboxylated MGP. Warfarin treatment caused a dramatic decrease in carboxylated MGP with a concomitant increase in uncarboxylated MGP (Figure 6A-F). mRNA expression levels of MGP were not affected by warfarin (Table 4).

These results suggest that warfarin interferes with calcification phenotype in the plaque by inhibiting carboxylation of MGP. We also measured mRNA expression levels of MMP-2 and MMP-9 which were also not significantly different by 4-weeks warfarin administration compared to baseline (Table 4).

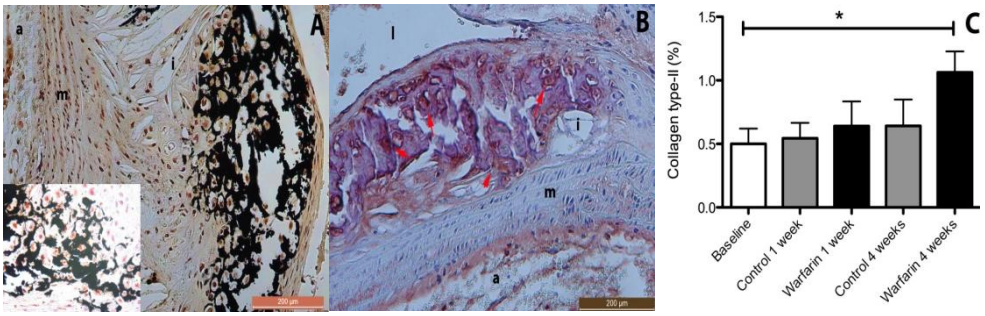


Figure 4. *Warfarin induces atherosclerotic calcification which induces a chondrogenic plaque phenotype. To characterize cells calcified or adjacent to the calcification area we closely examined stained von Kossa sections (A). Calcified cells in the atherosclerotic plaque displayed chondrocyte features. To confirm the presence of chondrocytes we stained for collagen type-II, a specific marker for chondrocytes. Areas in the plaque with suspected chondrocytes stained positive for collagen type-II (B). Quantification revealed that after 4 weeks of warfarin treatment a significant increase was measured (C). * P<0.05. a, adventitia; i, intima; l, lumen; m, media.*

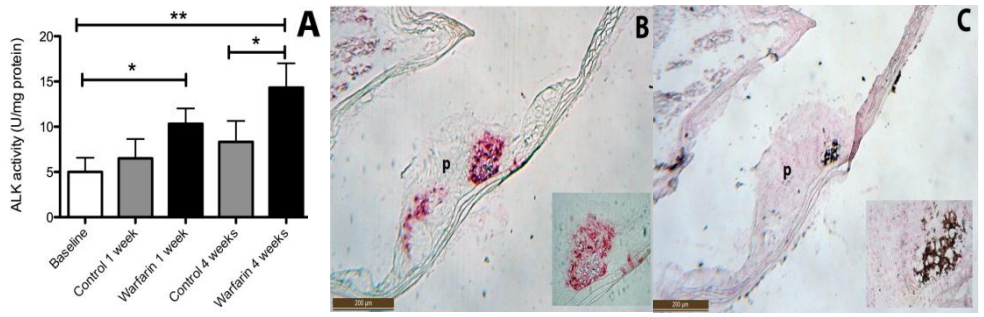


Figure 5. *Warfarin upregulates alkaline phosphatase activity in atherosclerotic plaque. To further characterize the calcified lesions we measured (A) and stained for alkaline phosphatase (C). Areas positive for von Kossa (B) often co-stained for ALK (C). Therefore we analyzed the ALK content of the different treatment groups (A). After one week of warfarin treatment a significant increase in ALK was already noticed, which further increased after 4 weeks of warfarin. ALK expression indicates an osteo–/chondrogenic differentiation of VSMCs. * P<0.05, **P<0.01. p, plaque.*

Table 4. mRNA expression levels of aortic tissue (n=3) of calcification biomarkers at control and warfarin treatment at 4 weeks time point.

	Control		Warfarin and K1		
Marker	**Mean**	**SD**	**Mean**	**SD**	***P* value**
SM22α	1.4	0.7	1.4	0.5	0.783
Runx2	2.0	0.7	1.9	1.1	0.946
BMP–2	1.3	0.6	1.4	0.3	0.967
BMP–4	2.2	1.6	2.2	0.9	0.825
MGP	1.3	0.7	1.9	0.4	0.292
VEGF	1.4	0.6	1.3	0.3	0.938
MMP–2	1.2	0.5	1.4	0.5	0.891
MMP–9	1.3	0.6	1.9	0.5	0.204

BMP indicates bone morphogenetic protein; MMP, matrix metalloproteinase; MGP, matrix Gla protein; VEGF, vascular endothelial growth factor.

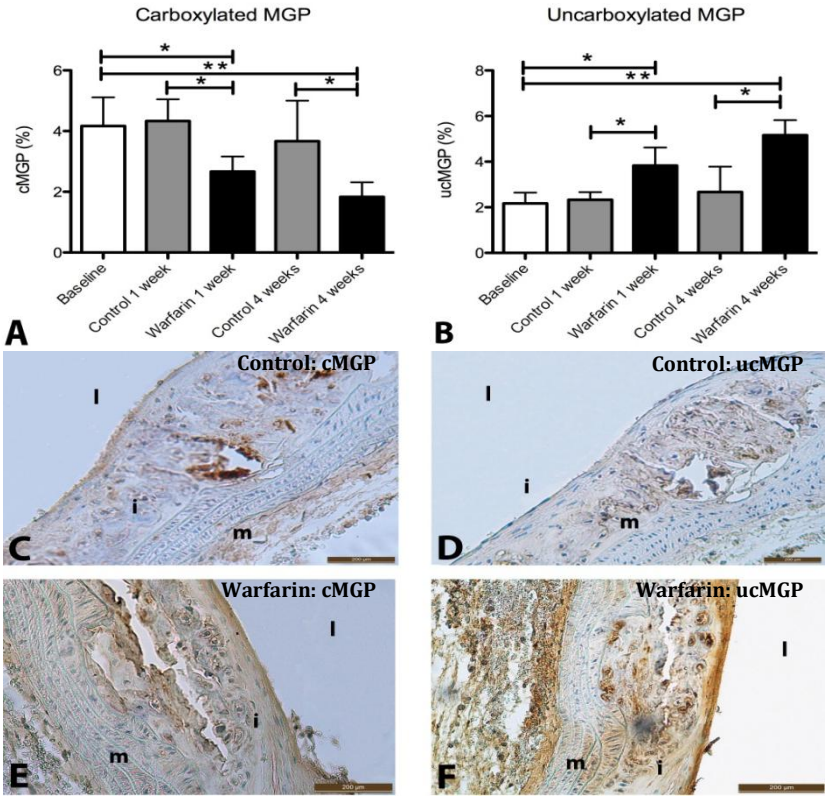


Figure 6 (previous page). *Warfarin affects carboxylation of MGP in atherosclerotic plaque. To confirm action of warfarin locally in the plaque we stained plaques for ucMGP and cMGP. UcMGP is the result of vitamin K-deficiency. Within one week of warfarin treatment, the amount of cMGP (A) decreased significantly and decreased even further after 4 weeks. The decrease in cMGP was accompanied by an increase in ucMGP (B). Immunohistochemistry for cMGP and ucMGP (E,F) showed increased amounts of ucMGP compared with cMGP in apoE$^{-/-}$ mice on warfarin, whereas apoE$^{-/-}$ mice on control diet had predominantly cMGP (C,D). * P<0.05, ** P<0.01. cMGP indicates carboxylated MGP; ucMGP, uncarboxylated MGP; i, intima; l, lumen; m, media.*

Vascular calcification has been associated with apoptosis, both in media and intima [6,29-32]. Therefore we quantified apoptosis using two methods. Apoptosis was measured by caspase-3 staining (Figure 7A) and by annexin A5-biotin (Figure 7B), which was injected intravenously 30 minutes before sacrificing the mice. Warfarin caused a significant increase of apoptosis after 4 weeks of treatment. Similar to the calcium deposits, apoptotic cells were predominantly observed in plaques. Positive caspase-3 staining was noticed both in the plaque core and shoulder (Figure 7C). Likewise, annexin-A5-biotin positive staining was observed in the core, shoulders and the fibrous cap of the plaque (Figure 7D, inset and 7E,H) and co-localization with Mac3 revealed that part of the annexin A5 positivity was associated with macrophages, especially in the shoulder of the plaque (Figure 7D, inset, and 7F). At baseline, annexin A5-biotin staining was not significantly present (Figure 7G). We also measured the ratio of plaque macrophage / VSMC number but this ratio was not significantly different between the groups (data not shown).

The intimal plaque vascular smooth muscle cells (VSMCs) are thought to be derived from the vascular media after having switched from a contractile into a synthetic phenotype. Therefore we measured VSMC number and elastin integrity in the medial layer underneath the atherosclerotic plaque. In warfarin treated animals, VSMCs were lower in number in the vascular media (Figure 8A,C), suggesting loss of elasticity and stability of the vascular media. Congruent with this finding, warfarin treated animals had significantly more outward remodeled plaques as demonstrated by disorganized tissue bulging outward through breaks of the elastic lamina (Figure 8B,D).

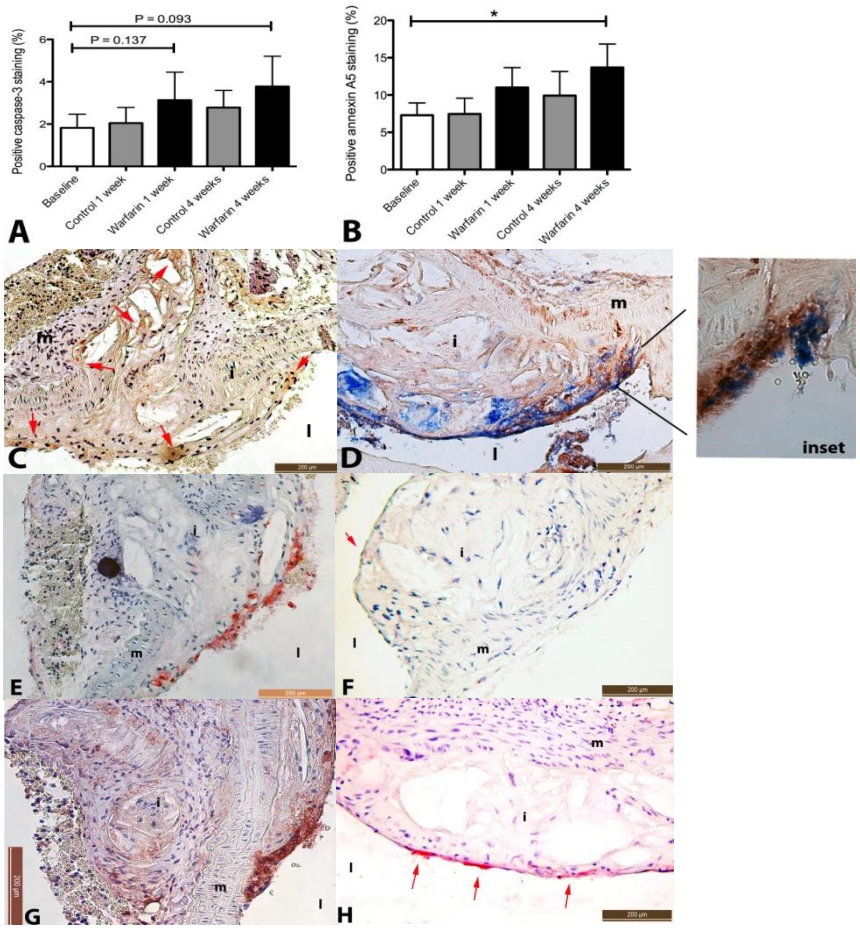


Figure 7. *Warfarin treatment of apoE$^{-/-}$ mice increases apoptosis in atherosclerotic plaque. Vascular calcification is closely linked to apoptosis. Therefore we stained sections for caspase-3. Moreover, all animals were injected with annexin A5-biotin, a protein with high affinity for phosphatidylserine (PS). Both cleaved caspase-3 (A,C) and annexin A5-biotin (B,D) increased in the warfarin treatment, with annexin A5 accumulation significantly increased after 4 weeks of warfarin treatment (B). Annexin A5 staining using AP vector blue confirmed PS externalization at different sites in the atherosclerotic plaques. Activated and apoptotic macrophages are known to express PS on their surface. We found co-localization of macrophages in the shoulder of the atherosclerotic plaque (Mac3; F) and PS (annexin A5 stained with AP vector red; E), indicating activated and apoptotic macrophages. In the inset annexin A5 (blue) localizes with Mac-3 staining (red/brown), indicating activated macrophages. Moreover, at baseline annexin A5 was only incidentally present at the surface of the plaque (G), whereas at the 4 weeks warfarin treatment, annexin A5 stained significantly positive (H). * P<0.05. i, intima; l, lumen; m, media.*

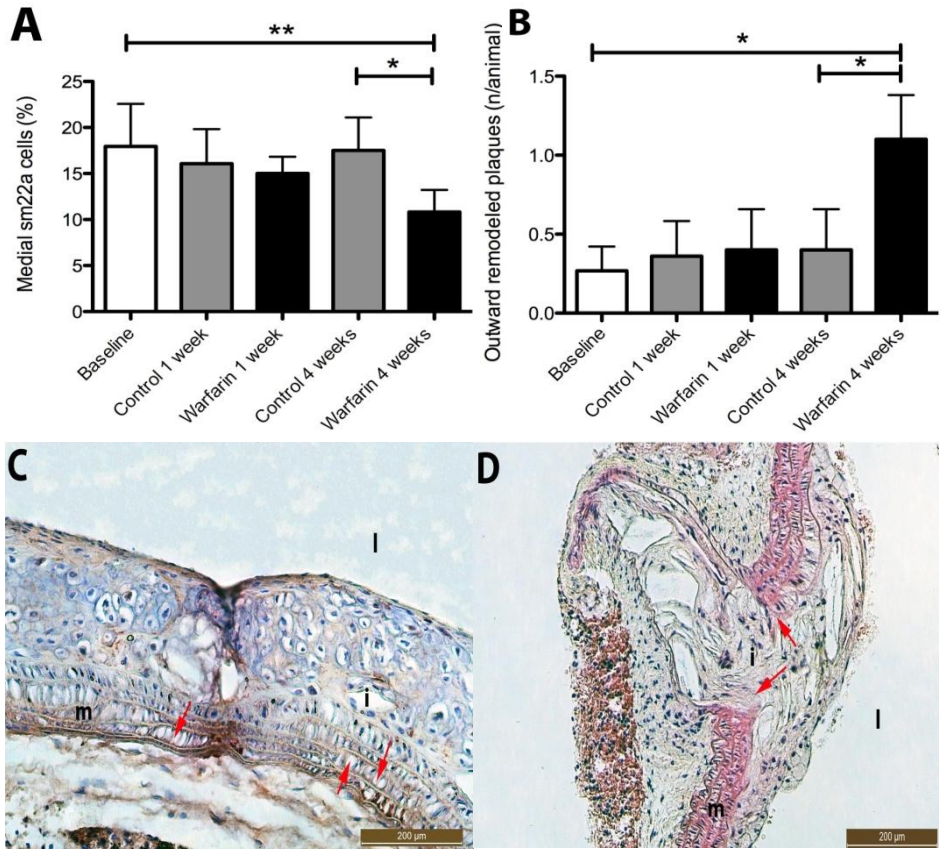


Figure 8. *Warfarin induces medial VSMC loss and increases outward remodeling of atherosclerotic plaques, both indications of an instable rupture-prone plaque phenotype. An unexpected feature of warfarin treatment was the significantly increased number of outward remodeled plaques (B,D). To explain this phenotype induced by warfarin we stained sections for the VSMC marker αSMactin. We found that warfarin treatment was associated with a significant loss of medial VSMCs (A,C). * P<0.05, ** P<0.01. i, intima; l, lumen; m, media.*

DISCUSSION

The present study demonstrates that VKA treatment is associated with accelerated calcification of atherosclerotic plaques in humans and is the first to demonstrate in a mouse model of atherosclerosis that VKA affects plaque phenotype negatively by enhancing features of plaque instability.

