Proteomic, circulating and functional biomarkers of cardiovascular disease

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Abstract

Cardiovascular disease is the leading cause of morbidity and mortality in the Western world, mainly through cerebrovascular and coronary artery related events. Cardiovascular disease is a chronic progressive disease with different stages. These stages can be assessed by a variety of biomarkers. Biomarker quantification can be used for different purposes: screening, prediction of disease recurrence, therapeutic monitoring, diagnosis and prognostication. Non-invasive, inexpensive diagnostic tests currently applied in clinical practice have a relative high rate of false positive and false negative results. Therefore further refinement of the diagnostic process could improve clinical care. Regarding prognostication the need for improvement also remains as current risk models only predict a small quantity of occurring cardiovascular events.

The concept of the cardiovascular continuum postulates that cardiovascular disease consists of a chain of events, is initiated by numerous cardiovascular risk factors and subsequently progresses through pathophysiological processes, ultimately leading to end-stage heart failure. For that reason cardiovascular diseases are chronic progressive conditions and can be divided into different stages, such as early tissue dysfunction or subclinical atherosclerosis prior to development of clinically overt disease. Biomarkers suitable for prognostication and diagnosis can differ at each stage. The general aim of this thesis was therefore the investigation of a variety of biomarkers in diagnosis and prediction of cardiovascular disease at different stages of the cardiovascular continuum, as covered by three different study cohorts contributing to this thesis. This included several approaches: the comparison of central and peripheral pulse pressure in middle aged hypertensive patients in regards of their prognostic potential; the application of established circulating, functional and structural biomarkers to the diagnostic process of coronary artery disease in stable angina patients; the development/refinement of a urinary proteomic biomarker for coronary artery disease and the examination of its diagnostic potential in stable angina patients. Biomarkers successful in the diagnosis of coronary artery disease were included in multiple biomarker models.

Aside from biomarker development for the general population, investigations of specific cohorts, such as patients with certain diseases and belonging to certain age groups or sharing specific biochemical features provided advances in the past. To estimate the potential of a biomarker in risk prediction association studies with surrogate biomarkers are applicable. We collected a cohort of middle-aged hypertensive patients to assess if central pulse pressure, derived from non-invasive assessment of arterial stiffness, could improve risk prediction. Central pulse pressure has been previously shown to have prognostic value in populations with end-stage renal failure, coronary artery disease and high prevalence of diabetes mellitus. Considering the prognostic information of peripheral pulse pressure in the elderly, the hypothesis that central pulse pressure could improve risk prediction is comprehensive and was investigated as part of this thesis. This was accomplished by comparing the strength of correlation between central or peripheral pulse pressure and these surrogate biomarkers. When compared to peripheral pulse pressure, central pulse pressure had stronger associations with aortic pulse wave velocity, carotid intima-media thickness, and left ventricular mass index, but equal association with the albumin:creatinine ratio. In contrast, after adjustment for age, mean arterial pressure, heart rate and hypertension status there was no significant difference between central and peripheral pulse pressure for prediction of listed surrogate biomarkers in multivariate analysis. These results suggested that central pulse pressure is unlikely to provide more prognostic information than peripheral pulse pressure in middle-aged hypertensive patients.

The diagnosis of coronary artery disease is clinically relevant in symptomatic patients, either acute or stable. The diagnosis of stable flow limiting coronary artery disease is especially challenging as non-cardiac as well as other cardiac conditions can mimic symptoms. Non-invasive diagnostic tools have either moderate sensitivities or specificities, or are not widely available. Therefore new biomarkers for the diagnosis of flow limiting coronary artery disease have the potential to improve current diagnostic strategies. This could be accomplished adjacent to existing biomarkers or by replacement of such, due to cost effectiveness, better discriminatory etc. As part of this thesis, a biomarker identification and validation study was conducted into urinary proteomics of coronary artery disease. First we tried to replicate a study conducted by our

research group in the past. Therein, an established coronary artery disease specific polypeptide pattern was unable to differentiate between patients with severe coronary artery disease and healthy controls despite strong cohort similarities to the original study. We therefore recalibrated the urinary polypeptide pattern using an enlarged biomarker discovery cohort and adjusted the pattern for lipid lowering and angiotensin converting enzyme inhibitor treatment effects. We calculated a score from the resulting polypeptide pattern, which identified coronary artery disease patients with a sensitivity of 79% and a specificity of 88% in a biomarker validation cohort. As the next step of biomarker development we performed a diagnostic validation study. The investigated clinical cohort consisted of stable angina patients with or without coronary artery disease. The new polypeptide pattern score was unable to differentiate between these two groups. The score however correlated strongly with coronary artery disease extent as measured by the Gensini score, implying that urinary proteomics in the diagnosis of coronary artery disease is promising, yet requires further effort before clinical employment.

In addition to the urinary proteomic biomarker development, a second diagnostic approach was selected. As coronary artery disease is a complex chronic disease, the combination of different biomarkers should result in a better discrimination between stable angina patients with or without coronary artery disease. This approach attempts to position the individual as precisely as possible on the cardiovascular continuum including serologic, functional vascular and imaging biomarkers of subclinical atherosclerosis. Serologic markers thereby present a plasma proteomic approach covering pathophysiological processes with known correlation or causative for coronary artery disease. Functional and structural changes of the peripheral vasculature resemble the coronary artery system. We investigated circulating biomarkers and vascular biomarkers separately. A variety of circulating biomarkers differentiated patients with severe coronary artery disease from healthy control subjects. When patients with stable angina and with or without coronary artery disease as diagnosed by coronary angiography were investigated no statistically significant differences could be detected for circulating biomarkers. In the same study a microvascular biomarker, the reactive hyperaemia index, and a macrovascular biomarker, the carotide plaque score, were able to differentiated between cases and controls.

Both markers either added separately or together improved the risk classification of exercise treadmill test results. This suggests that a multiple biomarker approach in the diagnosis of coronary artery disease in stable angina patients could be successful.

Different aspects of the cardiovascular continuum can be applied to diagnosis and prognostication of cardiovascular disease. In this regard we were able to show, that early processes such as endothelial dysfunction or later processes such as plaque formation can support the diagnostic process. However, randomly collected circulating biomarkers might be unable to do this. Our finding that central pulse pressure is unlikely to have more prognostic value in middle aged hypertensive patients underlines that biomarkers can be useful in specific patient collectives but not necessarily in all cohorts. Instead of applying established biomarkers, also new biomarkers can be developed. Urine proteomics showed great promise in this regard, as specific polypeptide patterns reflect coronary artery disease and are strongly correlated to its extent.

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Abbrevations

AAT	α-1-antitrypsin
ABI	ankle-brachial index
ACC	american college of cardiology
ACR	albumin:creatinine ratio
ADAMTS5	A disintegrin and metalloproteinase with thrombospondin motifs 5
AHA	american heart association
AIx	augmentation index
AL	anterolateral
AUC	area under the curve
BHF	British Heart Foundation
BMI	body mass index
BSA	Body surface area
bpm	Beats per minute
C-IMT	carotid intima-media thickness
CA III	carbonic anhydrase III
CAD	coronary artery disease
CE-MS	capillary electrophoresis coupled to mass spectrometry
CK-MB	creatine kinase isoenzym MB
CKD	chronic kidney disease
cPP	central pulse pressure
CRP	C-reactive protein
CVD	cardiovascular disease
DBP	diastolic blood pressure
DiCADu	Diagnosis of Coronary Artery Disease with Urine proteomics
DNA	deoxyribonucleic acid
DTS	Duke treadmill score
ECG	electrocardiogram
EDTA	Ethylenediaminetetraacetic acid
eGFR	estimated glomerular filtration rate
ELISA	enzyme-linked immunosorbent assay
en-RAGE	ligand for the receptor for advanced glycation end products
EPC	endothelial progenitor cell
ESC	european society of cardiology
ESH	european society of hypertension
ESI-TOF-MS	electrospray ionization time of flight mass spectrometry
ETT	exercise treadmill test
GJNH	Golden Jubilee National Hospital
GPBB	glycogen phosphorylase BB
hFABP	heart fatty acid binding protein
ICAM-1	inter-cellular adhesion molecule 1
IL-6	interleukin-6
IL-8	interleukin-8
IMT	intima-media thickness
InGenious HyperCare	Integrated Genomics, Clinical Research and Care in Hypertension
IQR	interquartile range

LDL	low-density lipoprotein
LVM	left ventricular mass
LVMI	left ventricular mass index
m/z	mass to charge ratio
MCP-1	monocyte chemotactic protein-1
MDRD	Modification of Diet in Renal Disease
MIP-1b	macrophage inflammatory protein-1 beta
ML	mediolateral
MMP-2	matrix metalloprotease 2
NCA	normal coronary arteries
NT-proBNP	N-terminal pro brain natriuretic peptide
PAI-1	plasminogen activator inhibitor 1
PAT	peripheral arterial tonometry
PL	posterolateral
ppm	parts-per-million
pPP	peripheral pulse pressure
psi	pound per square inch
PWA	pulse wave analysis
PWV	pulse wave velocity
RH-PAT	reactive hyperaemia peripheral arterial tonometry
RHI	reactive hyperaemia index
RM	reflection magnitude
RNA	ribonucleic acid
ROC	receiver operating characteristic
SBP	systolic blood pressure
SCORE	Systematic COronary Risk Evaluation
SD	standard deviation
SELDi	surface enhanced laser desorption/ionization
sRAGE	soluble receptor for advanced glycation end products
TNFa	tumor necrosis factor a
TOF	time of flight
VASCAB	VAScular function in Coronary Artery Bypass surgery patients
VCAM-1	vascular cell adhesion molecule 1
z	charge

Author's Declaration

I declare that, except where explicit reference is made to the contribution of others, that this dissertation is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution.

Ulf Neisius

Chapter 1 Introduction

The major aim of this thesis is the application of established and new biomarkers in disease diagnosis or prediction at different stages of the cardiovascular continuum. The introduction of the thesis will therefore recapitulate several relevant topics: biomarkers, cardiovascular disease (CVD), the cardiovascular continuum and biomarker development. Such an approach provides sufficient information to understand the hypotheses of the thesis and helps to explain the link between its parts.

At first, epidemiological, pathophysiological and clinical aspects of CVD will be summarized. Then biomarkers and the historic development of cardiovascular biomarkers will be described. As the latter was predominantly driven by risk prediction and because a specific biomarker is investigated for its potential in CVD prognostication as part of the thesis, the current clinical role of biomarkers in cardiovascular risk prediction is discussed. A more abstract description of biomarker development follows to provide the basics for chapter 6 which focuses on this topic. To explore the aspects of CVD chronicity the concept of the cardiovascular disease continuum is subsequently introduced and connects the two major parts of the thesis. The concept is then expanded to the CVD biomarkers continuum to emphasise the importance of certain biomarkers for its different stages. To complement this a short description of these biomarkers in the context of CVD diagnosis or prognostication follows.

The development of a new CVD biomarkers is an important part of this thesis. This is accomplished with protoemic methodologies, in particular with the help of discovery proteomics in urine. Therefore the principles of proteomics will be introduced in more detail at the end of the thesis.

1.1 Cardiovascular disease

The term cardiovascular disease (CVD) refers to a class of diseases that involves the heart or the blood vessels. In an epidemiological context CVD is the leading cause of morbidity and mortality in the Western world, mainly through cerebrovascular and coronary artery related events. In the United States and the United Kingdom, every third death was secondary to CVD in 2006 and 2009,

respectively [1, 2]. In the European Union it is the foremost cause of premature mortality and morbidity [3]. The overall financial cost of CVD in the European Union represents almost 192 billion Euro in indirect and direct healthcare costs [3]. Whilst cardiovascular mortality is decreasing in the Western world, CVD is increasing in many developing countries. In east or south-east Asia the risk of developing CVD appears even greater than in the developed world [4] and CVD is expected to become the dominant cause of death in those countries by 2020 [5].

Atherosclerosis represents a hardening of the arteries, which involves in particular atheromatous plaque formation, an asymmetric focal thickening of the intima as illustrated in Figure 1.1. These plaques are characterised by a deposition of lipids and fibrous elements in the inner layer of the artery wall. Atheromas are initiated by the retention of low-density lipoprotein (LDL) and other lipoproteins in the subendothelial matrix. This is supported by permeability changes of the endothelium triggered by haemodynamic forces acting on the endothelial cell surface. Trapped LDL becomes oxidised as a result of interactions with reactive oxygen species. As a consequence of this endothelial cells in close proximity to oxidised LDL produce pro-inflammatory molecules and anti-atherogenic substances such as nitric oxide are downregulated. Following this, monocytes invade the vessel wall where they develop into foam cells through uptake of oxidised LDL. Collections of such cells can be seen in autopsy specimens as ‘fatty streaks’. Further disease progression leads to an intermediate lesion consisting of layers of macrophages and smooth muscle cells. Infiltrating T-cells are also present in atherosclerotic lesions. A fibrous plaque develops when extracellular lipid deposits increase and smooth muscle cells and extracellular matrix accumulates in the intima resulting in a fibrous cap. This pathological structure is defined as a plaque, which can be stable or unstable. Stable plaques are rich in extracellular matrix and smooth muscle cells which usually overlay a core of lipid and necrotic debris. An unstable plaque [6] however has a very thin fibrous cap as a result of matrix degradation by various proteinases and an inhibition of *de novo* matrix secretion. As a result unstable plaques are prone to rupture and thrombus formation. Other features of an unstable plaque are a necrotic and sometimes calcified core, and an increased number of inflammatory cells with an accumulation of foam and mast cells [7] at the margin of the plaque, also called the shoulder area.

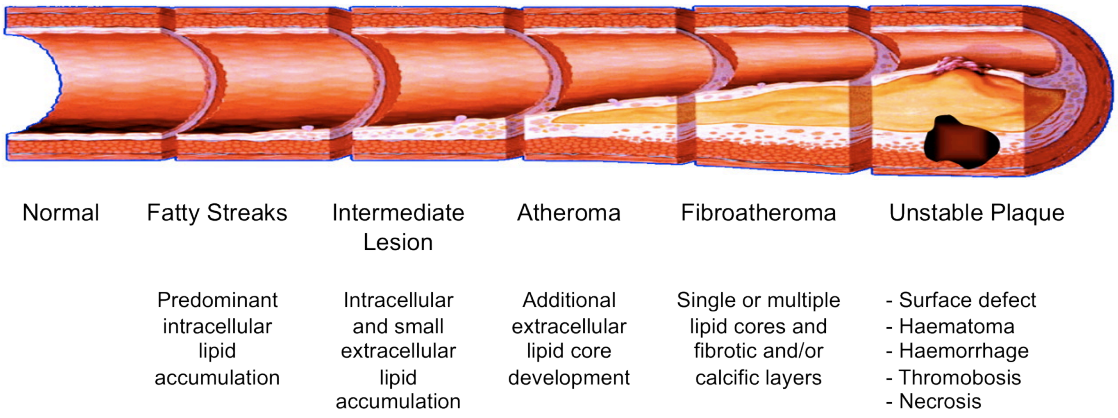


Figure 1.1 Stages of plaque development. Depicted is the stepwise development of unstable plaque. The artery structure, consisting of intima (inner layer), media (middle layer) and adventitia (outer layer), is illustrated with atheroma progression associated with intima thickening, positive remodeling and lumen obstruction. The different stages merging into each other are listed with their histological features below the illustration. The image was adapted from Apple et al. [8].

Atherosclerosis can affect the vessel lumen in different ways. Continuing growth of smooth muscle cells can lead to extensive coronary artery stenosis. In contrast, unstable plaques can cause myocardial infarction without obstruction of the coronary lumen prior to rupture [9]. “Positive remodeling”, a process by which the vessel diameter increases, allows a vessel wall plaque load of 40% to evolve before it affects the cross-sectional luminal area [10]. Therefore smaller plaques are not detectable by coronary angiography. Similar processes can even lead to coronary ectasia, a dilatation of the artery lumen [11].

Atherosclerosis is a chronic disease that remains silent for decades. The early onset of atherosclerotic changes was first reported in 1953 by Enos et al., who investigated coronary artery atherosclerosis in young US soldiers who died in the Korean war [12]. The atherosclerotic process therefore starts early in life. Fatty streaks, for instance, can already be present in fetuses, as shown by Napoli et al. [13]. In 10-14 year old adolescences, investigated by HC Stray [14], 50% had foam cells or fatty streaks in histological studies. Depending on the risk profile, 15-19 year old teenagers already had up to 25% of their arteries covered with fatty streaks or raised lesions in the Pathobiological Determinants of Atherosclerosis Study [15]. The same study showed in 657 individuals with an age of 30-34 years that the right coronary artery surface was covered with fibrous plaques in 5.5% of cases [16]. This is a 5.14% increase in prevalence when compared to the group aged 15-19 years. In 25% of individuals with an average

age of 40 years and dying of non-cardiac causes significant coronary artery disease (CAD) could be detected [17]. In an autopsy study investigating a cohort without CVD and an average age of 60 years, coronary artery plaque could be recorded in 25 % of cadavers [18]. The inter-experimental variation in study cohorts and different study designs explain the discrepancy between these two data sets. As shown by Zhdanov et al. [19] such findings are also influenced by ethnicities and the time of sample collection. Nevertheless atherosclerosis is characterised by an early onset and continuous progression.

Considering the chronic nature of disease progression, a clinical differentiation into subclinical non-symptomatic and clinical symptomatic disease can be made. Subclinical disease is characterised by existing atherosclerosis without any related symptoms. Symptoms of overt clinical disease include for instance chest pain, shortness of breath, neurological deficits, cognitive impairment or exercise related leg pain, depending on the affected vascular bed. Clinically relevant CVD can be further divided into acute syndromes and chronic disease states. Acute syndromes include acute coronary syndromes such as unstable angina and myocardial infarction or acute cerebral perfusion defects such as transitory ischaemic attacks and strokes. Stable disease forms are characterised by symptoms unchanged in quantity and quality over a longer time period.

Acute coronary syndromes represent the clinical manifestation of the critical phase of CAD. The most common underlying pathology is a plaque rupture or erosion leading to intra-arterial thrombus formation and the potential occlusion of the arterial lumen [20]. This causes insufficient blood supply to parts of the heart muscle and further complications like cardiac failure due to loss of myocardial contractility, valve dysfunction or changed hemodynamics; for example as a consequence of a ventricular septum defect. Acute cerebrovascular diseases, such as transitory ischaemic attacks or stroke, are usually caused by cerebral artery embolisation by a floating thrombus originating frequently in the carotid arteries. Other pathologies leading to strokes include local vasculopathies such as aneurysms and related complications like vessel wall rupture or thrombus formation. The subsequent hypoperfusion of cerebral tissue leads to a variety of symptoms depending on the supply area of the affected artery.

Stable clinical overt disease is characterised by persisting symptoms over a period of months, years or sometimes even decades. The dominant symptom of stable ischaemic heart disease is angina caused by transient myocardial ischaemia. This may represent the first presentation of ischaemic heart disease or it might manifest after an acute coronary syndrome. Angina can be divided into three different subtypes depending on their pathophysiology: stable angina, microvascular angina and vasospastic angina. In stable angina myocardial hypoperfusion occurs secondarily to obstructive coronary stenoses, affecting the coronary flow reserve and therefore leading to a mismatch of myocardial oxygen demand and supply when the subendocardial coronary flow reserve is completely utilised. The extent of symptoms is influenced by either dynamic vasomotion in the area of the stenosis or by microvascular coronary artery dysfunction [21]. Microvascular angina is exclusively caused by dysfunction of the coronary microcirculation. Patients with microvascular angina are characterised by angiographically normal or not flow limiting diseased epicardial coronary arteries, in the absence of other cardiac disease that could cause chest pain. Vasospastic angina is a consequence of coronary artery spasms. The difference between vasospastic and the other two angina forms is its typical occurrence at rest as opposed to the predominance of exercise or stress related onset in the other angina forms.

1.2 Cardiovascular biomarker development in risk prediction

Cardiovascular disease, especially its pathophysiological processes, can be quantified with the help of biomarkers. Considering the importance of the biomarker concept for this thesis and to provide a general overview, in the next subchapter its definition is explained and set into context of the historic development of cardiovascular biomarkers and risk prediction.

1.2.1 Biomarker definition

The term biomarker, biological marker, was established in 1989 as a Medical Subject Heading term meaning “measurable and quantifiable biological parameter (e.g. specific enzyme concentration, specific hormone concentration, specific gene phenotype distribution in a population, presence of biological

substance) which serves as index for health- and physiology-related assessments, such as disease risk, psychiatric disorders, environmental exposure and its effects, disease diagnosis, metabolic processes, substance abuse, pregnancy, cell line development, epidemiologic studies, etc." More recently, referencing a National Institutes of Health working group statement from 2001, a biomarker is defined as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention" [22]. This includes not only circulating molecules in the blood, for which the term is often used in the literature. Also genetic and cellular markers as well as measurements by a variety of imaging modalities or even physical examination abnormalities are therefore by definition biomarkers.

1.2.2 History of biomarkers in cardiovascular disease

William Kannel [23] made the concept of cardiovascular risk factors popular in the 1960's. In his initial broad definition, risk factors were utilised as predictors of cardiovascular disease. Factors such as hypertension and hypercholesterolaemia also cause cardiovascular disease, as proven later. According to Kannel's definition, however, a causal connection with the disease is not mandatory. For instance due to the shared pathophysiologic process peripheral artery disease is correlated with CAD, yet does not directly cause CAD. To prove that a risk factor is not only correlated with but also causative of a disease, extensive experimental studies and clinical interventional trials are necessary. As such it is a requisite to link a reduction of the risk factor with the attenuation of disease development or clinical prognosis. And of course, identification of causal factors will provide the greatest chances of improved prevention. Clinically, risk factors therefore help in the identification of patients benefitting from preventive measurements. The purpose of prevention strategies in patient management will be elucidated in the following subchapter.

1.2.3 Cardiovascular risk assessment and prevention

Medical prevention is defined as actions directed to avoid illness and promoting health to reduce the need for secondary and tertiary health care. Therefore it is different from intervention and treatment in that it is aimed at general

population groups with various levels of risk for a specific disease. The goal of prevention is to reduce risk factors and to enhance protective factors. Depending on the relation to disease onset, prevention can be divided into three classes; primary, secondary and tertiary prevention. Primary prevention deals with delaying or preventing the onset of disease. Secondary prevention relies on early detection of the disease process and application of intervention to prevent progression of disease. Tertiary prevention provides appropriate supportive and rehabilitative services to minimise morbidity and maximise quality of life during long-term disease or injury. These definitions are taken from medical subject headings of the U.S. National Library of Medicine. In CVD, primary prevention assessment [24] of cardiovascular risk is used to guide therapy with the intention of preventing a first episode of CAD or stroke as well as the development of peripheral artery disease or an aortic aneurysm. To do so, those with increased risk of clinical CVD manifestation have to be identified. Historically the focus in risk assessment has been unifactorial. Therefore clinical risk assessment was based on single risk factors instead of overall risk, based on a combination of such single factors. Traditional risk factors are smoking, blood pressure, sex, age, diabetes mellitus and hyperlipidaemia.

Over the last 20 years the concept of total risk has been developed. This recognises the multifactorial aetiology of CVD, as opposed to the risk derived from a single traditional risk factor. Additionally the overall CVD risk also incorporates the mal-synergistic effects of multiple risk factors. For example in the INTERHEART study [25], a standardised case-control study of acute myocardial infarction in 52 countries, smoking (single OR 2,87), diabetes mellitus (single OR 2.37) and hypertension (single OR 1.91), when presenting simultaneously predicted a myocardial infarction with an odds ratio approximately 100% larger than the added single odds ratios.

To facilitate risk assessment with traditional risk factors the Framingham risk score [26] was developed. The score is based on the Framingham and Framingham Offspring study and therefore represents only a small population in the Northeast of the United States of America. The accuracy of the Framingham risk equations is dependent on the background risk of the population to which they are being applied. The better the agreement between the local and the Framingham population, the more reliable is the score in the clinical practice.

To establish if the Framingham risk score accurately estimates cardiovascular risk in a European population, the risk algorithm was applied to the Second Northwick Park Heart Study for example [27]. The authors monitored a cohort of 50-64 year old individuals over a mean of 10.8 years for CAD events. Interestingly, the Framingham risk algorithm in this cohort was only able to predict approximately 11% of the occurring events. This demonstrates that despite similarities in living standards of American and European populations other factors not represented in the risk score account for its poor prognostic accuracy. Therefore a risk estimation system based on a large pool of representative European data was developed, the SCORE risk charts (Systematic COronary Risk Evaluation) [28]. In contrast to the Framingham cohort the SCORE risk charts were based on data from 12 European countries comprising 205,178 subjects with 2.7 million years of follow-up and 7934 cardiovascular deaths as end points. Specific risk charts for high and low risk areas of Europe incorporated differences between European regions. The fact that SCORE is only based on cardiovascular events as endpoints is a limitation. In contrast the Joint British Societies published risk charts to estimate risk to develop a first time atherosclerotic CVD event over a 10 year period [29]. Those charts estimate risk based on the risk factors: age, sex, smoking habit, systolic blood pressure (SBP) and the ratio of total cholesterol to HDL cholesterol. A regional approach was also chosen in Scotland with the ASSIGN score in 2006, aiming to better represent the specifics of the local population [30]. In contrast to previously mentioned scoring systems the ASSIGN score incorporates social deprivation as a cardiovascular risk factor. Social deprivation contributes independently to cardiovascular risk [31]. Hence the ASSIGN score reflects better on the Scottish disparity in socio-economic status and helps to avoid insufficient treatment provision to the socially deprived. This however illustrates the limitations of epidemiologic risk profiling. Risk can differ between regions, countries and continents due to different life styles, life expectancy and genetic predisposition. Furthermore populations differ regarding the time point of assessment. For instance a risk profile developed in 1970 will not remain accurate 40 years later due to advancement in medicine, changes in life style and socio-economic flux in the western world. Factors, which are not incorporated in the risk prediction system but still contribute such as nutrition, environmental pollution, co-morbidities and activity levels will have changed

during this time period as well. Therefore over- or underestimation of cardiovascular morbidity and mortality by an outdated risk estimation system depends on the specific population.

Risk prediction is usually established on the basis of hard endpoints. The Framingham score was based on fatal and non-fatal cardiovascular heart disease. This approach ignored the risk of other manifestations of atherosclerosis, such as stroke or aortic aneurysms. Additionally the definition of non-fatal cardiovascular heart disease was incongruous with most other published studies, and made comparisons difficult. The SCORE system on the other hand used fatal CVD events only, including all international classification of disease codes that could be reasonably assumed to be of atherosclerotic aetiology. This approach allows recalibration with the help of up-to-date mortality and risk factor prevalence data. For non-fatal events however the data quality does not allow this. Recalibration of the SCORE system was undertaken for countries like Germany and Belgium with a higher than average cardiovascular mortality. Primary prevention however not only aims at the prevention of fatal or non-fatal events but also at morbidity. In this context morbidity refers to a health condition that affects an individual. Therefore the aim of primary prevention refers also to the avoidance of clinical symptomatic cardiovascular disease. This coincides with prevention of cardiovascular events, yet is not inclusive.

Another problem with risk factors in CVD is the fact that clinicians treat individuals. As only 50% of individuals developing CAD have one or more traditional risk factors [32], a large number of patients will be missed in preventative screening. Attempts to overcome this dilemma by addition of further factors only improved marginally the prediction of cardiovascular endpoints. More than 100 risk factors have been proposed to improve risk assessment [33]. In the absence of strong supportive data for the majority of those 100 risk factors, only a few markers, specifically C-reactive protein (CRP), ankle-brachial index (ABI), leukocyte count, fasting blood glucose level, periodontal disease, carotid intima-media thickness (C-IMT), coronary artery calcium score as measured by electron-beam computed tomography, serum homocysteine level and lipoprotein(a) level have prognostic potential [34]. Especially for CRP evidence suggesting an added value to the Framingham risk score exists [35, 36].

Interestingly, several of these additional risk markers such as ABI, C-IMT and the coronary artery calcium score represent a direct or indirect assessment of progressed CVD and have therefore no causative link to the disease pathophysiology themselves. A term frequently used to describe these biomarkers is target organ damage, as they reflect on the functional impairment or structural damage of the vasculature. Another more accurate description for these risk markers is the term intermediate cardiovascular phenotype, as the combination of them reflects actual disease progress prior to clinically overt disease or end organ damage. The use of the term phenotype reflects the composite of observable characteristics in the cardiovascular system in line with the developmental biology definition of phenotype. Imitating the overall risk concept of prediction models such as Framingham, SCORE or ASSIGN the cardiovascular phenotype represents an overall assessment of target organ damage. Therefore it importantly reflects the state of the cardiovascular system, measured in a single individual at a specific time point.

The importance of assessing the intermediate cardiovascular phenotype can be explained by the individual responses to causative risk factors. For example, due to genetic predisposition an individual might be more prone to develop atherosclerosis in their coronary arteries compared with the average Framingham study participant although both individuals have an identical Framingham risk score. For instance a high coronary artery calcium score would indicate that, despite a low Framingham risk score, risk factor modification could be beneficial. Single target organ damage has therefore been established for instance in hypertension guidelines [37]. This could also solve an additional problem of risk prediction based on single cross-sectional profiles. Although atherosclerosis progresses with age, periods of quiescence and episodic augmentation in plaque inflammation and growth exist throughout disease. Periodic risk assessment, targeting such changes in plaque behaviour, could detect temporary deterioration in plaque stability and therefore improve risk prediction.

In summary, risk factor assessment covering either single or overall cardiovascular risk is a good tool to identify patients who would benefit from primary prevention. However several aspects of currently available strategies are amenable to improvement. Such enhancements would improve therapy

guidance and considering the global epidemic of CVD lead to a major impact on morbidity and mortality. Assessment of the intermediate cardiovascular phenotype has potential to contribute to such an effort, especially as it represents a more individualised approach in contrast to the population based risk factor assessment tools currently employed. Other options for improvement would be the development of totally novel biomarkers covering new aspects of CVD or through replacing older biomarkers with more efficient ones.

1.3 Biomarker Development

There is a wide range of cardiovascular disease biomarkers. Despite differeneces between biomarkers they share several features or rules, such as clinical tasks, test qualities, the definition of abnormal values, analysis variability or common development strategies. These will be explained in more detail in the following subchapter.

1.3.1 Clinical tasks of biomarkers

Biomarkers are established for different clinical tasks. Those cover mainly the areas of screening, prediction of disease recurrence, therapeutic monitoring, diagnosis and prognostication. A single biomarker can be used for different clinical tasks. Before employing a biomarker for certain tasks evidence from clinical research is required. For troponin for example this led to several fold higher cut-off levels for diagnosis of myocardial infarction in comparison to troponin levels useful in cardiovascular risk prediction [38]. For diagnostic purposes qualitative research studies of new biomarkers require an independent masked comparison of the new biomarker with an established standard biomarker in a suitable cohort of consecutive patients covering an appropriate disease spectrum [39]. As biomarkers are often less accurate in a second cohort in comparison to the cohort of initial assessment, it is necessary to replicate study results in an independent cohort. Standards for design and reporting of diagnostic [39] and prognostic [40] biomarker studies have been published.

1.3.2 Biomarker qualities

In general the accuracy of a biomarker is assessed by four test characteristics: sensitivity, the ability to detect disease when it is present; specificity, the

ability to rule out disease when it is absent; the positive predictive value, the proportion of subjects with positive test results and a correct diagnosis; the negative predictive value, the proportion of subjects with a negative test result and a correct diagnosis. This is illustrated in Figure 1.2.

		Condition (as defined by gold standard)		
		Positive	Negative	
Test outcome	Positive	True Positive (A)	False Positive (Type I Error) (C)	*Positive Predictive Value = A/(A+C)*
	Negative	False Negative (Type II Error) (B)	True Negative (D)	*Negative Predictive Value = D/(B+D)*
		Sensitivity = A/(A+B)	*Specificity = D/(C+D)*	

Figure 1.2 Diagram of biomarker test statistics for accuracy. Illustrated are the definitions of sensitivity, specificity, positive and negative predictive value.

Most biomarkers in CVD are continuously distributed quantitative variables. Therefore it is important to assess the information content of a biomarker over the scale of its biological measurements. This can be achieved with the use of receiver operating characteristic (ROC) curves [41]. ROC curves are a graphic realization of the relationship between sensitivity and specificity when biomarker levels are used to identify disease in a clinical setting. Therefore the sensitivity is plotted against the false positive rate, or one minus the specificity, for a binary classifier system. The points on the graph reflect on variations in the discrimination threshold. Therefore each threshold has a different chance to identify a diseased individual and to simultaneously categorise a healthy individual as such. An alternative statistic tool to evaluate test accuracy are the likelihood ratios [42]. These are clinically relevant as they represent the likelihood to obtain a positive test result in a diseased person, likelihood ratio for a positive test (LR+ = sensitivity/(1-specificity)), or to get a negative test result in a patient with disease, likelihood ratio for a negative test (LR- = (1-sensitivity)/specificity). According to these equations a likelihood ratio for a positive test with a value greater than one is linked to test results associated

with the presence of disease. In this calculation a likelihood ratio for a positive test, with a value smaller than 1 on the other hand implies that the test result is associated with the absence of disease. The more the likelihood ratio deviates from 1 the more likely it is that the test will detect presence or absence of disease. Therefore likelihood ratios above 10 and below 0.1 are considered to provide strong evidence to accept or exclude a diagnosis, respectively. An additional advantage of the likelihood ratios is their simple use in the Bayesian approach. The pre-test probability can easily be multiplied with the likelihood ratio to receive the post-test probability. Such an approach is necessary to adjust for disease prevalence. Even with a simultaneous high sensitivity and specificity, false positive tests will occur more frequently than true positive results if the disease prevalence is very low.

For biomarkers the term discrimination means the capacity to differentiate controls from cases in cross-sectional studies or to distinguish those who will develop a disease from those who will not in longitudinal studies. A frequently used measurement for discrimination is the c statistic, also known as the concordance index. It is equal to the area under the ROC curve. The c statistic stands for the probability that in two random individuals, one the control and the other the case; the biomarker precisely identifies the one without disease. However, as the c statistic is the metric of overall performance, two tests can have the same value, but at a specific threshold one biomarker might be superior. Another frequently used term in biomarker statistics is calibration. It defines the capacity of a biomarker to predict risk in comparison to the observed risk in subgroups of the population. If the difference between the predicted and the observed risk is too significant a biomarker can be deemed inappropriate, whereas a risk model can be recalibrated when it uniformly under- or overestimates the level of risk. A good example for the latter is the Framingham risk score in a Chinese population. It overestimates the risk and recalibration improves cardiovascular risk prediction substantially in this population [43].

1.3.3 Abnormal biomarker values

Another critical point in biomarker development is the definition of abnormal values. This is an important step prior to the introduction of a novel biomarker into the clinic. Attributing factors such as age, sex, ethnicity and prevalence of

disease require consideration if abnormal values are defined. Three strategies exist to characterise such values as illustrated in Figure 1.3.