Vascular calcification precipitates in media of arteries along elastic fibers, also known as Mönckeberg's sclerosis. Media calcification is closely linked with uremic risk factors. Calcification of intima is associated with atherosclerotic lesions. Calcium precipitates in close vicinity with lipid deposits and necrotic debris. It has been suggested that mechanisms of the two types of vascular calcification are distinct [33,34]. A recent study reported a significant relationship between VKA treatment and coronary calcium score in atrial fibrillation patients without CAD [13]. This study did not address localization of calcium deposits in coronary arteries and, hence, could not provide insight into the arterial site affected by VKA treatment. Animal studies suggest that VKA treatment causes medial calcification similar to Mönckeberg's sclerosis [25,35,36].

The present study investigated effects of VKA treatment on coronary calcium score in patients with suspected CAD, who underwent MDCT. Our study confirmed the relationship between VKA treatment and coronary calcification in this group of patients. Furthermore, MDCT offers the possibility to non-invasively assess lesions of the coronary arteries on morphology, degree of luminal stenosis and presence of calcium deposits [37]. Our data convincingly show that use and duration of VKA treatment correlate significantly with coronary plaque calcification. Although amount of coronary calcification was reported to have predictive value for cardiovascular events in subsets of patients [38,39], the actual impact of calcification on plaque stability is controversial. In our patient population we did not find a clinical relevant difference in the occurrence of cardiovascular events (coronary revascularization, acute coronary syndrome, cardiovascular death) between VKA users and non VKA users. There are a few reasons contributing to the absence of a difference in cardiovascular events. First, we studied a small patient population. Secondly, the follow-up is relatively short (2.9 ± 0.6 years) and it is not inconceivable that the follow-up period is too short to follow-up on cardiovascular event. Moreover, in case of the presence of coronary artery disease, patients were treated (life style changes, medication use) to prevent a cardiovascular event.

Recently it was postulated that increased risk for acute coronary events depends on size and location of calcium deposits. For instance, calcifications adjacent to or beneath the lipid necrotic core were assumed to be stabilizing [40].

On the other hand, elevated calcium scores were seen to be predictive of acute coronary events [41,42] and culprit plaques often contain more but smaller calcium deposits (so-called spotty calcification) than stable plaques [20,37,43]. From biomechanical studies it was inferred that micro-calcifications in the fibrous cap have a destabilizing effect [21]. MDCT allows to map calcification to vascular anatomy and to distinguish calcified spots at millimeter resolution, insufficient to pick up micro-calcifications and to pinpoint localization. In order to reveal impact of VKA treatment on plaque stability we studied effects of warfarin on plaque calcification and phenotype in the apoE$^{-/-}$ mouse model of atherosclerosis.

In this paper we demonstrate for the first time that warfarin increases plaque calcification in the apoE$^{-/-}$ model. Warfarin-induced plaque calcification starts already after 1 week of administration, indicating that vitamin K-dependent mechanisms operate in developing plaques to suppress and limit pro-calcifying processes. cMGP is a well-known suppressor of vascular calcification [44], and a deficiency was previously shown to result in medial calcification [4,5]. Transgenesis causing overexpression of MGP in apoE$^{-/-}$ mice inhibited calcification of atherosclerotic lesions [18] demonstrating MGP's modulating role in plaque calcification and indicating shared mechanisms by intimal and medial calcification. We observed a warfarin-induced downregulation of cMGP with concomitant upregulation of ucMGP in the plaque without affecting MGP-mRNA levels. Hence, we conclude that warfarin affects plaque calcification by inhibiting post-translational γ-carboxylation of MGP. This is in agreement with previous in vitro studies from our group demonstrating that warfarin treatment causes ucMGP production by VSMCs [10]. A recent study showed positive correlation between calcification of human coronary plaques and ucMGP expression in the plaque [45], strongly indicating that warfarin causes accelerated plaque calcification in human by a mechanism similar to that observed in the mouse model.

cMGP antagonizes BMP and is consequently linked to signaling networks regulating inflammation [46] and inducing VSMC differentiation [19] and apoptosis [47]. Thus, warfarin potentially affects plaque phenotype more profoundly than solely accelerating plaque calcification. We observed that warfarin treatment did not affect BMP-2 and -4 expression but increased collagen type-II and ALK expression concurring MGP regulated chrondrocytic trans-differentiation of VSMC [4,48].

Furthermore, warfarin caused increased plaque apoptosis and loss of VSMC, both have been linked to calcification [30,49] and progression towards unstable plaque [50,51]. Thus, the loss of VSMCs underneath the plaque seen in our model may link to the observed increased number of outward remodeled plaques. In addition, it was recently shown that warfarin treated rats displayed increased MMP-9 activity in the vasculature which related to elastin degradation and vascular calcification [52]. Outward remodeled plaques have been linked to vulnerability of the plaque to rupture [53].

CONCLUSION

We conclude that VKA ignite a cascade of responses leading to progressive calcification and destabilization of atherosclerotic plaques. Although the use of VKA thus may impart a risk factor for acute coronary events, the relatively safe historical profile of VKA suggests, however, otherwise. Detrimental effects of VKA could well be masked by their potent inhibitory effects on the coagulant system, an important determinant in atherothrombosis [54]. Nevertheless our findings support the growing need for alternative anticoagulants and underscore a need for anticoagulants that do not interfere with the vitamin K-cycle [55].

REFERENCES

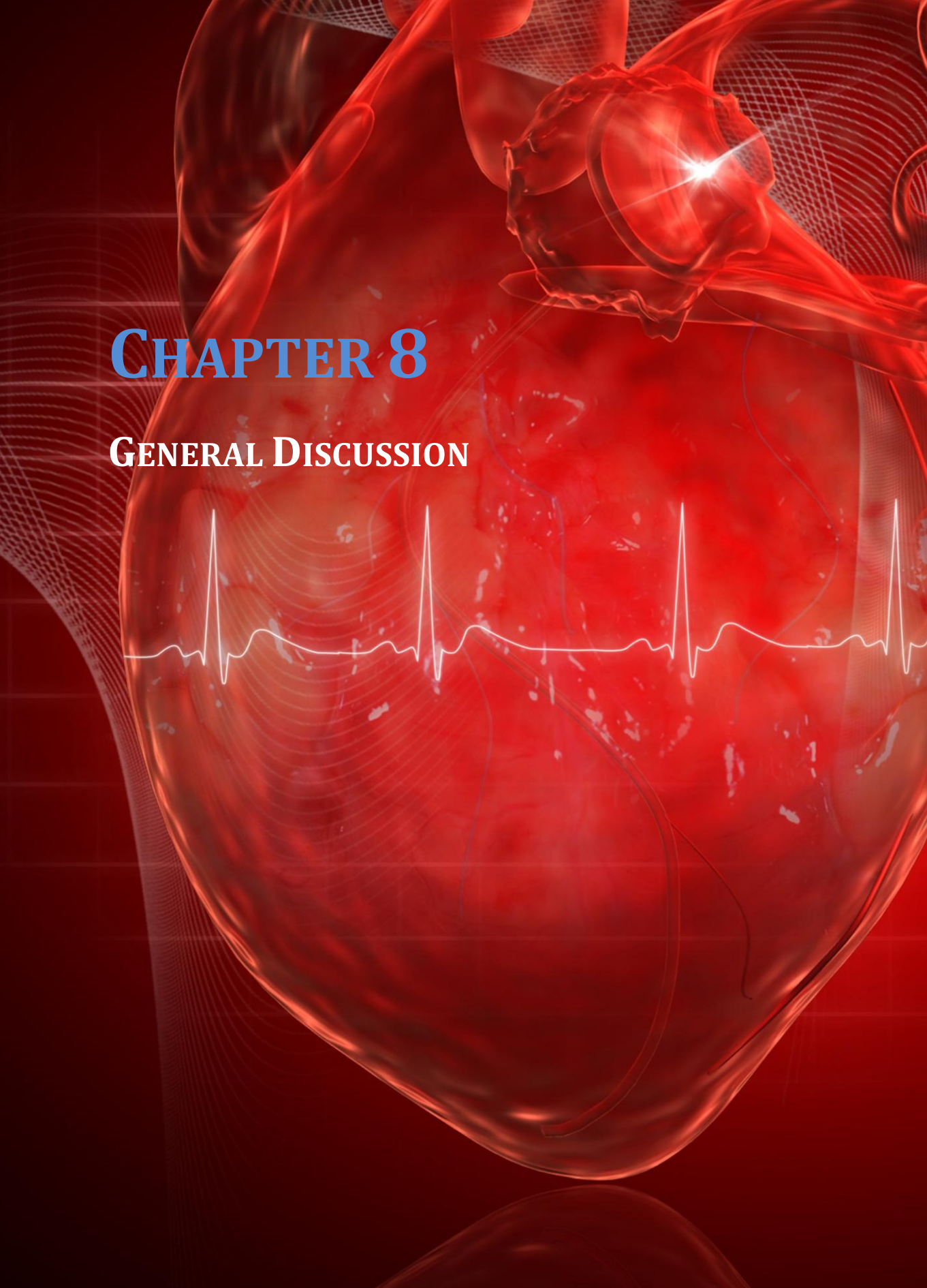
1. D'Andrea G, D'Ambrosio R, Margaglione M. Oral anticoagulants: Pharmacogenetics relationship between genetic and non-genetic factors. *Blood Rev* 2008;22:127–140.
2. Chatrou ML, Reutelingsperger CP, Schurgers LJ. Role of vitamin K-dependent proteins in the arterial vessel wall. *Hamostaseologie* 2011;31:251–257.
3. Price P, Williamson M. Primary structure of bovine matrix Gla protein, a new vitamin K-dependent bone protein. *J Biol Chem* 1985;260:14971–14975.
4. Luo G, Ducy P, McKee M, et al. Spontaneous calcification of arteries and cartilage in mice lacking matrix Gla protein. *Nature* 1997;385:78-81.
5. Murshed M, Schinke T, McKee M, et al. Extracellular matrix mineralization is regulated locally; different roles of two Gla-containing proteins. *J Cell Biol* 2004;165:625–630.

6. Schurgers LJ, Spronk HMH, Soute BAM, et al. Regression of warfarin-induced medial elastocalcinosis by high intake of vitamin K in rats. *Blood* 2007;109:2823–2831.
7. Zebboudj A, Imura M, Bostrom K. Matrix Gla protein, a regulatory protein for bone morphogenetic protein-2. *J Biol Chem* 2002;277:4388–4394.
8. Yao Y, Zebboudj A, Shao E, et al. Regulation of bone morphogenetic protein-4 by matrix Gla protein in vascular endothelial cells involves activin-like kinase receptor 1. *J Biol Chem* 2006;281:33921–33930.
9. Speer MY, Yang H-Y, Brabb T, et al. Smooth muscle cells give rise to osteochondrogenic precursors and chondrocytes in calcifying arteries. *Circ Res* 2009;104:733–741.
10. Schurgers LJ, Spronk HMH, Skepper JN, et al. Post-translational modifications regulate matrix Gla protein function: importance for inhibition of vascular smooth muscle cell calcification. *J Thromb Haemost* 2007;5:2503–2511.
11. O'Young J, Liao Y, Xiao Y, et al. Matrix Gla protein inhibits ectopic calcification by a direct interaction with hydroxyapatite crystals. *J Am Chem Soc* 2011;133:18406–18412.
12. Schurgers LJ, Aebert H, Vermeer C, et al. Oral anticoagulant treatment: friend or foe in cardiovascular disease? *Blood* 2004;104:3231–3232.
13. Weijs B, Blaauw Y, Rennenberg RJMW, et al. Patients using vitamin K antagonists show increased levels of coronary calcification: an observational study in low-risk atrial fibrillation patients. *Eur Heart J* 2011;32:2555–2562.
14. Rennenberg RJMW, van Varik BJ, Schurgers LJ, et al. Chronic coumarin treatment is associated with increased extracoronary arterial calcification in humans. *Blood* 2010;115: 5121–5123.
15. Koos R, Mahnken A, Muhlenbruch G, et al. Relation of oral anticoagulation to cardiac valvular and coronary calcium assessed by multislice spiral computed tomography. *Am J Cardiol* 2005;96:747–749.
16. Shanahan CM, Cary NR, Metcalfe JC, et al. High expression of genes for calcification-regulating proteins in human atherosclerotic plaques. *J Clin Invest* 1994;93:2393–2402.
17. Naik V, Leaf EM, Hu JH, et al. Sources of cells that contribute to atherosclerotic intimal calcification: an in vivo genetic fate mapping study. *Cardiovasc Res* 2012;94:545–554.
18. Yao Y, Bennett BJ, Wang X, et al. Inhibition of bone morphogenetic proteins protects against atherosclerosis and vascular calcification. *Circ Res* 2010;107:485–494.