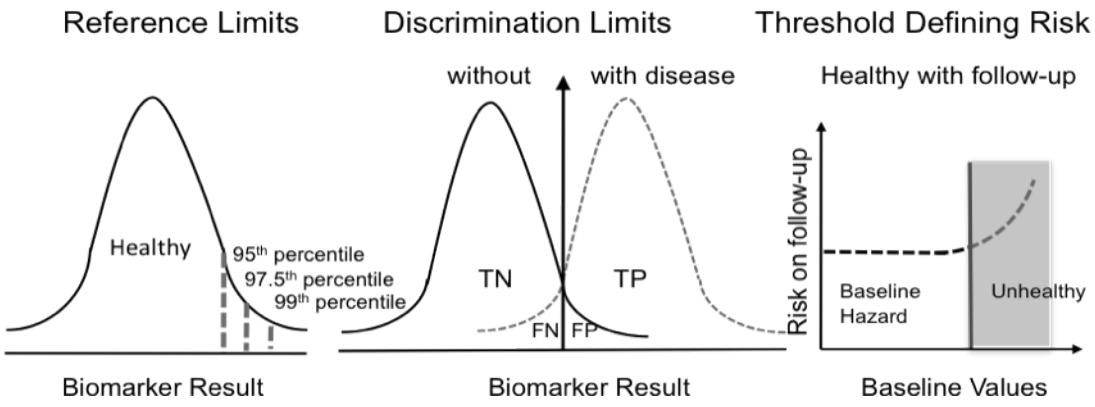


Figure 1.3 Definition of an abnormal biomarker. Depicted are three different approaches for definition of an abnormal biomarker value. Reference limits are based on statistical intercept points based on the distribution of reference sample values. Discrimination limits separate the distribution of patients with and without the disease (FN, false negative; FP, false positive; TN, true negative; TP, true positive). A risk threshold identifies the level after which the disease risk augments on follow-up. The illustration is modified from Vasan RS [44].

Reference limits are chosen arbitrarily by selecting for instance the 95th or 97.5th percentile in a sample without the disease of interest. The corresponding reference range is equal to the interval between the minimum and maximum reference values. The 2.5th and the 97.5th percentile are usually chosen as cut-off points resulting in a 95% reference interval for definition of normality. The reference interval can be altered to incorporate additionally consequences of false-negative or false–positive results. It might be for instance not acceptable to miss a diseased patient and therefore a lower reference limit than the 97.5th percentile is required. Other reasons to modify the reference range might be ethical, social, psychological and economic in nature. Problems which are derived from employing arbitrary reference limits include false positive individuals in the healthy population, the difference between a healthy individual and the average population in highly prevalent diseases such as hypertension or obesity, the fact that due to the definition unhealthy individuals can remain within the reference range and finally the error that a value change within the reference range is considered normal whilst it can represent disease. Discrimination limits are defined differently and instead of investigating the general population two specific cohorts are used to define limits. Of particular interest is the overlap between patients with and without disease examined in

cross-sectional studies. The strength of this approach is the ability to predict a diseased patient in an otherwise normal population. An example is the diagnosis of a non-ST segment elevation myocardial infarction with the help of troponin. Threshold biomarkers are a third strategy to characterise abnormal biomarker levels in disease. This approach is for instance used to predict adverse medical events in a selected cohort. Threshold levels are defined as values beyond which the incidence of an adverse event increases significantly. An example is the Framingham cardiovascular risk score. It triggers treatment in primary prevention when a certain level of risk is reached.

As mentioned above diagnostic and prognostic biomarker development differs substantially depending on the requirement of these indicators. Risk prediction biomarker development requires a firm study design and a representative population of subjects. Also the outcome must be characterised thoroughly, quantified accurately and detected completely to allow proper assessment of a biomarker. The discriminatory power of a biomarker can be assessed by statistical procedures such as logistic regression, Cox proportional hazard or parametric survival models. The first requirement for a biomarker in risk prediction is a statistically significant association with the outcome. Incorporation of a new biomarker in clinical practice also requires improvement to the predictive result of established risk models. Similarly to diagnostic biomarkers, cost, safety and acceptability need to be considered. For generalised usage a new biomarker needs to be easily measurable in a cost-effective manner. This usually involves a standardised and inexpensive commercial assay with low test variability and simple probe collection or analysis techniques.

1.3.4 Biomarker measurements

Biomarker requirements assume a perfect laboratory process and limited biological variability in bodily fluid related biomarkers or consistency and reproducibility for imaging biomarkers. The performance of bodily fluid related biomarkers depends on pre-analytical, analytical and post-analytical factors. Pre-analytical variability is based on factors such as the lack of standardised procedures for sample collection. Other contributing factors when assaying novel biomarkers are biological variability such as circadian rhythms or technical

problems such as inconsistency in patient preparation, specimen acquisition, probe handling and storage. Strategies against pre-analytical variability include phlebotomy protocols with standardised fasting state, body posture and collection time. Analytical variability on the other hand depends on the performance of the laboratory test, where its variability might derive from inter- or intra-laboratory variation. The former can occur due to different analytical platforms or differences in reagents used. The latter can derive from human error, usage of point-of-care testing devices such as bedside troponin tests instead of automated platforms, quality of control materials or number and types of control materials. The reasons for analytical variability are diverse and only a small collection is mentioned in this thesis. Due to internal quality control strategies in laboratories pre-analytical and post-analytical factors contribute most significantly to the total error when testing for biomarkers. According to Plebani [45] pre- and post-analytical errors contribute to the total error with 46-68.2% and 18.5-47%, accordingly. Furthermore, accuracy and precision are required to guarantee internal quality control. Guidelines have been recommend for this purpose [46]. The term accuracy refers to the agreement between the known amount of a standardised analyte and its measurement result on site. The term precision refers to the reproducibility of results. Post-analytic variability depends on transmission as well as the display of test results within a laboratory or hospital. Imaging related biomarkers such as ultrasonography or magnetic resonance imaging based markers are variable for different reasons. Those are influenced by inter- and intraobserver variability, biological variability and changes in measurement strategies. To overcome these problems quality control protocols have been proposed [47].

The development of new biomarkers holds several challenges. The vulnerable patient, destined to develop adverse cardiovascular events, can suffer from three abnormalities. These abnormalities are the vulnerable plaque, vulnerable myocardium and vulnerable blood [48]. As outcome is not only decided by plaque, but also by factors such as blood coagulabitliy and myocardial susceptiblity to develop fatal arrhythmia, the factors “vulnerable blood” and “myocardium” have to be considered when characterising the vulnerable patient. For instance the systemic thrombotic propensity influences the thrombotic response to vessel wall injury. In particular the activation status of

platelets, coagulation, and fibrinolysis is critical for the outcome of plaque rupture, documented by the protective effect of antiplatelet agents and anticoagulants in patients with high cardiovascular risk. Also the factor V Leiden, a variant of the coagulation factor V leading to hypercoagulability, is associated with an increased risk of myocardial infarction [49].

As the arterial wall and the myocardium is not easily accessible, biomarker development for those sites of disease posses as special challenge. Obstacles such as the blood-brain barrier in cerebrovascular disease can also restrict spatial access for sample acquisition. Furthermore the spatial resolution of blood related biomarkers is suboptimal as biomarkers originating in the vasculature can reflect on processes in the cardiac or peripheral artery system. It is therefore difficult to differentiate between processes which occur in the peripheral circulation versus the cardiac circulation. These limitations support the notion that biomarkers chosen to relate to a specific phenotype can be confounded by inaccuracies in the assessment of the disease phenotype. Furthermore, the complexity of CVD in itself may lead to a poor correlation between a biomarker and the clinical phenotype, as it reflects only a part of the whole disease process.

1.3.5 Development of new biomarkers

Technically, the development of new biomarkers utilises two possible approaches. A deductive, knowledge-based method relies on the understanding of pathophysiological processes of atherosclerosis. The inductive, unbiased strategy is based on investigation of large numbers of molecules with the use of newer high volume technologies and aims to identify patterns of molecules within a disease or indeed disease stage. Similar to the development of a new drug the evaluation of a novel biomarker should involve several stages of escalating stringency. Accordingly Hlatky et al. suggested six phases [50] as shown in Table 1.1.

Table 1.1 Phases of evaluation of novel biomarkers.

Stage	Summary
0) Biomarker discovery	Identification of a new biomarker, e.g. basic research or 'omics'.
1) Proof of concept	Difference between patients with or without outcome or disease
2a) Prospective validation (risk factor) 2b) Diagnostic validation (diagnostic factor)	Prediction of future adverse events in a prospective cohort Investigation of biomarker distribution in large reference group and disease group
3) Incremental value	Additional predictive information to established, standard risk factors
4) Clinical utility	Extent of change in risk prediction sufficient to justify change in recommended therapy
5) Clinical outcomes	Improvement of clinical outcome when biomarker is considered
6) Cost effectiveness	Clinical outcome improved to such extent that additional treatment and testing is justified

The table was adapted from Hlatky et al. [50].

Initially the new biomarker needs to be identified, for instance with the use of standardised technology platforms such as a proteomics platform. Next follows the validation of the new biomarker. In this context validation represents the investigation of whether a new biomarker is able to identify the outcome or phenotype of interest, otherwise known as a proof-of-principle study. At this point a replication of the study in an independent cohort is recommended. In diagnostic biomarker development this can be followed by statistical evaluation of the marker distribution within a reference sample and a cohort with disease. This can be achieved with epidemiological case control studies. At this stage the correlation between the biomarker levels and clinically assessed disease stages should be investigated. For the development of prognostic markers a prospective study to test the biomarker's predictive ability of hard outcomes such as

cardiovascular events should follow. Furthermore, the additional value and clinical merit of the marker over established markers needs verification. Then its clinical utility requires assessment. For instance it needs to be established if the novel marker changes contemporary therapeutic strategies. Lastly, it needs to be established that the impact of the risk marker on clinical practice changes outcome. Considering finite resources in the public health sector cost-effectiveness requires assessment as well. A biomarker without impact on clinical outcome thereby is unlikely to be cost-effective.

The usefulness of new biomarkers has been evaluated with different approaches. Taking for example cardiovascular risk prediction, most studies use a hazard ratio to measure the predictive ability of a new risk factor whilst controlling for Framingham risk factors. This strategy however gives no information about the biomarker's ability to improve classification of the group identified as intermediate risk by the Framingham or other risk scores. As such discrimination is insufficient to evaluate the clinical implications of a new risk factor. Even a risk factor with low discriminatory power may reclassify the intermediate risk group [51, 52]. Reclassification can be assessed by comparison of the proportion of individuals in the high-risk category identified by different models. This can be followed by the evaluation of the agreement between the predicted and the actual event rates, a process called calibration. Another approach is to calculate the Framingham or other risk scores and then to measure how well the new risk factor reclassifies the intermediate risk individuals, also called its classification accuracy. This is similar to the stratification capacity of a single biomarker assessing the part of a population categorised into risk groups. These approaches can be transferred to other clinical applications, for instance diagnostics, which include an intermediate category.

1.3.6 Surrogate biomarkers

A surrogate is someone or something that takes the place of another person or entity, a substitute. As diagnostic advances allow the identification or the monitoring of arterial wall or end-organ disease, the corresponding biomarkers have the potential to substitute end-point events in medical trials. This provides a different approach to assess the changes in cardiovascular risk [53]. A surrogate biomarker must fulfil several requirements to be a suitable substitute

for a morbid event end-point. Its extent should coincide with the incidence of the end point, not only as an epidemiological marker but also as a responder to therapy. Some surrogate biomarkers however might be sensitive for disease odds but not useful as a therapeutic target or vice versa. Furthermore direct measurements of CAD such as coronary angiography or coronary intravascular ultrasound cannot be considered surrogates for the presence of disease. The use of surrogate biomarkers in clinical trials can be limited when mortality or morbidity end points are not linked to disease progression as represented by the biomarker. For instance, cardiomyopathies, valvular heart disease or genetic arrhythmic disorders might contribute to morbid events, but lack a direct connection to atherosclerotic or atherothromobotic diseases. Therapy might decelerate, stop or reverse disease progression as assessed by the surrogate marker. However intervention might also cause simultaneously adverse effects like electrolyte disturbances. Therefore therapeutic efficacy in surrogate marker trials does not a priori include therapeutic safety and denote positive outcome.

1.4 The cardiovascular disease continuum

As pointed out in chapter 1.1 CVD is a chronic disease, commencing in childhood/adolescence and progressing throughout the whole life span of an individual. The velocity of this process will affect the CVD morbidity and mortality. Considering the long disease progression and plaque development summarized in Figure 1.1 the assumption can be made that the disease process occurs in different stages. Dzau and Braunwald [54] have postulated this progression in phases by introduceing the concept of the cardiovascular disease continuum in 1991. It was proposed that CVD consists of a chain of events, is initiated by numerous cardiovascular risk factors and subsequently progresses through pathophysiological pathways and processes with the final end-stage heart failure. In this context the authors assume that interventions at any stage of the continuum could alter its course and provide cardiovascular protection. At the time of publication clinical trials data was limited and the cardiovascular continuum needed further experimental confirmation to become validated. To summarise progress in this matter Dzau et al. published two additional papers in 2006 [55, 56]. And although the cardiovascular continuum was at first restricted to CAD, the concept was expanded to include related areas such as

cerebrovascular disease, peripheral vascular disease and renal disease to value the interdependence of those diseases (Figure 1.4).

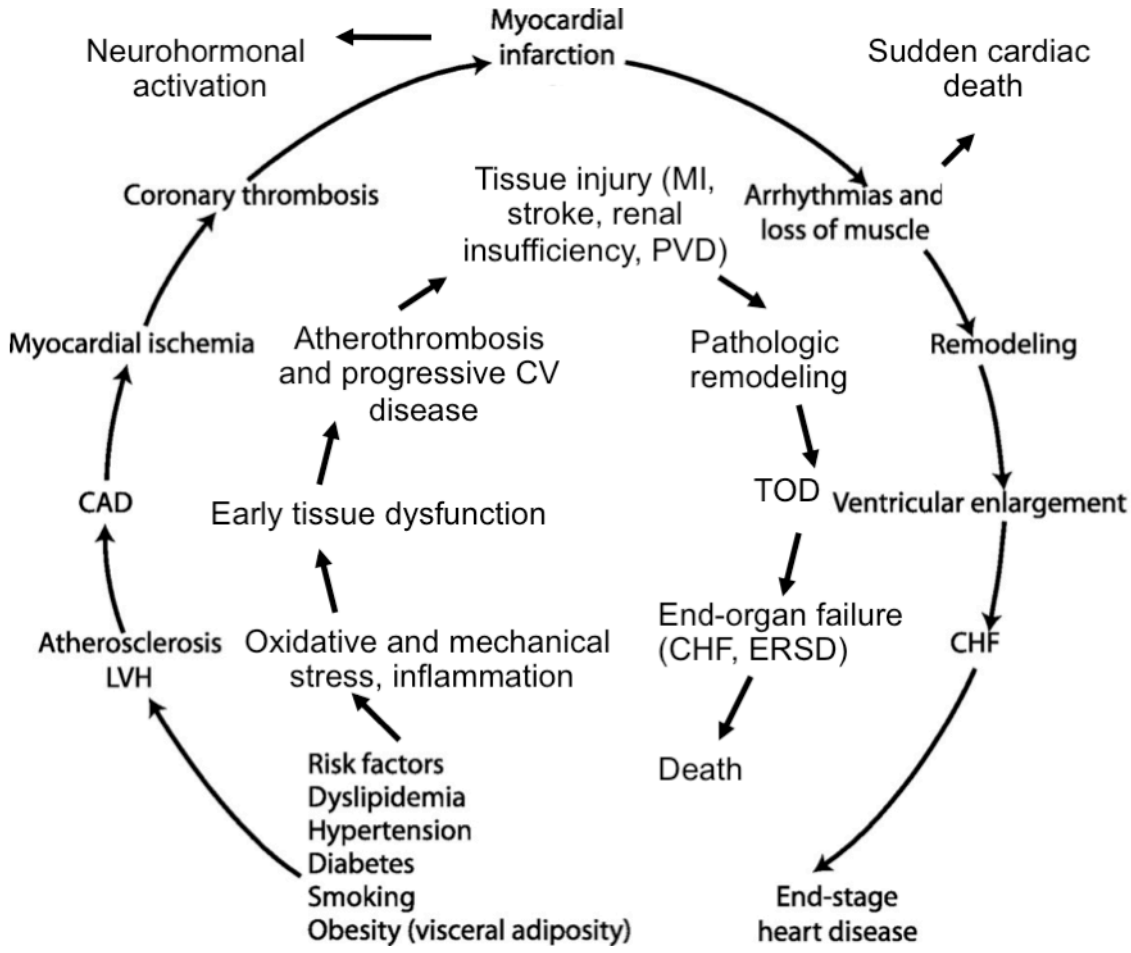


Figure 1.4 Cardiovascular disease continuum. The inner circle illustrates the cardiovascular and renal pathophysiological continuum, whereas the outer circle summarises Dzau's original cardiovascular continuum [53]. LVH; left ventricular hypertrophy; CV, cardiovascular; ESRD, end-stage renal disease; CHF, congestive heart failure; TOD, target organ damage.

The cardiovascular continuum concept implies the existence of early stages of target organ damage prior to development of clinically overt cardiovascular disease. Although several stages of the cardiovascular continuum such as myocardial infarction depend on single events, several processes relate to continuous, slow deterioration of the cardiovascular system occuring in the subsequent disease stages. Biomarkers detecting these stages have frequently had a role in traditional risk prediction, however many of these biomarkers do not provide additional information when added to established risk prediction models like Framingham [26], SCORE [28] and ASSIGN [30]. This is due to direct link between traditional risk factors and early stages of the cardiovascular continuum. For instance diabetes mellitus leads to endothelial dysfunction.

Having diabetes therefore implies a degree of endothelial dysfunction. On the other hand, an individualised risk assessment approach such as the intermediate cardiovascular phenotype allows for the placement of a patient on the cardiovascular continuum, independent of causative risk factors. The resulting position on the cardiovascular continuum could imply a need for therapeutic interventions.

In the cardiovascular continuum a more simplistic approach is therefore to consider a biomarker as an indicator of a trait predisposing to disease development, also called risk factor, at a certain time-point in disease progression or as an indicator of the velocity of disease progression. Consequently biomarkers can be grouped according to their function as antecedent biomarkers identifying a disease development risk, as screening biomarkers for subclinical disease detection, as diagnostic biomarkers for disease identification, as staging biomarkers for disease quantification or as prognostic biomarkers.

An example for a biomarker indicating the need for therapeutic intervention during an early disease stage is microalbuminuria, one of the most sensitive measures of kidney disease. Consequently, the European Society of Hypertension included microalbuminuria in the assessment of subclinical organ damage guiding hypertension treatment [37]. Microalbuminuria is therefore a good example of treatment guidance by individual intermediate phenotyping.

Whilst clinical events, like non-fatal or fatal myocardial infarctions, undoubtedly represent the best end points for clinical research, assessment of early disease stages might offer an alternative research tool. For instance pharmaceutical research using clinical outcomes as endpoints involves large-scale, expensive clinical trials lasting several years. Those trials have the ability to measure clinically relevant differences on the beneficial effect of drugs. Subclinical organ damage, the intermediate phenotype, however occurs much earlier in the continuum of cardiovascular disease, therefore it may be a more sensitive measure of the specific, differential actions of therapeutic agents [57]. Also alternative end-points have the potential to reduce the time frame or sample size of a clinical cohort, as effects can be detected much earlier in appropriate numbers when compared to traditional clinical endpoints. Whether intermediate

phenotypes offer an alternative to the traditional approach is currently under debate, however evidence supporting this alternative outcome measure exists. For instance regression of left ventricular hypertrophy [58] or decelerated progression of intima media thickness [59] is linked to improved outcome. Therefore intermediate phenotype assessment can play a role in clinical research. Examples for its usage are in small scale preliminary trials or in addition to clinical outcomes in traditional clinical trials. The latter could improve the understanding of drug effects on the cardiovascular system, whilst generating salient clinical data.

The different stages of the cardiovascular continuum can be detected with a variety of biomarkers. A selection along to the different stages is depicted in Figure 1.5 and will be discussed in subchapters, 1.5, 1.6 and 1.7. For the purpose of simplification, biomarkers will be divided into causative risk factors (antecedent biomarkers), biomarkers of the intermediated phenotype (screening biomarkers) and biomarkers of clinical overt cardiovascular disease (diagnostic biomarkers). This summary does not claim completeness, but will give a comprehensive overview of the most relevant CVD biomarkers in the context of the cardiovascular continuum. Biomarkers with special importance for the result chapters will be discussed in more detail.

Considering the variety of different stages and organs involved in the cardiovascular continuum, a characterisation of an individual with multiple biomarkers covering these different aspects of the continuum is a promising strategy in prognostication and diagnostics. This is supported by the hypothesis that phenotyping and disease staging on the cardiovascular continuum can be more precise if more information is available, especially in the context of a complex disease such as CAD. The relevance of such an approach is for instance confirmed by the finding that combined risk factor assessment is better than single evaluation of its components and that a combination of multiple serological markers improves cardiovascular risk stratification in some [60, 61], although not in all, studies [62]. Considering these benefits in prognostication a multiple biomarker strategy will be applied to the diagnosis of CAD as part of this thesis in chapters 4 to 6. Therefore many of the listed biomarkers are relevant for this thesis and are explained in detail. The multiple biomarker strategy will be applied to clinically overt CVD. Consequently all biomarkers

detecting earlier disease stages, as illustrated in Figure 1.5, are also potential diagnostic candidates. We subsequently aimed to include a large number of biomarkers in the investigation. The overview of CVD biomarkers will therefore not only represent biomarkers used for multiple biomarker assessment in the context of diagnosis but also for prognostication.

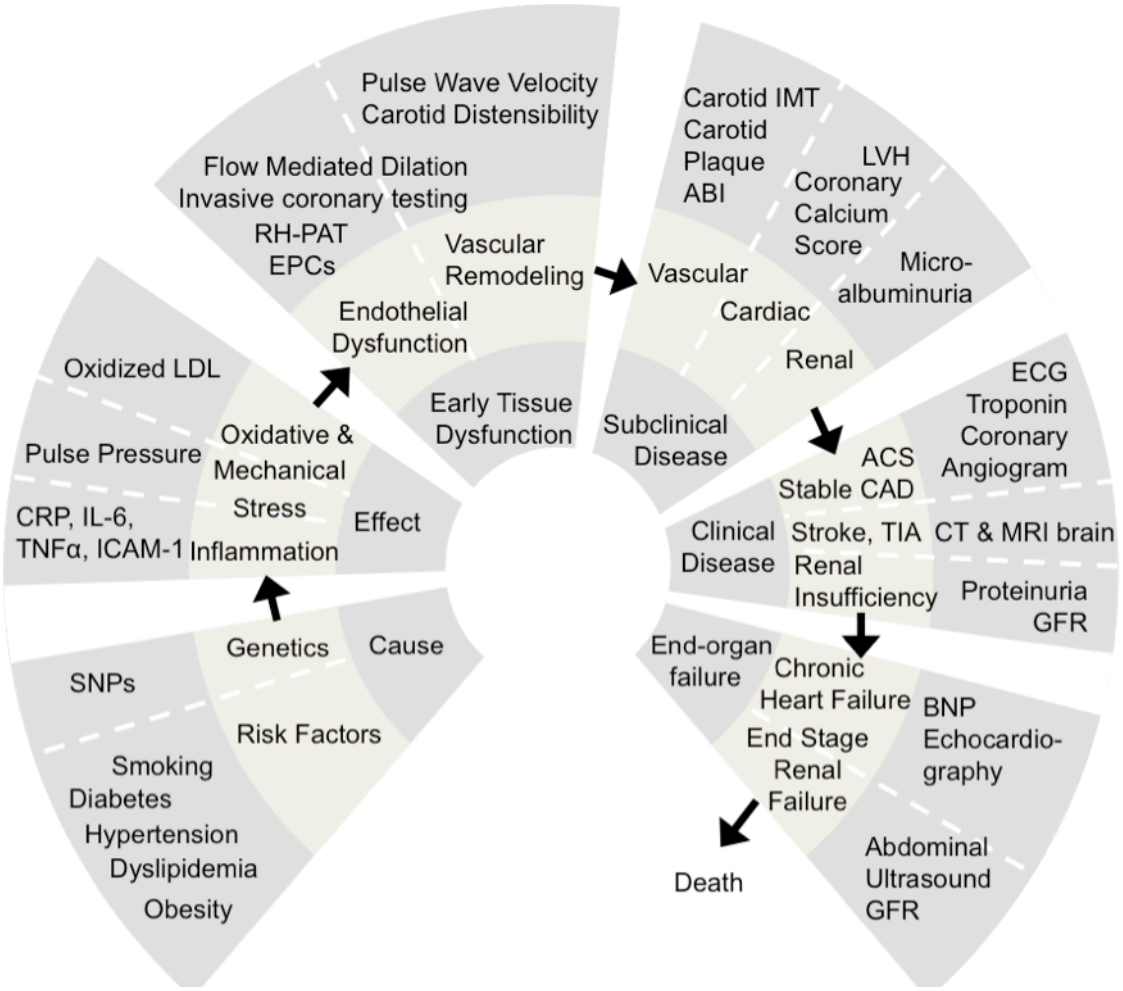


Figure 1.5 Biomarkers in the cardiovascular continuum. The inner circle states the stage of the cardiovascular continuum. The middle circle states the different pathophysiological process or categorises the cardiovascular continuum stage. The outer circle summarises a range of biomarkers quantifying the pathophysiological process. SNP, single nucleotide polymorphism; CRP, C-reactive protein; IL-6, interleukin 6; TNFα, tumour necrosis factor α; ICAM-1, intercellular adhesion molecule-1; LDL, low density lipoprotein; RH-PAT, reactive hyperaemia pulse amplitude tonometry; EPCs, endothelial progenitor cells; IMT, intima media thickness; ABI, ankle brachial index; LVH, left ventricular hypertrophy; ECG, electrocardiography; ACS, acute coronary syndrome; CAD, coronary artery disease; TIA, transitory ischaemic attack; CT, computer tomography; MRI, magnet resonance imaging; GFR, glomerular filtration rate; BNP, brain natriuretic peptide.

1.5 Risk factors in cardiovascular disease

The current concept of CVD implies that its process is initiated by risk factors such as dyslipidaemia, hypertension, diabetes, smoking and obesity. These

factors lead to oxidative and mechanical stress, endothelial dysfunction, inflammatory processes and ultimately vascular remodelling. The occurrence of these factors marks the beginning of the atherosclerotic process, and continues to be present throughout the cardiovascular continuum. Palinski et al. for example demonstrated oxidised LDL in fetal aorta walls of hypercholesterolaemic mothers; suggesting atherosclerosis represents a lifelong process [63].

Cardiovascular risk prediction estimates the onset of both non-fatal and fatal cardiovascular events normally in a 5-10 year time frame. Markers included in traditional risk factor models like the Framingham risk score, the SCORE tables, the Joint British Societies risk charts and the ASSIGN score are age, gender, total cholesterol, HDL cholesterol, blood pressure, diabetes, smoking and social deprivation. These elements are predominately causative risk factors for CVD. Age is not linked to the pathophysiology of cardiovascular disease, however epidemiologically aged individuals are more likely to develop cardiovascular events during a time period of 10 years. Other pathophysiological processes which sustain the development of atherosclerosis are inflammation, oxidative and mechanical stresses. For the purpose of simplicity markers supporting atherosclerosis will be designated as causative risk factors whereas markers of subclinical organ damage will be summarised as intermediate phenotypes. Considering the continuous progression of cardiovascular disease, as outlined in Figure 1.4, the dedication of biomarkers to stages of the cardiovascular continuum is rather arbitrary.

With the exception of oxidative stress risk factors of CVD listed below are investigated in each study cohort contributing to this thesis. This data allows for instance adjustment for contributing factors or comparison of groups such as cases and controls. For instance contributions of established factors to diagnosis or outcome have to be excluded to prove the value of an additional biomarker. The information about causative risk factors has therefore an important role in the analyses conducted as part of this thesis.

1.5.1 Hypertension

Pathologically raised blood pressure, also known as hypertension, is an established risk factor for all clinical manifestations of atherosclerosis. Hypertension is defined as persistent blood pressure equal or above 140 mmHg in systole and/or 90 mmHg in diastole. In Western societies particularly, more than 25% of the adult population are affected by hypertension. The risk of developing hypertension in these countries during a lifetime is in excess of 90% and is a common, independent predisposing factor for the development of CAD, stroke, peripheral artery disease and heart failure. In the Framingham cohort hypertension leads to a two to four fold increase in cardiovascular events in comparison to a normotensive person of the same age group. An elevated blood pressure is associated with the development of CAD in a continuously graded manner, without a critical threshold. Mortality in CVD doubles with each 20 mmHg systolic or 10 mmHg diastolic blood pressure (DBP) rise and this extends in the normal blood pressure range. For instance, in comparison to values below 130/85 mmHg, the combination of SBP 130-139 mmHg and DBP 85-89 mmHg are associated with a 1.6 and 2.5 fold hazard of CVD in men and women respectively.

Blood pressure can be altered by life-style factors like salt intake, physical activity, alcohol consumption and body weight. The modification of these life-style factors has however, a limited capacity to reduce blood pressure. As a concequence drug treatment is often necessary to reduce the overall cardiovascular risk in hypertensive patients. Treatment normally aims at blood pressures below 140/90 mmHg and for diabetic patients and those with established CVD below 130/80. The latter shows that the hypertension classification is highly dependent on the total cardiovascular risk of each individual, meaning that the hypertensive risk in CVD varies extensively depending on the burden of associated risk factors.

Essential hypertension, by definition hypertension of unknown cause, accounts for more than 90% of hypertension. It is influenced by several pathophysiological factors. Some examples for those are: increased sympathetic nervous system activity, persisting high sodium intake, abnormal function of resistant vessels including defects in the renal microvasculature, diabetes mellitus or insulin

resistance and obesity. Other hormon related examples are: overproduction of vasoconstricting and sodium-retaining hormones, inappropriate increase of renin secretion resulting in high levels of angiotensin II and aldosterone, insufficient amounts of vasodilators such as prostacycline or natriuretic peptides, up-regulated activity of vascular growth factors, alterations in adrenergic receptors with a positive ino- and chornotrophic effect on the heart and other pathophysiologic processes [64]. These factors underline the multifactorial origin of essential hypertension and the complex causes of cardiovascular disease, as most causative factors interact with each other. These interactions lead to a risk factor clustering, meaning that hypertension tends to occur in association with other atherogenic factors. In particular hypertension is metabolically linked to dyslipidaemia, glucose intolerance, abdominal obesity, hyperinsulinaemia, hyperuricaemia and others. In the Framingham study hypertension occured in less than 20% of study participants in isolation.

1.5.2 Diabetes

Type 1 and type 2 diabetes mellitus are characterised by hyperglycaemia. Other features of diabetes mellitus type 2 are hyperinsulinaemia and an excess of free fatty acids resulting from genetic and environmental factors. Type 1 diabetes mellitus is an autoimmune disease and comprises <10% of all diabetic patients. It is characterised by a loss of the pancreatic insulin-producing beta cells in the islets of Langerhans, which leads to a deficiency in insulin production. Insulin resistance characterises type 2 diabetes mellitus. It can occur simultaneously with reduced insulin secretion secondary to beta-cell dysfunction. Type 2 diabetes patients represent >90% of those with diabetes and atherosclerosis.

The link between elevated blood glucose levels and increased mortality and morbidity from vascular disease is well established. Coutinho et al. showed in a metaregression analysis covering a total of almost 100,000 individuals, that a fasting glucose level of 6.1 mmol/l in comparison to 4.2 mmol/l was associated with an relative cardiovascular event risk of 1.33 (95% CI, 1.06-1.67) [65]. The direct effect of hyperglycaemia on the vasculature is only partially understood, however hyperglycaemia directly impairs endothelial function [66]. Interestingly, high glucose levels augment protein kinase C dependent [67] endothelial nitric oxide synthase gene and protein expression [68]. Yet this upregulation also leads

to an increase in superoxide anions, which react with nitric oxide [68], suggesting an inactivation of nitric oxide by superoxide anions, which then leads to a production of further reactive oxygen species. Furthermore, protein kinase C activation by hyperglycaemia leads to cyclooxygenase 2 production. This is associated with an increase in thromboxane A2 and a reduction of prostacycline [67]. In summary, hyperglycaemia induces endothelial dysfunction and oxidative stress.

Epidemiologically diabetes has a high prevalence worldwide. Amos et al. estimated the global diabetes burden as 124 million people and projected a rise to 221 million people by the year 2010 [69]. The growing level of obesity and sedentary lifestyle, both major underlying risk factors for type 2 diabetes, is the main reason in developed and especially developing countries. Unfortunately, the process is continuing. This emphasises the growing worldwide importance of diabetes for cardiovascular mortality and morbidity, especially as diabetes mellitus amplifies cardiovascular risk [70]. Next to well established microvascular complications of diabetes such as diabetic nephropathy or retinopathy macrovascular complications such as CAD, peripheral artery disease and carotid vessel disease cause cardiovascular events.

Diabetic patients with either unstable angina or non-Q-wave infarction have a relative mortality risk of 1.57 in comparison to non-diabetic patients [71]. In patients with thrombolysed myocardial infarction diabetes portends a substantially worse 30-day and 1-year prognosis [72]. Approximately 30% of patients with an acute coronary syndrome have diabetes and as many as 40% have impaired glucose tolerance [73, 74]. On a population scale the 7 year incidence of first myocardial infarctions or death was 20% in diabetic patients in comparison to 3.5% in non-diabetic patients [75]. In the same study patients with previous myocardial infarction had a second event in 45% of the diabetic subgroup and in 18.8% of the non-diabetics subgroup. Therefore patients with diabetes and no cardiac history have the same total risk as non-diabetic patients with a history of myocardial infarction. As a consequence current guidelines consider diabetes equal to established CAD, mandating aggressive preventive treatment.

1.5.3 Dyslipidaemia

Lipid metabolism can be perturbed in different ways. In general dyslipidaemia is caused by an alteration in lipid function or increased levels compared to the physiologic state. These can be of genetic origin such as in familial hypercholesterolaemia, diet related, part of disease complexes such as the metabolic syndrome, secondary to other disease like end stage renal failure or a mixture of these causes. As it is possible to modify cholesterol levels with life style changes or with drug therapies hypercholesterolaemia has been thoroughly investigated by randomised trials [76]. Besides the most common forms of dyslipidaemia, the elevation of total cholesterol and LDL cholesterol, several other forms predispose to premature cardiovascular disease. An example is the so-called atherogenic lipid triad. It consists of the combined increase of very low density lipoproteins, triglycerides and small dense low density lipoprotein particles in association with reduced high density lipoprotein particles [77].

Elevated plasma cholesterol levels fulfil all criteria of a causative risk factor. In a meta-analysis involving 61 prospective observational studies consisting of almost 900,000 adults without previous CVD and with a baseline total cholesterol measurement a reduction of 1 mmol/L was associated with approximately a 56%, a 34% and a 17% reduction in ischaemic heart disease mortality at the age 40-49, 50-69 and 70-89 years, respectively [78]; interestingly the investigators were unable to establish a threshold of this effect. Consequently the cardiovascular risk increases continuously the higher the cholesterol level becomes. A total cholesterol of 8 mmol/L and an LDL cholesterol of 6 mmol/L for instance places a patient at a high total risk of CVD in the absence of other risk factors [77]. On the other hand, a 10% reduction in total cholesterol levels leads to a 25% reduction of CAD events over a 5 year period [76]. Similarly an LDL cholesterol reduction of 1 mmol/L is followed by a 20% decrease of CAD [76], and similar observations were made in primary prevention studies. In a meta-analysis, including 20 randomised trials and a total of 63899 individuals the relative risk for statin treatment in primary prevention was 0.93 (95% CI: 0.87 to 0.99, p=0.03) [79]. Therefore dyslipidaemia is not only correlated with cardiovascular risk but also causally linked as indicated by these intervention studies.