19. Nakagawa Y, Ikeda K, Akakabe Y, et al. Paracrine osteogenic signals via bone morphogenetic protein-2 accelerate the atherosclerotic intimal calcification in vivo. *Arterioscler Thromb Vasc Biol* 2010;30:1908–1915.
20. Ehara S, Kobayashi Y, Yoshiyama M, et al. Spotty calcification typifies the culprit plaque in patients with acute myocardial infarction: an intravascular ultrasound study. *Circulation* 2004;110:3424–3429.
21. Vengrenyuk Y, Carlier S, Xanthos S, et al. A hypothesis for vulnerable plaque rupture due to stress-induced debonding around cellular microcalcifications in thin fibrous caps. *Proc Natl Acad Sci USA* 2006;103:14678–14683.
22. Agatston AS, Janowitz WR, Hildner FJ, et al. Quantification of coronary artery calcium using ultrafast computed tomography. *J Am Coll Cardiol* 1990;15: 827–832.
23. McCollough CH, Ulzheimer S, Halliburton SS, et al. Coronary artery calcium: a multi-institutional, multimanufacturer international standard for quantification at cardiac CT. *Radiology* 2007;243:527–538.
24. Braunwald E, Antman EM, Beasley JW, et al. ACC/AHA 2002 guideline update for the management of patients with unstable angina and non-ST-segment elevation myocardial infarction--summary article: a report of the American College of Cardiology/American Heart Association task force on practice guidelines (Committee on the Management of Patients With Unstable Angina). *J Am Coll Cardiol* 2002;40:1366–1374.
25. Price P, Faus S, Williamson M. Warfarin causes rapid calcification of the elastic lamellae in rat arteries and heart valves. *Arterioscler Thromb Vasc Biol* 1998;18:1400–1407.
26. Dumont EA, Hofstra L, van Heerde WL, et al. Cardiomyocyte death induced by myocardial ischemia and reperfusion: measurement with recombinant human annexin-V in a mouse model. *Circulation* 2000;102:1564–1568.
27. Lutgens E, Daemen M, Kockx M, et al. Atherosclerosis in APOE*3-Leiden transgenic mice: from proliferative to atheromatous stage. *Circulation* 1999;99:276–283.
28. Chen N, Kircelli F, O'Neill K, et al. Verapamil inhibits calcification and matrix vesicle activity of bovine vascular smooth muscle cells. *Kidney Int* 2010;77:436–442.
29. Clarke M, Figg N, Maguire J, et al. Apoptosis of vascular smooth muscle cells induces features of plaque vulnerability in atherosclerosis. *Nat Med* 2006;12:1075–1080.
30. Clarke M, Littlewood T, Figg N, et al. Chronic apoptosis of vascular smooth muscle cells accelerates atherosclerosis and promotes calcification and medial degeneration. *Circ Res* 2008;102:1529–1538.

31. Ewence AE, Bootman M, Roderick HL, et al. Calcium phosphate crystals induce cell death in human vascular smooth muscle cells: a potential mechanism in atherosclerotic plaque destabilization. *Circ Res* 2008;103:28–34.
32. Nadra I, Mason J, Philippidis P, et al. Proinflammatory activation of macrophages by basic calcium phosphate crystals via protein kinase C and MAP kinase pathways: a vicious cycle of inflammation and arterial calcification? *Circ Res* 2005;96:1248–1256.
33. Amann K. Media calcification and intima calcification are distinct entities in chronic kidney disease. *Clin J Am Soc Nephrol* 2008;6:1599–1605.
34. Doherty T, Fitzpatrick L, Inoue D, et al. Molecular, endocrine, and genetic mechanisms of arterial calcification. *Endocr Rev* 2004;25:629–672.
35. Spronk HMH, Soute BAM, Schurgers LJ, et al. Tissue-specific utilization of menaquinone-4 results in the prevention of arterial calcification in warfarin-treated rats. *J Vasc Res* 2003;40:531–537.
36. Essalihi R, Dao H, Yamaguchi N, et al. A new model of isolated systolic hypertension induced by chronic warfarin and vitamin K1 treatment. *Am J Hypertens* 2003;16:103–110.
37. Motoyama S, Kondo T, Sarai M, et al. Multislice computed tomographic characteristics of coronary lesions in acute coronary syndromes. *J Am Coll Cardiol* 2007;50:319–326.
38. Raggi P, Shaw L, Berman D, et al. Prognostic value of coronary artery calcium screening in subjects with and without diabetes. *J Am Coll Cardiol* 2004;43:1663–1669.
39. Rennenberg RJMW, Kessels AGH, Schurgers LJ, et al. Vascular calcifications as a marker of increased cardiovascular risk: a meta-analysis. *Vasc Health Risk Manag* 2009;5:185–197.
40. Huang H, Virmani R, Younis H, et al. The impact of calcification on the biomechanical stability of atherosclerotic plaques. *Circulation* 2001;103:1051–1056.
41. Detrano R, Hsiai T, Wang S, et al. Prognostic value of coronary calcification and angiographic stenoses in patients undergoing coronary angiography. *J Am Coll Cardiol* 1996;27:285–290.
42. Detrano R, Guerci AD, Carr JJ, et al. Coronary calcium as a predictor of coronary events in four racial or ethnic groups. *N Engl J Med* 2008;358:1336–1345.
43. Fujii K, Carlier SG, Mintz GS, et al. Intravascular ultrasound study of patterns of calcium in ruptured coronary plaques. *Am J Cardiol* 2005;96:352–357.
44. Schurgers LJ, Cranenburg ECM, Vermeer C. Matrix Gla-protein: the calcification inhibitor in need of vitamin K. *Thromb Haemost* 2008;100:593–603.

45. Roijers RB, Debernardi N, Cleutjens JP, et al. Microcalcifications in early intimal lesions of atherosclerotic human coronary arteries. *Am J Pathol* 2011;178: 2879–2887.
46. Csiszar A, Smith KE, Koller A, et al. Regulation of bone morphogenetic protein-2 expression in endothelial cells: role of nuclear factor-kappaB activation by tumor necrosis factor-alpha, H2O2, and high intravascular pressure. *Circulation* 2005;111:2364–2372.
47. Hruska KA, Mathew S, Saab G. Bone morphogenetic proteins in vascular calcification. *Circ Res* 2005;97:105–114.
48. El-Maadawy S, Kaartinen M, Schinke T, et al. Cartilage formation and calcification in arteries of mice lacking matrix Gla protein. *Connect Tissue Res* 2003;44:272–278.
49. Proudfoot D, Skepper JN, Hegyi L, et al. Apoptosis regulates human vascular calcification in vitro: evidence for initiation of vascular calcification by apoptotic bodies. *Circ Res* 2000;87: 1055–1062.
50. Kolodgie FD, Narula J, Haider N, et al. Apoptosis in atherosclerosis. Does it contribute to plaque instability? *Cardiol Clin* 2001;19:127–139.
51. Tabas I. Macrophage death and defective inflammation resolution in atherosclerosis. *Nat Rev Immunol* 2010;10:36–46.
52. Bouvet C, Moreau S, Blanchette J, et al. Sequential activation of matrix metalloproteinase 9 and transforming growth factor beta in arterial elastocalcinosis. *Arterioscler Thromb Vasc Biol* 2008;28:856–862.
53. Motoyama S, Sarai M, Harigaya H, et al. Computed tomographic angiography characteristics of atherosclerotic plaques subsequently resulting in acute coronary syndrome. *J Am Coll Cardiol* 2009;54:49–57.
54. Borissoff JI, Spronk HM, Ten Cate H. The hemostatic system as a modulator of atherosclerosis. *N Engl J Med* 2011;364:1746–1760.
55. Chatrou ML, Winckers K, Hackeng TM, et al. Vascular calcification: The price to pay for anticoagulation therapy with vitamin K-antagonists. *Blood Rev* 2012;26:155–166.



CHAPTER 8

GENERAL DISCUSSION

CORONARY ARTERY DISEASE

Coronary artery disease remains one of the leading causes of morbidity and mortality in developed as well as in still developing countries, and is accompanied by high healthcare expenditures [1,2]. Although the process of initiation and progression of coronary atherosclerotic plaques has been extensively described [3], we are still not able to prevent end-organ damage of coronary atherosclerosis. Unfortunately, a myocardial infarction (MI) or sudden cardiac death is often the first symptom of already existing CAD [4]. Identification of these 'accidents waiting to happen' is a key goal for risk stratification in general and coronary artery imaging in particular [5].

Nowadays, we have access to various tools which can help us to identify patients at risk for a major adverse cardiovascular event (MACE). However, detecting the 'vulnerable patient' in daily clinical practice remains an ongoing challenge. This thesis provided more insight in risk stratification in patients with chest discomfort symptoms, suspect for CAD. Therefore, we combined the use of several (bio)markers with characteristics of CAD, as assessed with coronary CT-angiography (CCTA).

RISK PROFILING ALGORITHMS

Risk stratification is important and the premise in order to find individual patients with an increased risk of CAD and accordingly MACE. Detection of these patients in an early phase is important, since they may benefit from a tailored treatment to prevent adverse events [6]. Multiple clinical risk profiling algorithms have been developed to estimate the long-term cardiovascular risk in patients. Well known examples are the Framingham risk score (FRS), the PROCAM risk score and the SCORE risk score. These risk scores differ from each other in terms of included risk variables, outcome measurement and geographical location of included patients:

- The *Framingham risk score* predicts 10- year risk of developing cardiovascular disease (CVD) events including coronary heart disease, stroke, peripheral artery disease and heart failure. This score is gender specific and incorporates age, smoking, diabetes mellitus (DM), systolic blood pressure, treatment for hypertension, total cholesterol and high-density lipoprotein cholesterol [7].

- The *PROCAM risk score* predicts 10-year risk of developing acute coronary events including MI and sudden cardiac death. The risk score includes age, smoking, DM, systolic blood pressure, family history of premature MI, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol and triglycerides. The study was performed in middle-aged men of European descent [8].
- The *SCORE risk score* predicts 10-year risk of cardiovascular mortality. This score takes gender, age, systolic blood pressure, smoking and total cholesterol into account. The study included datasets from 12 European cohort studies, mainly carried out in general population settings [9].

In a previous study by our research group, these three risk score algorithms were compared in order to test their ability to predict for CAD, as assessed with CCTA, and to test their ability to predict MACE. The study population consisted of 1,296 patients who visited the cardiology outpatient department because of stable chest discomfort symptoms, suspect for CAD. All patients were followed-up for a mean of 19 ± 9 months for all cardiovascular events, including acute coronary syndrome (ACS), mortality and revascularization >90 days after CCTA. ROC-analysis to predict for any coronary plaque as well as for obstructive CAD showed significantly higher areas under the curve for FRS and SCORE compared to PROCAM. Moreover, in the low-risk FRS group, less patients with any plaque, obstructive CAD and cardiovascular events were found as compared to the low-risk PROCAM and low-risk SCORE group. Therefore, the FRS seems to be the safest risk stratification algorithm in patients with stable chest discomfort symptoms [10].

The most important disadvantage of the use of clinical risk profiling algorithms is that it is an epidemiologic concept, meant to be used in patient populations rather than in individuals. On the other hand, it also has compelling advantages. In addition to the fact that risk profiling is cost- and time-efficient, one of the major strengths is that multiple risk factors are combined in order to calculate the cardiovascular risk. When combined, risk factors increase the cardiovascular risk not in a linear but in an exponential way. Although risk factors are insufficient to identify absolute risk, they are strongly associated with the presence of CVD and explain 75-90% of events [11,12]. Therefore, risk profiling is recommended in clinical practice [13].

GENDER DIFFERENCES IN CORONARY ARTERY DISEASE

Although health statistics show that relatively more females die due to CVD as compared to males, CVD is still often considered as a male problem [14]. As a consequence, the risk of CVD is underestimated in women, resulting in underdiagnosis, undertreatment and even a decreased survival [15-17].

In chapter 2 of this thesis we described gender differences in clinical risk profile as well as gender differences in extent and degree of CAD. We found that men had a significantly higher coronary calcium score as compared to women. Men also had significantly more coronary plaques, irrespective the degree of CAD. Importantly, although women had more individual cardiovascular risk factors, their FRS was significantly lower compared to men, regardless the extent and degree of CAD. This suggests that relative to men, the FRS underestimates the extent and degree of CAD in women. All risk factors used to calculate the FRS have a gender specific weighting factor. The observation that women had a lower FRS may therefore be explained by the fact that male gender is an important risk factor in the FRS algorithm, resulting in much higher FRS. Our findings are supported by other studies, concluding that a majority of women are classified in the low risk Framingham category although a significant proportion of them had significant CAD [18,19]. Because the FRS underestimates the extent and degree of CAD in women, clinicians should consider all traditional risk factors in women, rather than only calculating the FRS.

In the future, it would definitely be of interest to develop a more precise risk stratification algorithm focused on women. In the recent 2011 AHA prevention guidelines for women, it has already been proposed to introduce an additional category of "ideal cardiovascular health" to the FRS [20].

NON-TRADITIONAL CARDIOVASCULAR RISK FACTORS

The discussed risk profiling algorithms focus on traditional risk factors, which are independently related to CAD and MACE [21]. Apart from these risk factors there are non-traditional cardiovascular risk factors including chronic inflammatory diseases [22], imbalance of the coagulation system, and chronic renal insufficiency [23].

It is well elucidated that end-stage renal disease is associated with an increased prevalence of coronary calcification and CAD. Moreover, CVD is the primary cause of mortality in these patients [24]. More recently, also earlier stages of chronic kidney disease (CKD) have been associated with a decreased prognosis [23]. Although several previous studies found an inverse association between renal function and calcification, it remains unclear if this association is independent from traditional cardiovascular risk factors [25,26].

We investigated whether mild to moderate CKD is independently associated with CAD beyond traditional cardiovascular risk factors in a large cohort (n=2,038) of patients with chest discomfort symptoms. As described in chapter 3, patients with mild CKD (eGFR 60-89 mL/min/1.73m^2) and moderate CKD (eGFR 30-59 mL/min/1.73m^2) significantly more often harbored coronary plaques compared to patients with a normal renal function (eGFR ≥90 mL/min/1.73m^2). Obstructive CAD, defined as coronary plaques with >70% luminal stenosis, was also significantly more often present in patients with mild CKD (OR 1.67, 95% CI 1.16-2.40) as well as in patients with moderate CKD (OR 2.36, 95% CI 1.35-4.13), both P<0.01. The coronary calcium score increased with decreasing renal function in accordance with previous studies. Interestingly, after adjustment for traditional cardiovascular risk factors, the association between impaired renal function and the presence of CAD were no longer statistically significant. Age, gender and smoking remained the only independent risk factors for obstructive CAD in this population. Question remains if there is added value of renal function over traditional cardiovascular risk factors in predicting MACE. Due to the relatively short follow-up time in this cohort, we were not yet able to answer this question.

BIOMARKERS IN CORONARY ARTERY DISEASE

Above we discussed risk stratification by means of (non-)traditional risk factors. Although traditional cardiovascular risk factors are validated for the diagnosis and treatment of CVD, they do not completely explain incident CVD. Moreover, underlying mechanisms for the associations between cardiovascular risk factors and CVD are not fully elucidated [27].

In general, biomarkers are a powerful tool to better understand the different areas of CVD including screening, diagnosis, prognosis and therapy [28]. Importantly, they have the potential to improve clinical risk stratification [29]. Therefore, there is considerable interest in the development of novel biomarkers in the area of inflammation, thrombosis, hemostasis and oxidative stress, in order to identify individuals at increased risk for MACE. Biomarker research can also contribute to disentangle novel pathways of disease [30]. Now, we will discuss high-sensitivity cardiac troponin as a biomarker and the relation between atherosclerosis, coagulation and the immune system.

The Meaning of An Elevated Cardiac Troponin Concentration

Cardiac troponins (cTn), both cardiac troponin T (cTnT) and cardiac troponin I (cTnI), are cardiac specific biomarker which is released into the blood as a result of cardiomyocyte injury. The introduction of cTn has led to major improvements in diagnosis and risk stratification in patients with symptoms of chest discomfort. Diagnosis of a MI requires detection of a rise and/or fall of a cardiac biomarker, preferably cTn, with at least one value above the 99th percentile upper reference limit in combination with at least one of the following criteria: ischemic symptoms, ECG changes (new ST-T segment changes or new left bundle branch block), development of pathologic Q waves, imaging evidence of new loss of viable myocardium or new regional wall motion abnormalities or identification of an intracoronary thrombus by angiography or autopsy [31]. The pattern of rise and fall is essential to differentiate acute elevations from chronic elevations. Chronic elevations are associated with structural heart disease and are present in patients with renal failure, heart failure and left ventricular hypertrophy [31]. Although cTn is a cardiac specific biomarker, elevations can also occur in alternative conditions such as sepsis, pulmonary embolism, stroke, pericarditis, myocarditis or cardiotoxic chemotherapy [31-33].

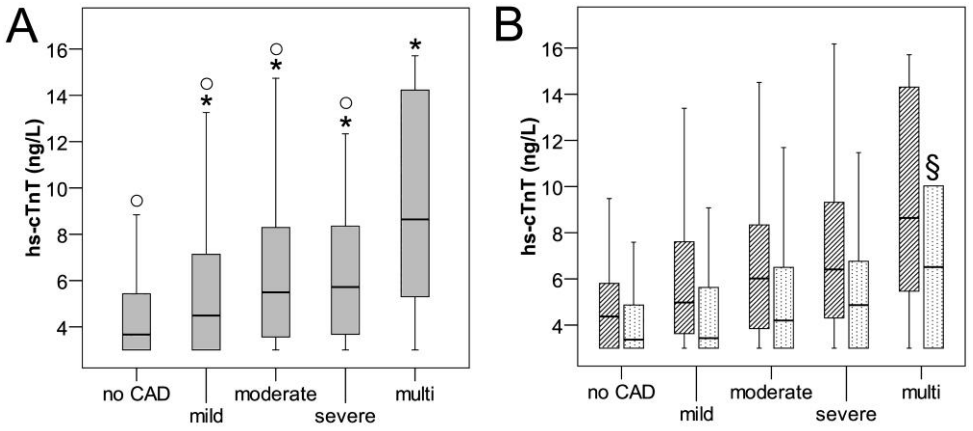
High-sensitivity Cardiac Troponin Immunoassays

Recently, high-sensitivity cardiac troponin (hs-cTn) immunoassays have become commercially available. Compared to the previous generation cTn immunoassays, these hs-cTn assays improved sensitivity and diagnostic accuracy in the lower range.

This may be important since previously undetectable cTn concentrations appeared to be associated with structural heart diseases and an increased mortality risk in the general population [34]. Moreover, recent studies in patients with suspected ACS showed that hs-cTn assays substantially improved the early diagnosis of an acute MI [35,36].

Association Between High-sensitivity Troponin and Severity of CAD

A previous study by our research group revealed that the severity of CAD is associated with increasing concentrations of hs-cTnT in patients (n=615) with stable chest pain symptoms, not suspicious for a MI [37]. The more severe CAD, the higher the hs-cTnT concentration (Figure 1A). We also found gender differences in hs-cTnT concentrations (Figure 1B), in accordance with previous research [38], which can be explained by pathophysiological differences in ACS between males and females. Endothelial dysfunction, microvascular disease and diffuse CAD are frequent causes of ischemia in females, while in male patients, plaque ruptures are usually responsible for repetitive thrombus embolization and subsequent increased cTnT concentrations [39]. Remarkably, high-sensitivity C-reactive protein (hsCRP) did not show a clear trend in this population, while N-terminal pro-B-type natriuretic peptide (NT-proBNP) showed a modest correlation with CAD (Figures 1C and 1D).



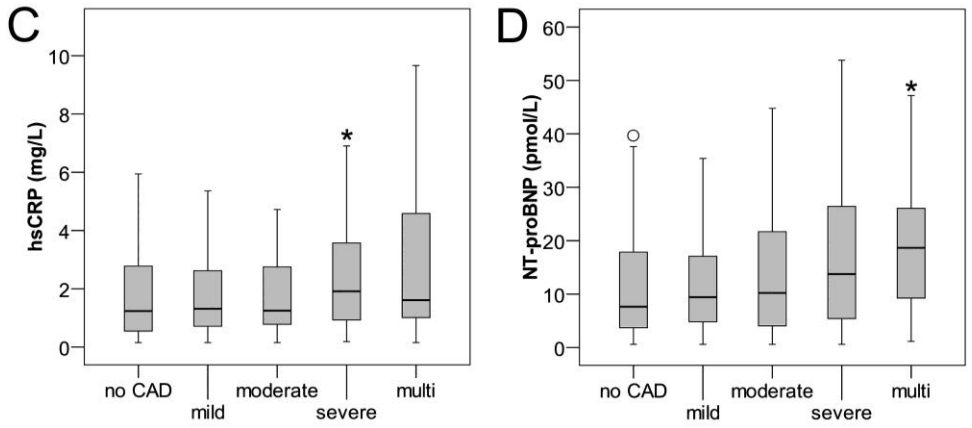


Figure 1 (partly previous page). *hs-cTnT concentrations show a progressive increase with increased severity of CAD (A), with a distinction between male gender (stripes) and female gender (dots) (B), in contrast to hsCRP (C) and NT-proBNP (D). Indicated are the significant differences between groups (*, as compared with the No CAD group; 0, as compared with the multivessel severe CAD group.) § indicates n=2 [37].*

Question remains what the explanation is for the observed increase in circulating cTn concentrations. Several mechanisms are postulated, including: 1) myocyte necrosis; 2) apoptosis; 3) normal myocyte turnover; 4) cellular release of proteolytic troponin degradation products; 5) increased cell wall permeability; and 6) formation and release of membraneous blebs [40]. Obstructive CAD does not necessarily have to be present, since non obstructive CAD can also lead to cTn release, possibly due to repeated plaque rupture with distal embolization [41].

Prognostic Value of High-sensitivity Troponin in Patients With Stable Chest Pain

Most prognostic studies have focused on the value of hs-cTnT in elderly or general population [34,42,43]. These studies found an association between elevated hs-cTnT and the incidence of heart failure, MI and mortality. The value of hs-cTnT in symptomatic patients with stable chest pain is less well known. In chapter 4 we described the prognostic value of hs-cTnT in stable chest pain patients and the additional value of hs-cTnT in combination with FRS, CCS and CCTA to predict MACE.

The composite endpoint of cardiac events included the occurrence of late coronary revascularization (>90 days), ACS and cardiac mortality. The mean follow-up time of the study was 2.2 years. Over three times as much events were found in patients with hs-cTnT concentrations in the 4th quartile, as compared to patients with hs-cTnT concentrations in the lower quartiles (HR 3.55, 95% CI 1.88-6.70, *P*<0.001). Survival analysis showed that hs-cTnT had significant predictive value on top of FRS, CCS and CCTA outcome. In conclusion, hs-cTnT seems to be a useful prognostic biomarker for stable chest pain patients presenting at the outpatient department. This is in line with a study showing that hs-cTn provides prognostic information for future MI and cardiovascular death in a stable high-risk population [44]. Interestingly, Omland et al found also a significant association between hs-cTnT, cardiovascular death and heart failure but not with MI in patients with stable chest pain [45]. Further research is needed to define the role of hs-cTn in different populations, including patients with symptoms, suspicious for ACS, presenting at the chest pain unit.

Chest pain units are developed to optimize care for patients with chest pain. The main goal is to filter those patients who need direct intervention and those who can safely be sent home, making patient care more cost-efficient. On the other hand, patient evaluation is still time-consuming and labor-intensive [46]. The future will bring us answers regarding the potential role for the new hs-cTn assays. Advantage will be that patients with a normal hs-cTn concentration can be safely discharged, possibly without additional testing. Disadvantage will be that a substantial part of the patients will present with an increased hs-cTn concentration due to a non-ACS cause. Potentially, these patients will undergo more tests. It will be a challenge for clinicians to deal with these developments.

ATHEROSCLEROSIS, COAGULATION AND THE IMMUNE SYSTEM

Atherosclerosis is a multifactorial vascular disorder [47], which has close interactions with the hemostatic, immune and inflammation system. In the vulnerable plaque concept, thrombosis plays an important role [48]. However, not only thrombin, but also neutrophils are important actors in the coagulation-inflammation interplay during atherogenesis.

Neutrophils, part of the innate immune system, appear to have pro-atherogenic and tissue destructing capabilities, through which they can contribute to plaque destabilization and subsequent plaque rupture [49,50].

Association Between Atherosclerosis and Thrombin Generation

Thrombin, the most central coagulation protein, is a strong pro-inflammatory mediator, which can induce an array of pro-atherogenic and plaque destabilizing effects [51]. Direct inhibition of thrombin attenuates the development of atherosclerosis and promotes lesion stability in ApoE-deficient mice [52,53]. In hypercoagulable mice, direct thrombin inhibition protects against severe plaque progression [54]. Less is known about the association between atherosclerosis and thrombin generation in humans.

In chapter 5 we provided novel clinical evidence indicating a positive independent association between thrombin formation in vivo and the presence and severity of CAD. Patients with CAD showed significantly higher thrombin-antithrombin complexes (TATc), which is a measure of in vivo thrombin formation, as compared with patients without CAD. Moreover, TATc concentrations were independently associated with the severity of both CCS and CAD, after adjustment for traditional cardiovascular risk factors. Despite this study was not designed to unravel the exact mechanism, these results suggest that thrombin plays a role in the pathophysiology of coronary calcification and CAD in humans. Although addition of TATc as a marker to the FRS improved the predictive value for the presence of CAD, it is too early yet to consider TATc as a general risk factor. Moreover, TATc measurement is of course no substitute for CCTA. Due to the small cohort and the short follow-up time we were unfortunately not yet able to analyze the relevance of TATc in predicting MACE. The future will show whether there is such a relationship.

Recently, new oral anticoagulants became available in daily practice, including direct factor-IIa (thrombin) inhibitors (e.g. Dabigatran). These drugs may be prescribed to patients with non-valvular AF with additional risk factors, in order to prevent cerebrovascular events and systemic embolization. A recent study investigated the effect of AF on atrial thrombogenesis [55]. Rapid atrial rates and AF in humans both resulted in increased platelet activation and thrombin generation.

Prothrombotic activation occurs to a greater extent in the left atrium compared with the systemic circulation. In addition, AF also induces endothelial dysfunction and inflammation. In this context, it would be interesting to investigate the effects of direct factor-IIa inhibitors on the extent and degree of CAD in patients with AF.

Association Between CAD and Markers of Extracellular DNA Formation

Formation of neutrophil extracellular traps (NETs), as a consequence of platelet-neutrophil interaction, is part of the innate immune response in order to eliminate infections. NET's are a network of extracellular DNA fibers, carrying histones and granule proteins, which are able to trap and kill bacteria [56]. Moreover, NETs can cause platelet activation, adhesion and aggregation in vitro [57]. NETs can generate red blood cell rich thrombi by binding red blood cells and platelets, which are associated with deep-vein thrombosis. Accordingly, it is not inconceivable that NET-platelet interactions and the subsequent thrombus formation may play a role in the pathogenesis of atherosclerosis and atherothrombosis [56]. However, there is only limited clinical evidence supporting this hypothesis.