1.5.4 Smoking

Worldwide tobacco use is one of the most important causes of CVD. Any form of tobacco consumption including chewing tobacco and second hand smoke is associated with a higher cardiovascular risk [80]. In the INTERHEART study, current smoking in comparison to no smoking history was associated with a higher risk of non-fatal myocardial infarction, the corresponding odds ratio was 2.95. Three years after smoking cessation, the odds ratio decreases to 1.87 [80]. Even 20 years after smoking cessation an elevated risk remained with an odds ratio of 1.22 [80]. More than 1 million men and 200,000 women die as a consequence of smoking in Europe each year, of which approximately one third die from CVD [81]. In this context it is not surprising that the INTERHEART study identified smoking as the traditional risk factor with the highest odds ratio for incidence of myocardial infarction, with an estimated 30% of all CVD cases secondary to smoking [25]. Therefore smoking is the most preventable CVD cause.

The adverse effect of tobacco consumption is related to the number of cigarettes smoked per day [80]. For instance a person smoking more than 20 cigarettes per day has a 6 fold higher risk for an acute coronary syndrome in comparison to someone consuming 2 cigarettes per day [80]. Especially if smoking is started at young age the total mortality risk is higher [82], which suggests a cumulative effect. In the atherosclerosis risk in the community study an increased carotid intima media thickening was associated with active and passive smoking over a three year period [83]. This finding underlines the direct effect of smoking on atherosclerotic processes. Smoking cessation is the most effective preventive measurement. It is associated with a 36% reduction of all cause mortality among patients with established CAD [84]. Even reduction of passive smoking leads to reduction of CAD incidence. As a result of the Scottish smoking ban in enclosed public places the admission rate for non smokers to hospitals with acute coronary syndromes was reduced by 21% during a 10 months period following the implementation of the legislation in comparison to the preceding 10 months [85].

1.5.5 Inflammation

A large number of circulating biomarkers representing inflammatory processes have been described. In chapter 4 of this thesis several of these markers are evaluated for their diagnostic capacity in symptomatic CVD. The following subchapter will therefore provide a detailed summary of the most relevant markers.

In general, inflammation is a complex biological response of vascularised tissues to harmful stimuli such as pathogens and chemicals or other irritants as well as mechanical injury. It constitutes the physiological attempt to remove harmful stimuli and to initiate healing processes. A chronic inflammatory state has been associated with atherosclerosis. For example, chronic inflammatory conditions of autoimmune origin such as rheumatoid arthritis, systemic lupus erythematosus and psoriasis are associated with a higher cardiovascular risk [77]. Pathophysiological processes of inflammation characterise all stages of the CVD progression and represent a link between plaque formation, acute plaque rupture and myocardial infarction with the consecutive arterial occlusion. A range of inflammation biomarkers such as CRP, interleukin-6 (IL-6) and lipoprotein-associated phospholipase A2 [86] have been investigated in atherosclerosis. In this regard the largest body of evidence is available for CRP. CRP is a circulating pentraxin composed of 5 23-kDa subunits and an acute phase reactant of hepatic origin. However, CRP is also synthesised in smooth muscle cells within diseased atherosclerotic arteries and is associated with multiple aspects of atherosclerosis such as adhesion molecule expression, effects on fibrinolysis and alteration of endothelial function [87]. Ridker et al. published one of the first studies reporting an association between baseline CRP and future myocardial infarctions in form of a nested case-control study [88]. A multitude of studies have been published since, all in general supporting the link between CRP and future cardiovascular events. A meta-analysis of 22 studies, all excluding baseline CAD, was published in 2009 [89]. It showed that CRP levels > 3.0 mg/dl in comparison to levels < 1.0 mg/dl have a 58% added risk for CAD incidence. Despite this association between CRP and cardiovascular events, the improvement of predictive accuracy of standard risk factors seems only modest. Several studies have shown that the addition of CRP to traditional risk factors only raises the c statistic modestly by 0.003 [90], 0.011 [91] or 0.015 [92]. In the

Women's Health Study adding CRP to the Framingham risk model reclassified 20% of intermediate-risk individuals [93]. Of those only 4% were reclassified upward from intermediate to high risk. Therefore CRP assessment would only have a small effect on treatment decisions. Moreover, during acute coronary ischaemia, raised CRP levels are predictive of longterm cardiovascular risk even if troponin levels are not raised [94]. This suggests an association between plaque vulnerability and inflammation in the absence of overt myocardial ischaemia. In the clinical setting CRP can be investigated with several validated, standardised and inexpensive, high-sensitivity assays. Regarding treatment guidance CRP might have a role in specific populations. The Jupiter Trial for instance assessed patients with normal lipid levels [95], here the authors were able to show that CRP identified patients benefiting from statin therapy. The Jupiter trail however did not address reclassification and was not reproducible in the ASCOT population [96].

Several other markers of inflammation are established. Only a number of them have shown clinical potential. Amongst those are the inflammatory cytokines IL-6 [97] and tumour necrosis factor α (TNFα), the inter-cellular adhesion molecule 1 (ICAM-1) [98, 99], vascular cell adhesion molecule 1 (VCAM-1) and P-selectin [100]. Although the evidence for risk prediction with these markers exists, investigations into clinical applications are not yet as advanced as research into CRP.

IL-6 is a pleiotrophic cytokine released by T-cells and macrophages with diverse humoral and cellular immunomodulatory effects. It possesses pro- and anti-inflammatory properties, is the main trigger for hepatic CRP secretion and is associated with CAD, especially in form of fatal events [101-103]. The link with fatal events might be related to the association of IL-6 and left ventricular impairment [104]. In prospective studies the odds ratio of IL-6 was quite variable ranging from 1.0 [105] to 3.0 [102]. However in a meta-analysis published in 2008 and incorporating 17 different prospective studies with clinical coronary outcomes the combined odds ratio was 1.61 per two standard deviation increase in baseline IL-6 [106]. Longterm elevation of IL-6 predicts therefore cardiovascular events in population screening [106]. IL-6 is also useful for outcome prediction in specific cohorts like patients with unstable CAD [107].

Furthermore, evidence exists for a diagnostic quality of IL-6 as Noto et al. observed a relation to the level of coronary stenosis in patients with CAD [108].

TNFα is a pro-inflammatory cytokine, important in the initiation of an inflammatory response. The cytokine is expressed by a variety of cells, including macrophages, foam cells, monocytes, T-cells, smooth muscle cells, adipocytes, and fibroblasts. TNFα contributes to the pathogenesis of atherosclerosis by enhancing arterial wall chemokine and adhesion molecule expression as well as by augmenting medial smooth muscle cell proliferation and migration [109]. TNFα also promotes infiltration of arterial plaque by inflammatory cells and stimulates additional cytokine production, which increase plaque instability and consecutively lead to thrombus formation [110]. Epidemiological evidence for an association between TNFα and CVD is inconsistent; some prospective case-cohort studies [111, 112] have reported a positive association, whilst others do not [113, 114]. Raised TNFα levels were related to recurrent coronary events in a cohort with previous myocardial infarction [115].

Soluble ICAM-1 is part of a glycoprotein receptor super-family that also includes VCAM-1 and platelet endothelial cellular adhesion molecules. These molecules function as mediators of leukocyte adhesion and migration to the vascular endothelium. They bind circulating leukocytes to the vascular endothelium and trigger leukocyte migration into the subendothelial space. Their expression and consecutively their concentrations are influenced by other inflammatory cytokines. These cytokines include interleukin-1, interleukin-4, TNFα, interferon-gamma, lipopolysaccharide and oxidised LDL. Increased levels of ICAM-1 have been found in atherosclerotic diseases including CAD [116], and ICAM-1 levels correlate with the extent of atherosclerotic lesions [117]. Soluble ICAM-1 is a predictor of myocardial infarction in prospective population based studies [118]. Adjustment for standard cardiovascular risk factors however abrogates the risk effect in other studies [99]. This might be due to an association of soluble ICAM-1 with coronary atherosclerosis progression leading to luminal narrowing. Yet soluble ICAM-1 is not associated with events of acute thrombosis or vessel occlusion [119]. In other cohorts such as patients with established CAD raised ICAM-1 concentration are linked to a higher risk of future coronary events, independent of established risk factors [120].

Soluble VCAM-1 is part of the immunoglobin superfamily. It can be detected on endothelial cells, lymphoid dendritic cells, tissue macrophages and renal tubular epithelial cells. It mediates leukocyte binding and trans-endothelial migration. Similar to ICAM-1,VCAM-1 is also associated with the extent of atherosclerosis [121]. For soluble VCAM-1 exists however no convincing evidence regarding the prediction of adverse cardiovascular events within a population [99, 122]. Yet VCAM-1 was associated with cardiovascular events in a cohort with documented CAD [123].

P-selectin is an adhesion receptor expressed on activated endothelial cells, which mediates the so called 'rolling' of leukocyte along the endothelium. The evidence supporting a role of P-selectin in prediction of cardiovascular events is sparse. Ridker et al. were able to show that raised P-selectin levels are associated with a higher incidence of cardiovascular events in a nested case-control study in women independent from traditional cardiovascular risk factors [100].

1.5.6 Oxidative stress

Oxidative stress biomarkers will not be investigated as part of this thesis. They are briefly summarised to cover the full spectrum of the CVD biomarker continuum, as illustrated in Figure 1.5.

The term oxidative stress describes the systemic disproportion between reactive oxygen species and the body's natural ability to counter them or the damage they cause. The imbalance leading to oxidative stress usually derives from an excessive production of reactive oxygen species, a group of molecules produced in aerobic cells including oxygen and its derivates such as superoxide and hydrogen peroxide. Despite their potential harm on cellular structures like proteins, lipids and DNA, reactive oxygen species also act as secondary messengers in intracellular signalling cascades important in other processes such as senescence and apoptosis. The more deleterious effects of reactive oxygen species are linked to hypertension and atherosclerosis. In situations like ischaemia or postischaemic reperfusion phagocytic cells release reactive oxygen species leading to further tissue damage. In the vasculature the predominant source of reactive oxygen species is a family of NADPH oxidases. These NADPH

oxidase enzymes produce constantly low levels of reactive oxygen species and their activity can be increased by stimuli such as inflammatory cytokines, mechanic stress and hormones such as angiotensin-II. Low levels of reactive oxygen species contribute to the development of atherosclerosis in different ways. Reactive oxygen species for instance react with nitric oxide. Consequently they decrease nitric oxides quantity and beneficial effects and simultaneously lead to the formation of highly reactive substances such as peroxynitrite. Furthermore reactive oxygen species can lead to lipid oxidation. Oxidative modified LDL has a more proatherosclerotic effect than its native form. Oxidation of LDL occurs predominantly in the vessel wall where it leads to an activation of many inflammatory and atherogenic pathways. Oxidated LDL has several deleterious effects including endothelial apoptosis, activation of inflammatory cells, decrease of endothelial nitric oxide synthase, vascular smooth muscle cell proliferation and augmentation of reactive oxygen species generation in endothelial cells. Therefore oxidative stress, either as the direct effect of reactive oxygen species or as oxidative modified LDL, plays a pivotal role in the early stages of atherosclerosis. Efforts to counter oxidative stress with vitamin supplements [124] as well as epidemiologic investigations [125] have shown no benefit on cardiovascular mortality. Considering the existing evidence for the connection between oxidative stress and atherosclerosis, the lack of benefit might for example be due to inefficient vascular wall penetration by antioxidants in these studies.

Several biomarkers for oxidative stress have been proposed. Especially oxidised LDL and antibodies raised against it were investigated in more detail. Oxidised LDL is produced during lipid peroxidation whilst free oxygen radicals extract a hydrogen atom from a carbon-hydrogen bond, a constituent part of a polyunsaturated fatty acid. This leads to generation of reactive species altering lipid and protein components of LDL. As the protein and lipid modifications are random, oxidised LDL is not a homogeneous entity, it rather represents a collection of chemical modifications of both lipids and apoB-100 proteins. Therefore it has been recommended that differentially oxidised LDL versions can be detected by oxidation specific antibodies, for instance the murine monoclonal antibodies DLH3, 4E6 and E06. Oxidised LDL levels are elevated in patients subsequently developing myocardial infarctions [126]. The quoted study

by Meisinger et al. examined only 88 cases and 258 controls. However in the combined Health Professionals Follow-up Study and the Nurses' Health Study, two prospective studies including 50,966 subjects with a follow-up of six to eight years, oxidised LDL did not predict cardiovascular events after adjustment for triglycerides, LDL and HDL cholesterol as published by Wu et al. [127]. Yet, oxidised LDL levels have been shown to predict the incidence of re-infarctions in secondary prevention cohorts [128]. Oxidised LDL might therefore have a role in prognostication in such cohorts. This is supported by the finding that the concentration of oxidised LDL is linked with a high Framingham cardiovascular risk score, as Holvet et al. observed [129]. Oxidised LDL is associated with other markers of CVD such as C-IMT [130] and coronary endothelial dysfunction [131]. Other markers of oxidative stress are F-2 isoprostane, plasma levels of oxidative modified tyrosins, glutathione and the ratio of reduced to oxidised glutathione.

1.5.7 Uric acid

Uric acid represents the final product of nucleic acid metabolism, in particular purine metabolism. Chemically it is a heterocyclic organic compound with the correct name 7,9-dihydro-1H-purine-2,6,8(3H)-trione and a molecular weight of 168 Daltons. In individuals with normal renal function uric acid is excreted in the urine.

Elevated uric acid levels are correlated with a higher CAD risk independent of traditional risk factors. In a meta-analysis covering 26 eligible studies and a total of 402,997 individuals by Kim et al. [132], after adjustment for confounding factors the pooled risk ratio for incidence of CAD was 1.09 (95% CI: 1.03-1.16) and for mortality 1.16 (95% CI: 1.01-1.30). In the same analysis an increase of 1 mg/dL in blood uric acid concentration led to a 12% higher CAD mortality. The presence of hyperuricaemia is especially deleterious in women. Another meta-analysis published by Wheeler et al. [133] showed a higher risk ratio of 1.12 in men and of 1.22 in women comparing hyperuricaemic with normal individuals.

Being a byproduct of hydrogen peroxide generation, uric acid is established as an anti-oxidant reagent [134]. It may therefore protect against vascular inflammation and dysfunction. Yet uric acid has also antioxidant properties in the presence of LDL cholesterol, as shown in human plasma by Patterson et al.

[135]. Uric acid also supports vascular smooth muscle cell proliferation where it has a mitogenic effect [136]. Furthermore, uric acid augments the up-regulation of CRP in endothelial and vascular smooth muscle cells [137].

1.6 Biomarkers of the intermediate phenotype

As discussed in chapter 1.3, the intermediate cardiovascular phenotype is a collective term for subclinical organ damage quantified by a multitude of biomarkers. Such are correlated with developing atherosclerosis and cardiovascular disease.

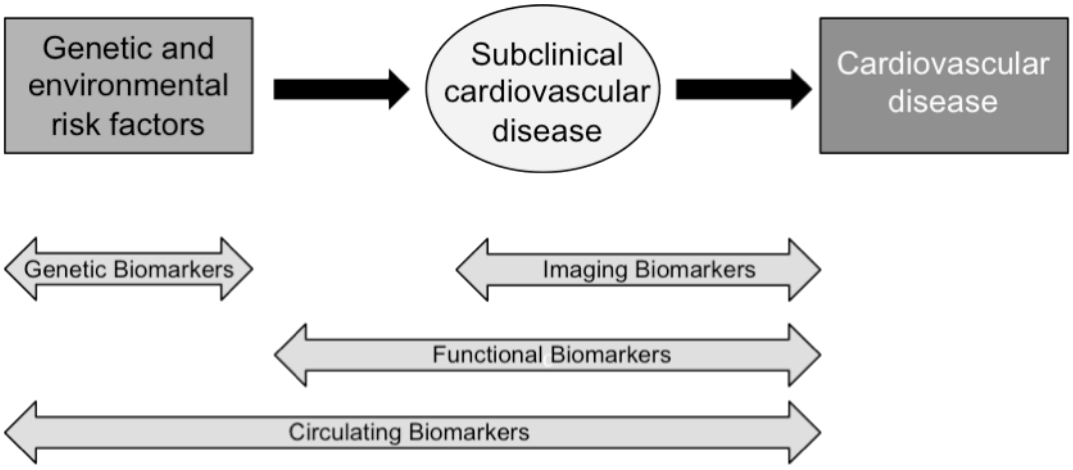


Figure 1.6 Biomarker types in the cardiovascular continuum. Depicted are stages at which genetic, circulating, functional and imaging biomarkers are most informative.

Subclinical CVD in particular is defined as detectable target organ damage either within the vasculature or the heart without previous or ongoing symptoms. Established markers of target organ damage are left ventricular hypertrophy, microalbuminuria, increase C-IMT and an elevated ankle brachial index. More recently the coronary calcium score has been developed as a biomarker of CAD. This list is not exclusive as every maker measuring CVD progression can be considered as a biomarker of the intermediate phenotype. Functional and circulating biomarkers can detect early stages of the cardiovascular continuum. Imaging modalities cannot detect the very early stages of subclinical CVD, as depicted in Figure 1.6.

Biomarkers of the intermediate phenotype are examined in all study cohorts contributing to this thesis. This includes all mentioned below biomarkers with

the exception of endothelial progenitor cells, ankle-brachial index and assessment of asymptomatic CAD. The three biomarkers are briefly summarised to explain the full spectrum of the CVD biomarker continuum, as illustrated in Figure 1.5.

1.6.1 Vascular function and structure

Exposure to causative risk factors is quite similar in different vascular beds. Therefore peripheral vascular function and structure resembles that of the coronary arteries. As a consequence, assessment of wall structure or endothelial function in the non-cardiac arteries allows an estimation of the state of global cardiac circulation. Peripheral vascular function and structure assessment is therefore a cardinal component of intermediate phenotyping.

1.6.1.1 Endothelial function

The endothelium provides a variety of physiological functions accomplished via secretion of a multitude of bioactive substances. These effect vasomotion, inhibition of platelet aggregation, thrombus generation and maintenance of endothelial permeability in the physiologic state. Endothelial function is linked to cardiovascular risk factors via pro-oxidative genes in the vascular wall. Their activation leads to a production of reactive oxygen species, which stimulate the endothelium to release transcriptional and growth factors, proinflammatory cytokines, adhesion molecules and chemoattractant substances [138]. Thereby the endothelium regulates platelet activity, vascular tone, angiogenesis and leukocyte adhesion. All of these components are involved in the transition from normal endothelial function to endothelial dysfunction. Endothelial dysfunction presents a systemic disorder and is a key variable in the pathogenesis of atherosclerosis and its complications. It is not only determined by the individual risk factor burden, but also represents an integrated index of atherogenic and atheroprotective factors in a single individual. One of the first factors which contributes to endothelial dysfunction is a reduced nitric oxide bioavailability. This is a result of inhibition and uncoupling of endothelial nitric oxide synthase and an augmented consumption of nitric oxide secondary to increased levels of reactive oxygen species. As a consequence all aspects of endothelial function become deranged. Examples are an abnormal vasomotor activity, an increasingly

pro-coagulant endothelial surface, a pro-inflammatory environment and ultimately plaque formation and atherosclerosis. Most of the established risk factors like hypertension, dyslipidaemia, diabetes, insulin resistance, smoking and aging are associated with endothelial dysfunction [139, 140]. Furthermore the number of risk factors present is linked to the extent of endothelial dysfunction, suggesting an additive effect. Endothelial function also predicts disease progression [141]. Therefore endothelial dysfunction is linked to CVD by strong clinical evidence. In cohorts with peripheral vascular disease [142] endothelial function as measured by brachial flow-mediated dilatation (FMD) is an independent predictor of cardiovascular events. However in patients with significant cardiovascular risk [143] or in elderly patients [144] brachial FMD had either no or only minimal contribution to prognostication after adjustment for traditional risk factors. In the Multi-Ethic Study of Artherosclerosis, a population-based cohort study of adults free of clinical CVD at baseline, brachial FMD was an independent contributor to cardiovascular event prediction [145]. However the relevance of endothelial function measured by flow mediated dilatation for discrimination and reclassification remains unclear in this study, as the the C statistic of the Framingham risk score was not improved by addition of brachial FMD and the biomarker even inappropriately reclassified 23% of individuals who experienced events whilst correctly reclassifying 29% of individuals [145]. More studies with either no or incremental prognostic information from measurements of FMD are summarized in a recent review by Charakida et al. [146].

Endothelial function can be assessed with invasive measurements. Intra-arterial infusions of specific endothelium-dependent vasodilators such as acetylcholine, methacholine, bradykinin, substance P or inhibitors of NO synthase can alter endothelial function in experimental settings. Acetylcholine for instance induces endothelium-dependent dilatation by stimulating the nitric oxide synthase and consecutively nitric oxide release. Therefore intracoronary acetylcholine infusion dilates epicardial arteries in healthy individuals. This is measured by comparing the coronary artery diameter before and after infusion of acetylcholine by quantitative angiography. Endothelial dysfunction on the other hand is diagnosed when epicardial constriction is observed. This is explained by a balance shift favouring the acetylcholine effect on direct smooth muscle cell contraction in opposition to its effect on the endothelium. In other vascular beds

such as the brachial or femoral circulation, only a diminished vasodilator effect is observed in endothelial dysfunction. Endothelium-independent vasodilatory agents such as nitroglycerine or sodium nitroprusside characterise the dilatory capacity, as they donate NO directly to the smooth muscle cells. Therefore they are used as a control stimulus in invasive experimental settings.

Non-invasive measurements of endothelial function have been developed to avoid arterial cannulation and procedural adverse effects. Imaging techniques applied include ultrasound, positron emission tomography and magnetic resonance imaging for peripheral or coronary artery assessment. The best-validated technique is the ultrasound based endothelium dependent flow mediated vasodilatation of the brachial artery [147] illustrated in Figure 1.7., which is also the most widely used non-invasive ultrasound technique to examine endothelial function [146].

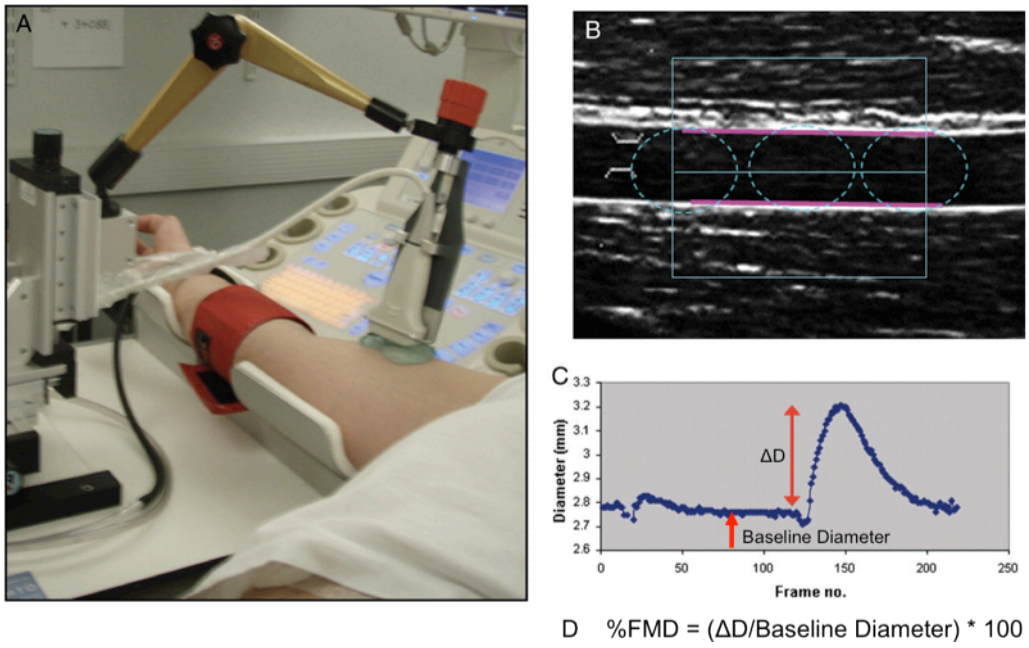


Figure 1.7 Flow-mediated dilatation (FMD). Depicted is the experimental set-up for a brachial FMD measurement including the recommended positioning of the sphygmomanometer cuff and the use of an ultrasound probe holder (A). A B-mode ultrasound image of the far and near wall of the brachial artery is depicted with additional placement of the region of interest box using an edge detection analysis software for flow-mediated dilatation analysis (B). The consecutive measurement of the brachial artery diameter, as generated by edge detection software, allows assessment of the vasodilatory response (C). From the difference between maximum and baseline diameters (∆D) %FMD can be calculated (D). The figure was adapted from Charakida et al. [146].

The reactive hyperaemia response to a temporary arterial occlusion is used to create a flow increase related shear stress to the endothelium. The stress activates endothelial nitric oxide synthase and leads to a vasodilator response. Flow mediated dilatation is measured as the difference in diameter of the brachial artery before and after the shear stress, as depicted in Figure 1.7. The extent of this response is representative of endothelial function.

Anderson et al. [148] established a significant correlation between invasively measured endothelial function in the coronary artery tree and non-invasive peripheral brachial flow mediated dilatation. The technique has several pitfalls, such as its high operator dependency, its transient variability leading to a poor reproducibility and incongruence to published techniques and protocols. Therefore other techniques such as reactive hyperaemia pulse amplitude tonometry [149] were developed. The latter was used as part of this thesis due to an uncomplicated measurement process requiring only a short learning curve, the relative good independency of test results from the performing operator and the well standarized investigation technique. The technique is explained in more detail in chapter 2.6.7 and illustrated in figures 2.11, 2.12 and 2.13.

Whilst flow mediated dilatation measures a mixture of macro- and microvascular endothelial function other techniques have been developed to measure exclusively microvascular endothelial function. For instance reactive hyperaemia peripheral arterial tonometry described in subchapter 2.6.7 and important for chapter 5 measures predominantely microvascular function. A completely isolated microvascular function measurement technique is laser Doppler imaging in combination with iontophoresis. This method will be explained in more detail to complete the overview of common endothelial function measurement techniques.

Laser Doppler flowmetry is a technique enabling the monitoring of skin microvascular blood flow. The qualities of a laser beam change after contact with red blood cells in the cutaneous microvasculature and the emerging beam can be detected by a photodiode. The fraction of shifted light depends on the concentration of moving erythrocytes, whilst the shift in frequency depends on their average flow velocity, according to the Doppler principle [150]. Several techniques can be utilised in laser Doppler flowmetry to estimate endothelial

function, such as direct delivery of acetylcholine through iontophoresis or micro-dialysis [151]. Other available techniques are post-occlusive hyperaemia or local skin heating.

Due to its frequent use in research iontophoresis will be explained in more detail. Iontophoresis, also called electromotive drug administration, is a method where small quantities of drugs are delivered into or through the skin by application of small electric charges. A substance is propelled into the skin by a charge identical to its own created in an iontophoretic chamber. The most common agonists for microvascular endothelial function testing in the skin are the endothelium-dependent and –independent vasodilators acetylcholine and sodium nitroprusside, respectively. After drug delivery the skin surface is scanned by a laser beam and moving red blood cells back-scatter light including a shift in frequency by an amount proportional to their velocity. These Doppler shifts are recorded and processed. This data can be reproduced with a colour-coded image representing two dimensional skin perfusion, as shown in Figure 1.8. This relative blood flow measurement is called the laser Doppler flux. Baseline images are obtained and compared with images taken in prespecified time intervalls after drug administration. The median laser Doppler flux in the area enclosed by the iontophretic chamber is calculated for each image. The ratio between the highest flux values after drug administration in comparison to the baseline measurement represents the flow change [152].

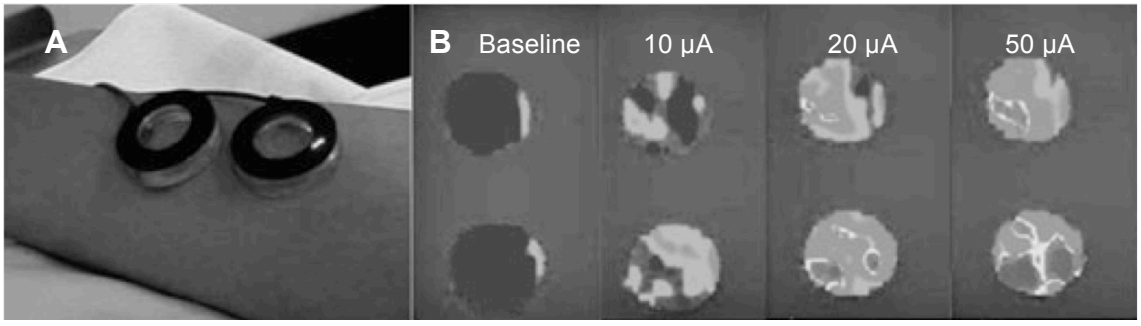


Figure 1.8 Laser Doppler imaging in combination with iontophoresis. Iontophoresis chambers are positioned on a proband's forearm (A). Microvascular response to sodium nitroprusside (upper images) and acetylcholine (lower images) as measured by laser Doppler imaging are depicted at baseline and in with increasing electric currents applied to the iontophoresis chamber (B). The figure was adapted from Khan [153].

The flow increase secondary to acetylcholine iontophoresis is reduced in different disease states such as diabetes [146, 154], hypercholesterolaemia and

hypertension [155]. Also an impaired response improves after treatment with nebivolol [156] and therefore represents a biomarker capable to display treatment effects. Microvascular endothelial function assesment by cutaneous laser doppler flowmetry coupled to iontophoresis is also related to the cardiac microvascular function, as the magnitude of the cuteanous flow increase by acetylcholine and sodium nitroprusside is strongly correlated with the coronary flow reserve during adenosin infusion as published by Khan et al. [157].

1.6.1.2 Endothelial progenitor cells

Endothelial progenitor cells (EPCs) were originally described by Asahara et al. in 1997 as bone marrow-derived cells capable of neovascularization [158]. Nowadays EPCs can be subdivided into two groups, cells that can differentiate into endothelial cells which probably derive from differentiated endothelial cells with clonal proliferation potential and cells of hematopoetic origin supporting new vessel formation with their angiogenic properties [159].

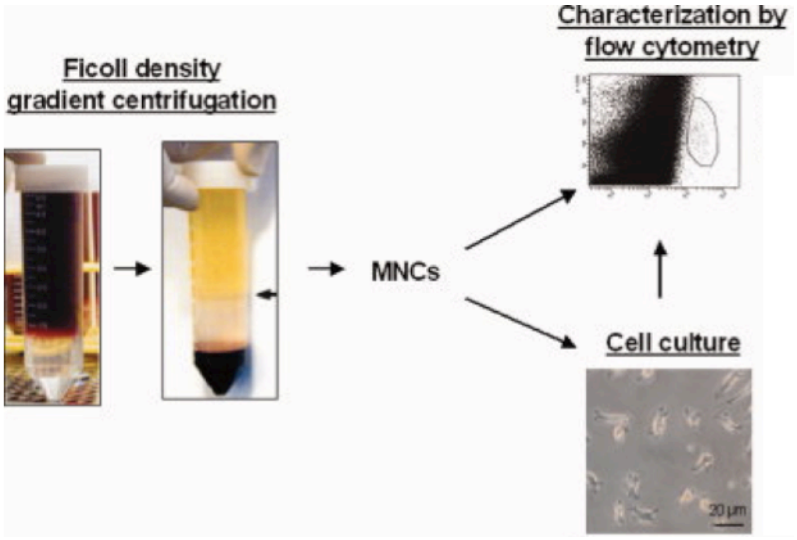


Figure 1.9 Endothelial progenitor cell isolation and characterisation strategy from peripheral circulating blood. Schematic illustration of standard techniques as cell culture and flow cytometry. MNC, mononuclear cell. The figure was adapted from Moebius-Winkler et al. [167].

EPCs are collected in the peripheral circulation and measured either by cytometry or cell culture, as illustrated in Figure 1.9. EPC quantification and functional assessment has potential as a biomarker in cardiovascular disease severity, prognosis and therapy response. In cohorts with increased

cardiovascular risk, such as patients with diabetes mellitus [160] or hypertension [161], the EPC number in the peripheral circulation is reduced whilst EPC function is often impaired. Hill et al. were also able to show that a lower number of circulating EPCs is associated with a higher Framingham risk score [162]. In established CVD, such as symptomatic CAD [163] or stroke [164] EPC numbers are likewise reduced. The EPC quantity however has been found to be increased in acute coronary syndromes such as myocardial infarction [165] or unstable angina [166] suggesting a mobilisation of EPCs during acute ischaemic events.

Furthermore, EPCs are related to cardiovascular outcome. Werner et al. [163] observed after adjustment for age, sex and cardiovascular risk factors that increased EPC levels are associated with a reduced risk for death from cardiovascular causes, as well as a reduced number of first major cardiovascular events, revascularization or hospitalization. The authors only investigated individuals with angiographically diagnosed CAD. In a more heterogenous cohort including healthy control subjects this finding is reproducible [168]. Thus, the EPC number measured in the peripheral circulation is a biomarker for diagnosis and prognostication in CAD.

1.6.1.3 Vascular Remodelling

Chronic alterations of hemodynamic conditions in the vasculature such as high blood pressure or increased shear stress [169] precipitate structural changes in the vessel wall. Vascular remodelling represents an active structural modification of the arteries; this includes changes of at least four different cellular processes – production or degradation of extracellular matrix, cell growth, cell death and cell migration. Those processes are controlled by dynamic interactions between vasoactive substances, hemodynamic stimuli and locally generated growth factors. Effects of this vascular remodelling are for instance structural changes, neointima formation, an increased ratio of wall to lumen width or changes in luminal dimension and functional changes such as reduced arterial compliance or increased stiffness [170]. Inward remodelling is usually caused by reduced blood flow and results in a reduced vessel diameter. Outward remodelling typically occurs in response to increased blood flow and leads to increased vessel diameter. Physiological examples for these remodelling

types are, for instance, the outward remodelling of conduit arteries in the limbs and the heart caused by repetitive increases in blood flow during exercise or the inward remodelling of the femoral arteries secondary to chronic disuse of the lower extremities as a result of spinal cord injury. Vascular remodelling is highly relevant to the atherosclerotic process, and its alterations contribute to the pathophysiology of vascular disease. By 1987 Glagov et al. [10] described outward remodelling as a compensatory mechanism to maintain coronary lumen diameter. Additionally histological studies observed a positive relationship between coronary artery dilatation and lipid core size or macrophage quantity in the plaque. This suggests a higher vulnerability of plaque in expansively remodelled vessels [171]. In small resistance vessels vascular remodelling may be the trigger for development of hypertensive target organ damage. Small resistance arteries with hyperplasia are more responsive to vasoconstrictor substances leading to reduction in vascular reserve. This contributes to tissue ischaemia, especially if surrounding arteries are narrowed. Also small artery remodelling is more common in hypertensive individuals providing a link to left ventricular hypertrophy.

Vascular remodelling leads to changes in vascular function, which may precede anatomic evidence of atherosclerosis. Therefore non-invasive assessment of vascular function is a potential early disease biomarker. Markers such as arterial compliance, elasticity or stiffness are related to the relative proportion of elastin to collagen fibres in the vessel walls. As aging produces arterial wall changes similar to those in atherosclerosis, all methods need correction for age. When considering different methods, it is important to distinct between large conduit arteries and arterioles causing flow resistance as well as small distal arteries and branch points which are the source of pulse wave reflection. In large arteries reduced compliance is a response to elevated pressure, aging or atherosclerotic disease. Large artery walls contain varying amounts of elastin and collagen, which determine their functional behaviour. The collagen renders the vessel wall pressure-dependent. This leads to a stiffening of the arterial wall when transmural pressures increase. Chronic stiffening is caused by alteration of the arterial wall structure with an increased collagen to elastin ratio. Stiffening of large conduit arteries and the aorta accelerates pulse wave velocity and increases pulse pressure. In smaller arteries compliance changes and calibre size

reflect altered vascular smooth muscle cell function. Smooth muscle concentric remodelling and cellular or collagen infiltration can contribute to this process. Arterial stiffening at the wave reflection sites leads to an alteration of the pressure wave curve by augmentation of the reflected wave. Non-invasive measurement techniques focus on either the large arteries or the reflection sites and some on a mixture of both.