In chapter 6 we showed that elevated levels of markers of extracellular DNA traps are independently associated with the extent and severity of CAD. Levels of double-stranded DNA (dsDNA), nucleosomes and MPO-DNA complexes were significantly increased in patients with severe CAD, while high plasma nucleosome levels were independently associated with severe CAD even after adjustment for confounding factors. Moreover, nucleosomes, dsDNA, MPO-DNA complexes and TATc levels were independent predictors of the extent of CAD after adjustment for confounding variables. These observations demonstrate that the formation of NETs might be important in the atherosclerotic process. However, more evidence is needed to confirm this and further research is required to investigate the possible contribution of NETs in development of MACE. Far ahead of these matters, but interesting, is a recent study on colchicines [58].

Colchicine is a drug, prescribed to treat gout, with anti-inflammatory actions on different cells including neutrophils. The objective of the study was to determine whether a low-dose colchicine is able to reduce the risk of cardiovascular events in patients with clinically stable CAD.

Patients received 0.5 mg colchicine/day in addition to standard secondary prevention therapies including aspirin and statins. After a median follow-up period of 3 years, 5.3% of patients on colchicine suffered an event (ACS, out of hospital cardiac arrest, noncardioembolic ischemic stroke) compared to 16.0% of patients not on colchicine. Therefore, colchicine appeared effective for the prevention of CVD events in patients with stable CAD. This study is a potentially breakthrough for anti-inflammatory therapy in CAD. It seems at least that colchicine is a worthy candidate drug for future trials regarding patients with stable CAD.

CALCIFICATION AND PLAQUE MORPHOLOGY BY CORONARY CT-ANGIOGRAPHY

CCTA has evolved as a widely available, highly accurate non-invasive diagnostic imaging tool for the assessment of CAD. A coronary CT-scan typically consists of two parts. First, a non-contrast-enhanced calcium score scan is performed to determine the calcium score (Agatston score), which is a measure for the amount of calcium hydroxyapatite in the coronary arteries [59]. Secondly, CCTA is performed to analyze the coronary tree for presence, extent, severity and morphology of coronary plaques. In terms of plaque morphology we distinguish calcified plaques, non-calcified plaques and mixed plaques [60]. These mixed plaques contain both a calcified as well as a non-calcified component.

Implications and Pitfalls of Calcium Score

The calcium score is a proven measure of severity of CAD; a good correlation has been found between the calcium score and the total amount of CAD, as assessed by histology [61,62]. Moreover, an increased calcium score is correlated with an increased risk of obstructive CAD and accordingly MACE [63,64]. The calcium score is even considered a better predictor of MACE than the FRS, although it is recognized that CAD can be present in patients with a zero calcium score [65-67]. However, obstructive CAD in absence of coronary calcification is rare. In our database, containing >4,500 CT-cases, only 1% of patients with a calcium score of zero had obstructive CAD as assessed with CCTA and confirmed by invasive coronary angiography (ICA) [68]. CAD in these patients is caused by non-calcified plaques.

The Calcium Challenge in Coronary CT-angiography

Although CCTA is a reliable and proven technique identify coronary calcification, there are a few important drawbacks. Due to limitations in spatial resolution it is difficult, maybe even impossible, to pick up micro-calcifications and to determine the exact location of these micro-calcification.

Despite recent advances in reconstruction techniques and the introduction of dual energy CT, the presence of coronary calcification leads to high levels of signal attenuation, which can cause blooming artifacts [69]. A high calcium score leads to an increased risk of blooming artifacts. As a result, it will be more difficult to reliable assess the CCTA, possibly leading to a non-diagnostic scan [70,71]. The choice whether to proceed with CCTA in the presence of extensive coronary calcification remains therefore controversial [72]. Question is if there is additional value of CCTA in these patients with a high calcium score. An alternative approach could be to refrain from CT-angiography and refer these patients for perfusion imaging or ICA. ICA is still the standard of reference for assessment of CAD severity [73]. Advantage of this approach is that patients are not repeatedly exposed to ionizing radiation and iodinated contrast during both CCTA and ICA.

According to the most recent appropriate use criteria for cardiac computed tomography, it is appropriate to perform CCTA in symptomatic patients if the calcium score is below 400 Agatston units [74]. In case of a calcium score >400, it is uncertain whether CCTA can offer additional value. The National Institute for Health and Clinical Excellence (NICE) recently acknowledged the role of coronary calcium scoring in their guideline “Chest pain of recent onset”. They recommend initial calcium scoring in patients with chest pain with a low to intermediate risk of CAD. In case of a zero calcium score, no further cardiac testing is needed because obstructive CAD has been ruled out with a high degree of accuracy. When the calcium score is between 1-400, CCTA or myocardial perfusion imaging is recommended. Patients with a calcium score >400 can be directly referred for ICA, because CCTA will not likely be informative in this category [73].

In literature however, there is ongoing debate on the use of certain cut-off values for calcium score as a gatekeeper for the performance of CCTA. Different cut-off values, including values of calcium score >400, are suggested, but there is still no consensus [75-78].

These higher cut-off values are fed by the observation that a substantial part of patients with such a high calcium score do not suffer from ICA proven obstructive CAD. Our data show that of all patients with a calcium score >400 who underwent ICA, 37,5% did not have plaques with ≥50% luminal stenosis, as assessed with ICA. Even more extreme is the small part of people with a very high calcium score (> 1,000 Agatston units), only showing minor wall irregularities during ICA.

The Effects of Vitamin K-antagonists on Calcification

Previously, vascular calcification was considered as a passive, degenerative process. However, nowadays we know that it is an active, well-regulated process [79], resulting from an imbalance between calcification promoting and inhibiting factors. Matrix Gla Protein (MGP) is a strong, vitamin K dependent, inhibitor of calcification. As can be expected, vitamin K deficiency has shown to lead to calcification [80]. More recently, some studies showed an association between VKA treatment and arterial calcification [81,82]. VKA therapy is a cornerstone in the treatment of venous thrombosis and frequently prescribed to patients with atrial fibrillation (AF), in order to prevent thromboembolic events in general and ischemic stroke more specific [83].

In chapter 7 we investigated the effect of VKA on coronary calcification in both patients and ApoE$^{-/-}$ mice. We compared 133 patients on VKA to 133 age-, gender- and FRS-matched non-VKA users. Patients on VKA were divided into three tertiles, based on duration of VKA treatment. The mean calcium score increased significantly in VKA users as the duration of VKA treatment increased, while the calcium score in non-VKA users did not change. Moreover, the proportion of calcified plaques increased significantly with prolonged VKA use, a trend which also was not present in non-VKA users. In ApoE$^{-/-}$ mice, VKA treatment significantly increased the frequency and extent of vascular calcification, including micro-calcification of the intima. Moreover, VKA treatment ignite a cascade of responses, resulting in increased apoptosis and outward plaque remodeling, which are features of plaque vulnerability.

The findings of this study support the need for alternative anticoagulation drugs, which are not interfering with the vitamin K cycle. Examples of these so-called novel oral anticoagulant drugs are direct factor-Xa inhibitors (e.g. Rivaroxaban, Apixaban) and direct factor-IIa (thrombin) inhibitors (e.g. Dabigatran).

These drugs could offer an interesting alternative to VKA treatment, because they do not require frequent blood tests for monitoring while offering similar efficacy results. Lacking the inhibitory effect on the vitamin K cycle, adverse side effect like vascular calcification and fractures can be avoided. If this will be demonstrated, the advantages over VKA will be even greater [84].

Vitamin K2: a Possible Fighter of Calcification?

Coronary calcification is a progressive process. A few studies in which patients underwent serial CCTA reported the progression of CAD, including increase in number and extent of calcified plaques [85]. An interesting hypothesis in order to decrease the progression rate of coronary calcification is administration of vitamin K2. In a previous study, a vitamin K2 rich diet was associated with a reduced cardiovascular mortality rate. Moreover, it was inversely related with severe aortic calcification [86]. Supplementation of vitamin K2 in animals resulted in regression of existing arterial calcification [87]. Currently, we are testing this hypothesis in a randomized, double-blind, placebo-controlled trial of patients with CCTA-established coronary calcification.

Characteristics of a Vulnerable Plaque

Non-calcified plaques with low plaque density (low attenuation plaques) are described as vulnerable [88]. Other characteristics of culprit vulnerable plaques, associated with ACS, include positive vascular remodeling and spotty calcification [88-90]. In a prospective CCTA study, it was confirmed that patients demonstrating positively remodeled coronary segments with low attenuation plaques were at higher risk of ACS developing over time when compared with patients having lesions without these characteristics [91]. Recently, we investigated whether the use of a semi-automatic plaque quantification algorithm (reporting volumetric and geometric plaque characteristics) provides additional prognostic value for the development of future ACS as compared with conventional CCTA assessment (calcium score, extent and degree of luminal stenosis by eyeballing). Therefore, we selected 25 patients who developed ACS after CCTA and 101 randomly selected controls who did not develop ACS.

There were no significant differences between the groups regarding conventional CCTA assessment results and baseline characteristics. Using the semi-automatic plaque quantification, patients who developed ACS had a higher total plaque volume (mm^3), total number of plaques and a higher non-calcified plaque volume (mm^3), $P \leq 0.001$. Semi-automatic plaque quantification provided additional prognostic value over both clinical risk profiling as well as conventional CCTA assessment. Therefore, it can improve risk stratification in patients with chest discomfort symptoms, suspect for stable CAD [92].

The Controversy Between Calcified Plaques and Plaque Vulnerability

In our study, described in the previous paragraph, the calcium score was not different between the ACS group and the control group. Moreover, calcification was not associated with the occurrence of future ACS.

Interesting is the observation that the calcium score is associated with extent and degree of CAD and also act as a predictor of MACE, although it is not recognized as a characteristic of plaque vulnerability in the majority of CCTA-studies. Spotty calcifications may be a characteristic of plaque vulnerability, but are too small to provide a huge increase in calcium score. Therefore, the exact role of calcification with respect to plaque vulnerability remains controversial.

Coronary calcification may represent an attempt to protect threatened myocardium by strengthening weakened atherosclerotic plaque prone to rupture [93]. Relative stability of calcified lesions has been demonstrated in an intravascular ultrasound study [94]. In short term, this attempt will lead to plaque stabilizing. However, plaque rupture often occurs at the interface between the calcified and the non-calcified component [95]. Due to more extensive calcification these weak points may be eliminated with a decreasing risk of plaque rupture [93]. This hypothesis partly supports the theory of plaque vulnerability due to spotty calcification. Calcifications adjacent to or beneath the lipid necrotic core were assumed to be stabilizing, while micro-calcifications in the fibrous cap were assumed to be destabilizing [96,97].

SOCIAL ECONOMIC CONSIDERATIONS

As stated previously, CVD is accompanied by high healthcare costs. In the USA, 15% of the current total health expenditures are related to CVD, which is more than any other major diagnostic group. Between 2013 and 2030, total direct medical costs of CVD will increase from $320 billion to $818 billion, while indirect costs (attributable to lost productivity) will increase with 52%, from $203 billion to $308 billion [2].

The number of people who need medical care is constantly rising; a trend that will continue in the (near) future as a consequence of conditions like aging, obesity and DM. Currently, already 34.6% of the adult US population is obese (body mass index ≥30 kg/m^2), while another 33.6% is overweight. Among children (2-19 years of age), 16.9% is obese and another 14.9% is overweight [2]. These numbers are worrisome, especially since obesity is not only associated with marked excess mortality, but also with excess morbidity, including an increased risk on conditions like DM, CVD, asthma, cancer and degenerative joint disease.

These numbers pose a challenge for health care professionals. In order to keep our healthcare affordable, it is important to be as cost-effective as possible. In the diagnostic process it means that clinicians need to choose the most appropriate test for a particular patient. The proper use of risk stratification algorithms and biomarkers could be the initial step to make a deliberately decision about the further diagnostic approach. Importantly, a biomarker will only be cost-effective if it provides information which can change the further diagnostic approach and clinical management, thereby improving clinical outcome [29]. Another important aspect regarding cost-effectiveness is to gain as much information as possible from a certain test, in order to avoid unnecessary diagnostic tests. Due to recent technological improvements, CCTA is increasingly able to provide information regarding functional analysis rather than just anatomical analysis [98]. Fractional flow reserve measurement on CT (CT-FFR) is one of the most recent development in order to measure the hemodynamic relevance of a coronary plaque [99]. Moreover, software is available which can provide more accurate information about atherosclerotic plaque characteristics beyond the conventional assessment method [92]. Integrating all this information may contribute to an increased cost-effectiveness.

DIRECTIONS FOR FUTURE RESEARCH

Future research and developments are needed in order to further optimize risk stratification in patients with chest discomfort symptoms. This research would consist of several pillars.

One of the pillars is the development of a risk stratification algorithm, incorporating not only cardiovascular risk factors but also biomarkers may further improve risk estimation. Future research should determine which combination of biomarkers will be most suitable. This algorithm could possibly be further extended with imaging results, for example the coronary calcium score. Moreover, the risk factors in such an algorithm must be up to date. The body mass index for example is not included in the current algorithms, but may play an important role.

Another pillar of future research is to further decipher underlying patho-physiologic mechanisms of atherosclerosis, and the association of atherosclerosis with other systems including the hemostatic, inflammation and immune system. Biomarker studies can help us to better understand these mechanisms.

REFERENCES

1. Celermajer DS, Chow CK, Marijon E, et al. Cardiovascular disease in the developing World: prevalences, patterns, and the potential of early disease detection. *J Am Coll Cardiol* 2012;14:1207–1216.
2. Go AS, Mozaffarian D, Roger Vl, et al. Heart disease and stroke statistics -- 2013 update: a report from the American Heart Association. *Circulation* 2013;127:6–245.
3. Libby P. Current concepts of the pathogenesis of the acute coronary syndromes. *Circulation* 2001;104:365–372.
4. Myerburg RJ, Junttila MJ. Sudden cardiac death caused by coronary heart disease. *Circulation* 2012;125:1043–1052.
5. Braunwald E. Epilogue: what do clinicians expect from imagers? *J Am Coll Cardiol* 2006;47:101–103.

6. Smith SC Jr, Benjamin EJ, Bonow RO, et al. AHA/ACCF secondary prevention and risk reduction therapy for patients with coronary and other atherosclerotic vascular disease: 2011 update. *J Am Coll Cardiol* 2011;23:2432–2446.
7. D'Agostino RB Sr, Vasan RS, Pencina MJ, et al. General cardiovascular risk profile for use in primary care: The Framingham Heart Study. *Circulation* 2008;117:743–753.
8. Assmann G, Cullen P, Schulte H. Simple scoring scheme for calculating the risk of acute coronary events based on the 10-year follow-up of the prospective cardiovascular Munster (PROCAM) study. *Circulation* 2002;105:310–315.
9. Conroy RM, Pyorala K, Fitzgerald AP, et al. Estimation of ten-year risk of fatal cardiovascular disease in Europe: The SCORE project. *Eur Heart J* 2003;24:987–1003.
10. Versteylen MO, Joosen IA, Shaw LJ, et al. Comparison of Framingham, PROCAM, SCORE, and Diamond Forrester to predict coronary atherosclerosis and cardiovascular events. *J Nucl Cardiol* 2011;18:904–911.
11. McGill HC Jr, McMahan CA, Gidding SS. Preventing heart disease in the 21st century: implications of the Pathobiological Determinants of Atherosclerosis in Youth (PDAY) Study. *Circulation* 2008;117:1216–1227.
12. Greenland P, Knoll MD, Stamler J, et al. Major risk factors as antecedents of fatal and nonfatal coronary heart disease events. *JAMA* 2003;290:891–897.
13. Greenland P, Alpert JS, Beller GA, et al. 2010 ACCF/AHA guideline for assessment of cardiovascular risk in asymptomatic adults. *Circulation* 2010;122:584–636.
14. World Health Organization Statistical Information System 2009: www.who.int/whosis
15. Enriquez JR, Pratap P, Zbilut JP, et al. Women tolerate drug therapy for coronary artery disease as well as men do, but are treated less frequently with aspirin, β-blockers, or statins. *Gender Med* 2008;5:53–61.
16. Lahoz C, Mantilla T, Taboada M, et al. Gender differences in evidence-based pharmacological therapy for patients with stable coronary heart disease. *Int J Cardiol* 2009;133:336–340.
17. Gottlieb S, Harpaz D, Shotan A, et al. Sex differences in management and outcome after acute myocardial infarction in the 1990s. A prospective observational community-based study. *Circulation* 2000;102:2484–2490.
18. Nair D, Carrigan TP, Curtin RJ, et al. Association of coronary atherosclerosis detected by multislice computed tomography and traditional risk-factor assessment. *Am J Cardiol* 2008;102:316–320.

19. Lakoski SG, Greenland P, Wong ND, et al. Coronary artery calcium scores and risk for cardiovascular events in women classified as “low risk” based on Framingham risk score: the multi-ethnic study of atherosclerosis (MESA). *Arch Intern Med* 2007;167:2437–2442.
20. Mosca l, Benjamin EJ, Berra K, et al. Effectiveness-based guidelines for the prevention of cardiovascular disease in women -- 2011 update: a guideline from the American Heart Association. *Circulation* 2011;123:1243–62.
21. Grundy SM, Pasternak R, Greenland P, et al. Assessment of cardiovascular risk by use of multiple-risk-factor assessment equations. *Circulation* 1999;100:1481–1492.
22. Chung CP, Oeser A, Raggi P, et al. Increased coronary-artery atherosclerosis in rheumatoid arthritis: relationship to disease duration and cardiovascular risk factors. *Arthritis Rheum* 2005;52:3045–3053.
23. Go AS, Chertow GM, Fan D, et al. Chronic kidney disease and the risk of death, cardiovascular events, and hospitalization. *N Engl J Med* 2004;351:1296–1305.
24. Foley RN, Collins AJ. End-stage renal disease in the United States: an update from the United States Renal Data System. *J Am Soc Nephrol* 2007;18:2644–2648.
25. Budoff MJ, Rader DJ, Reilly MP, et al. Relationship of estimated GFR and coronary artery calcification in the CRIC Study. *Am J Kidney Dis* 2011;4:519–526.
26. Ix JH, Katz R, Kestenbaum B, et al. Association of mild to moderate kidney dysfunction and coronary calcification. *J Am Soc Nephrol* 2008;19:579–585.
27. Balagopal PB, de Ferranti SD, Cook S, et al. Nontraditional risk factors and biomarkers for cardiovascular disease: mechanistic, research, and clinical considerations for youth. *Circulation* 2011;123:2749–2769.
28. Vasan R. Biomarkers of cardiovascular disease: molecular basis and practical considerations. *Circulation* 2006;113:2335–2362.
29. Hlatky MA, Greenland P, Arnett DK, et al. American Heart Association expert panel on subclinical atherosclerotic diseases and emerging risk factors and the stroke council. Criteria for evaluation of novel markers of cardiovascular risk. *Circulation* 2009;119:2408–2416.
30. Ridker PM, Brown NJ, Vaughan DE, et al. Established and emerging plasma biomarkers in the prediction of first atherothrombotic events. *Circulation* 2004;109:6–19.
31. Jaffe AS. Third universal definition of myocardial infarction. *Clin Biochem* 2013;46:1–4.
32. Agzew Y. Elevated serum cardiac troponin in non-acute coronary syndrome. *Clin Cardiol* 2009;32:15–20.

33. Korff S, Katus HA, Giannitsis E. Differential diagnosis of elevated troponins. *Heart* 2006;92:987–993.
34. De Lemos JA, Drazner MH, Omland T, et al. Association of troponin T detected with a highly sensitive assay and cardiac structure and mortality risk in the general population. *JAMA* 2010;304:2503–2512.
35. Reichlin T, Hochholzer W, Bassetti S, et al. Early diagnosis of myocardial infarction with sensitive cardiac troponin assays. *N Engl J Med* 2009;361:858–867.
36. Keller T, Zeller T, Peetz D, et al. Sensitive troponin I assay in early diagnosis of acute myocardial infarction. *N Engl J Med* 2009;361:868–877.
37. Laufer EM, Mingels AM, Winkens MH, et al. The extent of coronary atherosclerosis is associated with increasing circulating levels of high sensitive cardiac troponin T. *Arterioscler Thromb Vasc Biol* 2010;30:1269–1275.
38. Mingels A, Jacobs L, Michielsen E, et al. Reference population and marathon runner sera assessed by highly sensitive cardiac troponin T and commercial cardiac troponin T and I assays. *Clin Chem* 2009;55:101–108.
39. Sbarouni E, Georgiadou P, Voudris V. Gender-specific differences in biomarkers responses to acute coronary syndrome and revascularization procedures. *Biomarkers* 2011;16:457–465.
40. White HD. Pathobiology of troponin elevations: do elevations occur with myocardial ischemia as well as necrosis? *J Am Coll Cardiol* 2011;57:2406–2408.
41. Ahmed W, Schlett CL, Uthamalingam S, et al. Single resting hsTnT level predicts abnormal myocardial stress test in acute chest pain patients with normal initial standard troponin. *JACC Cardiovasc Imaging* 2013;6:72–82.
42. Otsuka T, Kawada T, Ibuki C, et al. Association between high-sensitivity cardiac troponin T levels and the predicted cardiovascular risk in middle-aged men without overt cardiovascular disease. *Am Heart J* 2010;159:972–978.
43. deFilippi CR, de Lemos JA, Christenson RH, et al. Association of serial measures of cardiac troponin T using a sensitive assay with incident heart failure and cardiovascular mortality in older adults. *JAMA* 2010;304:2494–2502.
44. Kavsak PA, Xu L, Yusuf S, et al. High-sensitivity cardiac troponin I measurement for risk stratification in a stable high-risk population. *Clin Chem* 2011;57:1146–1153.
45. Omland T, de Lemos JA, Sabatine MS, et al. A sensitive cardiac troponin T assay in stable coronary artery disease. *N Engl J Med* 2009;361:2538–2547.

46. Miller TD, Bailey KR. Will high-sensitivity troponin improve the evaluation of patients with chest pain in the emergency department? *JACC Cardiovasc Imaging* 2013;6:83–85.
47. Ross R. Atherosclerosis: an inflammatory disease. *N Engl J Med* 1999;340:115–126.
48. Finn AV, Nakano M, Narula J, et al. Concept of vulnerable/unstable plaque. *Arterioscler Thromb Vasc Biol* 2010;30:1282–1292.
49. Drechsler M, Megens RT, van Zandvoort M, et al. Hyperlipidemia-triggered neutrophilia promotes early atherosclerosis. *Circulation* 2010;122:1837–1845.
50. Ionita MG, van den Borne P, Catanzariti LM, et al. High neutrophil numbers in human carotid atherosclerotic plaques are associated with characteristics of rupture-prone lesions. *Arterioscler Thromb Vasc Biol* 2010;30:1842–1848.
51. Borissoff JI, Spronk HM, ten Cate H. The hemostatic system as a modulator of atherosclerosis. *N Engl J Med* 2011;364:1746–1760.
52. Bea F, Kreuzer J, Preusch M, et al. Melagatran reduces advanced atherosclerotic lesion size and may promote plaque stability in apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol* 2006;26:2787–2792.
53. Zhou Q, Bea F, Preusch M, et al. Evaluation of plaque stability of advanced atherosclerotic lesions in apo E-deficient mice after treatment with the oral factor Xa inhibitor rivaroxaban. *Mediators Inflamm* 2011;2011:432080.
54. Borissoff JI, Otten JJ, Heeneman S, et al. Genetic and pharmacological modifiations of thrombin formation in apolipoprotein E-deficient mice determine atherosclerosis severity and atherothrombosis onset in a neutrophil-dependent manner. *PLoS One* 2013;8:e55784.
55. Lim HS, Willoughby SR, Schultz C, et al. Effect of atrial fibrillation on atrial thrombogenesis in humans: impact of rate and rhythm. *J Am Coll Cardiol* 2013;61:852–860.
56. Drechsler M, Döring Y, Megens RT, et al. Neutrophilic granulocytes – promiscuous accelerators of atherosclerosis. *Thromb Haemost* 2011;106:839–848.
57. Fuchs TA, Brill A, Duerschmied D, et al. Extracellular DNA traps promote thrombosis. *Proc Natl Acad Sci USA* 2010;107:15880–15885.
58. Nidorf SM, Eikelboom JW, Budgeon CA, et al. Low-dose colchicine for secondary prevention of cardiovascular disease. *J Am Coll Cardiol* 2013;61:404–410.
59. Agatston AS, Janowitz WR, Hildner FJ, et al. Quantification of coronary artery calcium using ultrafast computed tomography. *J Am Coll Cardiol* 1990;15:827–832.

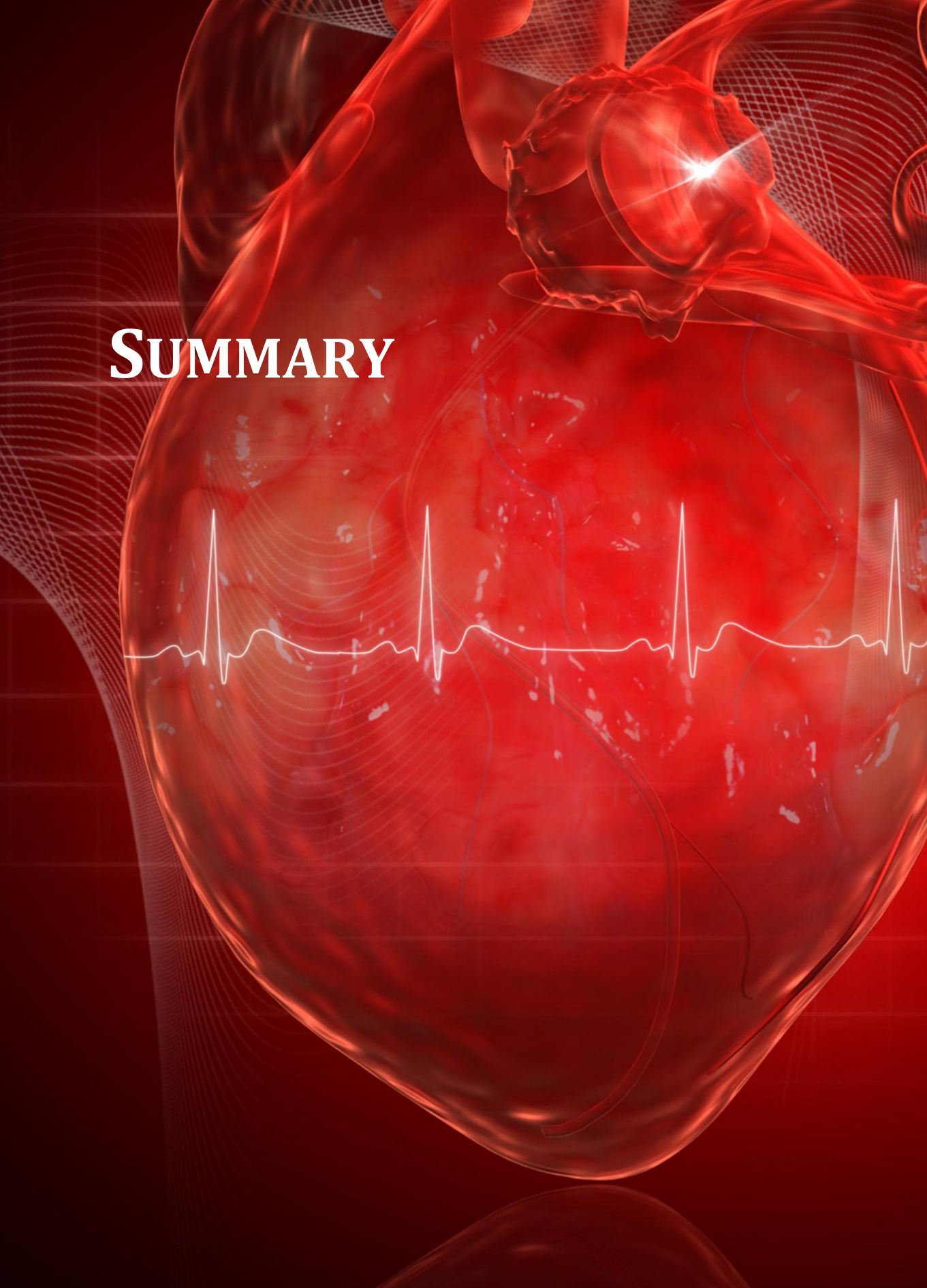
60. Raff GL, Abidov A, Achenbach S, et al. SCCT guidelines for the interpretation and reporting of coronary computed tomographic angiography. *J Cardiovasc Comput Tomogr* 2009;3:122–136.
61. Rumberger JA, Simons DB, Fitzpatrick LA, et al. Coronary artery calcium area by electron-beam computed tomography and coronary atherosclerotic plaque area. A histopathologic correlative study. *Circulation* 1995;92:2157–2162.
62. Sangiorgi G, Rumberger JA, Severson A, et al. Arterial calcification and not lumen stenosis is highly correlated with atherosclerotic plaque burden in humans: a histologic study of 723 coronary artery segments using nondecalcifying methodology. *J Am Coll Cardiol* 1998;31:126–133.
63. Rumberger JA, Brundage BH, Rader DJ, et al. Electron beam computed tomographic coronary calcium scanning: a review and guidelines for use in asymptomatic patients. *Mayo Clin Proc* 1999;74:243–252.
64. Detrano R, Guerci AD, Carr JJ, et al. Coronary calcium as a predictor of coronary events in four racial or ethnic groups. *N Engl J Med* 2008;358:1336–1345.
65. McEvoy JW, Blaha MJ, Defilippis AP, et al. Coronary artery calcium progression: an important clinical measurement? *J Am Coll Cardiol* 2010;56:1613–1622.
66. Alexopoulos N, Raggi P. Calcification in atherosclerosis. *Nat Rev Cardiol* 2009;6:681–688.
67. Morita H, Fujimoto S, Kondo T, et al. Prevalence of computed tomographic angiography verified high-risk plaques and significant luminal stenosis in patients with zero coronary calcium score. *Int J Cardiol* 2012;158:272–278.
68. Joosen IA, Versteylen MO, Das M, et al. Letter in response to Andrea K.Y. Lee et al. "normalcy rate of computed tomographic coronary angioghraphy". *Int J Cardiol* 2012;158:299–300.
69. Zhang S, Levin DC, Halpern EJ, et al. Accuracy of MDCT in assessing the degree of stenosis caused by calcified coronary artery plaques. *Am J Roentgenol* 2008;191:1676–1683.
70. Ong TK, Chin SP, Liew CK, et al. Accuracy of 64-row multidetector computed tomography in detecting coronary artery disease in 134 symptomatic patients: influence of calcification. *Am Heart J* 2006;151:1–6.
71. Achenbach S. Computed tomography coronary angiography. *J Am Coll Cardiol* 2006;48:1919–1928.
72. Abbara S, Arbab-Zadeh A, Callister TQ, et al. SCCT guidelines for performance of coronary computed tomographic angiography. *J Cardiovasc Comput Tomogr* 2009;3:190–204.