From the available assessment tools, aortic pulse wave velocity is the best established marker of large artery stiffness. In general, arterial stiffness assessed by pulse wave velocity is associated with traditional CV risk factors and atherosclerotic disease. Recently Vlachopoulos et al. published a meta-analysis on the basis of 17 independent studies, here they documented that an increase in pulse wave velocity by 1 m/s leads to a 15% higher all-cause mortality [172]. Data is also available suggesting that pulse wave velocity improves prognostic discrimination and allows reclassification [173, 174]. Other markers of large artery function such as carotid distensibility [173] or pulse wave reflection parameters [174] do not relate to CVD outcomes in general populations. Augmentation index (AIx) and central pulse pressure (cPP) however are predictive of cardiovascular events in selected cohorts such as end-stage renal failure patients [175] and patients with established CAD [176].

1.6.1.4 Carotid intima-media thickness and plaque

Several modalities are available to image atherosclerosis. The most commonly applied in the peripheral vasculature is ultrasonography. Especially assessment of the carotid artery by ultrasound is well established. Carotid ultrasound is routinely used to evaluate ischaemic cerebrovascular signs like bruit and related symptoms. However in clinical studies C-IMT is most frequently used for risk stratification and to identify subclinical atherosclerosis. C-IMT represents the combined thickness of the intimal and medial layers of the artery wall. Pignoli et al. [177] demonstrated that C-IMT measurement by B-mode ultrasound is in line with in vitro or in situ findings. Measures at autopsy and by direct measurement were comparable to those acquired in living subjects by ultrasound. Although the carotid artery consists of several segments most frequently the common carotid artery is assessed at the far wall. Increased C-IMT is probably the predecessor of an atherosclerotic plaque. Therefore it predates changes such as

plaque calcification and in comparison to coronary calcium, can be seen as a biomarker of early atherosclerosis [178]. As a result Adams et al. [179] were able to show a correlation (R^2<0.10) between the extent and severity of CAD and C-IMT. The association of C-IMT with cardiovascular risk factors such as cholesterol [180], blood pressure or smoking [181] is well established. As recently reviewed by Simon et al. [178] C-IMT is also an independent predictor of CAD and stroke; accordingly, the absolute risk for a myocardial infarction, stroke or CVD in total ranged from 0.7% to 2.2%, 0.4% to 2.2% or 1.8% to 3.2%, respectively. A meta-analysis by Lorenz et al. [182] involving eight large, prospective studies and including 37,197 asymptomatic subjects suggested after adjustment for age and sex a relative risk of 1.26 or 1.32 per standard deviation for myocardial infarction or stroke, respectively. Also C-IMT progression is a predictor of cardiovascular events [59]. The relative risk for coronary events was 3.1 for each 0.03 mm increase per year of the C-IMT. As investigated by Folsom et al. [183] C-IMT has potential to improve predictive accuracy. In the MESA cohort its addition to traditional risk factors increased the c statistic for predicting cardiovascular or coronary events.

At the moment the use of C-IMT is limited to research. Therein it is the most common non-invasive measurement in cardiovascular epidemiology studies. As a surrogate end point it has become popular in randomised trials of new cardiovascular therapies [184]. Whilst C-IMT has been proven useful in a variety of studies, it is necessary to recognise that comparison between different study sites is difficult due to lack of sufficient standards. Such would be required for widespread clinical screening, especially as sub-millimetre differences differentiate low- from high-risk groups.

Carotid plaque rather than C-IMT might be the more important feature for risk prediction [185]. The presence of carotid plaque is more closely related to the prevalence of CAD and to cardiovascular risk factors compared to C-IMT [186]. In a general population without established CAD carotid plaque area in comparison to C-IMT was a better predictor of myocardial infarctions [187]. Also the plaque area progression in a longitudinal study by Spence et al. [188] was a good predictor of cardiovascular events. Furthermore, in patients with established

CAD both carotid plaque thickness and plaque area are better predictors of cardiovascular events in comparison to C-IMT [189, 190].

Plaque volume can be quantified with computed tomography [191], magnet resonance imaging [192] or three dimensional ultrasonography. These technologies are however time consuming and expensive. Other assessment strategies of plaque size or extent are plaque thickness or area quantification, defined as the sum of the maximal thickness or the sum of the total area of all plaques on two dimensional ultrasound images, respectively [189, 190]. Both were successfully used in a prognostic study [187]. Plaque thickness or area investigations depending on two dimensional ultrasonography are however only estimating the plaque extent. Therefore other semiquantitative measurements such as the Rotterdam plaque score [193] are also applicable as biomarkers for carotid plaque extent. The Rotterdam plaque score has several advantages such as its relative independence from picture quality and a small interobserver variability. The score was therefore used in this thesis as summarized in chapter 2.6.2 and illustrated in Figure 2.7.

1.6.1.5 Ankle-brachial index

ABI is a non-invasive, functional biomarker that indirectly provides information on the presence of peripheral arterial disease. The ABI is the ratio of the SBP in the lower legs, ideally at the ankle, compared to the SBP in the arm. An increased ratio is an indirect indicator of peripheral vascular disease. As individuals with peripheral arterial disease frequently suffer from atherosclerosis in other arterial beds, an abnormal ABI can serve as a biomarker for overall cardiovascular risk. A meta-analysis of 16 cohort studies demonstrated that an ABI greater than 0.90 in comparison to an ABI equal or smaller than 0.90 is associated with an almost two-fold higher risk of cardiovascular mortality and major coronary events independently from traditional risk factors [194]. The authors also suggested that the addition of the ABI to the Framingham risk score enabled a reclassification of 4% in men and of 10% in women with intermediate risk.

1.6.1.6 Asymptomatic coronary artery disease

On the basis of radiological advances during the past two decades, techniques have been developed which are able to assess the coronary system non-invasively. In contrast to carotid ultrasound these modalities detect existing CAD and not atherosclerosis of the peripheral vasculature. Due to their superior image quality cardiac computed tomography and magnetic resonance imaging are preferred. According to a meta-analysis by Schuetz et al. [195] computed tomography is more accurate than magnetic resonance imaging with a higher sensitivity and specificity, 97.2 vs. 87.1% and 87.4 vs 70.3%, respectively. Cardiac computer tomography results also have prognostic value [196]. Individuals with normal findings rarely develop adverse cardiovascular events. Greater CAD severity detected by cardiac computed tomography is related to an higher incidence of adverse CVD events. The quantity of coronary artery calcification can also be assed by computed tomography. Coronary artery calcification is correlated with plaque burden of the coronary arteries and is therefore a specific marker of later stages of CAD. Coronary calcium scores have a strong association with incidence of cardiovascular events in a number of observational studies [197, 198]. The coronary calcium score also improves risk prediction. In the Multi-Ethnic Study of Atherosclerosis [183] the c-statistic of 0.77 of the Framingham score was improved to 0.81 by addition of the coronary calcium score. A similar result was reported in the Rotterdam study [199] where the coronary calcium score improved the c-statistic of the Framingham score from 0.72 to 0.76. The coronary calcium score also improves risk classification, in particular for intermediate Framingham risk individuals. In the Multi-Ethnic Study of Atherosclerosis the net reclassification index was 25%. In the Rotterdam Study it was 14% and in a third population-based cohort study, the Heinz Nixdorf Recall Study, it was 22% [200]. Despite these findings the coronary calcium score is not currently recommended as a screening tool because of the related radiation exposure [201]. Should the magnitude of radiation exposure decrease in the future as a result of technologic advances in computed tomography, this policy might change.

1.6.2 Left ventricular hypertrophy

Left ventricular hypertrophy is the thickening of the myocardium of the left ventricle. Left ventricular hypertrophy detected by ECG [202], echocardiography [203, 204] or magnetic resonance imaging [205] is a strong predictor of cardiovascular morbidity and mortality. Good evidence exists that left ventricular hypertrophy is an independent cardiovascular risk predictor [203, 206]. Moreover improvement of outcome is correlated with its reversal on medical treatment [58]. Left ventricular hypertrophy is therefore an established surrogate end point biomarker. Hypertension is the most common cause of left ventricular hypertrophy and despite the association with hypertension [207], left ventricular hypertrophy is also a good predictor of cardiovascular outcome in general population cohorts like Framingham [203, 206]. The prognostic value of left ventricular hypertrophy is maintained in patients with established CAD [206] or hypertension [208]. In a meta analysis of 20 studies covering 48,545 participants left ventricular hypertrophy predicted cardiovascular morbidity or mortality, and the associated risk ratios were 2.3 or 2.5, respectively [209].

A variety of antihypertensive drugs interfere with the progression of left ventricular hypertrophy. Left ventricular mass (LVM) reduction by such pharmaceutical interventions predicts an improved prognosis independent from blood pressure and the agents used, as shown in the LIFE echocardiography sub-study [58]. Furthermore, the study showed that development or maintenance of left ventricular hypertrophy during antihypertensive therapy is related with an higher incidence of major cardiovascular events. According to a meta-analysis of 80 double-blinded clinical trials, angiotensin receptor blockers cause the biggest reduction of left ventricular hypertrophy with similar blood pressure control [210].

1.6.3 Microalbuminuria

Microalbuminuria is defined as albumin concentration of 30 to 300 mg/l in a spot urine sample. Respectively macroalbuminuria stands for albumin amounts bigger than 300 mg/l. Larger amounts with more than 1 g/l protein in the urine represent proteinuria. All are associated with a higher risk for onset of CAD in population based cohort studies [211, 212], with overall mortality in heart

failure [213] and diabetes [214, 215]. The direct link between proteinuria and cardiovascular risk is not completely understood. One possible explanation is an adverse effect of urinary protein excretion on traditional risk factors in that subjects with albuminuria are more likely to be smokers, to be hypertensive, to be diabetic and to have dyslipidaemia [211, 215]. Furthermore renal function decline is associated with increased renal production of deleterious vasoactive substances like angiotensin II [216] contributing to atherosclerosis of the general vasculature.

1.6.4 Markers of myocardial damage

Some serological biomarkers with diagnostic or prognostic value in CVD are not directly linked to the atherosclerotic process. CVD can cause cardiovascular injury or stress, which can induce the production of related biomarkers such as troponin or B-type natriuretic peptide, respectively. B-type natriuretic peptide, a 32 amino acid polypeptide, is predominantly released by the ventricle of the heart in response to excessive stretching of cardiomyocytes. Similar to other natriuretic peptides, it has vasodilatory, natriuretic and antihypertrophic effects. The name B-type natriuretic peptide was chosen because it was first extracted from porcine brain tissue. It is secreted in parallel with a 76 amino acid, biologically inactive N-terminal fragment. Both peptides are the product of the cleavage of pro-brain natriuretic protein into the mature natriuretic peptide and the N terminus mentioned above. Levels of both peptides are elevated in individuals with heart failure and established CVD [217, 218]. Levels in healthy individuals are lower, but even variations in the levels of brain natriuretic peptide within the reference range can hold clinical information. In the Framingham Offspring Study brain natriuretic peptic plasma concentrations above the 80th percentile, in men 20.0 pg per millilitre and in women 23.3 pg per millilitre, were associated with a 76 percent higher cardiovascular risk [219]. A recent meta-analysis summarising 40 long-term prospective studies and analyzing a total of 87,474 participants demonstrated a combined risk ratio of 2.82 for the upper third of natriuretic peptide concentrations compared with the bottom third, after adjustment for standard risk factors [220]. In secondary prevention populations N-terminal pro-brain natriuretic peptide improves discrimination, and different authors have shown that it raises the c statistic for traditional cardiovascular risk factors from 0.65 [62] or 0.66 [221] to 0.69. Yet,

in primary prevention cohorts the c statistic improvement is either minimal or insignificant in large prospective cohort investigations [61, 91, 222]. Similar reclassification in the context of secondary prevention with N-terminal pro brain natriuretic peptide (NT-proBNP) provides a net reclassification improvement of 0.11 (p=0.03) [221], whereas in primary prevention it does not significantly improve reclassification [91].

Troponin can be found in skeletal and cardiac muscle, but not in smooth muscle. It consists of three regulatory proteins: troponin C, I and T. Cardiac versions of troponin I and T are the gold standard biomarker for diagnosing myocardial infarction in clinical scenarios suggestive of an acute coronary syndrome. However, cardiac troponins are also markers for any kind of heart muscle damage. Therefore, levels are raised in myocarditis or trauma as troponin is released in response to cardiomyocyte necrosis. Small elevations in cardiac troponin are not associated with the risk for myocardial infarction in asymptomatic individuals with stable CAD and normal heart function [223], suggesting a mechanism other than atherothrombosis-mediated myocardial troponin release. Possible processes are coronary microvascular dysfunction, cardiomyocyte apoptosis, or subclinical structural or functional cardiac abnormalities.

Troponin levels measured by standard assays with a lower detection limit of 0.010 ng/ml are, however, associated with cardiovascular mortality, in primary prevention studies [61, 224]. The number of patients with troponin levels above the detection limit was small in these studies; in the Rancho Bernardo Study [224] only 4.1% of participants had a concentration above the lower detection limit. The improvement of predictive accuracy with these assays was non-significant [61]. The use of more sensitive troponin assays with a lower detection limit of 0.003 ng/ml leads to a higher percentage of study participants with valid measurements. In the Atherosclerosis Risk in Communities Study, 66.5% of the 9698 participants had concentration above the detection limit [225]. When high sensitivity troponin assays were used, levels were not only correlated with cardiovascular mortality but also improved the predictive accuracy significantly. In the Dallas Heart Study [226], a general population study, troponin T was associated with all-cause mortality after adjustment for traditional risk factors, including CRP, N-terminal pro-brain-type natriuretic peptide level and chronic

kidney disease (CKD). In addition, the c-statistic of traditional risk factors was improved significantly, from 0.832 to 0.868 for the end point cardiovascular mortality [226]. Similar results were detected in the Atherosclerosis Risk in Communities Study, where the addition of troponin T to traditional risk factors improved risk prediction with a significant c-statistic increase from 0.710 to 0.724 for cardiovascular mortality and incidence of myocardial infarction [225]. Cardiac troponin is also independently associated with adverse outcomes in other clinical scenarios, such as patients after acute coronary syndromes [227], patients with stable asymptomatic CAD [223] and patients with chronic heart failure [228].

1.7 Clinically relevant cardiovascular disease

The appearance of symptoms divides subclinical from clinically relevant cardiovascular disease. Symptom onset can be gradual, as in stable angina pectoris, or sudden, as in acute coronary syndromes. Furthermore, cardiac end-organ failure in the form of acute or chronic heart failure can cause symptoms. These disease stages are part of the cardiovascular continuum and can be assessed by a variety of diagnostic or prognostic biomarkers.

A patient cohort with stable angina is investigated in several chapters of this thesis. The clinical definition, epidemiology, diagnosis and prognostic relevance of angina are therefore discussed in more detail. Acute coronary syndromes and end organ failure are briefly summarised to complement the CVD biomarker continuum, as illustrated in Figure 1.5.

1.7.1 Stable angina

Stable angina is the term used to describe a clinical syndrome characterised by discomfort or pain in the chest, jaw, shoulder, back, arm or, less frequently, in the epigastric area. It is typically described as tightness or pressure, and it is elicited by exertion or emotional stress. The syndrome is confined to processes involving myocardial ischaemia. This differentiates it from other pathological processes such as gastric oesophageal reflux disease, structural lung diseases or musculo-skeletal chest wall problems, which can present with similar symptoms. Although frequently related to atherosclerotic flow limiting CAD, myocardial

ischaemia can also be induced by hypertrophic or dilated cardiomyopathies, aortic stenosis or microvascular dysfunction. The prevalence of angina is strongly associated with age. It rises from 0.1–1% in women aged 45–54 years to 10–15% in women aged 65–74 years. In men, it rises from 2–5% at the age of 45–54 years to 10–20% at age 65–74 years. In the Framingham Heart Study, the onset of angina symptoms was associated with a 33.4% and 17.8% 10-year risk for myocardial infarction in men and women, respectively [229].

A thorough medical history is the cornerstone of the diagnosis of angina pectoris. Although a physical examination and objective tests are necessary to confirm angina pectoris, a diagnosis can be made with high probability on the basis of the history alone [230]. Although a normal ECG is common, it may show an abnormal repolarisation pattern or abnormalities due to previous myocardial infarctions. Other tests are necessary to confirm myocardial ischaemia in suspected stable angina. An exercise ECG is cost-effective and frequently available. Numerous publications, including several meta-analyses, exist on the performance of exercise ECG for the diagnosis of CAD. According to Gianrossi et al. [231] the usage of exercise induced ST segment depression to define a positive exercise test produces a mean sensitivity of 68% and a mean specificity of 77%. As a consequence of this suboptimal result, exercise ECG testing was excluded from the diagnostic CAD pathway in the United Kingdom, as documented by the updated NICE guidelines for chest pain with recent onset [232].

Nevertheless, the diagnosis of hemodynamically significant CAD with an exercise ECG requires a Bayesian approach in which pre-test estimates in addition to the results of the exercise ECG are used to calculate a post-test probability. Pre-test probabilities in relation to age, gender and clinical presentation are depicted in Table 1.2, a modification of the literature review by Diamond and Forrester [230]. The authors classified chest pain as typical, atypical and non-cardiac to introduce a qualitative grading [233]. Accordingly, typical chest pain is defined as substernal pain that is triggered by exertion or emotional stress and relieved by rest and/or nitroglycerin. As listed in Table 1.2, atypical chest pain meets two of the mentioned three criteria, and non-cardiac chest pain meets one.

Based on this information, the Canadian Cardiovascular Society established a severity classification grading systems (Table 1.3) [234].

Table 1.2 Coronary artery disease probability in patients presenting with chest pain.

Age, years	Gender	Typical Angina Pectoris	Atypical Angina Pectoris	Nonanginal Chest Pain	Asymptomatic
30-39	Male	Intermediate	Intermediate	Low	Very Low
	Female	Intermediate	Very Low	Very Low	Very Low
40-49	Male	High	Intermediate	Low	Very Low
	Female	Intermediate	Low	Very Low	Very Low
50-59	Male	High	Intermediate	Intermediate	Low
	Female	Intermediate	Intermediate	Very Low	Very Low
60-69	Male	High	Intermediate	Intermediate	Low
	Female	High	Intermediate	Intermediate	Low

Clinical Chest Pain Classification	
Typical (definite)	Meets three of the following characteristics 1) Substernal chest discomfort of characteristic quality and duration 2) Provoked by exertion or emotional stress 3) Relieved by rest and/or GTN
Atypical (probable)	Meets two of the above characteristics
Non cardiac chest pain	Meets one of the above characterstics

Clinical chest pain characteristics and the corresponding definitions are listed. The ordinary pre-test risk quantification translates into a probability of having coronary artery disease: High 90%; intermediate, 10–90%; low, 10%; and very low, 5%.

The use of pre-test probabilities has the advantage of combining the actual diagnostic test with additional information, especially regarding disease prevalence and symptom severity.

Table 1.3 The Canadian Cardiovascular Society grading scale for angina severity.

Grade	Definition
Class I	Angina only during strenuous or prolonged physical activity
Class II	Slight limitation, with angina only during vigorous physical activity
Class III	Symptoms with everyday living activities
Class IV	Inability to perform any activity without angina or angina at rest

The results of exercise ECG testing are greatly dependent on the patient's physical capacity. It can be inconclusive in situations where the heart rate during exercise remains <85% of the calculated maximum rate in the absence of ischaemia or angina symptoms. This can be due to unfitness or limitation secondary to non-cardiac diseases, such as osteoarthritis, or to equivocal ECG changes. According to current guidelines [235] an inconclusive test should be followed by a further non-invasive diagnostic test unless a very low pre-test probability is given. A list of possible test modalities with corresponding sensitivities and specificities is presented in Table 1.4.

The long-term prognosis of patients with stable angina depends on treatment, traditional cardiovascular risk factors and other comorbidities. In the PEACE study [236], for instance, the rate of cardiovascular death was <1% per annum. In stable angina cohorts with a high cardiovascular risk, such as the diabetic population in the MICRO-HOPE trial [237], the cardiovascular mortality can be >2%. Correspondingly, an annual cardiovascular mortality of >2% is often considered high, 1–2% as intermediate and <1% as low. To set these values into context, a 10-year cardiovascular mortality of >5% is considered appropriate to start primary prevention drug treatment according to contemporary guidelines [238].

Table 1.4 Test characteristics for investigations used in the diagnosis of stable angina.

	Diagnosis of Coronary Artery Disease	
	Sensitivity, %	Specificity, %
Exercise ECG	68	77
Exercise Echo	80-85	84-86
Exercise Myocardial Perfusion	85-90	70-75
Dobutamin Stress Echo	40-100	62-100
Vasodilator Stress Echo	56-96	87-100
Vasodilatro Stress Myocardial Perfusion	83-94	64-90
CT angiogram	97.2	87.4
MRI angiogram	87.1	70.3

Data provided corresponds to publications by Schuetz et al. [195] for structural imaging and by Fox et al. [235] for stress related test modalities.

Risk stratification by clinical assessment can be improved by stress testing. Exercise ECGs not only allow the diagnosis of transient myocardial ischaemia as a simple binary response but also provide prognostic information. In patients with known or suspected CAD, different features of the exercise ECG testing have proven to be prognostic markers. Examples are the maximum exercise capacity and clinical or electrocardiographic ischaemia during exercise. Given that no randomised trials of exercise ECG testing have been published, the available evidence is based on observational studies. Using standardised exercise protocols, exercise capacity has been measured by exercise duration, maximum heart rate, maximum achieved workload (in Watts), among others. The 5-year survival is higher in patients with a better exercise tolerance for expected [239] or established [240] CAD and no more than mildly impaired left ventricular function. Combinations of several variables derived from an exercise ECG provide more information than a single marker. Examples for such variables are exercise time, extent of ST-segment depression or elevation and symptom occurrence during exercise. A well-validated score including these variables is the Duke treadmill score (DTS) [239]. A high DTS risk score (≥5) was associated with a 99% 4-year survival rate in patients with suspected CAD. The group with a low DTS risk score (≤-11) had a 4-year survival rate of only 79%. Therefore, an

exercise ECG – and, in particular, the DTS – provides prognostic information in addition to its diagnostic capacity. Interestingly, the DTS is also useful for the diagnosis of haemodynamically-relevant CAD and provides information regarding the extent of the disease [241]. In detail, 60% of low-risk patients had no significant stenosis, in comparison with 33% and <1% of moderate and high risk patients, respectively. In summary, the combination of exercise tests and clinical parameters is an effective method for differentiating high and low risk groups within a population presenting with symptoms suggestive of CAD.

1.7.2 Acute coronary syndromes

The term acute coronary syndrome refers to a clinical situation induced by coronary artery obstruction and incorporates a spectrum of events ranging from unstable angina to non-ST-segment elevation myocardial infarction and ST-segment elevation myocardial infarction. These subtypes share a widely common pathophysiological process, atherosclerotic plaque rupture or erosion with different degrees of superimposed thrombosis and distal embolisations. As such, an event might be life threatening and a patient presenting with an acute coronary syndrome requires immediate treatment to interrupt disease progression, cardiac muscle death and ischaemic complications such as ventricular arrhythmias. The main trigger for the diagnostic cascade is chest pain. An electrocardiogram can classify the subtypes of acute coronary syndromes and thereby has a major influence on therapy. In patients with acute chest pain and persistent ST-segment elevation, the main objective is immediate removal of the coronary artery occlusion by fibrinolytic therapy or primary angioplasty. In patients with acute chest pain but without persistent ST-segment elevation, the initial therapeutic strategy aims to reduce ischaemia and improve symptoms in parallel with patient monitoring using serial electrocardiograms and measurement of markers of myocardial necrosis. Traditional markers of myocardial necrosis are cardiac enzymes such as creatine kinase, its isoenzyme MB and myoglobin. Troponin is currently the gold standard for detecting acute myocardial necrosis. Initial troponin levels rise within approximately 4 hours in patients with myocardial ischaemia, and troponin levels remain elevated after an ischaemic event for up to two weeks due to proteolysis of the contractile apparatus. On the other hand, small increases in troponin levels can resolve within three days in non ST-segment elevation myocardial necrosis, and thus the

diagnosis of unstable angina is indicated if serial troponin measurements remain in the normal range. Unstable angina can also present as a rapid symptom deterioration in patients with previously stable angina or a new onset of severe angina leading to marked limitations of ordinary activity within 2 months of the initial presentation [235]

Non-invasive, and especially invasive, imaging is important in the assessment of acute coronary syndromes. Relevant non-invasive imaging techniques are echocardiography, which allows assessment of wall motion abnormalities and differential diagnoses of acute chest pain, stress imaging in patients with normal electrocardiograms and troponin levels to assist with the diagnosis of CAD, or modalities such as cardiac computer tomography that can directly visualise coronary artery stenoses. Invasive imaging in the form of coronary angiography remains the gold standard for assessing the presence and severity of CAD.

1.7.3 End-organ failure in cardiovascular disease

Heart failure can be understood “as an abnormality of cardiac structure or function leading to failure of the heart to deliver oxygen at a rate commensurate with the requirements of the metabolizing tissues, despite normal filling pressure” [242]. Clinically, heart failure can be defined as a syndrome in which patients develop symptoms and signs resulting from abnormal cardiac structure or function, including breathlessness, ankle swelling and fatigue. Typical signs on physical examination are an elevated jugular venous pressure, pulmonary crackles and a displaced apex beat. As these symptoms and signs can occur in other disease than heart failure, the demonstration of an impaired heart function by echocardiography or other imaging modalities is essential for the diagnosis of heart failure. Also electrocardiographic features with evidence of left ventricular hypertrophy or Q waves suggesting loss of viable myocardium can be helpful. An alternative approach to echocardiography for examining ventricular function is the measurement of natriuretic peptides in the peripheral blood stream.

1.8 Proteomics

As described in subchapters 1.2.3 and 1.7.1 contemporary prognosis and diagnosis algorithms are identifying only a portion of individuals who develop or have clinical significant CAD. The clinical process of risk stratification and CAD diagnosis requires therefore further refinement. In addition to the application of existing biomarkers to new diagnostic or prognostic hypotheses, the development of novel biomarkers is a rational approach to this clinical requirement. Two different strategies are available for the development of new CVD biomarkers. The first strategy is based on existing knowledge, a deductive method, whilst the second strategy is more unbiased and inductive. These two strategies are rather complementary than mutually exclusive. The deductive approach uses knowledge emerged from single gene, metabolite or pathway investigations to identify new biomarkers. This implies a direct understanding of the biological processes and the evolution of their sequelae. In contrast, the unbiased approach relies on evaluation of large quantities of molecules with the help of recent technological advances with the goal to characterize a biomarker profile specific for a disease or a particular disease stage. Such an assessment can be carried out with different molecules, such as proteins. The research field related to the latter is known as proteomics.

Proteomics has several advantages over the other 'omics' strategies. Assuming an individual is progressing through the cardiovascular continuum, the genome remains largely unchanged whereas the protein structure of tissues and organs alters considerably. As a consequence, the status of the proteome representing structural and physiologic changes in the cardiovascular system are more appropriate to estimate the situation of an individual.

Especially the application of discovery proteomics represents an unbiased approach. Discovery proteomics, capable of assessing large numbers of proteins simultaneously, is an innovative approach to biomarker development. In light of the importance for this thesis the concept of proteomics in general as well as discovery proteomics and urine proteomics in particular will be explained in more detail in the following sub-chapter. At the end of the sub-chapter several proteomic studies will be summarized to emphasise the value of proteomic research in CVD.

1.8.1 The proteome

The term proteome represents the collection of all proteins in a cell, organ or organism. Proteins are biochemical compounds consisting of one or more polypeptides. The understanding of protein structure and interaction is therefore crucial in physiology and pathology. One gene can produce different proteins as a result of alternative splicing, the use of multiple transcription start sites, polyadenylation and editing of pre-mRNA, or posttranslational protein modification. On average, 5–7 protein isoforms exist for each open reading frame in the human genome, representing a total of 100 to 200 thousand proteins. If one considers the enormous range of 600,000 immunoglobulins that exist with only small differences in their epitope binding domain, this totals approximately 1,000,000 proteins encoded by only 30,000 known human genes [243]. Considering this large number of different proteins, proteomic research will provide new inside into physiological and pathological processes. Proteomic approaches can be applied across a range of data set sizes, from the assessment of single proteins or protein complexes to the assessment of entire patient populations.

1.8.2 Basic principles of proteomics

The goal of proteomics is a “comprehensive, quantitative description of protein expression and its changes under the influence of biological perturbations such as disease or drug treatment” [244]. Proteins can be investigated by a multitude of techniques including physical separation and array technology. It is possible to immobilise specific proteins, small molecular compounds, antibodies and peptides on solid surfaces by a range of methods [245]. The methodology used depends mainly on the research objective. Two objectives of proteomic research are for instance protein identification and quantification. An example for a methodology useful in identification of known proteins is Western blotting [246]. To this goal tissue homogenate or extract is separated by gel electrophoresis depending on its three dimensional structure or, in the case of denatured proteins, on the length of the polypeptide. In a consecutive step proteins are transferred to a membrane followed by detection of target proteins with specific antibodies. This allows the positive identification of proteins of interest in a semi-quantitative manner. A similar approach for identification of known

proteins is the enzyme-linked immunosorbent assay (ELISA) [247]. The quantity and presence of an antigen in a sample is measured with a three-step approach. First, antigens from a sample are attached to a specific surface. Then, an antibody against an antigen of interest, which is also linked to an enzyme whose presence can be detected in the final stage of the technique, is applied to the surface. If the antigen of interest is present amongst the antigens bound to the surface, this will interact with the enzyme-linked antibody. Subsequently, all unbound antibodies are removed in a washing step. Finally, to confirm the presence of the antigen of interest, the substrate for the enzyme linked to the antibody is added. This produces a detectable signal, such as a colour change, in the presence of the enzyme, resulting in the positive identification of the target protein.

Identification of unknown proteins is, however, more elaborate and usually requires several methods connected with each other. Such techniques are high-resolution 2-dimensional gel electrophoresis, high performance liquid chromatography, surface chromatography by protein adsorption to activated surfaces, or, with the help of peptide ionization procedures, mass spectroscopy. Sodium dodecyl sulfate polyacrylaminde gel electrophoresis and, later, two-dimensional gel electrophoresis laid the foundation of today's proteomics. Two-dimensional gel electrophoresis separates proteins on the basis of two intrinsic protein characteristics such as the isoelectric point and the molecular mass. First, molecules are fractionated one-dimensionally by electrofocusing where proteins migrate to their isoelectric point in a pH gradient. In the second dimension proteins are separated by sodium dodecyl sulfate polyacrylaminde gel electrophoresis in a dimension that is perpendicular to that of the electrofocusing. Thereby, proteins migrate on the basis of their molecular mass. In the last step of 2-dimensional gel electrophoresis proteins can be stained, semi-quantified and mechanically isolated for further processing. This, in comparison to one-dimensional gel electrophoresis, provides the advantage of a more effective separation, as it is improbable to find molecules that are similar in both their isoelectric point and molecular mass. Another separation technique of samples containing protein mixtures is chromatography. It represents a collective term for several laboratory techniques utilising a so-called mobile and stationary phase. Depending on various constituents, the components of the

mixture travel at different speed through the stationary phase. The technique can be used to prepare samples for further analysis, e.g. prior to mass spectrometry, or as an analytic tool. An example is liquid chromatography, which uses a liquid as the mobile phase. The widely used high performance liquid chromatography, for instance, packs the stationary phase on very small particles and uses high pressures to press the mobile phase through the stationary phase.

Usually protein mixture separation is followed by content assessment for which most proteomic studies use mass spectrometry as this enables detection and identification of proteins. Its main feature is the measurement of the mass-to-charge ratio of charged particles. Samples first undergo vaporization followed by ionization, before they can be further separated in an electromagnetic field. Last, proteins or peptides get quantitatively and qualitatively analysed by a detection unit. Mass spectrometry can be used in multiple research settings such as the identification of unknown compounds, structure evaluation of a compound or quantification.

1.8.3 Complexity of proteomic analyses

The results of proteomic studies are often difficult to interpret and appear at a first glance to be inconsistent between studies. Primarily, this is due to the large number of available proteomic platforms. These have recently been reviewed by Tuñòn et al. [248]. Different proteomic platforms are characterised by different sensitivities. Also platforms differ regarding the molecular weight range, which they are capable to detect. Therefore results can diverge depending on the method used even if the same samples were processed. Consequently, knowledge about the performance of each platform is important for the interpretation of results. Simplified, most platforms use three consecutive steps: protein digestion, protein separation and identification, usually accomplished using a combination of two methods such as the previously described liquid chromatography and mass spectrometry.

The common principle of most proteomic platforms is consistent with so-called bottom-up analyses, requiring a conversion of proteins into peptides. This is in contrast to top-down proteomics where intact protein ions are introduced into

the mass analyser. For the former, proteins of a sample are digested into smaller peptides. Features simplifying the analyses are peptide length, optimally 7-35 residues long, protonation of proteins, a low charge state and a high mass-to-charge ratio [249]. In the majority of cases proteins are fragmented with the enzyme trypsin [250]. Trypsin exclusively cleaves polypeptides C-terminal to arginine and lysine residues. The resulting peptide mixture is then separated, e.g. by liquid chromatography. The peptides are then identified and sequenced allowing to identify the original protein they came from [251]. Top-down proteomics, on the other hand, provides access to complete protein sequences and has the ability to localise and characterise a protein's post-translational modifications [249]. It is also time saving as a protein digestion is no longer required.

Proteomic methodologies are challenging for a variety of reasons. 2-dimensional gel electrophoresis, for instance, under-represents high or low mass proteins [252]. More importantly, 2-dimensional gel electrophoresis is very time-consuming due to the consecutive processing of each identified protein. To exemplify this, Thelen et al. [249] estimated the time required to identify 200 protein spots excised from two-dimensional gels by liquid chromatography coupled to mass spectroscopy as 14 days. Therefore attempts were made to improve existing techniques by increasing the flow rates in the liquid chromatography phase [253] or by establishing new techniques such as the one-step direct transfer technique [254]. The latter uses a novel target plate to transfer analytes from one-dimensional gel electrophoresis onto the plates in one step. This allows direct analyses by matrix assisted laser desorption/ionization coupled to mass spectrometry. Therefore steps like staining, protein extraction and liquid chromatography become unnecessary and as a result the proteomic analysis is dramatically accelerated.

Another good example for the limitations of proteomic methodologies is the widespread use of the enzyme trypsin for bottom-up analyses. This leads to several enzyme related disadvantages. The enzyme's thermostability is not good and it is susceptible to autolysis at alkaline pH values. Furthermore, lysine and arginine residues, the cleavage points of the enzyme, are randomly distributed throughout the proteome. Therefore, peptides can be either too short or too long for further analysis by mass spectrometry. This highlights the problem of

insufficient protein sequence coverage [249]. Attempts to overcome this issue are the use of chymotrypsin with cleaveage points C-terminal to phenylalanine, tyrosin, tryptophane and leucine. Also, so-called sequencing endoproteases exist, which cleave only C-terminal to specific residues such as lysine, arginine, glutamic acid or aspartic acid. Chymotrypsin [255] or sequencing endoproteases [256] yield improved sequence coverage when used in combination with trypsin.