73. Skinner JS, Smeeth L, Kendall JM, et al. Chest Pain Guideline Development Group. NICE guidance. Chest pain of recent onset: assessment and diagnosis of recent onset chest pain or discomfort of suspected cardiac origin. *Heart* 2010;96:974–978.
74. Taylor AJ, Cerqueira M, Hodgson JM, et al. 2010 appropriate use criteria for cardiac computed tomography. *J Am Coll Cardiol* 2010;56:1864–1894.
75. Palumbo AA, Maffei E, Martini C, et al. Coronary calcium score as gatekeeper for 64-slice computed tomography coronary angiography in patients with chest pain: per-segment and per-patient analysis. *Eur Radiol* 2009;19:2127–2135.
76. de Agustin JA, Marcos-Alberca P, Fernández-Golfin C, et al. Should computed tomography coronary angiography be aborted when the calcium score exceeds a certain threshold in patients with chest pain? *Int J Cardiol* 2012 [Epub ahead of print].
77. Meng L, Cui L, Cheng Y, et al. Effect of heart rate and coronary calcification on the diagnostic accuracy of the dual-source CT coronary angiography in patients with suspected coronary artery disease. *Korean J Cardiol* 2009;10:347–354.
78. Arbab-Zadeh A, Miller JM, Rochitte CE, et al. Diagnostic accuracy of computed tomography coronary angiography according to pre-test probability of coronary artery disease and severity of coronary arterial calcification. The CORE-64 international multicenter study. *J Am Coll Cardiol* 2012;59:379–387.
79. Demer LL, Tintut Y. Vascular calcification: pathobiology of a multifaceted disease. *Circulation* 2008;117:2938–2948.
80. Vermeer C. Vitamin K: the effect on health beyond coagulation – an overview. *Food Nutr Res* 2012;56:5329–5334.
81. Weijs B, Blaauw Y, Rennenberg RJMW, et al. Patients using vitamin K antagonists show increased levels of coronary calcification: an observational study in low-risk atrial fibrillation patients. *Eur Heart J* 2011;32:2555–2562.
82. Rennenberg RJMW, van Varik BJ, Schurgers LJ, et al. Chronic coumarin treatment is associated with increased extracoronary arterial calcification in humans. *Blood* 2010;115:5121–5123.
83. Hart RG, Pearce LA, Aguilar MI. Meta-analysis: antithrombotic therapy to prevent stroke in patients who have nonvalvular atrial fibrillation. *Ann Intern Med* 2007;146:857–867.
84. Fusaro M, Crepaldi G, Maggi S, et al. Bleeding, vertebral fractures and vascular calcifications in patients tretaed with warfarin: hope for lower risks with alternative therapies. *Curr Vasc Pharmacol* 2011;9:763–769.

85. Hoffmann H, Frieler K, Schlattmann P, et al. Influence of statin treatment on coronary atherosclerosis visualized using multidetector computed tomography. *Eur Radiol* 2010;20:2824–2833.
86. Geleijnse JM, Vermeer C, Grobbee DE, et al. Dietary intake of menaquinone is associated with a reduced risk of coronary heart disease: the Rotterdam Study. *J Nutr* 2004;134:3100–3105.
87. Seyama Y, Horiuch M, Hayashi M, et al. Effect of vitamin K2 on experimental calcinosis induced by vitamin D2 in rat soft tissue. *Int J Vitam Nutr Res* 1996;66:36–38.
88. Motoyama S, Kondo T, Sarai M, et al. Multislice computed tomographic characteristics of coronary lesions in acute coronary syndromes. *J Am Coll Cardiol* 2007;50:319–326.
89. Ehara S, Kobayashi Y, Yoshiyama M, et al. Spotty calcification typifies the culprit plaque in patients with acute myocardial infarction: an intravascular ultrasound study. *Circulation* 2004;110:3424–3429.
90. Fujii K, Carlier SG, Mintz GS, et al. Intravascular ultrasound study of patterns of calcium in ruptured coronary plaques. *Am J Cardiol* 2005;96:352–357.
91. Motoyama S, Sarai M, Harigaya H, et al. Computed tomographic angiography characteristics of atherosclerotic plaques subsequently resulting in acute coronary syndrome. *J Am Coll Cardiol* 2009;54:49–57.
92. Versteylen MO, Kietselaer BL, Dagnelie PC, et al. Additive value of semi-automated quantification of coronary artery disease using cardiac CT-angiography to predict for future acute coronary syndrome. *J Am Coll Cardiol* 2013;61:2296–2305.
93. Wexler L, Brundage B, Crouse J, et al. Coronary artery calcification: pathophysiology, epidemiology, imaging methods, and clinical implications. *Circulation* 1996;94:1175–1192.
94. Ehara S, Kobayashi Y, Yoshiyama M, et al. Spotty calcification typifies the culprit plaque in patients with acute myocardial infarction: an intravascular ultrasound study. *Circulation* 2004;110:3424–3429.
95. Abedin M, Tintut Y, Demer LL. Vascular calcification: mechanisms and clinical ramifications. *Arterioscler Thromb Vasc Biol* 2004; 24:1161–1170.
96. Huang H, Virmani R, Younis H, et al. The impact of calcification on the biomechanical stability of atherosclerotic plaques. *Circulation* 2001;103:1051–1056

97. Vengrenyuk Y, Carlier S, Xanthos S, et al. A hypothesis for vulnerable plaque rupture due to stress-induced debonding around cellular microcalcifications in thin fibrous caps. *Proc Natl Acad Sci USA* 2006;103:14678–14683.
98. Ko SM, Choi JW, Hwang HK, et al. Diagnostic performance of combined noninvasive anatomic and functional assessment with dual-source CT and adenosine-induced stress dual-energy CT for detection of significant coronary stenosis. *Am J Roentgenol* 2012;198:512–520.
99. Min JK, Leipsic J, Pencina MJ, et al. Diagnostic accuracy of fractional flow reserve from anatomic CT angiography. *JAMA* 2012;308:1237–1245.

Summary

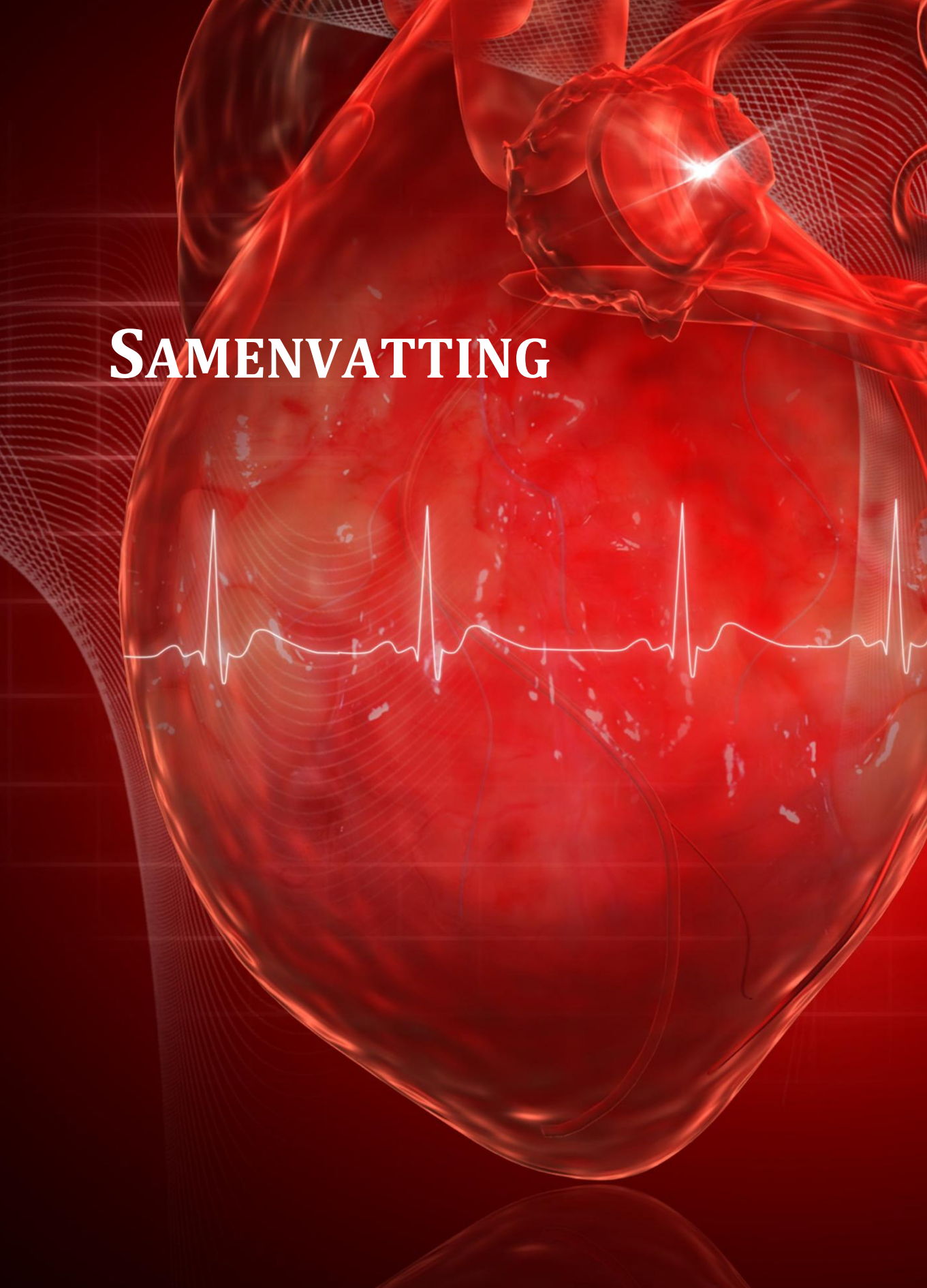


Cardiovascular disease, including coronary artery disease, is still one of the major causes of mortality and morbidity in Western and developing countries. After decades of decline, it is expected that the proportion of cardiovascular disease will increase again, accompanied by high healthcare costs. Therefore, cardiovascular disease is a rising major global health problem. Atherosclerosis, a multifactorial chronic inflammatory disease, is the main cause of coronary artery disease. Atherosclerosis is not limited to the coronary arteries, but is a systemic disease, which can result in complications like myocardial infarction, stroke and peripheral artery disease.

With the use of coronary CT-angiography it is possible to visualize characteristics of coronary artery disease, especially the presence, extent, degree and morphology of coronary plaques. Coronary CT-angiography has a reasonable diagnostic accuracy to detect obstructive coronary artery disease and a proven prognostic value to predict major adverse cardiovascular events.

In this thesis, we related characteristics of coronary artery disease, as measured with coronary CT-angiography, with (bio)markers, as a first step in order to improve risk stratification in patients with suspected coronary artery disease. Furthermore, we tried to gain more insight into underlying mechanisms between atherosclerosis, coagulation and immunology.

SAMENVATTING



Hart- en vaatziekten, waaronder coronairlijden, is een van de belangrijkste oorzaken van ziekte en overlijden in zowel de Westere wereld alsook in landen die zich sterk aan het ontwikkelen zijn. Jarenlang is er een afname van hart- en vaatziekten geweest, maar de verwachting is dat hart- en vaatziekten weer sterk gaan toenemen, wat gepaard gaat met een toename van de gezondheidszorgkosten. Hierdoor blijven hart- en vaatziekten een belangrijk, wereldwijd gezondheidsprobleem.