Further complexity is added by the fact that proteomic studies can be performed on a wide range of biological specimens including whole tissue samples but also cells and biofluids [248]. Although they are unlikely to fully represent the proteome of certain organs such as the heart or the vasculature, samples that can be obtained noninvasively or minimally invasively, such as urine and blood, appear ideal for clinical purposes. This situation is in contrast to genetic studies where DNA is the same or at least very similar between different cell types. Therefore genomic studies can be performed on DNA extracted from a wide range of specimens with very similar results. The results of proteomic studies on the other hand depend heavily on the specimen used.

The dynamic nature of the proteome has direct implications on sample handling and processing. Post-translational modifications can occur ex vivo, for example due to hypoxia if a sample is not immediately snap frozen or otherwise processed. The abundance of proteases in tissue and biofluids, such as blood, will also change the proteomic make-up of specimens from the time of sampling. For clinical purposes, very strict criteria for sample processing are necessary so that proteomic studies can be interpreted with confidence [257].

Finally, proteins undergo complex post-translational modifications, with phosphorylation, acetylation and glycosylation being the most common ones [251]. These modifications can change protein function dramatically but are not easy to detect comprehensively with existing proteomic platforms. A combination of methods can be employed to detect a larger range of post-translational modifications, but interpretation of the results remains challenging. Combinations of different post-translational modifications and more rare modifications, such as prenylation or S-nitrosylation, remain analytically demanding.

Similar to other omics data or large biomarkers quantities in system medicine it is important to highlight problems with standard statistical methods in proteomics. Most univariate statistical methods (e.g. Student's t-test) are not well suited for large datasets. For instance, repetitive testing of significance of different variables leads to an accumulation of the error in repeated testing and ultimately to high false positive rates.

1.8.4 Proteome quantification

In addition to qualitative protein identification, methodologies for quantitative protein analyses are available. For these, gel based and gel free sample preparation is possible. Considering the time constrains of gel based proteomics, gel free quantitative proteomics is more suitable. It can be divided into subcategories based on metabolic or chemical labelling and label-free preparation [258]. Especially the latter preparation has great potential for use in medical diagnostics, as it is a straight forward and inexpensive quantification strategy. This proteomic technology can be divided into two methods. One uses the peptide's mass spectroscopy signal as a direct quantitative measure whereas the other infers quantity indirectly from so-called spectrum counts; the number of peptide-to-spectrum matches obtained for each protein. The peptide-to-spectrum matches, in comparison to peptide count and sequence coverage, offers the higher dynamic range of quantification and is more reproducible [259]. An advantages of relative quantification with spectrum counts is its simplicity since it only requires comparison of spectrum counts for each protein, easily generated during database searches for protein identification [260]. In bottom-up experiments, label free quantification is usually accomplished by integrating the ion intensities of each detected peptide over its chromatographic elution profile. Such peak areas from liquid chromatography mass spectrometry correlated (R^2 = 0.991) linearly with the concentration of the measured peptide [261]. This correlation was proven over a wide range from 10 fmol to 100 pmol. The integrated signal response of individual peptides can therefore be easily used to compare peptide quantity in different samples. Variation in protein abundance can then be estimated by aggregation of differences measured for all peptides. This can be accomplished by averaging peptide fold changes or summation of peptide responses. Similar to spectrum counting this allows comparison of large data sets.

1.8.5 Discovery proteomics

Mass spectrometry based proteomics in “discovery” mode can identify thousands of proteins in tissues, cells and biofluids [248]. The aim of discovery proteomics is the identification of new biomarkers and pathways, for instance, in cardiovascular disease. Next to mentioned complexities of proteomic research, further problems arise with proteomic biomarkers. With extremely complex samples, such as human plasma, certain analytical barriers exist. For instance, a large number of proteins identified with proteomic methods occur due to inter-individual variations in protein abundance and not as a result of the disease investigated. Hence, robust methods are necessary to clearly identify proteins that are disease related.

Other problems related to the use of the plasma proteome include three factors. First, few high-abundance proteins dominate the blood. Albumin, for instance, constitutes over 50% of the total plasma protein, with a concentration of 35 to 60 mg/mL in humans [262]. The 22 most abundant proteins constitute almost 99% of the whole plasma protein mass [263]. Secondly, an enormous number of different protein exist in the blood stream. Estimates vary from 10 000 to 1 000 000 unique proteins contained in the blood. The higher number takes into account that many proteins will only vary slightly due to proteolytic processing, posttranslational modifications, single-nucleotide polymorphisms and splice variants. Third, the dynamic range in concentrations of these proteins makes analysis difficult. This range can span from femtomoles per liter of blood to concentrations >600 μmol/L [262]. A large number of biologically interesting molecules that are relevant to a disease process, such as cardiovascular disease, are low-abundance proteins. For example, troponin, usually found in the nanomolar range, and TNFα, usually found in the femtomolar range even when elevated in pathologic states.

Liquid-chromatography coupled to tandem mass spectrometry, especially when merged with an antecedent peptide or protein fractioning step, is currently thought to be the only technology capable of detecting and identifying thousands of peptides and proteins in tissue and plasma samples [264, 265]. In biomarker discovery studies, high sensitivity and completeness of peptide/protein identification are central requirements as disease process

related proteins are only detectable at low levels. In comparison to proteomic assessment of tissues affected by the disease, e.g. atherosclerotic carotid arteries, this is more prominent in samples indirectly in contact with the disease processes, e.g. body fluids or peripheral blood. In “discovery” proteomics, electrospray ionization is the standard ionization procedure, which is ideally matched to online liquid chromatography tandem mass spectrometry [251]. Matrix-assisted laser desorption/ionization mass spectrometry can also be integrated into multidimensional separation procedures, however only with significant reduction in process speed and assessment accuracy for high complex peptide mixtures [266]. As both are crucial in biomarker discovery, electrospray ionization is the preferred method. Also, robustness and reproducibility of liquid chromatography tandem mass spectrometry methods have been investigated extensively. This led to the development and publication of reference data sets as well as system performance metrics for monitoring of the different process steps [267, 268]. Two-dimensional gel electrophoresis followed by mass spectrometry is also established in biomarker discovery [269], but as previously discussed the method is time consuming and only able to identify a few hundred proteins in biologic samples.

The sample type selection, e.g. plasma, tissue or biofluids, as well as the experimental design, have substantial influence on the achievable result. For instance different proteomic platforms are characterised by different sensitivities, allowing identification of peptides and proteins only in a certain range of molecular weight. Therefore, the chosen methodology has considerable influence on the result and makes comparison of results between platforms difficult. Furthermore, the presence of high abundance proteins in plasma is a major complicating issue for proteomic analysis. Related peptides are detectable in every fraction independent of the separation method and their peptide ion intensities usually dominate the mass spectra. To diminish the impact of these proteins on the detected plasma proteome, abundant plasma proteins can be remove by immunoaffinity depletion columns, which are able to extract up to 14 or more proteins [270-272]. Unfortunately the protein removal also involves proteins or peptides of interest, as albumin binds lower-molecular-weight proteins and thereby protects them from kidney clearance. It has therefore been shown that non-specific losses occur with the use of

immunaffinity depletion columns [273]. This leads to a reduction in sample complexity by 10 to 20-fold [274].

A standard proteomic biomarker discovery experiment compares peptide/protein abundance of case samples against control samples. First, proteins are reduced and alkylated to cut off disulfide bonds and to obstruct cysteine residues. Then, proteins are usually cleaved into peptides with the enzyme trypsin. Next, the resulting peptide solution is applied to, for instance, a reversed-phase chromatographic column with direct connection to a mass spectrometer. Prior to entering the mass spectrometer, peptides are ionised by electrospray. The mass and charge of the intact peptides will be detected by a high-performance mass spectrometry system. Intact peptide ions fragment further in the mass spectrometry system through the mechanic interaction with gas. This produces ionised fragments which can be used to obtain information on the peptide sequence [251]. The volume of data obtained during a single measurement is enormous; for example 4000 single scans in mass spectrometry and 30 000 single scans in tandem mass spectrometry scans. Therefore, data processing is facilitated by the widespread availability of robust software packages and application of modern information technologies is critical to mass spectrometry in biomarker discovery. A standard instrument mass spectrometry scanning cycle starts with the acquirement of a full-scan mass spectrum over a period of approximately 1 second. The mass spectrum constitutes the mass-to-charge ratio and intensities of the ions detected during the mentioned time interval of the chromatographic separation. This process is repeated over the entire course of the liquid chromatography tandem mass spectrometry analysis, which lasts, on average, between 60 to 180 minutes. The time required to achieve this depends on the complexity of the sample analysed. The more complex the sample, the longer the time required. Furthermore, modern mass spectrometry instruments automatically select ions for fragmentation and sequencing during a scan. This is called a “data dependent” experiment [251]. An integrated data processor identifies peptide mass and charge in the mass spectrum. A predefined number of these proteins is then selected for further fragmentation. However, a modern mass spectrometer is only capable of sequencing up to 20 ions, depending on the data acquisition speed and the sensitivity of the particular instrument. This limits the quantity of sequenced ions, especially as the number of ions of

complex biological samples exceeds the capacity of a modern mass spectrometer. Also, not all mass spectrometry spectra provide an interpretable sequence. To improve the yield, additional separation stages can be deployed before the online reverse phase liquid chromatography. These techniques are usually carried out at peptide level with strong cation exchange [275], high pH reverse phase [276] or off-gel electrophoresis [277]. Also protein separation techniques can be used like 1-dimensional gel separation. Although these multidimensional separation techniques decrease the sample complexity they have a clear disadvantage; they are time consuming.

Instead of identifying a single disease biomarker and unfolding its peptide sequence data, analysis of mass spectra can be used differently. With quantification of protein expression patterns liquid chromatography mass spectrometry can produce a “peptide finger print” of certain diseases. This also requires the comparison of protein expression between normal and diseased tissues. However, there is no longer the need to extensively separate complex bio samples for protein identification and therefore, the time requirement is drastically diminished. Additionally, comparative data processing allows the elimination of high abundance proteins. Therefore extraction of such proteins is no longer necessary prior to sample separation. This protein signature approach follows a multiple biomarker strategy. Therefore experiments, if such markers have discriminatory power or offer incremental predictive value, are fundamentally different to the search of an unknown single biomarker. For instance, some of the markers will be correlated to the outcome due to random chance. Therefore, a replication of the result is necessary. A simple approach is to test the marker in an independent sample of individuals. This can be a split sample from the original cohort or an entirely independent cohort of subjects.

1.8.6 Urine proteomics

Urine is the result of plasma ultrafiltration in the kidneys with the purpose of waste product elimination, for example urea and metabolites. Despite the kidney’s small size, accounting only for 0.5% of the total body mass, about 350-400 ml of plasma is filtrated per 100 g kidney tissue every minute [278]. Thereby, 150 to 180 litres of ultrafiltrate are generated daily under normal physiologic conditions. Most components of the ultrafiltrate, such as water,

glucose, amino acid and inorganic salts, are selectively reabsorbed leaving less than 1% of the ultrafiltrate for excretion with the urine. The selection of serum proteins filtrated at the glomeruli depends on their size and charge [279]. At the end of the passage through the glomeruli, abundant serum proteins, such as albumin, transferrin, immunoglobulin light chain, vitamin D binding protein and others, are reabsorbed predominantly by endocytic receptors like megalin and cubilin in the proximal renal tubules [280, 281]. This leads, despite additional tubular protein secretion, to a very low urine protein concentration in healthy individuals; on average less then 100 mg/l per day.

1.8.6.1 History

Despite this, urine still contains a substantial quantity of different peptides and proteins. In 2001 Spahr et al. published one of the earlier attempts to characterise the urinary proteome [282]. They used trypsin digested urine of healthy male subjects and performed liquid chromatography tandem mass spectrometry for data-dependent ion selection and fragmentation to identify 124 different proteins. While the study was not intended for biomarker discovery it demonstrated that urine contains a wide range of different proteins and associated information. The authors also showed a possible analysis approach. Three years later, the number of peptides identified in the urine increased approximately three fold. After immunoaffinity subtraction of highly abundant proteins, 1400 spots were detected on two-dimensional gel electrophoresis [283]. Of those, only 30% were identified, revealing 150 unique protein annotations. Hence, an estimate of 500 proteins in addition to the previously subtracted proteins, were present in the samples. Then, in 2006, Adachi et al. [284] were able to increase this number to 1543 by combination of one-dimensional gel electrophoresis and reverse phase liquid chromatography coupled to mass spectrometry. More recently, a total of 116 689 peptides and proteins were identified in 3687 different urine samples with the help of capillary electrophoresis coupled to mass spectrometry (CE-MS) [285].

1.8.6.2 Advantages and disadvantages

For clinical proteomic studies, the use of urine has several advantages over blood or other biofluids. First, urine is non-invasively available in large amounts.

Despite the mentioned low protein concentration, sufficient protein quantity can be collected from a single sample. Also, repeated sampling of the same patient for disease surveillance is possible when urine samples are used. Furthermore, urinary peptides and proteins are, in general, soluble. As a result, the step of solubilization and its influence on the proteomics analysis is omitted. Due to the bias for lower molecular mass compounds generated by the kidney, usually below 30 kDa, no further digestion step is necessary for mass spectrometry analysis [286]. Also, the urinary protein content is stable due to its "stagnation" for several hours in the bladder. Therefore, proteolytic degradation by endogenous proteases is completed by the time of urination. This is in contrast to the situation in blood, in which protease activity will lead to protein degradation [287]. The urine proteome content appears to be stable when stored for up to 6 hours at room temperature or up to 3 days at 4°C [288, 289]. Additionally, the urine proteome remains stable, when stored at -20°C, for several years [286]. Finally, the glomerular barrier only restricts passage of large proteins. Therefore almost 30% of all urine protein in healthy kidneys derives from plasma [290]. As the consequence urine contains polypeptides originating from a large number of biochemical pathways within the body. These are not only plasma proteins but also kidney or genitourinary tract proteins [283]. As a consequence urinary proteomics has been successfully piloted in the diagnosis of renal disease [291], kidney transplant rejection [292], CKD [293] and urothelial cancer [289]. In contrast to blood, protease activity in urine is low, leading to a more stable sample. The urinary proteome or peptidome is less complex, and analyses are technically less demanding and thereby more reproducible in urine compared to plasma [290]. There are also disadvantages of urine as the source for proteome analysis. Urine differs widely in protein and peptide concentration due to differences in fluid intake. This, however, can be overcome by standardization based on creatinine concentration [294] or based on peptides generally present in the urine [295]. Also, the definition of disease-specific biomarkers in the urine is complicated by circadian fluctuations in the urinary proteome due to variations in diet, metabolic or catabolic processes, or exercise [296]. This reduces the reproducibility of the urine proteome analysis even if the method itself is highly reproducible. Fortunately, these variations are limited to a fraction of the urinary proteome and a large portion remains unaffected [297].

1.8.6.3 Techniques

Most established mass spectrometry techniques have been applied to urinary proteome analysis in the past, including two-dimensional gel electrophoresis mass spectrometry, liquid chromatography mass spectrometry, surface enhanced laser desorption/ionization (SELDI) time of flight (TOF) mass spectrometry (Figure 1.10) and capillary electrophoresis mass spectrometry [298].

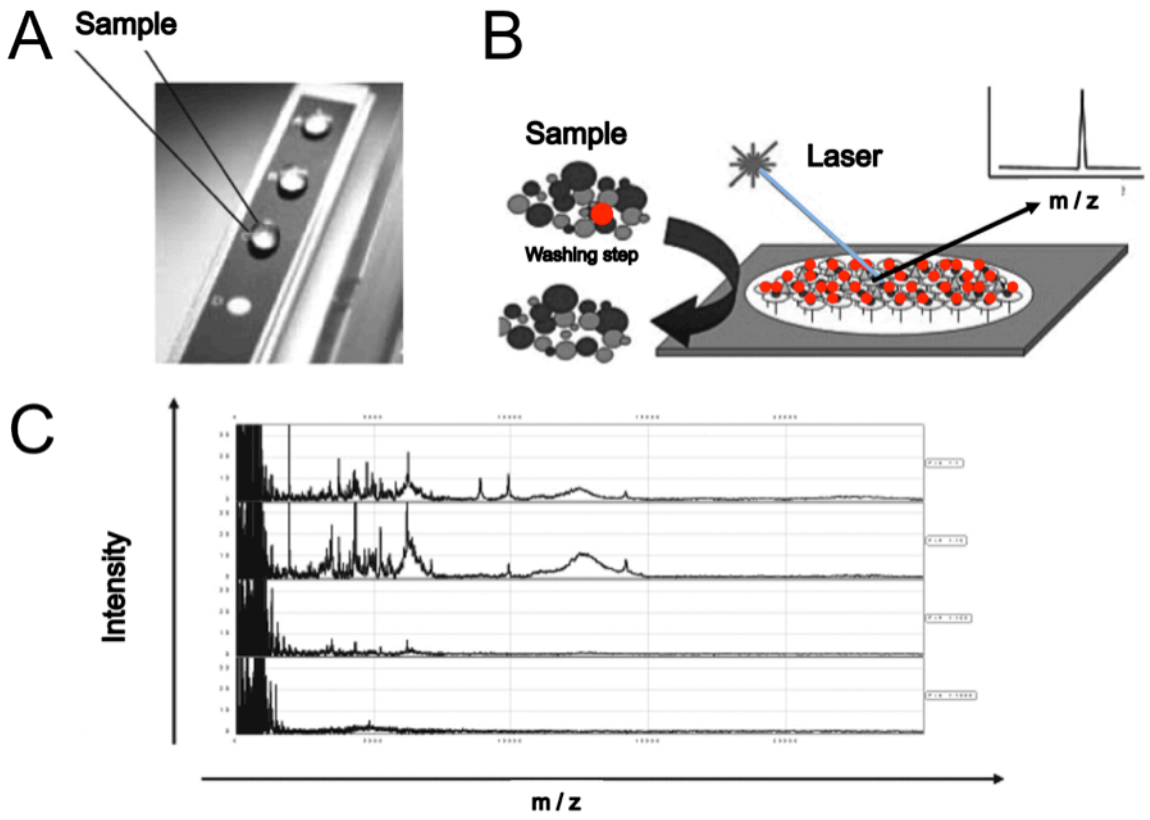


Figure 1.10 Surface-enhanced laser desorption ionization mass spectrometry (SELDI-MS). A sample is placed on a chip surface (A). After several washing steps (B), only a few proteins (red) stay bound to the surface; these are subsequently analysed using low-resolution MS. The laser application to the chip is illustrated in B. In C a typical SELDI-MS spectrum from urine is depicted. The figure is adapted from Fliser et al. [299].

The optimal approach to analyse the urine proteome would be a mass-spectrometry based platform with consecutive validation by ELISA and clinical application. The closest approaches for biomarker discovery to this optimal platform are SELDI-TOF and CE-MS, as they can be applied in biomarker discovery and validation as well as in clinical studies. Advantages and disadvantages of these techniques are summarised in Table 1.5, which also

includes liquid chromatography and 2-dimensional gel electrophoresis coupled to mass spectrometry.

Table 1.5 Advantages and disadvantages of different mass spectrometry-based proteomics methodologies for use in clinical proteomics applications.

Platform	Advantages	Disadvantages
SELDI-TOF	- Easy usage - High throughput - Automation - Small sample volume	- Restriction to selected proteins - Low resolution mass spectrometry - No comparability - Sensitive towards interfering compounds
Capillary electrophoresis mass spectrometry	- Automation - High sensitivity - High throughput - Small sample volume - Multidimensional	- Unsuitable for large molecules (>20 kDa)
Liquid chromatography mass spectrometry	- Automation - Multidimensional - High sensitivity - Large molecule (>20 kDa) detection - Biomarker sequencing	- Tryptic digestion - Time-consuming - Sensitive towards interfering compounds - Medium throughput
2-dimensional gel electrophoresis mass spectrometry	- Large molecule detection - Estimation of real molecular weight - Biomarker sequencing	- Unsuitable for small molecules (<10 kDa) - No automation - Time-consuming - Medium throughput - Moderate comparability

The table was adjusted from Decramer et al. [286]. SELDI-TOF, surface enhanced laser desorption/ionization time of flight.

The SELDI platform condenses the proteome complexity selectively. Proteins are adsorbed to different active surfaces: a hydrophilic matrix, a reverse-phase material or antibodies with a particular protein affinity. Then, the unbound proteome is removed by a washing step and a matrix, capable of energy absorption or sample laser vaporization and ionization, is added [299]. Subsequently, a urine sample undergoes mass spectrometry analyses, including time of flight analysis leading to SELDI-TOF, as illustrated in Figure 1.10. The

advantage of SELDI-TOF includes its ability to investigate a variety of samples in a short time and its simple usage [286]. Consequently, SELDI-TOF was applied in a variety of biomarker definition studies [300]. A disadvantage of the technique is its susceptibility to artifact generation [301]. The reasons for this are manifold and contributing factors and difficulties with calibration and lack of precision to determine the analytes' molecular masses. Also, only a tiny proteome fraction binds to the absorbing surface, diminishing the information content of the biological sample even if several chip surfaces are used. Furthermore, the extent of surface binding varies depending on sample concentration, pH, salt content and the presence of interfering compounds such as lipids. Another disadvantage of the SELDI-TOF technique is its lack of direct interfacing with mass spectrometry instruments for sequencing [286].

In capillary electrophoresis coupled to tandem mass spectrometry the proteome of a sample is separated in a single step. The corresponding apparatus is illustrated in Figure 1.11. The separation is based on the rate of protein migration through a gel matrix in an electric field. In comparison to other platforms its advantages are predominantly its fast separation step, of approximately one hour, and its high resolution [302]. It is also quite robust and uses inexpensive capillaries as opposed to expensive liquid chromatography columns [303]. Furthermore, most buffers and analytes are compatible with the method [253] and it establishes a constant flow avoiding elution gradients. Therefore elution gradients are no longer interfering with mass spectrometry [304]. Due to its speed, reliability and reproducibility, as well as its cost efficiency, capillary electrophoresis mass spectrometry is best suited for high throughput analysis of clinical samples [305].

The method is characterised by its high resolution and is therefore an ideal platform for analysis of complex biofluids that contain several thousands of different peptides and proteins. There is a degree of intra-individual variability of the urinary proteome, for example, depending on fluid intake and time of collection, so that the complex polypeptide patterns can only be interpreted when compared with data on the normal urinary proteome in healthy subjects. Hence, data become normalised in comparison to levels of polypeptide markers that are commonly found in urine based on the experience from analysis of thousands of samples in previous and ongoing studies.

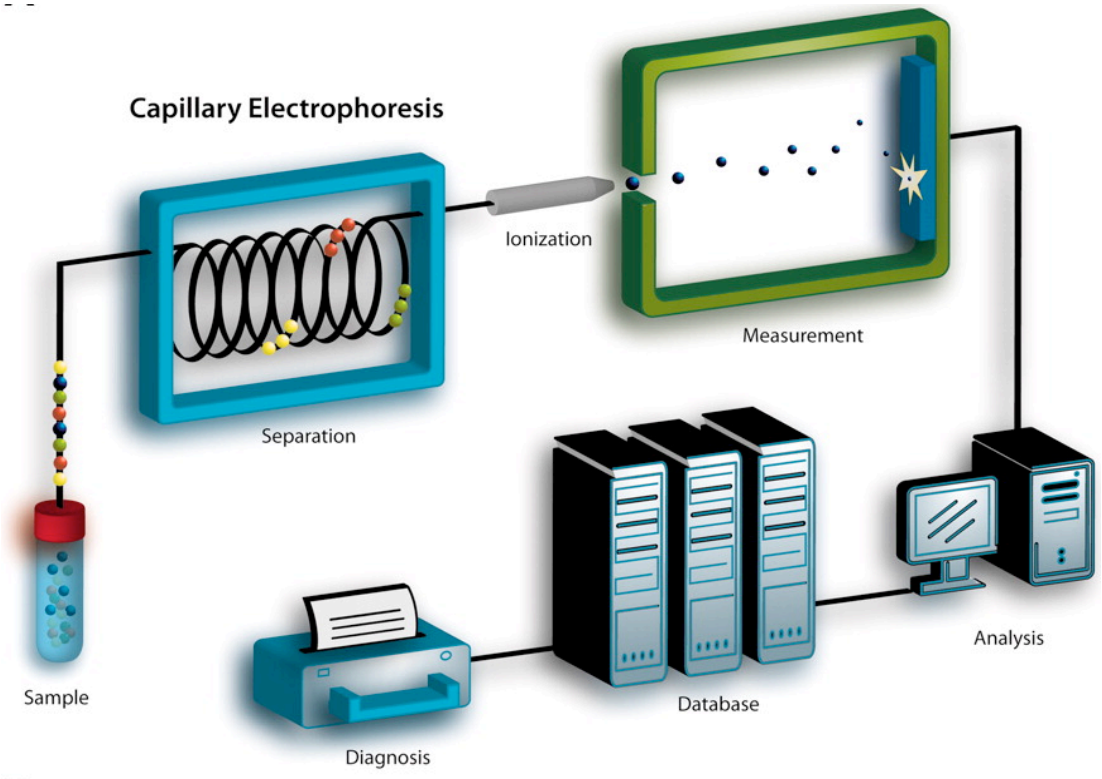


Figure 1.11 Illustration of the on-line coupling of capillary electrophoresis to the mass spectrometer (CE-MS). Urine polypeptides are elecrophoretically separated, ionized on-line by high voltage application and analysed in the mass spectrometer. This apparatus yields a mass spectrogram of mass per charge plotted against migration time. The acquired data is then interpreted automatically and allows detection of disease specific polypeptide patterns. The illustration is adapted from Fliser et al. [299].

A disadvantage of capillary electrophoresis, although not as extensive as in liquid chromatography, is that high molecular weight proteins cannot be easily analysed, as large proteins tend to precipitate at low pH levels that are generally present in running buffers. However, after digestion, protein fragments can be analyzed instead of the full-length protein. As mentioned before, the urinary proteome of individuals with normal renal physiology contains mostly low molecular weight proteins [285]. A further limitation of capillary electrophoresis is the relatively small sample volume that can be loaded onto a capillary, resulting in decreased detection sensitivity. Improvement of the electrophoresis to mass spectrometry, coupled to better ionization and protein delivery from the capillary end to the mass spectrometry instrument through nano-ion spray, makes this issue less relevant. Also, augmentation of the detection limit of mass spectrometers, enabling detection in the molar range, renders the issue of sensitivity less important [306]. Sequencing of potential biomarkers defined by this platform can be accomplished by interfacing capillary electrophoresis with tandem mass

spectrometry [307] or by subsequent targeted sequencing using liquid chromatography [308]. The latter approach is preferred as the low amount of sample volume loaded on a capillary limits the success rate of other methods.

Two-dimensional gel electrophoresis has been successfully applied to protein separation for more that thirty years and still remains popular. Historically, the development of mass spectrometry implied a major innovation in protein identification of gel spots. The first step is the proteolytic in-gel digestions by, for instance, exposure of an excised gel fragment to trypsin [309] with consecutive extraction of proteolytic fragments form the gel. At least three fragments are necessary to identify a protein from a protein database. If a match is found, it can later be verified by tandem mass spectrometry sequencing or by other techniques such as Western blotting. The major limitations of the two-dimensional gel electrophoresis are low reproducibility, extensive time requirements and difficulties to automate the process. A further feature of the technique is its limitation to proteins between 10 and 200 kD, leaving it the method of choice for analysis of medium-sized or large proteins in the discovery phase of biomarker definition.

Liquid chromatography is a very effective fractionation method and it is compatible with all versions of mass spectrometers. It is capable to process large amounts of analytes on a liquid chromatography column [310] and provides high sensitivity. The use of different media in two independent sequential separation steps enables multidimensional fractionation. Examples for this are the multidimensional protein identification technology [311] or two-dimensional liquid chromatography separation [312]. Limitations of the technique include its difficulty with comparative analysis, partially due to the variability in multidimensional separations, and the duration of a single samples analysis, which takes several days. Also, proteins larger than 10 kDa cannot be analyzed. To avoid this problem, analytes can be cleaved by a protease such as trypsin. The digestion step, however, increases the complexity of the resulting mixture and leads to an undersampling of tandem mass spectrometry and therefore an incomplete analytic coverage [313]. Another disadvantage is the interference of compounds like lipids and detergents with the separation process affecting the sensitivity of liquid chromatography. For instance, a comparison of two-dimensional liquid chromatography with fluorescence two-dimensional

difference gel electrophoresis by Komatsu et al. [314] showed that each technique identified predominately different proteins. Therefore, many techniques individually or in combination are useful in biomarker detection, however only a few seem suitable for comparative analysis of hundreds of samples and therefore in clinical diagnostics. As a consequence, capillary electrophoresis online coupled with mass spectrometry was used for this thesis.

Urinary proteomics, a multiple marker approach measuring a range of peptides and peptide fragments, has the potential to assess a multitude of pathways involved in the pathogenesis of complex diseases such as cardiovascular diseases. However, limitations of multiple marker strategies also apply to proteomics. If more than one protein/peptide is used to define a disease specific proteome pattern, there is a risk of overfeeding models with too many biomarkers relative to the sample size. As a consequence, a diagnostic model will not be translatable to other cohorts or the general population. Therefore, strict criteria for the use of proteomics as a clinical diagnostic tool should be observed [257]. A good characterization of the technical platform and precise measurements with the platform are mandatory. To reduce biological variability, urine collection and preparation requires standardization [298]. Furthermore, it is necessary to use suitable statistical methods in combination with a defined clinical hypothesis [290]. Although methods correcting for large false positive rates have been developed, univariate analyses are only a compromise for network or omics statistics. Multivariate analyses are better suited to data sets with a high quantity of variables and few observations. In multivariate analysis those variables reflecting on the major data set variance replace most of the others. Typical pitfalls for multivariate analyses are overfitting of the resulting model to the data and inclusion of strong outliers. The former can be avoided by using variables, which improve the prediction in an independent cohort. The latter has a strong influence on the whole model and therefore leads to a loss of transferability to other cohorts. Also, as a variety of the underlying hypotheses for statistical evaluation, such as even data distribution or data set comparability, are not granted, proteomic findings require validation using independent blinded sample set. This replication in clinical diagnostics should be carried out in at least one independent cohort with blinding of the investigator to the diagnosis of study participants.

1.8.7 Proteomics in cardiovascular disease

Proteomics of atherosclerosis can be performed on a wide range of biological specimens including whole tissue samples but also cells and biofluids [248]. For clinical purposes, easily accessible samples like urine or blood appear ideal, although they are only indirectly presenting the proteome of certain organs like the vasculature or myocardium. Furthermore, unlike genomic studies in which the extracted DNA is similar in a wide range of specimens, results of proteomic studies depend heavily on the specimen used.

Proteomic analyses of whole tissue samples have been carried out in the past. Examples for such tissues are carotid atheroma obtained from endarterectomy or coronary plaque from post mortem investigations. For instance, You et al. [315], collected human tissue from explanted hearts or from autopsies. By comparing affected with non-affected arteries they were able to identify ferritin light chain as a protein enriched in diseased coronaries. Messenger RNA levels were decreased in the pathologic tissue. Leppeda et al. [316] used endarterectomy tissue to examine protein differences in unstable and stable plaque. The authors used minced endarterectomy specimens to compare protein content of stable and unstable plaque by 2-dimensional gel electrophoresis. Protein content was extracted and analysed by matrix assisted laser desorption/ionization coupled to mass spectrometry. Unstable plaque contained smaller quantities of proteins, such as heat shock protein 20 and 27. On the other hand, proteins like fibrinogen fragment D was found in greater abundance in unstable plaque. Heat shock protein 27 is also strongly diminished in the supernatant of carotid atherosclerotic plaque when compared with normal carotid arteries, as shown by Martin-Ventura et al. by 2-dimensional electrophoresis [317].

Instead of directly assessing atherosclerotic tissues with the help of proteomics, it is also possible to investigate circulating inflammatory cells. This approach allows the researcher to inquire about processes involved in immune cell activation that contribute to atherosclerosis development. To this end, Barderas et al. [318] conducted a two-dimensional gel electrophoresis on the protein content of circulating monocytes in patients with acute coronary syndrome and compared them with stable CAD patients. The inflammatory cells of patients

with acute CAD contained higher levels of cathepsin D, heat shock protein 60 and 70 and protein S100A8. A further indirect approach to assess processes involved in CAD on proteome level was performed by Salgado-Somoza et al. [319]. The authors compared the proteome of epicardial adipose tissue with subcutaneous tissue in patients with CVD by two-dimensional gel electrophoresis. Samples were acquired in 55 patients either during cardiac artery bypass or valvular replacement surgery. Seven protein differences were identified and further investigated using matrix-assisted laser desorption/ionization coupled to mass spectrometry. The identified proteins were predominantly related to oxidative stress. For instance, antioxidant enzymes like catalase were less abundant in epicardial adipose tissue. A reduced ability to neutralise reactive oxygen species in the epicardium augments oxidative stress levels in close proximity to the coronary arteries and might therefore contribute to the development of CAD.

Expanding the idea of proteomic analysis of atherosclerosis supernatant as performed by Martin-Ventura et al. [317], the next logical step is assessment of plasma samples in patients with CAD. This was performed by Donahue et al. [320]. Using liquid chromatography coupled to electrospray ionization tandem mass spectrometry, they compared plasma samples of patients with angiographically confirmed CAD with disease free control subjects. The authors pooled samples obtained from 53 patients in each group for analysis, to allow detection of CAD relevant protein signals. Although this approach increases the risk for error, the authors were able to identify disease markers similar to the findings of other groups [321]. Examples of such proteins include: collagens, fibrinogen, α-2-antiplasmin, apo-lipoproteins, and complement factors. Dardé et al. [321] chose a different approach to analyse plasma samples in CAD. They compared patients with acute coronary syndrome with stable CAD patients and healthy controls using two-dimensional gel electrophoresis. This allowed identification of 33 proteins differentially expressed in acute coronary syndrome. These proteins could be classified into coagulation proteins, proteins participating in metabolism and/or lipid transport, inflammation and immune response and other proteins. Additionally, the authors performed longitudinal assessment of plasma proteins in the two diseased groups on the day of admission and days 4, 60 and 180 following admission. This showed significant

changes in plasma protein content in plasma samples obtained on follow-up appointments. This underlines the dynamic nature of the plasma proteome. The dynamics, in relation to CAD progression, was also investigated by Jing et al. [322]. The authors compared plasma samples taken at week six and twelve after birth of wild type and apolipoprotein E knock-out mice fed with a high fat diet. These samples were investigated with quantitative proteomics using liquid chromatography coupled to matrix-assisted laser desorption/ionization or electron spray ionization mass spectrometry. Similar to previously mentioned studies [320, 321], they identified fibrinogen fragments and apolipoproteins in the knock out model.

Although the listed research is mainly descriptive, the congruence with current pathophysiologic knowledge and the repeatability of its results shows the value of proteomics in atherosclerosis research.

1.8.8 Urine proteomics in cardiovascular disease

Urine proteomics analysis has been applied to the diagnosis of CAD. Zimmerli et al. [323] developed a urinary polypeptide pattern capable to differentiate between patients with severe CAD requiring coronary artery bypass surgery and healthy controls. At total of 88 patients with CAD and 32 subjects with no history of angina, CAD, or peripheral artery disease were recruited. To rule out centre specific bias and medication effects further healthy controls from another population (Hannover, Germany) and patients before and after treatment with ramipril (Nuernberg, Germany) were used to refine the proteome pattern. A total of 370 urine samples from 88 patients and 282 controls were analyzed. Eleven patients with CAD were excluded due to missing (n=2) and insufficient (n=9) urine samples. The urinary polypeptide content was measured with capillary electrophoresis coupled to mass spectrometry. A multiple biomarker pattern identifying CAD patients was defined. This consisted of 15 different peptides and for the purpose of this thesis is therefore named CAD_{15} score. The score was calculated by using a linear classifier algorithm to sum the product of a classification coefficient and the signal for each of the 15 peptides. By defining the classification threshold as a CAD score = 13, sensitivity and specificity for discrimination between presence and absence of disease was 98% and 83% in the training set, respectively. The 15 peptides were sequenced with the help of

capillary electrophoresis coupled to tandem mass spectrometry. In a second step the CAD score was evaluated in a different patient subgroup in a blinded manner. The CAD score had a sensitivity of 98% and a specificity of 83% for differentiation between CAD patients and healthy individuals in this cohort. This study was a successful application of urine proteomics to the development of CAD specific biomarkers.