Atherosclerose is een multifactoriele chronische ontstekingsziekte. Het is de belangrijkste oorzaak van coronairlijden. Het komt echter niet alleen in de coronairvaten voor, maar is een systemische ziekte die uiteindelijk kan leiden tot een hartinfarct, herseninfarct of perifeer vaatlijden.

Door middel van cardiale CT-angiografie is het mogelijk om meer inzicht te krijgen in de aanwezigheid, ernst en mate van coronairlijden. Bovendien is het mogelijk om coronaire plaques beter te karakteriseren wat betreft morfologie. CT-angiografie is geschikt voor het aantonen van obstructief coronairlijden. Daarnaast heeft het ook een bewezen waarde in het voorspellen van ongewenste cardiovasculaire events.

In dit proefschrift hebben we kenmerken van coronairlijden, zoals we die bepaald hebben met CT-angiografie, gerelateerd aan (bio)markers. Doel hiervan is om de risicostratificatie van patiënten met een verdenking op coronairlijden te verbeteren. Daarnaast hebben we ook meer inzicht proberen te krijgen in de onderliggende mechanismen tussen atherosclerose, bloedstolling en immunologie.

Dankwoord

Ruim vier jaar geleden begon ik aan een avontuur, waarvan ik geen idee had waar het zou eindigen. Nu, vier jaar later, is dit avontuur concreet vastgelegd in dit proefschrift en daarmee tot een einde gekomen. Inmiddels ben ik alweer in een nieuw avontuur beland, de opleiding tot cardioloog. Allereerst wil ik echter een aantal mensen bedanken die een belangrijke bijdrage hebben geleverd aan de totstandkoming van dit proefschrift.

Mijn speciale dank gaat uit naar mijn beide promotoren, Prof. dr. L. Hofstra en Prof. dr. J.E. Wildberger, en mijn co-promotoren, Dr. B.L.J.H. Kietselaer en Dr. M. Das.

Beste Leo, jij was degene die mij in Maastricht liet blijven voor mijn wetenschapsstage. De officiële opdracht van deze wetenschapsstage was 'iets met muizen', maar gelukkig deelden we de mening om die muizen in te ruilen voor CT-angiografie. Dat was immers veel 'spannender en sexier', om het maar meteen met jouw termen samen te vatten. Naast promotor ben je een bron van inspiratie en motivatie. Niemand anders kan zo motiveren als jij. Helaas koos je er voor om elders te gaan werken, zodat zomaar even binnenvallen in 'The Office' er niet meer bij was. Anderzijds heeft dit tot nog meer vrijheid geleid, en daarmee tot de mogelijkheid van verregaande eigen inbreng.

Beste Joachim, vanaf het eerste kennismakingsmoment was er al direct een klik tussen ons. Altijd stond je deur open om me met raad en daad bij te staan. Daarnaast ben je wellicht de meest kritische reviewer die ik ken, in de positieve zin van het woord. Waar anderen een manuscript al snel 'mooi' vinden, ben jij degene die het 'mooi' vindt, met een aantal tips en verbeterpunten. Je ziet altijd mogelijkheden om een draft nog beter te maken, waarbij je een enorm oog voor detail hebt, zodat zelfs per abuis vermelde foutieve jaartallen in de referentielijst niet onopgemerkt bleven. Ik hoop dat we samen nog vele artikelen mogen publiceren.

Beste Bas en Marco, dank voor jullie hulp en medewerking om samen te komen tot een alsmaar groeiende lijst van publicaties. Let's keep the train rolling… Dank ook voor de gezellige tijd samen!

Mijn dank gaat eveneens uit naar Prof. dr. H.J.G.M. Crijns. Dank voor alle tijd die u hebt vrijgemaakt voor ons reguliere overleg. Dank ook voor al uw ideeën en enthousiasme betreffende CT-angiografie.

De leden van de beoordelingscommissie, Prof. dr. Stephane R.B. Heymans (voorzitter), Prof. dr. Erik A.L. Biessen, Prof. dr. A. de Roos, Dr. Bram A. Kroon en Dr. Koen Nieman wil ik bedanken voor het beoordelen van dit proefschrift.

Mark en Ward, CT-iconen van het eerste uur. Dank voor jullie introductie in de wondere wereld van de CT. Ook jullie hebben Maastricht helaas voortijdig verlaten, maar gelukkig komen we elkaar nog met enige regelmaat tegen op de cardiac CT-courses. Hopelijk vinden ook jullie nog tijd om te promoveren; jullie verdienen het!!!

Mathijs, je eerste woorden na het ondertekenen van je contract blijven formidabel. "Wordt er nog iets verwacht van mij?" Jazeker, aan het werk… Je bent de beste collega die iemand zich kan wensen. Een dag niet gelachen is een dag niet gewerkt. Wat hebben we samen een lol gehad in 'The bad cave', hoewel de boventoon uiteraard gewoon keihard werken was. Altijd een luisterend oor; altijd tijd om mee te denken en mee te discussiëren. Samen vormden we een succesvol duo. Mooi ook dat mensen zich ongerust gingen maken als we niet samen waren op onze werkplek; we werden dan ook door menigeen gezien als tweelingbroers op research gebied. We waren de spreekwoordelijke 'Jut & Jul' van de CT.

Dank ook aan alle CT-laboranten; we hebben jullie jarenlang verveeld door te vragen bloed af te nemen bij patiënten, extra formulieren in te vullen en dergelijke. Daar stond tegenover dat we er samen een leuke tijd van hebben gemaakt en dat we gezamenlijk coronary CT-angiografie naar een hoger platform hebben weten te brengen.

Dank ook aan de gehele 'Troponine crew' (Prof. Marja P. van Dieijen-Visser, Alma, Eline) voor de prettige en vruchtbare samenwerking. Echter, ten gevolge van de introductie van de high-sensitive troponine in de dagelijkse praktijk heb ik wel al vele extra consulten moeten verrichten… ☺☺☺ … Gelukkig staat er alweer een nieuwe generatie promovendi klaar om onze intensieve samenwerking te continueren, wat ook zeker tot nieuwe papers zal gaan leiden.

Daarnaast ook dank aan de collega's van de biochemie. Prof. Hugo ten Cate, Henri, Rene, Leon, Chris, dank voor alle inspirerende overlegmomenten. Jullie verrijkende kennis blijft me intrigeren. Mooi dat we samen een brug hebben geslagen tussen basic research en daily clinical practice.

Dear Julian, thank you very much for everything my friend. Although we only met each other a few times 'face to face in real life', it was an honor and pleasure to work with you in the different research projects we performed. During the years, we proved how successful collaboration via e-mail and Skype can be. I wish you much fun and success with your new job.

Dr. P. Polak en Dr. W.A.A.J. van Ekelen, beste Peter en Wim, dank voor jullie enorme gastvrijheid en leerzame periode tijdens mijn allereerste (keuze) co-schap in de cardiologie. Jullie hebben me nog enthousiaster voor het vak gemaakt dan ik al was. Ook de 'jonge garde', Ramon, Hans, Rutger en Stijn, dank voor al jullie enthousiasme en interesse tijdens de weekenddiensten in Geldrop.

Ook een speciaal, welverdiend woord van dank voor Barry. Je was altijd beschikbaar als een soort 'Master Mind' als we weer eens een technische vraag hadden over onze database of Access in het algemeen. Daarnaast was het altijd leuk om, onder het genot van een kopje koffie, bij te praten over de stand van zaken. Bovendien heb je me door al je kennis en kunde op het gebied van design en vormgeving enorm geholpen bij de uiteindelijke uitstraling van dit proefschrift. Dank daarvoor, en alvast heel veel succes met de afronding van je eigen proefschrift.

Sibel, mijn opvolgster op research gebied. Ik wens je heel veel succes en plezier de komende jaren in het realiseren van je proefschrift. Onze gezamenlijke tijd bij de CT was kort, maar ik heb er alle vertrouwen in dat je het tot een goed einde weet te brengen.

Verder wil ik Tim Leiner, Frank Schiphof, Patty Nelemans, Jeroen Kooman, Georgi Nalbantov, Angela Maas, Casper Mihl, Erik Biessen, onze CT-studenten en de collega's van de BioBank Maastricht danken voor de samenwerking in de afgelopen jaren. Ook dank aan alle collega's die ik hier niet persoonlijk genoemd hebt. Het waren bijzondere jaren!

List of Publications

Laufer EM, Mingels AM, Winkens MH, Joosen IA, Schellings MW, Leiner T, Wildberger JE, Narula J, Van Dieijen-Visser MP, Hofstra L. The extent of coronary atherosclerosis is associated with increasing circulating levels of high sensitive cardiac troponin T. *Arterioscler Thromb Vasc Biol* 2010;30:1269–1275.

Versteylen MO, Joosen IA, Hofstra L. Monocoronary system with malignant course of the LAD. *Heart* 2010;96:1049.

Versteylen MO, Joosen IA, Shaw LJ, Narula J, Hofstra L. Comparison of Framingham, PROCAM, SCORE, and Diamond Forrester to predict coronary atherosclerosis and cardiovascular events. *J Nucl Cardiol* 2011;18:904–911.

Versteylen MO, Joosen IA, Winkens MH, Laufer EM, Snijder RJ, Wildberger JE, Crijns HJ, Narula J, Hofstra L. Combined use of exercise electrocardiography, coronary calcium score and cardiac CT angiography for the prediction of major cardiovascular events in patients presenting with stable chest pain. *Int J Cardiol* 2013;167:121–125.

Versteylen MO, Takx RA, Joosen IA, Nelemans PJ, Das M, Crijns HJ, Hofstra L, Leiner T. Epicardial adipose tissue volume as a predictor for coronary artery disease in diabetic, impaired fasting glucose, and non-diabetic patients presenting with chest pain. *Eur Heart J Cardiovasc Imaging* 2012;13:517–523.

Mingels AM*, Joosen IA*, Versteylen MO, Laufer EM, Winkens MH, Wildberger JE, Van Dieijen-Visser MP, Hofstra L. * Equal contribution. High-sensitivity cardiac troponin T: risk stratification tool in patients with symptoms of chest discomfort. *PLoS ONE* 2012;7:e35059.

Joosen IA, Versteylen MO, Das M, Kietselaer BL. Letter in response to Andrea K.Y. Lee et al. “Normalcy rate of computed tomographic coronary angiography”. *Int J Cardiol* 2012;158:299–300.

Weijs B, Pisters R, Haest RJ, Kragten JA, Joosen IA, Versteylen MO, Timmermans CC, Pison L, Blaauw Y, Hofstra L, Nieuwlaat R, Wildberger JE, Crijns HJ. Patients originally diagnosed with idiopathic atrial fibrillation more often suffer from insidious coronary artery disease compared to healthy sinus rhythm controls. *Heart Rhythm* 2012;9:1923–1929.

Schurgers LJ, Joosen IA, Laufer EM, Chatrou ML, Herfs M, Winkens MH, Westenfeld R, Veulemans V, Krueger T, Shanahan CM, Jahnen-Dechent W, Biessen E, Narula J, Vermeer C, Hofstra L, Reutelingsperger CP. Vitamin K-antagonists accelerate atherosclerotic calcification and induce a vulnerable plaque phenotype. *PLoS ONE* 2012;7:e43229.

Joosen IA, Schiphof F, Versteylen MO, Laufer EM, Winkens MH, Nelemans PJ, Kooman JP, Hofstra L, Wildberger JE, Leiner T. Relation between mild to moderate chronic kidney disease and coronary artery disease determined with coronary CT angiography. *PLoS ONE* 2012;7:e47267.

Borissoff JI*, Joosen IA*, Versteylen MO, Spronk HM, ten Cate H, Hofstra L. * Equal contribution. Accelerated in vivo thrombin formation independently predicts the presence and severity of CT angiographic coronary atherosclerosis. *JACC Cardiovasc Imaging* 2012;5:1201–1210.

Versteylen MO, Kietselaer BL, Dagnelie PC, Joosen IA, Dedic A, Raaijmakers RH, Wildberger JE, Nieman K, Crijns HJ, Niessen WJ, Daemen MJ, Hofstra L. Additive value of semiautomated quantification of coronary artery disease using cardiac computed tomographic angiography to predict future acute coronary syndrome. *J Am Coll Cardiol* 2013;61:2296–2305.

Borissoff JI*, Joosen IA*, Versteylen MO, Brill A, Fuchs TA, Savchenko AS, Gallant M, Martinod K, ten Cate H, Hofstra L, Crijns HJ, Wagner DD, Kietselaer BL. * Equal contribution. Elevated levels of circulating DNA and chromatin are independently associated with severe coronary atherosclerosis and a prothrombotic state. *Arterioscler Thromb Vasc Biol* 2013;33:2032–2040.

Versteylen MO, Joosen IA, Hofstra L. Reply to Letter to the Editor "Quantification of atherosclerotic coronary plaque. The missing link between elevated biochemical markers and adverse outcomes in the 'vulnerable' patient?" *J Am Coll Cardiol* 2013 [ePub ahead of print].

Joosen IA, Versteylen MO, Eerdekens R, Das M, Maas AH, Wildberger JE, Crijns HJ, Kietselaer BL, Hofstra L. Gender differences in clinical risk profile and CT-angiographically defined coronary artery disease. *Submitted*.

Versteylen MO, Manca M, Joosen IA, Schmidt DE, Das M, Hofstra L, Crijns HJ, Biessen EA, Kietselaer BL. CC chemokine ligands in patients presenting with stable chest pain: association with atherosclerosis and future cardiovascular events. *Submitted*.

Brandsma DS, Versteylen MO, Joosen IA, Mihl C, Das M, Wildberger JE, Crijns HJ, Kietselaer BL. Coronary anomalies with and without an inter-arterial course as detected by cardiac CT-angiography: differences in coronary dimensions and clinical follow-up. *Submitted*.

Versteylen MO, Joosen IA, Kietselaer BL, Wildberger JE, Crijns HJ, Hofstra L. Gender difference in the prognostic value of cardiac computed tomographic angiography. *Submitted*.