1.9 Aims and objectives

A multitude of biomarkers exist and reflect CVD. These can be circulating, functional and derived from different imaging modalities. The aim of this PhD was the assessment of a biomarker variety in CVD. Such biomarkers can be used for screening, prediction of disease recurrence, therapeutic monitoring, diagnosis and prognostication. Whilst time constrains and limited resources excluded the former three in a PhD thesis, especially diagnosis and to some extent prognostication are approachable. Considering the phases of biomarker development the thesis covers biomarker discovery, proof of concept studies and a diagnostic validation study.

- One of the major CVD risk factors is hypertension. Hypertensive patients are therefore a good cohort to investigate early stages of the cardiovascular continuum. Hypertension affects especially the structure of the vasculature. Therefore functional vascular markers such as peripheral pulse pressure (pPP) play a prognostic role in hypertensive patients. pPP has however only prognostic value in the elderly. Central pulse pressure (cPP) on the other hand was shown to be superior to pPP in several studies. We therefore investigated if cPP could improve outcome prediction for CVD in younger patients, as recruited for the InGenious HyperCare study. As we were unable to do this in a prospective study, a different approach was selected. To decide if cPP might be a better CVD outcome predictor in comparison with peripheral pulse pressure, both were correlated with surrogate biomarker related to hypertension: left ventricular hypertrophy, carotid intima-media thickness, aortic pulse wave analysis and microalbuminuria.

- Processes like inflammation or myocardial tissue injury play a major role in the cardiovascular continuum. Both processes are represented by circulating

biomarkers. As these markers aid CVD prognosis we hypothesised that circulating biomarkers could support the diagnosis of CAD in patients with angina like symptoms. We therefore examined the discriminating capacity of several markers in two setting: comparison of extensive CAD with healthy controls and comparison of stable angina patients having either flow limiting CAD or normal coronary arteries as represented in the VASCAB and DiCADu study, respectively. As inflammation or myocardial injury is more prominent in severe CAD we first tested circulating biomarkers in the VASCAB cohort and adjusted the biomarker selection accordingly for the DiCADu study. This represents a proof of concept study for the diagnosis of CAD.

- Later stages of the cardiovascular continuum are represented by structural and functional changes of the vasculature. We therefore hypothesised that patients with flow limiting CAD can be characterised by such markers. To establish if non-invasive vascular phenotyping could aid the diagnosis in a clinical relevant setting the DiCADu study participants were investigated. The study compared patients with angina like symptoms and either flow limiting CAD or normal coronary arteries. Several micro- and macrovascular biomarkers were investigated, and we assessed if those markers could add diagnostic information to the results of exercise treadmill testing. This represents a proof of concept study for the diagnosis of CAD.

- Instead of investigating established CVD biomarkers in new diagnostic or prognostic settings, novel biomarkers can be developed. For this purpose we used an inductive, unbiased approach: discovery proteomics. In particular we conducted several steps of the development of a urinary polypeptide pattern for the diagnosis of CAD. Urine was the biofluid of choice as it has several advantages over other biofluids. First we tried to replicate results of a study previously conducted by our group in an independent cohort consisting of patients with established severe CAD in the VASCAB study. We then recalibrated the urine proteome pattern within a training set and tested its diagnostic value in a validation set. Finally, we assessed the diagnostic capacity of the resulting polypeptide pattern in patients with stable angina collected for the DiCADu study representing less extensive CAD. This represents a biomarker identification and diagnostic validation study.

Chapter 2 Material and Methods

2.1 Study cohorts

2.1.1 The VASCAB study

The VAScular function in Coronary Artery Bypass patients (VASCAB) study was a cross-sectional cohort study. The study aimed for the assessment of biomarkers derived from the peripheral vasculature and the collection of biologic samples in stable patients with severe CAD prior to a first bypass surgery. Furthermore, a healthy control cohort without evidence of CAD was collected for comparison with patients. Recruitment took place from October 2006 until October 2009. The study was approved by the West of Scotland Research Ethics Committee.

Patients with severe CAD were recruited from pre-operative cardiothoracic clinics in the Western Infirmary Glasgow. Patients were approached after their surgical assessment when inclusion criteria identified from medical records were fulfilled. These criteria are summarised in Table 2.1. If the patient was interested in study participation a questionnaire and an information sheet were provided, and a study visit in the clinical research facility of the British Heart Foundation (BHF) Glasgow Cardiovascular Research Centre was arranged on the day of admission prior to bypass surgery. Details of the questionnaire are provided in section 2.2. In total 126 patients were recruited.

Part of the control cohort consisted of patients undergoing varicose vein surgery. Patients on the vascular surgical ward in Garnavel General Hospital, Glasgow were checked for inclusion and exclusion criteria, as stated in Table 2.1, and approached on the day before surgery. Patients who were willing to participate were provided with a questionnaire and the possibility of a study visit at the BHF Glasgow Cardiovascular Research Centre was discussed. The visits were scheduled 1 to 4 weeks after the operation. In total 15 patients were recruited.

Table 2.1 In- and exclusion criteria of the VASCAB study.

Inclusion Criteria

- Written informed consent
- Adulthood
- Chronic stable chest pain (Cases)
- Referral for coronary artery bypass grafting (Cases)

Exclusion Criteria

- Unstable angina, acute coronary syndrome or myocardial infarction
- Kidney or other organ transplantation
- Heart failure stage D (AHA/ACC criteria)
- Any malignant concomitant disease or history of malignant disease within the last five years
- Systemic inflammatory disease, such as autoimmune disease, connective tissue diseases, collagenosis, Crohn's disease.
- Treatment with oral steroids or any other immunosuppressive drug
- Severe known liver disease (ALT or gamma-GT above three-fold of upper normal limit)
- Proteomic analysis will not be possible in patients with clinical or laboratory signs of acute infection, especially urinary tract infection, at the time of the study visit.

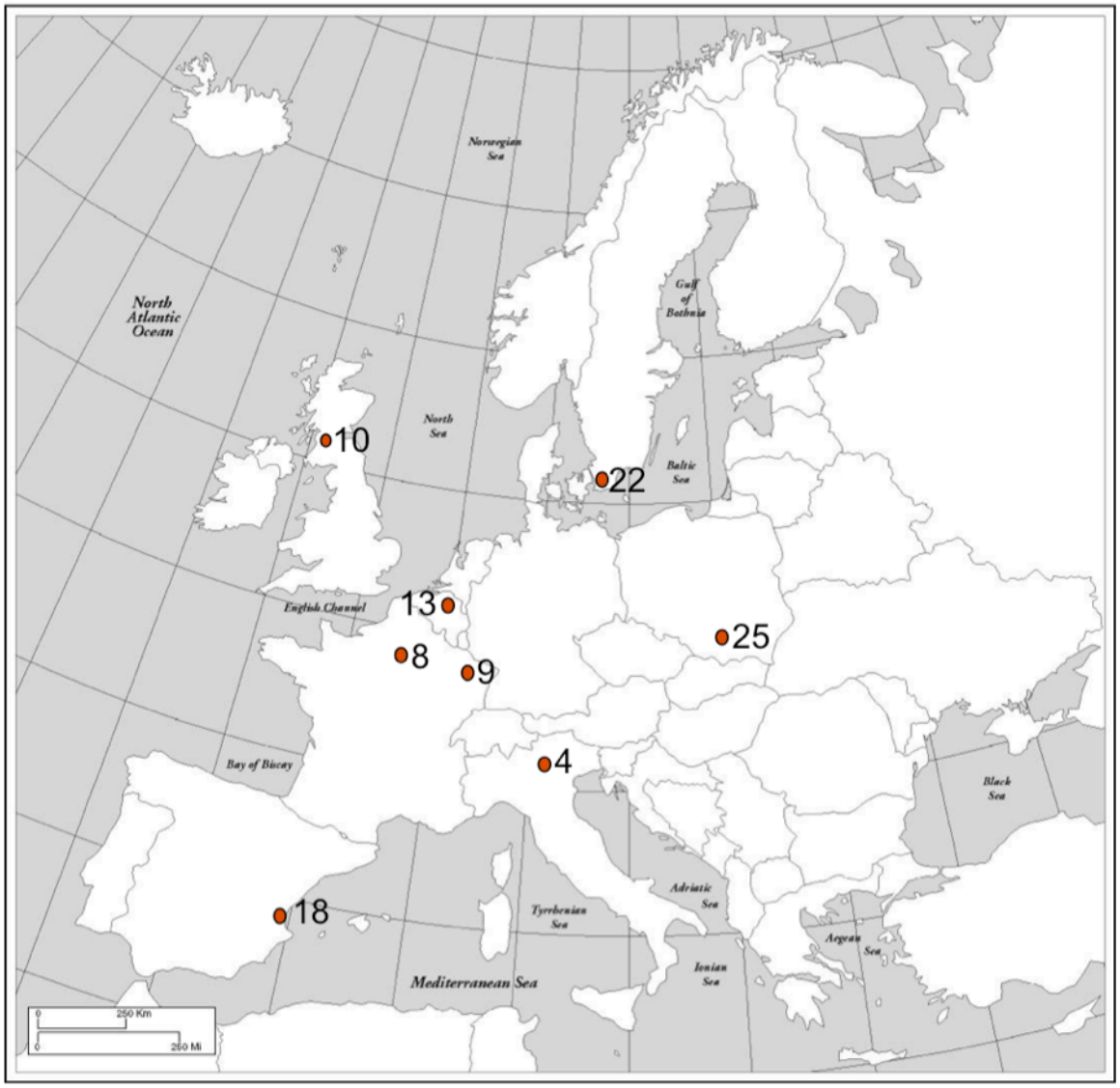
The second part of the control cohort consisted of healthy volunteers either approached in local fitness clubs or via advertisement on the University of Glasgow website. Information sheets and questionnaires were provided and study visits in the BHF Glasgow Cardiovascular Research Centre were arranged. In total 64 participants were recruited.

For the purpose of this thesis, the VASCAB cohort was used for two tasks. Considering the severity and extent of CAD in the VASCAB study blood samples were used to screen for circulating biomarkers capable to differentiate between cases and controls in chapter 4. Considering the chronic progressive nature of the cardiovascular continuum we hypothesised that circulating biomarkers would be more deranged in patients with severe three vessel CAD in comparison to earlier disease stages. With the same rationale VASCAB study urine samples were used to first revalidated and then recalibrate a urine proteome pattern specific for CAD in chapter 6.

2.1.2 The InGenious HyperCare family cohort

The Integrated Genomics, Clinical Research and Care in Hypertension (InGenious HyperCare) study is an European multicentre family-based genome wide association study investigating families with high prevalence of hypertension. The study is part of a Network of Excellence funded by the European Union. Participating centres, as relevant for this thesis, are depicted and listed in Figure 2.1. The study centre Glasgow was one of 19 centres who recruited families into the study. Cardiovascular phenotyping and sample collections, as relevant for this thesis, are summarised in Table 2.2. Local research ethics committees approved the study. The West of Scotland Research Ethics Committee was responsible in Glasgow. The complete data set as available in the centralised database in august 2010 was used for this thesis. Access to the data was granted by the coordinator of the InGenious HyperCare Network of Excellence, Prof Alberto Zanchetti.

In Glasgow recruitment of families started with the identification of index patients. Index patients were approached in hypertension clinics at the Western Infirmary Glasgow from February 2008 to February 2010 and at Stobhill Hospital, Glasgow from August 2009 to February 2010. Index patients were identified by assessment of medical records and according to in- and exclusion criteria (Table 2.2). Appropriate patients were approached and interviewed in regards of in- and exclusion criteria as well as family structure and availability of family members for study enrolment. As summarised in Table 2.2, inclusion criteria for index patients were diagnosis of hypertension before the age of 50 years and current blood pressure ≥ 160/95 mmHg on two occasions or treatment with at least two antihypertensive drugs. To qualify a family for study inclusion a further family member with blood pressure ≥ 140/90 mmHg on two occasions or treatment with at least one antihypertensive drug and identical age criteria was required. The minimum family size was four, and normotension was defined as blood pressure < 140/90 mmHg. Study participants on treatment for hypertension were graded as hypertensive. Members of at least two generations had to participate per family. Examples of eligible family structures are depicted in Figure 2.2. The primary aim of the InGenious HyperCare study was a genome wide association study in hypertension.



No	Participating Centre	Country
4	Universita degli Studi di Brescia	I
8	Institut National de la Sante et de la Recherche Medical, Paris	F
9	Centre Hospitalier Universitaire de Nancy	F
10	University of Glasgow	UK
13	Katholieke Universiteit Leuven	B
18	Fundacion para la Investigacion Biomedica L Docencia La Cooperation Int., Valencia	E
22	Lunds Universitet	SE
25	Jagiellonian University Med. College, Kraków	PL

Figure 2.1 InGenious HyperCare Study Centres in Europe. Numbers represent study centres as listed in the table section. Visualised are only study centres relevant for this thesis.

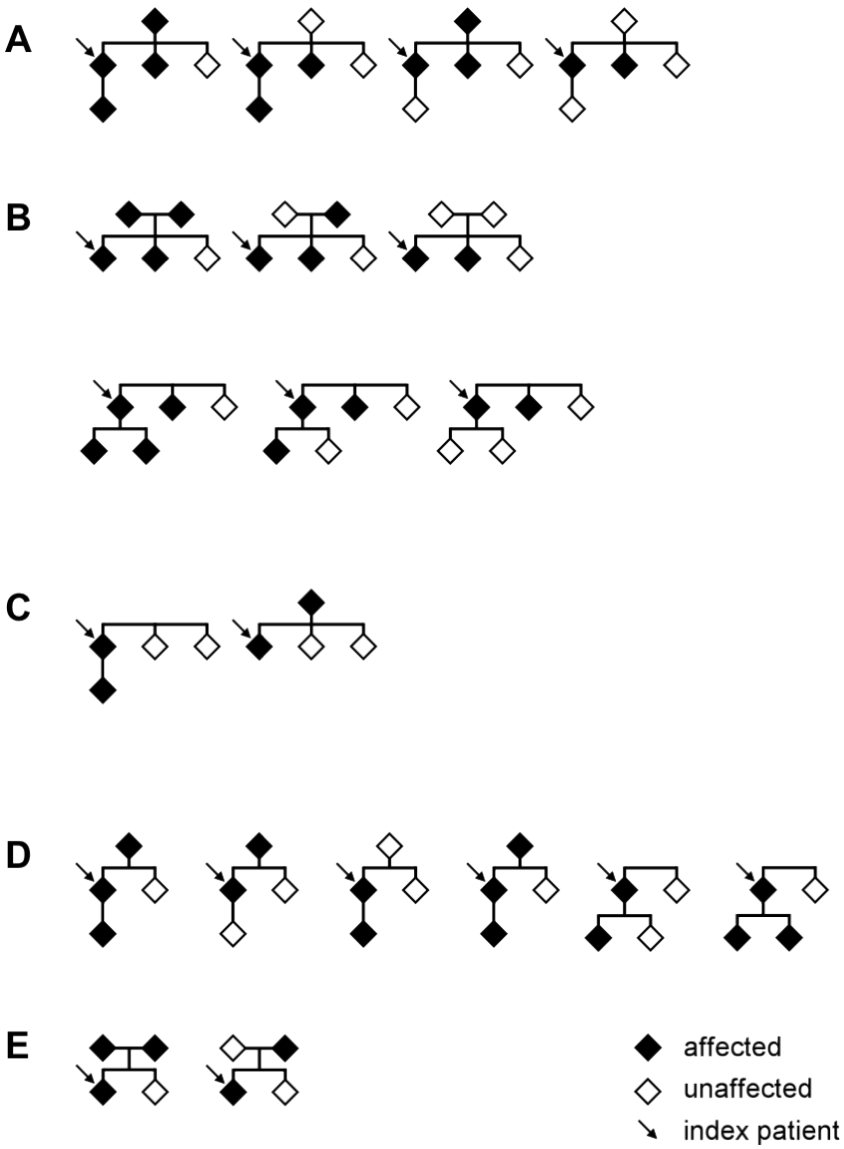


Figure 2.2 Family structures for the InGenious HyperCare study. Examples of possible family size and structure for involvement in the InGenious HyperCare study are depicted. Five or four members over three generations (A and D) and five or four members in two generations (B, C and E) are possible.

Information material for index patients and family members was provided to index patients during the first encounter. Individuals were asked to discuss study participation with relatives, and contact details of relatives were collected. After a minimum of seven days index patients were phoned, and if a sufficient number of family members agreed to participate individual or paired study appointments were arranged. Study visits were either carried out in the BHF Glasgow Cardiovascular Research Centre or the Glasgow Clinical Research Facility in the Tennent Institute. In Glasgow 42 families, including 195 participants were recruited. Two families did not complete their study visits. All participants gave informed consent. Biomarkers assessed in the InGenious

HyperCare study are summarised in Table 2.2. I recruited all study participants and performed all clinical investigations.

Table 2.2 In- and exclusion criteria of the InGenious HyperCare study.

	Index patient	Affected family member	Unaffected family member
Inclusion Criteria			
≥ 18 years	X	X	X
≤ 60 year at study enrollment	X		
Diagnosis of essential hypertension < 50 years	X		
SBP ≥ 160 or DBP ≥ 95 mmHg if untreated or ≥ 2 antihypertensive drugs	X		
SBP ≥ 140 or DBP ≥ 90 mmHg if untreated or ≥ 1 antihypertensive drugs		X	
≥ 3 participating first degree relatives with ≥ 1 affected before age of 50 years and ≥ 1 from a different generation	X		
written informed consent	X	X	X
Exclusion Criteria			
Secondary Hypertension	X	X	
Previous clinical complications of hypertension as angina, MI, stroke, TIA, peripheral artery disease	X		
Renal disease	X		
Kidney or other organ transplantation	X	X	X
Type 1 diabetes mellitus	X		
Heart failure stage D (AHA/ACC criteria)	X		
Malignant concomitant diseases or history of malignant diseases within the last five years	X	X	X
Clinical or laboratory signs of acute infection	X	X	X
Systemic inflammatory diseases	X	X	X
Steroids or any other immunosuppressive drug	X		
Severe liver disease	X	X	X
Known pregnancy	X	X	X
Current alcohol consume of more than 21 drinks/week	X	X	X

The terms affected and unaffected family members refer to the diagnosis of essential hypertension.

For the purpose of this thesis, the InGenious HyperCare cohort was used to investigate central pulse pressure for its prognostic value, as described in chapter 4. In line with in- and exclusion criteria the study cohort covered earlier stages of the cardiovascular continuum as illustrated in Figure 2.4.

2.1.3 The DiCADu study

The Diagnosis of Coronary Artery Disease with Urine proteomics (DiCADu) study was a cohort study in patients who underwent elective coronary angiography in the Golden Jubilee National Hospital (GJNH) in Clydebank.

The primary aim was to validate a urinary proteome pattern for diagnosis of CAD in patients with stable chest pain with and without significant CAD. The secondary aim was the testing of other emerging biomarkers in the same clinical setting. Study visits were carried out in the BHF Glasgow Cardiovascular Research Centre or the Glasgow Clinical Research Facility. The study was approved by the West of Scotland research ethics committee and conducted as summarised in Figure 2.3.

Patients who were admitted to the GJNH for assessment of angina like chest pain by elective coronary angiography between January 2009 and June 2010 were screened for eligibility to participate in the study. Patients were eligible to participate if either normal coronary arteries or significant CAD was diagnosed on coronary angiography. Normal arteries were defined as the absence of artery narrowing whereas significant CAD was present if at least one stenosis was >75%. Patients were in- or excluded based on criteria displayed in Table 2.3.

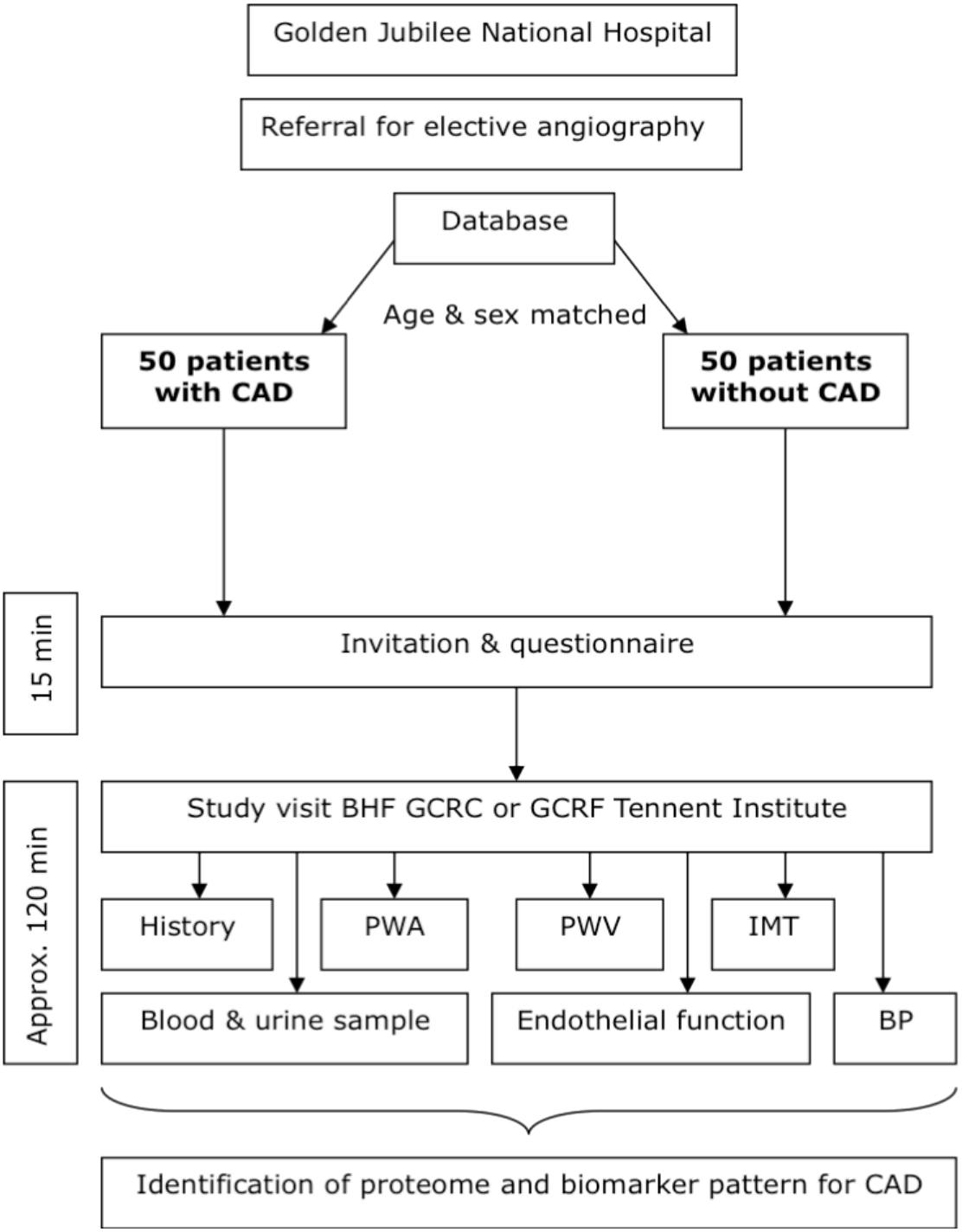


Figure 2.3 Flowchart of the DiCADu study. CAD, coronary artery disease; BHF GCRC, British Heart Foundation Glasgow Cardiovascular Research Centre; GCRF, Glasgow Clinical Research Facility; PWA, pulse wave analysis; PWV, pulse wave velocity; IMT, intima-media thickness; BP, blood pressure.

Table 2.3 In- and exclusion criteria for the DiCADu study.

Inclusion Criteria

- Written informed consent
- Adulthood
- Chronic stable chest pain prior to angiography (≥6 weeks)
- Referral for elective coronary angiography

Exclusion Criteria

- History of established CAD
- Unstable angina, acute coronary syndrome or myocardial infarction
- Kidney or other organ transplantation
- Heart failure stage D (AHA/ACC criteria)
- Any malignant concomitant disease or history of malignant disease within the last five years
- Systemic inflammatory disease, such as autoimmune disease, connective tissue diseases, collagenosis, Crohn's disease.
- Treatment with oral steroids or any other immunosuppressive drug
- Severe known liver disease (ALT or gamma-GT above three-fold of upper normal limit)
- Proteomic analysis will not be possible in patients with clinical or laboratory signs of acute infection, especially urinary tract infection, at the time of the study visit.

Patients were invited to the DiCADu study by letter if three factors were given: medical records did not disclose any of the exclusion criteria listed in Table 2.3, a contact telephone number was available, and the distance between the home address and research centre was acceptable. Study information material was provided with the invitation letter. After a minimum of 7 days patients were called and an appointment was scheduled in case of interest. Then participants received a formal confirmation, a questionnaire and a consent form by mail. Questionnaires were either completed before or at the beginning of the study visit. Study visits took place between February 2010 and January 2011. Patients undergoing angiography in 2010 were selected in smaller number to improve age and sex matching of participants admitted to the GJNH between January 2009 and December 2009. The average age of recruited cases and controls and their gender distribution was assessed. Patients, admitted to the GJNH between January 2010 and June 2010, were selected to balance existing discrepancies. Especially older and female cases as well as younger and male controls were required. In total 93 out of 260 invited patients participated.

Participants underwent a standardised assessment during study visits including the biomarkers summarised in Table 2.5. Exercise tolerance test and myocardial perfusion scan results prior to coronary angiography were extracted from medical records.

For the purpose of this thesis, the DiCADu cohort was used for two different tasks. The first task was to test a urine polypeptide patterns established with the help of the VASCAB cohort. The study cohort was recruited for this purpose. A clinical scenario was chosen which would benefit from additional diagnostic biomarkers, as discussed in detail in chapter 6. The second task was to test a multiple biomarker approach for the diagnosis of CAD. For this purpose vascular phenotyping, as described in chapter 5, and measurement of circulating biomarkers, as described in chapter 4, were carried out. In addition to test each biomarker group individually this also allowed multiple biomarker analysis is described in chapter 5 and 6. To avoid the risk of overfeeding models with too many biomarkers relative to the sample size only biomarkers capable of differentiating between cases and controls were considered.

2.1.4 Connection between different study cohorts

The three studies InGenious HyperCare, DiCADu and VASCAB cover populations at different stages of the cardiovascular continuum as depicted in Figure 2.4. The cases of the three studies cover almost the whole spectrum of the cardiovascular continuum preceeding the onset of severe tissue injury, such as myocardial infarction or heart failure, especially as tissue injuries are exclusion criteria in all three studies. Although the study populations overlap and the study recruitment criteria are focusing on specific criteria, such as familiar hypertension, angina like chest pain or CAD requiring coronary artery bypass grafting, the studies provide the unique opportunity to compare biomakers between the different stages of the cardiovascular continuum or to correlate biomakers with CAD extent. This is possible as several biomarkers were investigated in all three studies, allowing analysis of pooled data. Such investigations will be carried out in particular in chapter 4 and 6.

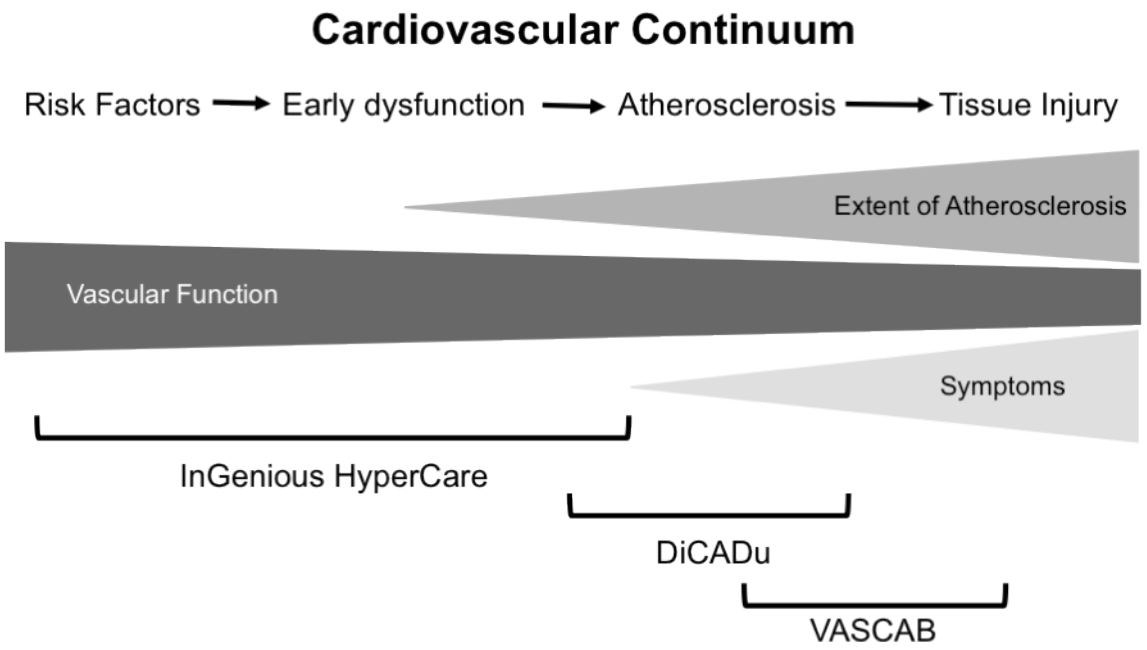


Figure 2.4 The relation of study populations to the cardiovascular continuum. Illustrated are the different stages of the cardiovascular continuum in relation to vascular function, atherosclerosis extent and associated symptoms. The studies InGenious HyperCare, DiCADu (cases) and VASCAB (cases) contributing to the thesis are linked to these process and the different stages of the cardiovascular continuum.

2.1.5 Recruitment and biomarker assessment

I contributed to the three studies to different extent. To clarify this further, each study will be discussed subsequently and is summarized in Table 2.4.

Table 2.4 The author's contribution to studies covered in the PhD thesis.

	VASCAB	InGenious HyperCARE	DiCADu
Study design	-	-	100%
Ethics Application	-	-	100%
Study participant recruitment	15%	100%*	100%
Study participant screening/investigations	15%‡	100%*	100%‡
Data analysis presented in thesis	100%‡	100%	100%‡

The table provides a summary of the authors contribution to the VASCAB, InGenious HyperCare and DiCADu thesis. *For InGenious HyperCare this represents only patients recruited in Glasgow. ‡ Urine proteome analysis and calculation of related biomarker were carried out by collaborators at mosaiques diagnostics GmbH, Hannover, Germany.

The VASCAB study was initiated before the start of this PhD thesis. The main part of the VASCAB study recruitment was carried out by Dr Jane Dymott as part of her MD thesis. Approximately 15% of the overall study recruitment and patient screening were carried out by myself. This involved predominantely healthy controls. Biomarkers investigated for the thesis are summarized in Table 2.5. All listed biomarkers were investigated by myself in 15% of the whole study cohort with the exception of standard routine biochemistry measurements and urine proteomics. Additionally I performed several analyses in the whole VASCAB study cohort for this thesis: batch analysis of serum samples and offline analysis of carotid ultrasound pictures. I organized casenote access to analyse CAD extent in VASCAB cases. Data analysis in this thesis involving the VASCAB cohort with the exception of urine polypeptide pattern calculations were performed by myself.

Ethics approval for the InGenious HyperCare study was already obtained when my PhD started. Consequently study design and protocol were already in place. However the complete recruitment of study participants for the Glasgow study site was carried out by myself. This required screening of approximately 2000 hypertension clinic appointments. Study visits including investigations listed in Table 2.5 were carried out by myself with the exception of standard laboratory urine and blood sample assessment. Data analysis as reported in this thesis was also performed by myself. At first I defined a hypothesis which could be investigated with the InGenious HyperCare cohort and analysed the Glasgow study centre data in this regard. On the basis of these pilot data access to the whole study cohort was granted by the coordinator of the InGenious HyperCare Network of Excellence, Professor Alberto Zanchetti. In the whole InGenious HyperCare study cohort results were consistent with pilot data and are presented in chapter 3.

Table 2.5 Overview of phenotypes that were measured in the VASCAB, InGenious HyperCare and DiCADu study.

Studies	VASCAB	InGenious HyperCare	DiCADu
History	x	x	x
Physical examination		x	
Body mass index and Waist-hip ratio	x	x	x
Resting blood pressure	x	x	x
Electrocardiogram	x	x	x
Echocardiography		x	
Pulse wave analysis and velocity	x	x	x
Carotid Intima-Media Thickness	x	x	x
Carotid distensibility			x
Endothelial function assessment			x
Standard haematology and biochemistry	x	x	x
Urinary albumin excretion	x	x	x
Urine samples for proteomics	x		x
Circulating biomarkers of inflammation, and heart function	x		x

The DiCADu study, constituting the largest part of this thesis with result chapter 4, 5 and 6, was designed by myself. I wrote the ethics application and obtained ethics approval. The complete patient recruitment and investigations as summarized in Table 2.5 with the exception of urine proteomic analysis and serum sample analyses were carried out by myself. I organized database access

to quantify CAD extent and casenote access to analyse exercise treatmill tests. I carried out data analysis as presented in this thesis with the exception of urine polypeptid pattern calculations.

2.2 Questionnaires

The VASCAB and InGenious HyperCare study questionnaires are identical. They covered a variety of clinical and demographic information: family status, family history of CVD, nutrition, smoking, alcohol consumption, cardiovascular risk factors, past medical history, menopausal status, history of hormone replacement therapy in women, diabetic organ damage and current medication. In addition, the DiCADu study questionnaire contained a chest pain section adapted from the Seattle Angina Questionnaire [324] as well as an extended menstrual status and hormone replacement therapy section. All three questionnaires are available in the appendix of this thesis.

2.3 Anthropometric data

Weight was measured on a standard electric scale. Height was measured in subjects standing without shoes. Body surface area (BSA) was calculated from height and weight according to the Dubois & Dubois formula [325].

$$BSA_{\text{Dubois \& Dubois}}\ [m^2] = 0.007184 * \text{weight}\ [kg]^{0.425} * \text{height}\ [cm]^{0.725}$$

Body mass index (BMI) was calculated according to BMI $[kg/m^2]$ = weight [kg] / (height $[m])^2$. Waist and hip measurements were taken with subjects wearing light clothes. Waist circumference was defined as the smallest circumference around the abdomen in a relaxed participant, usually at the level of the umbilicus. Hip circumference was defined as the largest measured circumference at the levels of the buttocks. Waist to hip ratio was defined as the quotient of these measurements. For the DiCADu study bioimpedance was measured in parallel to measuring body weight with the Tanita Body Composition Analyser BC-418 MA (Tanita Corporation, Arlington Heights, USA).

2.4 Blood pressure

Participants had refrained from smoking, eating and drinking alcohol or caffeinated beverages for at least 2 hours prior to the examinations and rested in a sitting position for 5 minutes in a quiet room before measurements were obtained. Brachial blood pressure and heart rate were measured in 1-minute intervals with an automated digital oscillometric sphygmomanometer (Omron, Model 705IT; Omron Corporation, Shimogyo-ku, Kyoto, Japan). For each arm the mean of the 2nd and 3rd reading was calculated and the higher value of both arms was used for further analysis. For measurement of blood pressure in supine position participants rested for at least 15 minutes, and the arm with higher sitting blood pressure was selected.

2.5 Electrocardiogram

In all studies 12-lead electrocardiograms (ECG) were acquired with participants being in supine position. Measurements were performed with a Burdick® Atria® 6100 ECG (Cardiac Science, Bothell, USA) and recordings were standardised at 25 mm/sec and 1 mV/cm. Tracings covered 2.5 seconds in each chest and limb lead and a rhythm strip was recorded for 10 seconds in the 2nd limb lead. Electrocardiograms were analysed for criteria of left ventricular hypertrophy after exclusion of cardiac conduction abnormalities. The Sokolow-Lyon, Lewis and Cornell Voltage indices were calculated as illustrated in Figure 2.5.

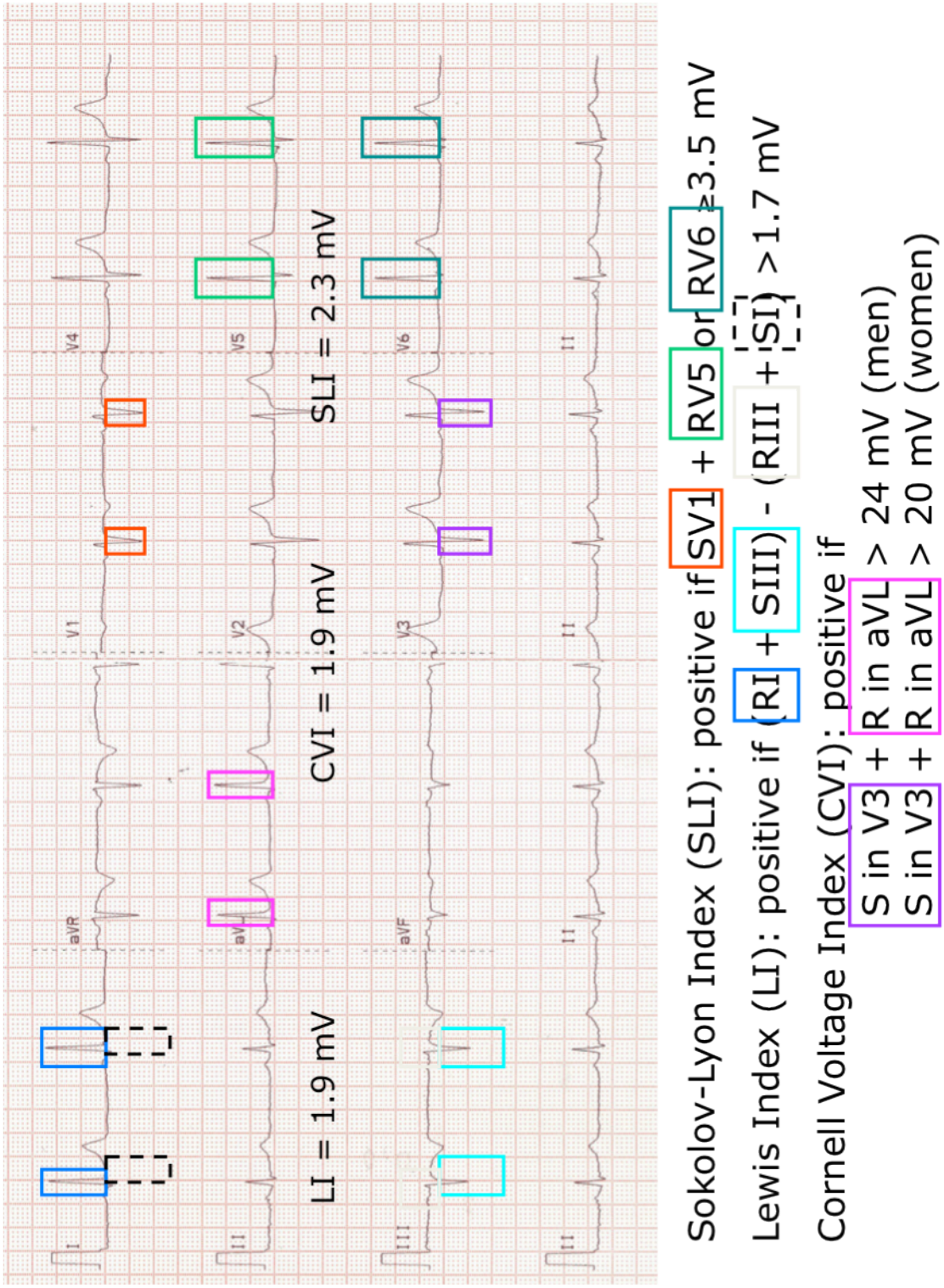


Figure 2.5 Standardised ECG and left ventricular hypertrophy indices. The formulas for the Sokolow-Lyon index, the Lewis index and the Cornell Voltage index are linked to the corresponding ECG waves by colour coding. The term 'positive' is used to highlight the cut-off-points for left ventricular hypertrophy. The original recording is from the male InGenious HyperCare participant A2100473204.

2.6 Biomarkers of vasculature function and structure

2.6.1 Carotid Intima-Media Thickness

Measurement of C-IMT was performed by ultrasonography (Acuson Sequoia C512, Siemens, Erlangen, Germany) with an 8 MHz linear-array transducer. Left and right common carotid arteries were examined in anterolateral (AL), posterlateral (PL) and mediolateral (ML) directions. C-IMT was measured in the far wall, 1 cm proximal of the carotid bulb in a plaque free region in accordance with the Mannheim consensus [326]. ECG signals were stored simultaneously to define the systolic and diastolic phase of the cardiac cycle. Offline measurements were performed semi-automatically at end diastole on B-mode images using Image-Pro Plus software, version 3.0 (Media Cybernetics, Bethesda, USA). Depending on image quality up to six different measurements corresponding to the AL, ML and PL recording directions on both common carotid arteries were used to average the C-IMT, as illustrated in Figure 2.6.

2.6.2 Assessment of carotid plaque

Offline B-mode common carotid artery images in AL, ML and PL direction were assessed for plaque presence and extent. Corresponding to the Mannheim carotid IMT consensus [326] plaque was defined as a "focal structure that encroaches into the arterial lumen of at least 0.5 mm or 50% of the surrounding C-IMT value or demonstrates a thickness > 1.5 mm as measured from the media-adventitia interface to the intima-lumen interface". As published by Hollander et al. [193] and van der Meer et al. [327] the plaque score had values from 0 to 6. The number of sites with a detected plaque (left- and right-sided common carotid artery, bifurcation and internal carotid artery) is divided by the total number of sites with an available ultrasonographic image and then multiplied by six. Participants of the DiCADu study were not assessed when less then two sites were available. The corresponding carotid plaque score is illustrated in Figure 2.7.

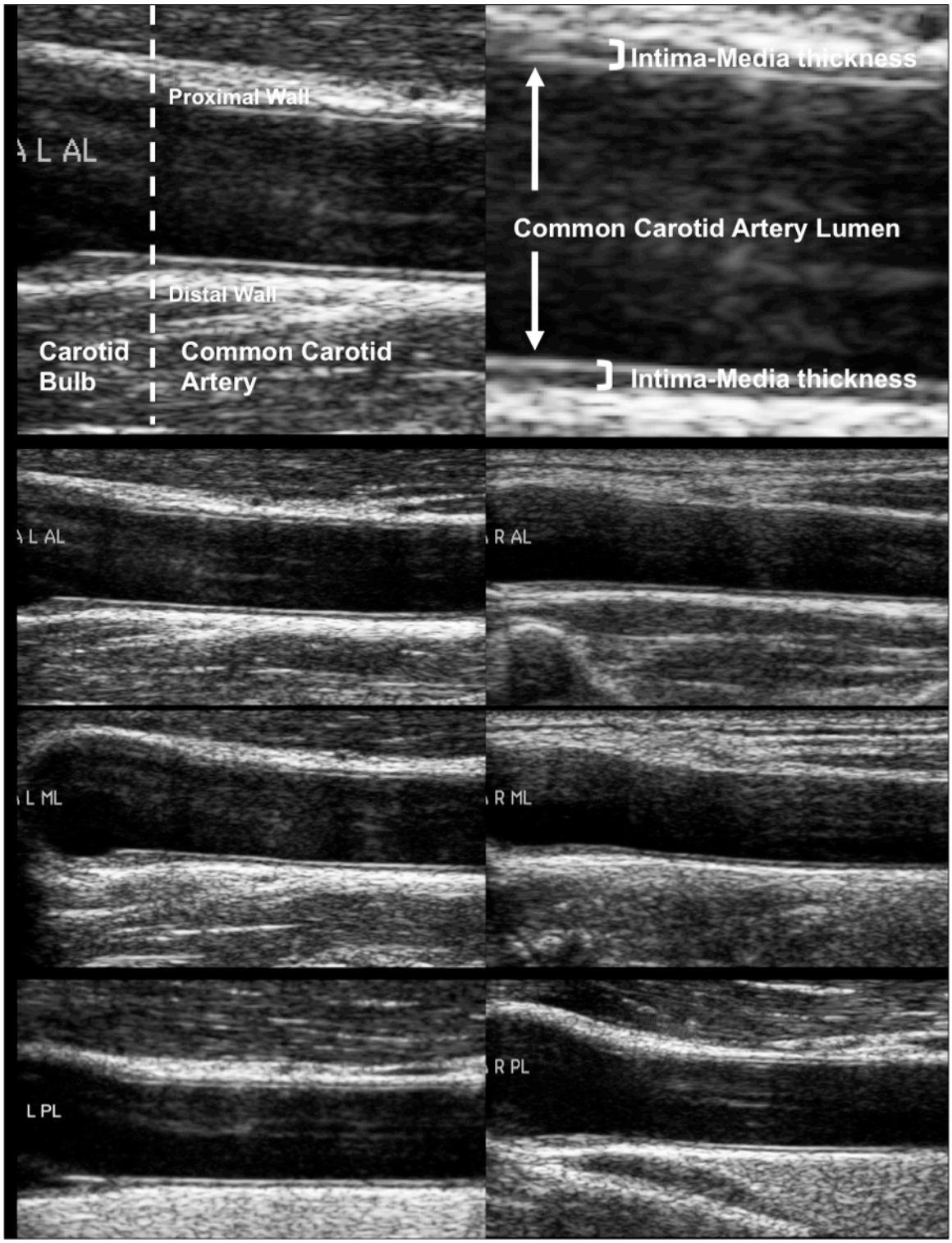
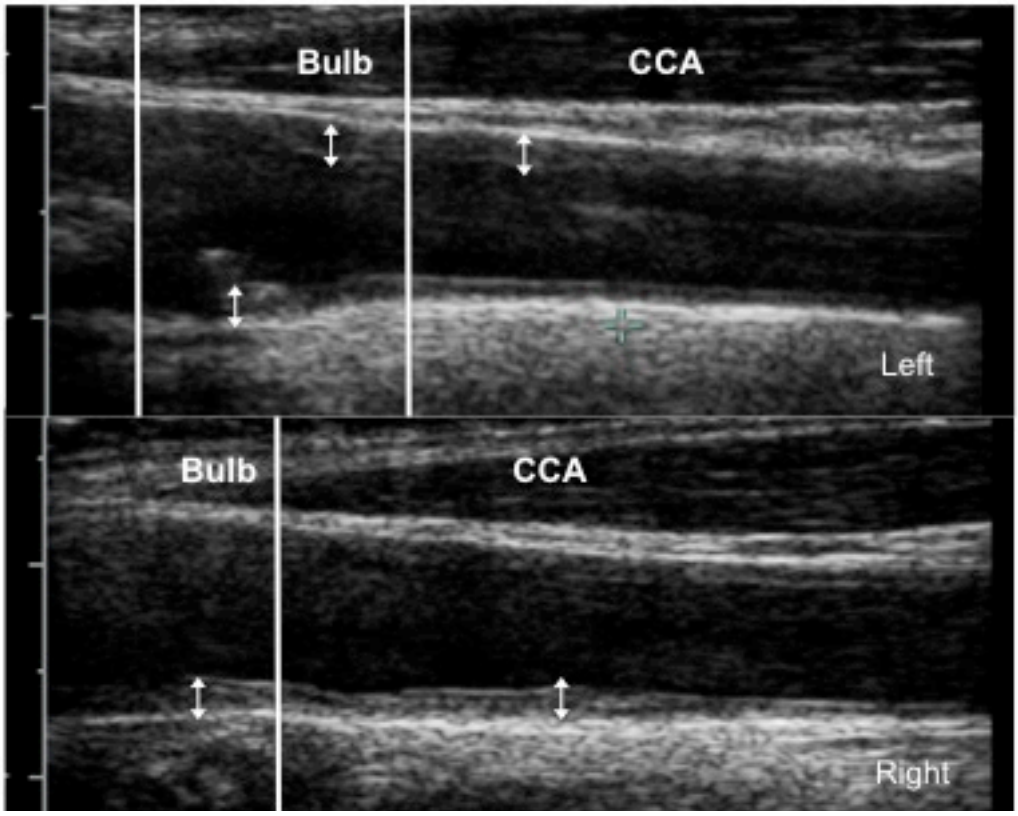


Figure 2.6 B-mode pictures of the carotid artery. Pictures were taken in anterolateral (AL), mediolateral (ML) and posterlateral (PL) direction at either the right (R) or the left (L) carotid artery. The original recording is from the InGenious HyperCare participant A2100483102.

A



B

Mannheim Consensus Plaque Definition:

1) Focal structure that encroaches
a) into the arterial lumen of at least 0.5 mm or
b) 50% of the surrounding IMT value or
2) Focal structure that demonstrates a thickness > 1.5 mm as measured from the media-adventitia interface to the intima-lumen interface

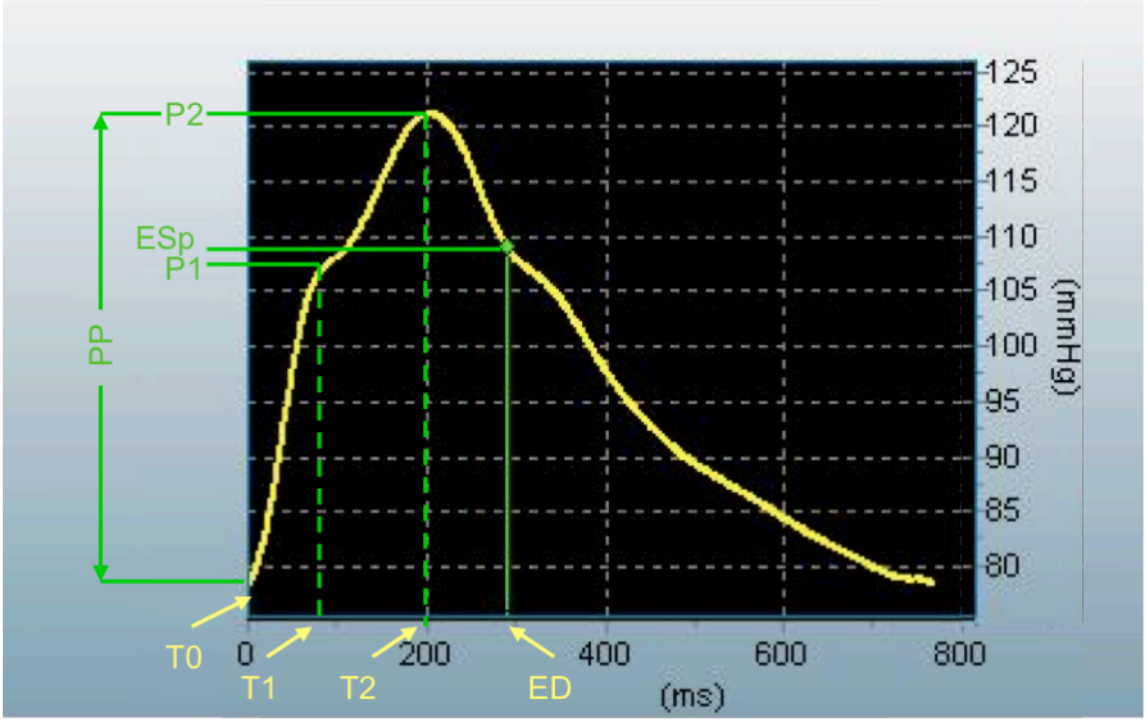
C

$$Carotid\ Plaque\ Score = \frac{Number\ of\ affected\ vessel\ segments \times 6}{Number\ of\ visualized\ vessels\ segments}$$

Figure 2.7 Illustration of the carotid plaque score. Areas with plaque burden are highlighted with arrows in B-mode common carotid artery images (A). The carotid plaque definition according to the Mannheim carotid IMT consensus is listed (B). The carotid plaque score equation is shown in C. Regarding the carotid pictures in panel A this leads to a carotid plaque score of 4.8 (number of affected vessel segments: 4, number of visualised vessel segments: 5). Depicted ultrasound images derive from DiCADU participant 1-0016-B.

2.6.3 Pulse wave analysis

For pulse wave analysis (PWA) participants were rested for 20 minutes in supine position at a temperature 23-24 °C. Radial arterial pressure waveforms were recorded with a Millar piezo-resistive pressure transducer (Millar SPT 301, Millar Instruments, Houston, US) coupled to a SphygmoCor device (AtCor Medical, Sydney, Australia). The corresponding central (ascending aortic) waveform was generated by the SphygmoCor software (version 7.0) using a generalised and validated [328] transfer function. Supine peripheral blood pressure measurements were used for calibration.



$$AIx\ (AG/PP) = \frac{P_2 - P_1}{SBP - P(T_0)} \times 100$$

Figure 2.8 Pulse wave analysis. Depicted are an aortic pressure curve as calculated by the SphygmoCor device and relevant parameters for calculation of the augmentation index (AIx). ESp, endsystolic pressure; ED, ejection duration; PP, pulse pressure; AG, augmentation; SBP, systolic blood pressure. The original tracing is from the InGenious HyperCare participant A2100211004.

The central AIx was calculated from the aortic pressure waveforms, as shown in Figure 2.8. The AIx was always adjusted for the heart rate, as the heart rate has a strong effect on the index [329]. Aortic SBP and DBP were derived from the aortic pressure waveform under the assumption of a constant mean arterial pressure. The mean arterial pressure was calculated with the integration of the radial waveform. Only measurements of good quality, defined as an operator index ≥80 determined by the software of the SphygmoCor device were used for PWA. cPP was defined as the difference between aortic SBP and DBP. Peripheral pulse pressure (pPP) was defined as the difference between brachial SBP and DBP in supine position. The pulse pressure ratio was calculated as cPP/pPP. Pulse pressure amplification was defined as pPP/cPP.

2.6.4 Pulse wave reflection

To quantify the maximum of the forward and backward pressure waves in the aortic root the triangulation method as reported by Westerhof et al. [330] was used. The parameters shown in Figure 2.9 were used to calculate the forward and backward pressure waves based on central pressure curve measured by the Sphygmocor device. The formula [331] for the forward and backward pressure amplitudes is depicted in Figure 2.9. In this context the aortic flow wave is assumed to be triangular. The shape of the triangle can be reconstructed with pressure/time ratios extracted from the central pulse wave. To adjust for the magnitude of the pressure wave the reflection magnitude (RM) was calculated as the ratio of the backward and forward pressure amplitudes.

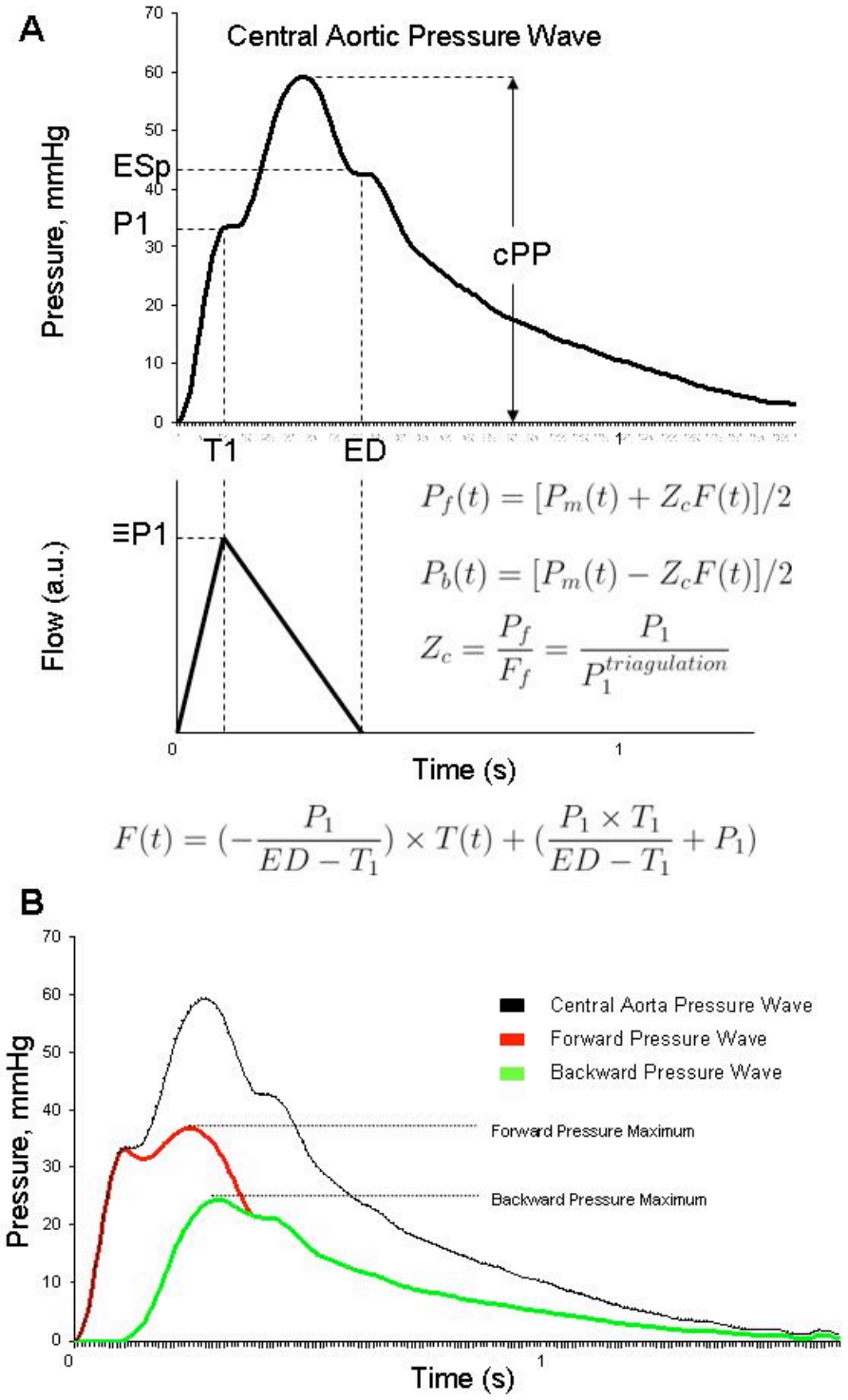


Figure 2.9 Calculation of aortic forward and backward pressure waves. The central pulse wave and a corresponding triangular aortic flow wave [330] are shown in A. Equations for calculation of forward and backward pressure waves are given (A). B illustrates an aortic flow wave with corresponding forward and backward pressure waves. cPP, central pulse pressure; ESp, end-systolic pressure; P1, pressure at T1; Pf, forward pressure wave; Pb, backward pressure wave; Zc, aortic characteristic impedance; F, flow; T1, time at first inflection point; ED, time point marking the start of diastole (early diastole). The original tracing was taken from the DiCADu participant 1-0001-B.

2.6.5 Pulse wave velocity

Aortic pulse wave velocity (PWV) was measured using the SphygmoCor device according to the manufacturers' protocol. Three surface electrodes were positioned on both arms and the abdomen for continuous ECG tracing. The distances "common carotid artery to suprasternal notch" ($d_{ster\text{-}car}$) and "femoral artery to suprasternal notch" ($d_{ster\text{-}fem}$) were measured with a measuring tape. To avoid measurement errors the linear distance instead of the body surface distance was used. The travel distance was estimated by the subtracted distance method [332], as shown in the following equation:

$$\text{Travel Distance}_{\text{subtracted distance}} = d_{ster\text{-}fem} - d_{ster\text{-}car}.$$

Femoral and carotid pulse wave tracings were obtained consecutively with the Millar pressure transducer in parallel to ECG recordings for 10 seconds. PWV was determined from time delay differences between the QRS complex onset on an ECG and the upstroke of the pressure tracing measured at the femoral and carotid arteries. PWV was determined from the foot-to-foot time interval of waveforms [333]. Measurements were rejected if the standard deviation of single heart beat intervals was bigger than 10% during the recording period. The measurement and definition of PWV are illustrated in Figure 2.10. Instead of the SphygmoCor device centres in Brescia and Paris used the Complior (Colson, Garges les Genosse, France), and the centre in Nancy used the PulsePen device (DiaTecne s.r.l., Milan, Italy) in the InGenious HyperCare study. For these centres PWV based on direct carotid femoral distance was converted to PWV based on subtracted distance with the rule of proportion.

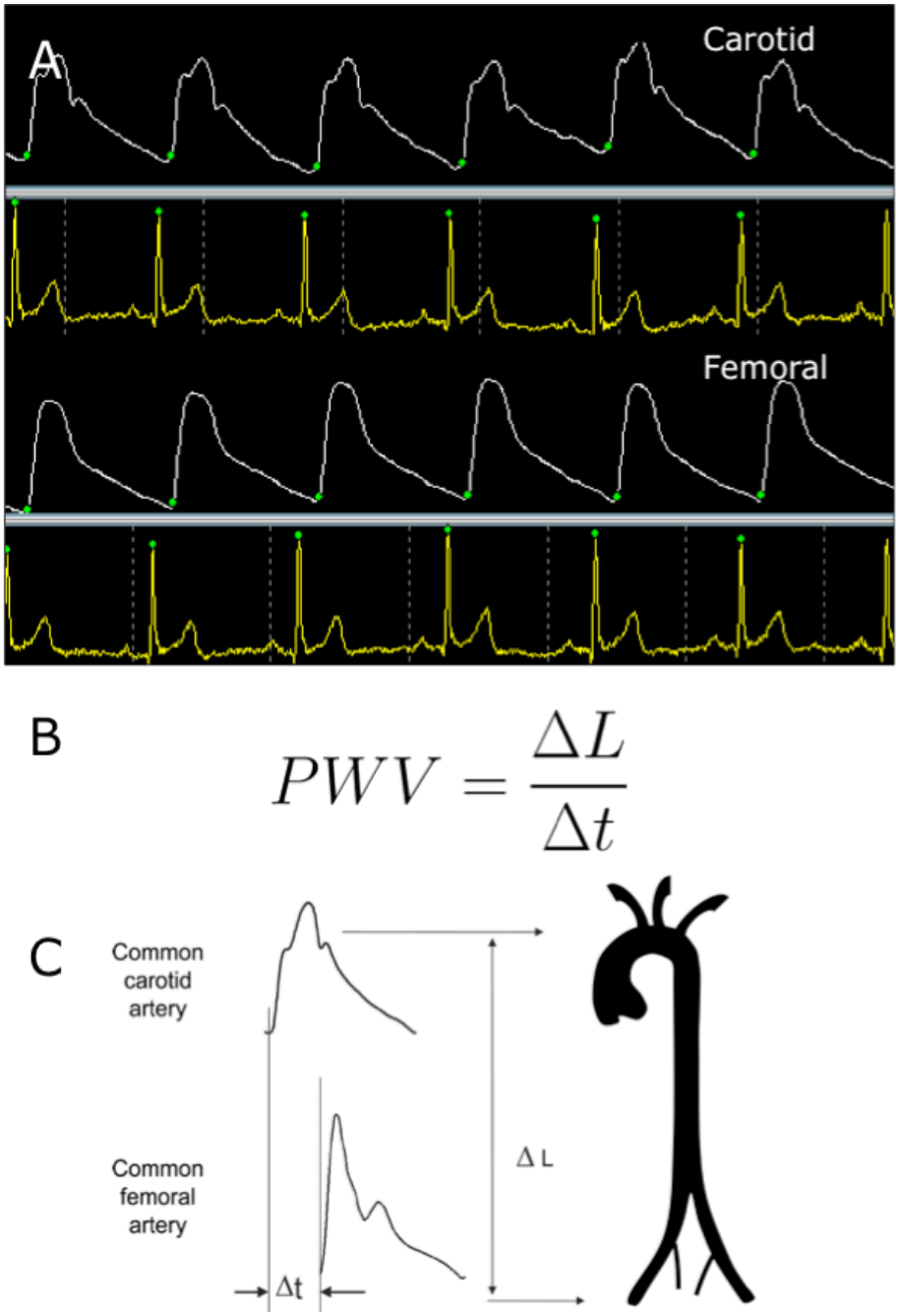


Figure 2.10 Pulse wave velocity measurement. Depicted are pulse wave tracings at the common carotid artery and the femoral artery (white tracing) in comparison to the R-wave (spike of the yellow tracing) as provided by the SphygmoCor device (A). The pulse wave velocity equation (B) and an explanation of corresponding parameters (C), as published by Laurent et al. [334], are illustrated. The drawing (C) sketches the aorta and its main continuation arteries. The original tracing is from the DiCADu participant 1-0043-B.

2.6.6 Carotid distensibility

Diameter changes in the common carotid arteries were recorded during the cardiac cycle to assess carotid distensibility. Perpendicular to the common carotid artery wall, as visualised on ultrasonographic B-mode, an M-mode line was recorded over several heart cycles. To avoid an artificial distensibility reduction, no pressure was applied exceeding the weight of the 8 MHz linear-array transducer. Minimal and maximal carotid diameters were measured offline on the right and left common carotid artery at a plaque-free site, 1 cm below the bulb with Image-Pro Plus software, version 3.0 (Media Cybernetics, Bethesda, USA). The minimum and maximum diameters during a heart cycle were defined as diastolic and systolic diameters, respectively. Depending on picture quality, M-mode pictures from AL, PL and ML directions were evaluated. The measurements of the left and right common carotid artery were averaged and subsequently the average of both sides was used for calculation of the distensibility coefficient. The distensibility coefficient was calculated according to the following equation [335]:

$$\text{Distensibility coefficient } [10^{-3}/\text{kPa}] = (2\Delta D/D) / \Delta P$$

In this equation ΔD represents the diameter change of common carotid artery during systole, D represents the end-diastolic diameter and ΔP stands for the pulse pressure. The distensibility coefficient is illustrated in Figure 2.11. Due to anatomical proximity the central blood pressure measurements as provided by PWA were used in the calculation. As a further index of artery stiffness the cross-sectional compliance [336] was calculated by the following equation:

$$\text{Cross-sectional Compliance } [m^2 * kPa^{-1} * 10^{-7}] = \pi * (D_{systolic}{}^2 - D^2)/(4\Delta P)$$

In this equation $D_{systolic}$ represents the maximum systolic diameter, π is approximately 3.14159 and further abbreviations are identical to the distensibility coefficient equation.

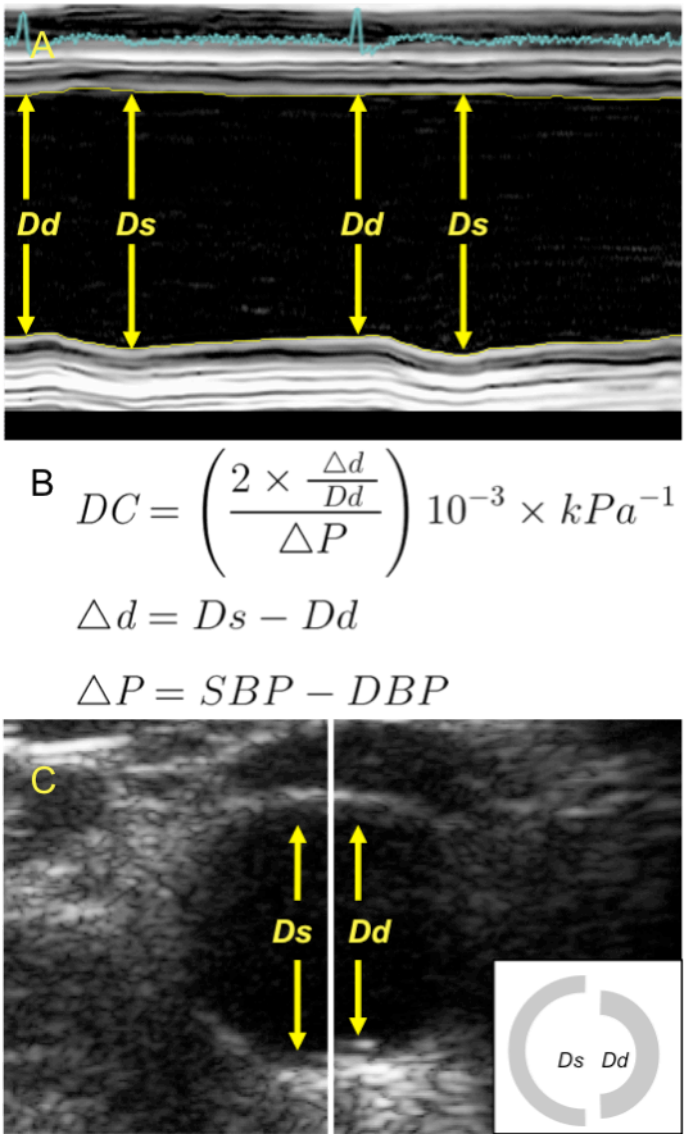


Figure 2.11 The distensibility coefficient. Depicted are relevant measurements on M-mode images of the common carotid artery (A) and the distensibility coefficient equation (B) as well as Cross-sectional B-mode image of the common carotid artery (C) in systole (left side) and diastole (right side). DC, distensibility coefficient; Dd, diastolic diameter; Ds, systolic diameter; SBP, systolic blood pressure; DBP, diastolic blood pressure.

2.6.7 Peripheral arterial tonometry

Peripheral arterial tonometry (PAT) was performed with the Endo-PAT2000 device (Itamar Medical Ltd., Caesarea, Israel). Beat-to-beat finger volume changes were captured by plethysmographic finger cuffs on the index fingers of both hands. The features of the device are illustrated in Figure 2.12. After an equilibration period of at least 5 minutes to assess the baseline PAT signal a blood pressure cuff (Hokanson SC12, Bellevue, USA) was inflated to at least 200 mmHg or pressures 60 mmHg above the SBP for exactly five minutes on the upper right arm. To ascertain that the brachial artery was completely occluded,

the on screen scale of the PAT signal was increased to maximum amplitude. The cuff pressure was then released and the hyperaemia PAT signal was recorded, as shown in Figure 2.13. The ratio between the PAT signal after hyperaemia and at baseline adjusted for the control finger was defined as the reactive hyperaemia index (RHI). The corresponding equation is illustrated in Figure 2.14. Due to the experimental setup the test can be described as reactive hyperaemia PAT (RH-PAT). To avoid interference of factors such as outside temperature, physical activity, drug intake and smoking, recordings were performed in a temperature controlled room at 23-24 °C, after 20 minutes of rest in supine. Participants were fasted and refrained from smoking for at least 2 hours.

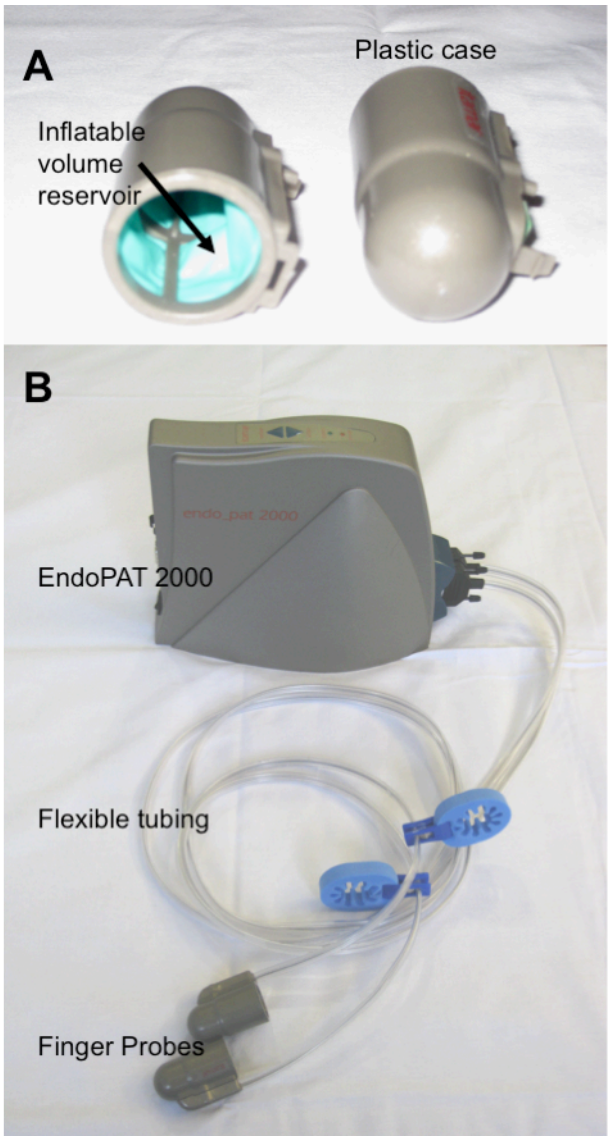


Figure 2.12 Illustration of the EndoPAT 2000 device. Shown are finger probes (A) and the device setup (B). The green inflatable volume reservoirs fitted to the index finger enable to exert a uniform pressure field around the entire finger surface. This allows measurement of pulsatile volume changes and the application of counter pressure for avoidance of venous distension.

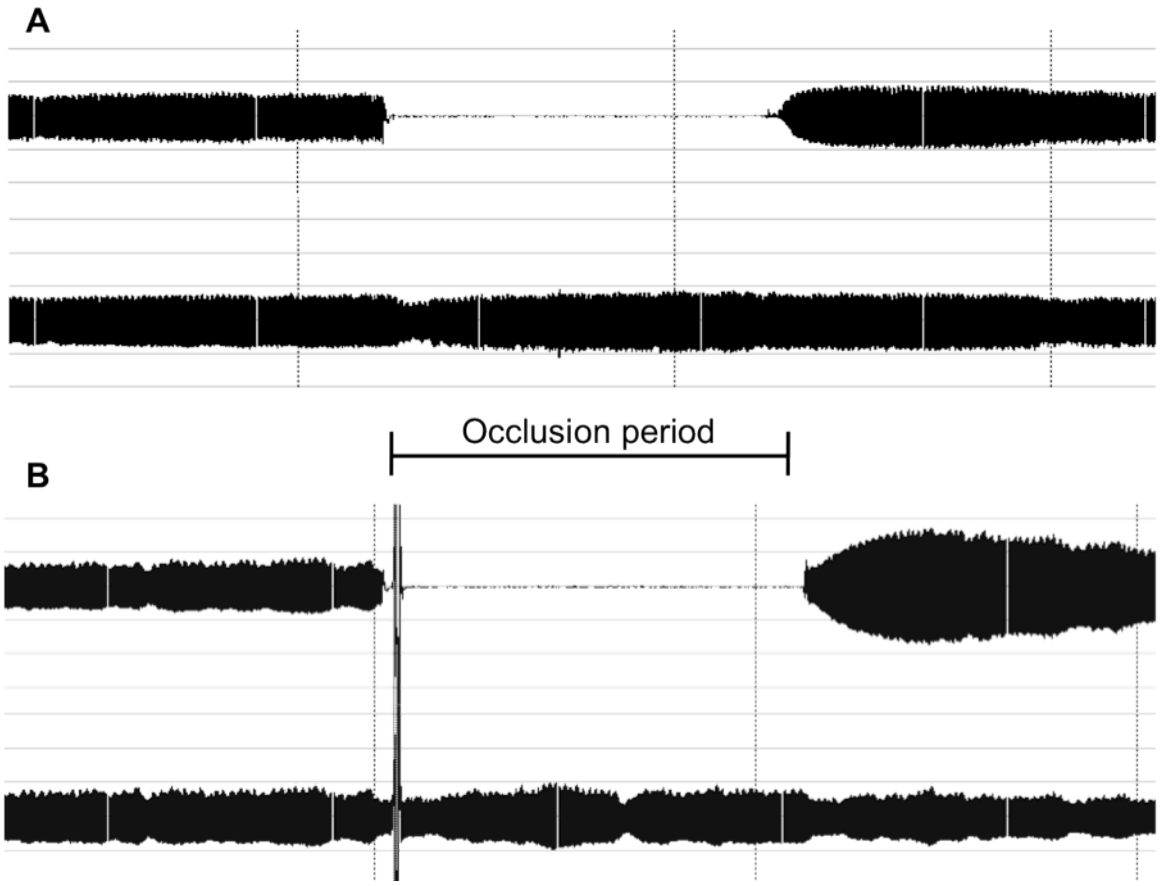


Figure 2.13 Examples of EndoPAT 2000 measurements. Comparison of a pathologic (A) and a normal (B) hyperaemia responses as recorded by fingertip plethysmography. Original tracings are from DiCADu study participant 1-0007-B (A) and 2-0071-B (B).

$$RHI = \frac{PAT^{h}_{occluded}}{PAT^{h}_{control}} \times \frac{PAT^{b}_{control}}{PAT^{b}_{occluded}}$$

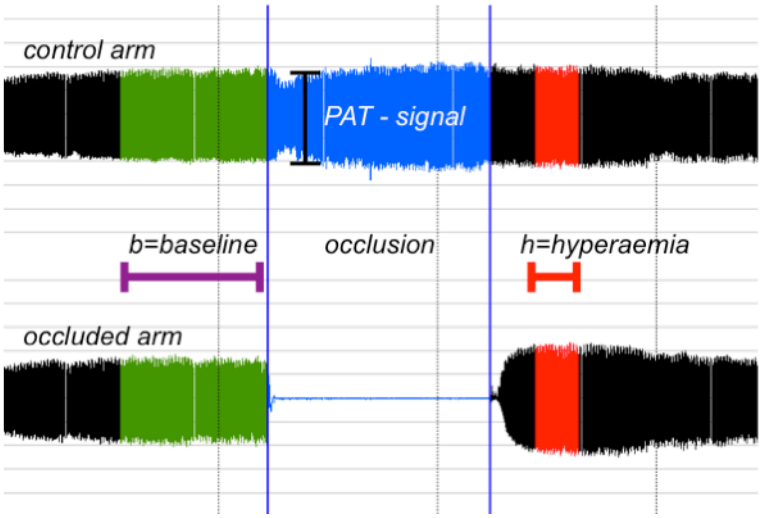


Figure 2.14 Calculation of the reactive hyperaemia index. Time intervals corresponding to PAT signals in the equation are colour coded. The interval 60-120 seconds post cuff release

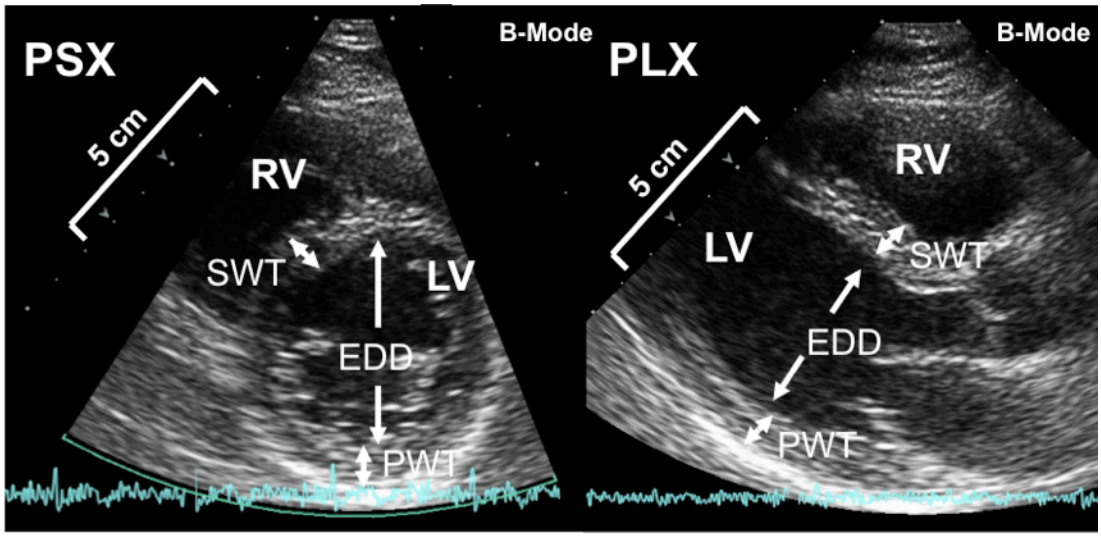
is best linked to coronary microvasculature dysfunction as published by Bonnetti et al. [337]. RHI, reactive hyperaemia index. The original tracing is from participant 1-0007-B of the DiCADu study.

2.7 Echocardiography

Disposable ECG electrodes were attached to the abdomen, left and right shoulder. Participants were placed in partial left decubitus position with a mildly elevated examining table head. For the multicentre InGenious HyperCare study recordings were made with an echocardiograph equipped with 2.5 to 3.5 MHz transducer with M-mode, two dimensional, and Doppler capabilities according to current guidelines [338]. In Glasgow images for offline analysis were recorded with a 1-4 MHz linear-array transducer (Siemens 4V1c Sector Array, Siemens, Erlangen, Germany) on an Acuson Sequoia C512 ultrasound device (Siemens, Erlangen, Germany). Offline analysis was conducted on a KinetDX CWS 3000 workstation (Siemens, Erlangen, Germany).

Echocardiography followed a standardised procedure. The examination protocol consisted of the feature covered in the minimal standard digital acquisition protocol for transthoracic echocardiography [339], and additional colour Doppler myocardial imaging and mitral annulus Doppler tissue imaging were performed in 4 different wall segments of the left ventricle. Each recording covered at least 5 heartbeats.

In accordance with current guidelines for chamber quantification [340] left ventricular mass was calculated with the formula recommended by the American Society of Echocardiography (Figure 2.15). Required parameters were measured at the level of the left ventricular minor axis close to the mitral valve leaflet tips either from M-mode pictures in the long or short parasternal acoustic window or directly from two dimensional images if no perpendicular placement of the M-mode beam was possible. To adjust for body size, left ventricular mass index (LVMI) was calculated by dividing LVM by height$^{2.7}$ [341].



$$LV\,mass = 0.8 \times (1.04[(LV\,EDD + PWT + SWT)^3 - (LV\,EDD)^3]) + 0.6\ g$$

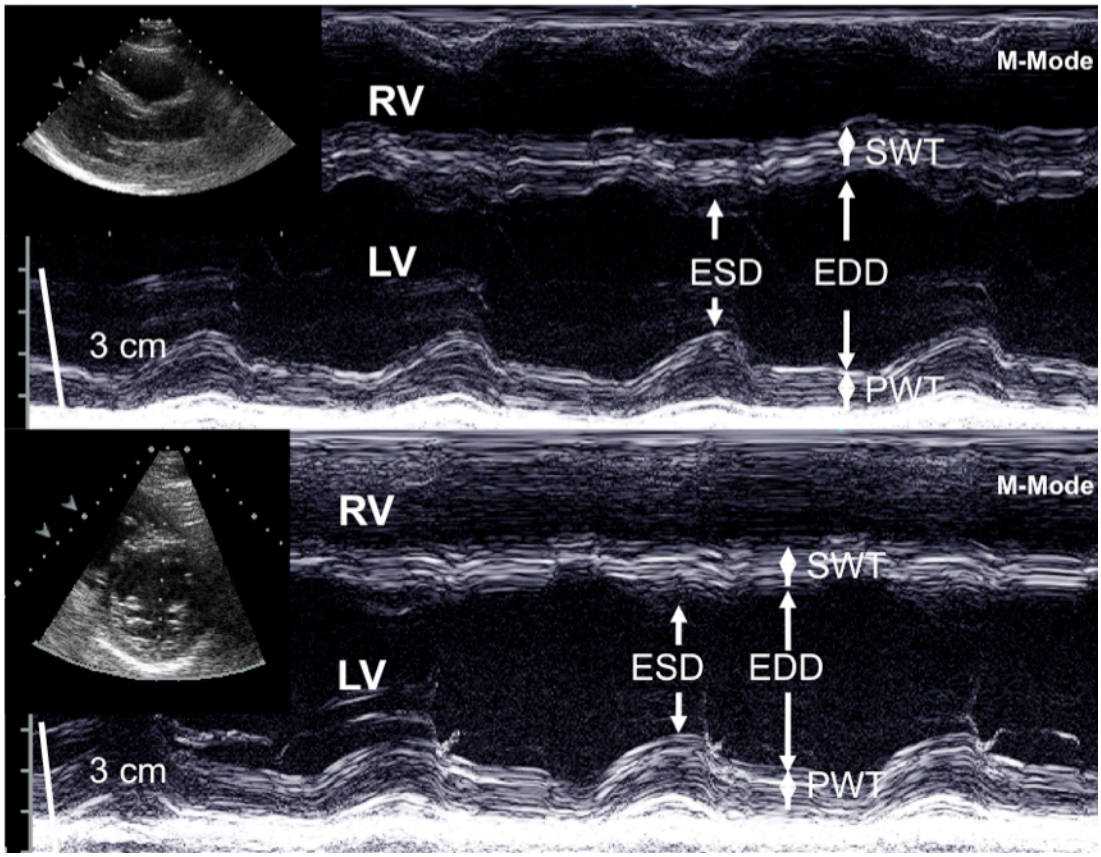


Figure 2.15 Calculation of left ventricular mass. Depicted are measurements of left ventricular (LV) end-diastolic diameter (EDD) and end-systolic diameter (ESD) on M-mode and B-mode pictures either guided by a parasternal short axis (PSX) or a long axis (PLX) image. In all pictures septal wall thickness (SWT) and posterior wall thickness (PWT) present end-diastolic extension. The original tracing is from the InGenious HyperCare participant A2100453103.

2.8 Exercise treadmill testing

Results from exercise treadmill tests prior to coronary angiography were extracted from medical records. Recordings were performed on locally available treadmills at different hospitals in Glasgow. All test followed the standard Bruce-protocol [342]. In this context, patients were asked to exercise until 85% of their age related target heart rate (220 – patient age [bpm]) were accomplished. Exercise was continued until onset of limiting symptoms, abnormalities of rhythm or marked and progressive ST-segment deviation. In line with current guidelines [343], a positive exercise test result was defined as a horizontal or down-sloping ST-segment depression or elevation for at least 60 to 80 ms after the end of the QRS complex, greater than or equal to 1 mm in leads without pathologic Q-waves (excluding lead aVR). The spatial resolution on the ECG y-axis was 1 mV/cm. The extent of the ST-segment deviation was measured by locally available exercise electrocardiogram software, manually revised during assessment of medical records and recorded to the nearest 0.1 mV. The largest value for ST-segment deviation in any lead, except aVR, during exercise or in recovery was used in the analysis. Pre-test probability of CAD was assessed based on age, gender and symptoms [230] as summarised in Table 1.2. Missing data were complemented with questionnaire and interview information.

The Duke treadmill score (DTS) was used to estimate the prognostic value of the exercise treadmill testing. The score was calculated by the following formula:

$$DTS = t_{exercise}\,[min] - (5 * \Delta ST_{max}\,[mm]) - (4 * TAI)$$

In this equation $t_{exercise}$ represents the exercise duration in minutes on the full Bruce protocol, ΔST_{max} stands for the maximum ST-segment deviation in millimetres during or after exercise and the TAI is an abbreviation of the treadmill angina index. The TAI equals 0 if exercise angina was absent, 1 if exercise angina occurred and 2 if angina caused exercise termination. The relation of the DTS to cardiovascular risk published by Mark et al. [239] is shown in Table 2.6.

Table 2.6 The Duke treadmill score in relation to cardiovascular risk.

Risk of Death	Duke treadmill score	Annual cardiovascular mortality*
Low	≥5	0.5%
Moderate	-10 to +4	~2%
High	<-10	≥5%

The source of the presented data is a publication by Mark et al. [239].

2.9 Coronary Angiography

Patients in the DiCADu study had coronary angiography via the right radial artery in the GJNH Clydebank. Angiograms were carried out and graded by single operators. Based on local standards patients were categorised in reference to the coronary artery with the biggest stenosis. Six categories were applied: normal coronary arteries (no vessel wall irregularity), plaque disease with vessel wall irregularities only, narrowing <25% of the artery lumen, mild CAD (25-50% stenosis), moderate CAD (50-75% stenosis) and severe CAD (>75% stenosis). For the DiCADu study patients with severe CAD were recruited as cases and those with normal coronary arteries or vessel wall irregularities served as controls.

For the VASCAB study coronary angiograms were performed in referring hospitals of the Greater Glasgow and Clyde area. The decision for bypass surgery was based on severity and extent of CAD as well as co-morbidities. Each patient was discussed in the local multiple disciplinary team meeting and treated according to current guidelines [344-347].

To quantify the overall CAD extent in DiCADu and VASCAB study patients the scoring system suggested by Gensini [348] was used. The corresponding Gensini score is illustrated in Figure 2.16. Diagnostic angiographic reports in medical records were used to evaluate location and severity of stenoses in the coronary arteries. Stenoses of less than 25%, 25-49%, 50-74%, 75-94%, 95-99% and 100% were equated with 1, 2, 4, 8 and 16 accordingly. Depending on the artery

segment and dominance of the left or right coronary artery those were multiplied with factors from 0.5 to 5 [348] to implement the functional significance of the area supplied by that segment. The artery segment scores were finally added to obtain the Gensini-score.

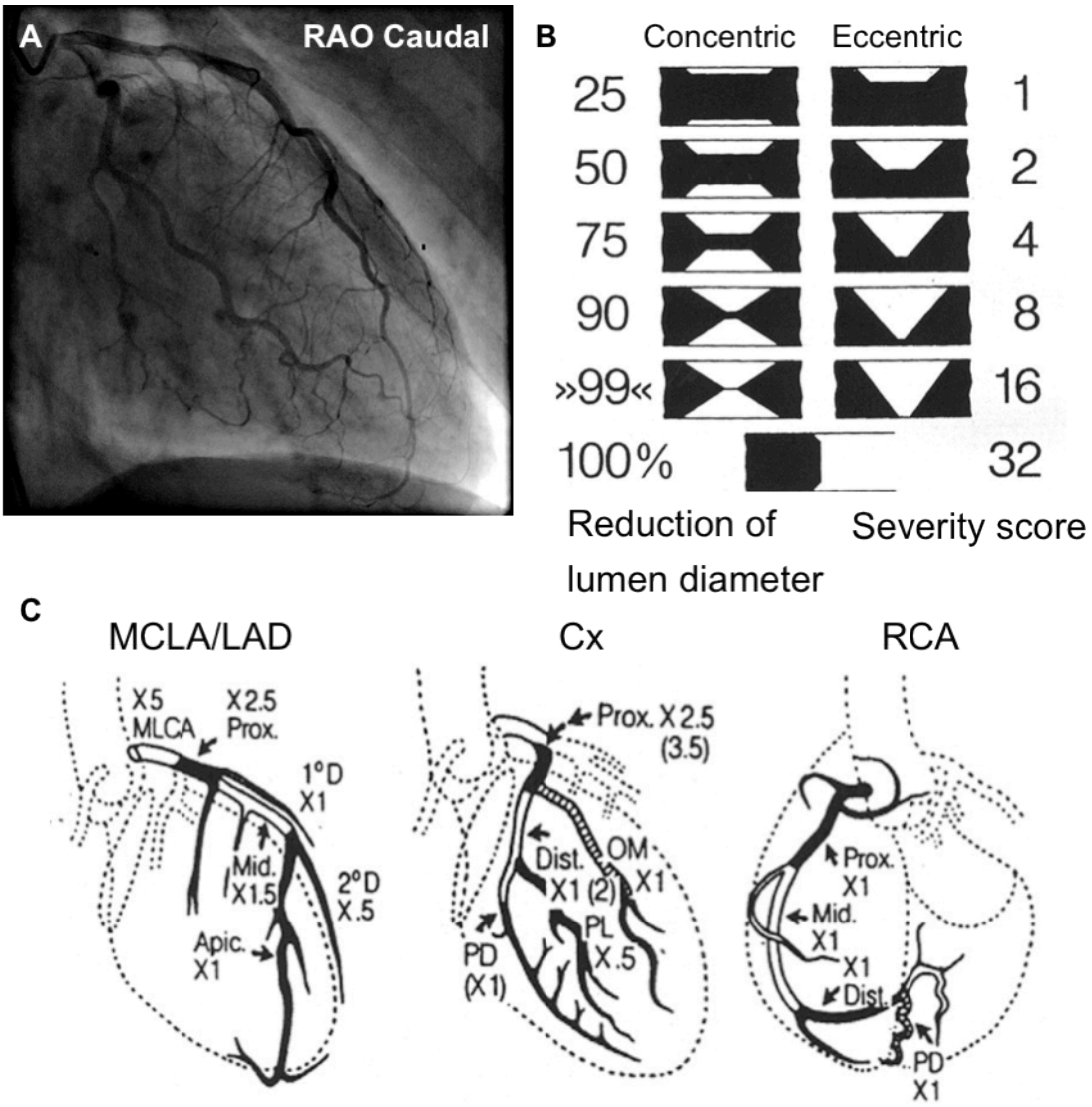


Figure 2.16 Illustration of the Gensini Score. Normal coronary angiogram in RAO caudal projection (A). Roentgenogaphic appearance of concentric and eccentric lesions resulting in 25, 50, 75, 90, 99 or 100% obstructions (B). The right column in B indicates the relative severity of the according lesions (e.g. 4 for 75%). Panel C depicts sketches of the three main coronary arteries and their adjacent branches (MLCA, main left coronary artery; LAD, left anterior descending artery; Cx, circumflex artery; RCA, right coronary artery; PD, posterior descending artery; PL, posterolateral artery; OM, obtuse marginal artery; 1°D, first diagonal artery; 2°D, second diagonal artery; Prox, proximal segment; Mid, middle segment; Dist, distal segment). Abbreviations are followed by multiplying factors to underline the functional significance of the areas supplied by the particular segment. The Gensini score derives from the multiplication of the severity scores with significant factors and the summation of all coronary artery lesions. Panel B and C are modified from Gensini [348].

2.10 Serological biomarkers

Blood samples were collected after at least three hours of fasting. For local laboratory use serum and whole blood (EDTA) was sampled with the Vacutainer® system (BD, Franklin Lakes, USA). The same collection system was used in the VASCAB and DiCADu study for analysis of additional biomarkers. In the InGenious HyperCare study Sarstedt Monovettes (Sarstedt, Nuembrecht, Germany) were use for this purpose. Serum and EDTA plasma samples were spun immediately after collection at 3000 G for 10 minutes and the supernatant was kept at -80 °C. For storage CryoPure tubes (Sarstedt, Nuembrecht, Germany) were used in the InGenious HyperCare study and the 1.5 ml Screwcap MCT tube (Alphalaboratories, Eastleigh, UK) in the DiCADu and VASCAB study.

2.10.1 Local laboratory

For all studies blood samples for routine parameters, such as full blood count, C-reactive protein, urea and electrolytes, uric acid, liver enzymes, cholesterol levels and fasting glucose were collected according to local standards and analysed in laboratories at Gartnavel General Hospital, Glasgow. Estimated glomerular filtration rate (eGFR) was calculated with the MDRD formula [349]:

$$eGFR\ [mL/min/l.73\ m^2] = 175 \times (\text{Serum creatinine [mg/dL]})^{-1.154} \times (\text{Age [y]})^{-0.203}$$

(× 0.742 if female).

2.10.2 ELISA

For the DiCADu study IL-6 and NT-proBNP were quantified in a subgroup. NT-proBNP was measured with the Roche Elecsys electrochemiluminescence immunoassay (Roche Diagnostics, Indianapolis, USA) and IL-6 was measured with the R&D System Quantikine HS (R & D Systems Inc., Minneapolis, USA).

For NT-proBNP measurements 15 µl of sample antigen containing biofluid, a biotinylated monoclonal NT-proBNP-specific antibody, a monoclonal NT-proBNP-specific antibody labeled with a ruthenium complex and streptavidin-coated microparticles were mixed and incubated for 9 minutes. The reaction mixture was aspirated into a measuring cell where the microparticles are magnetically captured onto the surface of an electrode. Unbound substances are removed in a

washing step. Then chemiluminscent emission is induced by voltage application to the electrode. The brightness of the emission is measured by a photomultiplier. Results are determined via a calibration curve, which was previously generated.

For IL-6 measurements reagents and working standards were prepared. 100 µl of assay diluents were added to each well of a microplate pre-coated with capture antibodies for IL-6. Then 100 µl of Standard solution, sample fluid or control fluid were added to each well. Wells were covered with an adhesive strip and incubated for 2 hours at room temperature. Afterwards each well was washed four times by addition of 400 µl wash buffer and removal of liquid at each step. Then 200 µl of horseradish peroxidase labeled antibody is added. The plates are again incubated for 2 hours at room temperature. Unbound detection antibody is washed away with four washing steps as described above. 200 µl of substrate solution are added to each well and incubated under light protection for 20 minutes at room temperature. Finally 50 µl of stop solution is added to each well and the optical density of each well was determined using a microplate reader. The use of standard solutions allowed the estimation of IL-6 concentrations.

2.10.3 Luminex

For the VASCAB study batch analysis of additional biomarkers was performed in an age and sex matched subgroup of 40 patients with severe CAD and 40 healthy control subjects. To enlarge the investigated cohort four additional CAD patients with similar age distribution were added. Using WideScreen® BeadPlex™ Multiplex Assays (Merck, Darmstadt, Germany) and the Luminex® xMAP® Technology platform (Luminex Corporation, Austin, USA) the following serum biomarkers were measured with the Luminex® 100™ analyser (Luminex Corporation, Austin, USA) according to the manufacturer's protocol: IL-6, interleukin 8 (IL-8), monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein-1 beta (MIP-1β), TNFα, E-selectin, ICAM-1, leptin, osteopontin, P-selectin, soluble receptor for advanced glycation end products (sRAGE), adiponectin, cystatin C, ligand for the receptor for advanced glycation end products (en-RAGE), plasminogen activator inhibitor 1 (PAI-1) and VCAM-1.

The technique is based on 5.6 micron-sized polystyrene microspheres and a flow cytometry-like measuring process. The microspheres can be coated with varying capturing reagents, contain different intensities of two fluorochroms and thereby have distinguishable spectral addresses. A third fluorochome coupled to a reporter molecule is used for quantification of the protein binding at the microsphere surface. Individual microsphere assessment occurs in a rapid flowing fluid stream by two separate lasers as depicted in Figure 2.17.

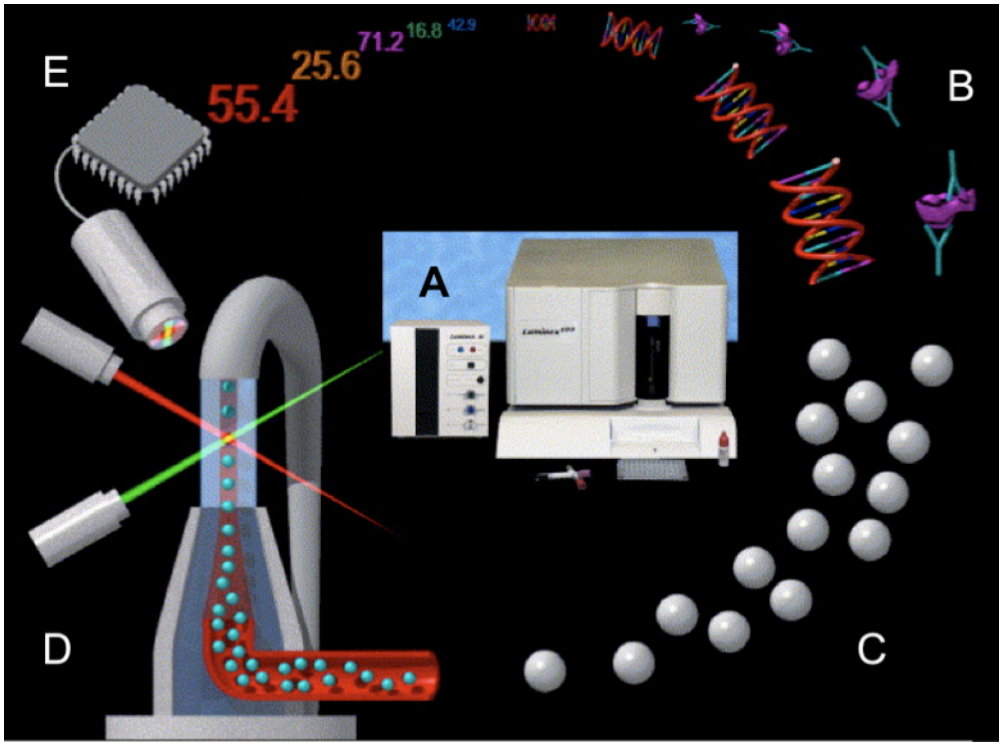


Figure 2.17 The Luminex xMAP system and its key components. Depicted are the Luminex 100 analyzer (A) in the centre and clockwise from top-right: DNA and antibody coated proteins as examples for possible biomolecular reactants (B), microspheres (C), a sketch of the rapid flowing fluid stream and two detection lasers (one for the microsphere fluorochromes and one for the reporter fluorochrome) (D) and the high-speed digital signal processing unit (E). Modified from Dunbar [350].

Standard reagents in the WideScreen Human CVD Panel 2, 3 and 6 kit (Merck, Whitehouse Station, USA) were prepared according to the manufactures instruction. Assay buffer was added to Pre-wet Filter Plates and liquids were removed by vacuum. Eight different standard dilutions for each assessed biomarker were prepared after the manufactures instruction. Corresponding concentrations for generation of standard curves are listed in Table 2.7.

Table 2.7 Biomarker dilutions for standard curve calculation with the Luminex xMAP system.

Panel 2 Standard	IL-6 (pg/ml)	IL-8 (pg/ml)	MCP-1 (pg/ml)	TNF-α (pg/ml)	MIP-1β (pg/ml)	
8	472	470	2080	251	1328	
7	157	157	693	84	443	
6	52	52	231	28	148	
5	17	17	77	9.3	49	
4	5.8	5.8	26	3.1	16	
3	1.9	1.9	8.6	1.0	5.5	
2	0.64	0.64	2.9	0.33	1.8	
1	0.21	0.21	0.95	0.11	0.60	
Panel 3 Standard	E-selectin (ng/ml)	ICAM-1 (ng/ml)	Leptin (ng/ml)	Osteopontin (ng/ml)	P-selectin (ng/ml)	sRAGE (ng/ml)
8	750	200	32	500	400	100
7	250	67	11	167	133	33
6	83	22	3.6	56	44	11
5	28	7.4	1.2	19	15	3.7
4	9.3	2.5	0.40	6.2	4.9	1.2
3	3.1	0.82	0.13	2.1	1.6	0.41
2	1.0	0.27	0.044	0.69	0.55	0.14
1	0.34	0.091	0.015	0.23	0.18	0.046
Panel 6 Standard	PAI-1 (ng/ml)	Cystatin C (ng/ml)	EN-RAGE (ng/ml)	Adiponectin (ng/ml)	VCAM-1 (ng/ml)	
8	21	200	6.1	300	48	
7	7.0	67	2.0	100	16	
6	2.3	22	0.68	33	5.3	
5	0.78	7.4	0.23	11	1.8	
4	0.26	2.5	0.075	3.7	0.59	
3	0.086	0.82	0.025	1.2	0.20	
2	0.029	0.27	0.0084	0.41	0.066	
1	0.010	0.091	0.0028	0.14	0.022	

Shown are standard dilutions of listed biomarkers as provided by the manufacturer.

Test samples for panel 2 and 3 were 5-fold diluted or for panel 6 300-fold diluted. After 10 μl of blocking buffer, 30 μl of test sample or standards were added to the filter plates in duplicates together with 70 μl of beads. The plates were covered with tin foil and shacked at 750 rpm for 60 minutes at room temperature. The plates were washed with 100 μl assay buffer. Supernatant was removed by vacuum. These two steps were repeated once. Then 40 μl of detection antibodies were mixed to each well followed by 60 minutes of incubation under the above conditions. Next 20 μl of streptavidin phycoerytherin was added to each well and each plate was shacked for another 30 minutes

under the above conditions. Each plate was washed and vacuumed twice before the beads were resuspended in 100 µl assay buffer. For analysis 50 µl from each well were run on a Luminex 100 machine. In each inserted probe 50 beads were measured and the resulting median fluorescence intensity was compared to the standard curve generated with the standard dilutions. Last the results were multiplied by the dilution factor.

2.10.4 Randox

Stored serum samples were used to measure cardiac Troponin 1, myoglobin, creatine kinase isoenzym MB (CK-MB), carbonic anhydrase III (CA III), glycogen phosphorylase BB (GPBB) and heart fatty acid binding protein (hFABP) with the Randox Evidence Investigator and a complementing cardiac array (Randox Laboratories Ltd., Crumlin, United Kingdom) which consist of a sandwich chemiluminescent immunoassay. Each of the specific antibodies was immobilised in an ordered array arrangement on one of the 9 mm^2 biochips supplied by the manufacturer.

In a first step, samples were diluted with assay buffer and applied to a single biochip. Arranged on a carrier, biochips were incubated at 37 °C at 370 rpm in a thermoshaker for 60 min. The diluent was removed with a washing step, the horse radish peroxidase labelled antibodies were added and incubated as described. Following a further washing step, 250 mL of a 1:1 mix of luminol and peroxide was added and incubated for 2 min. After this the carrier tray was read using digital imaging technology. As antibodies are labelled with horse-radish peroxidase light signals generated from each Biochip test region were detected with a super cooled charge coupled device camera. Signal strength was consecutively compared to calibration data. Each single biochip on the carrier contained an array of discrete test regions covered with immobilised antibodies specific to the biomarkers of interest. The biochips were provided in carriers containing 3 × 3 biochips. Each carrier was arranged in a handling tray that allowed simultaneous handling of 6 carriers.

2.11 Urinary biomarkers

2.11.1 Urine dipstick

Standard dip stick analysis was carried out using Multistix 10 SG reagent strips for urinalysis (Siemens Healthcare Diagnostics Inc., Tarrytown, USA) according to the manufacturer's protocol. Dip stick analysis was used as a basic screening tool for proteinuria, leucocyturia, haematuria and nitrite in the urine.

2.11.2 Urinary albumin excretion

For all studies urine samples for urinary albumin, urinary creatinine and urinary protein were collected according to local standards and analysed in certified biochemistry laboratories at the General Gartnavel Hospital, Glasgow. The urinary albumin:creatinine ratio was calculated as ACR [mg/mmol] = urinary albumine [mg/L] : urinary creatinine [mmol/L].

2.11.3 Urinary Proteomics

Each study participant had 3 x 1000 µl aliquots of urine stored at -80°C at the BHF Glasgow Cardiovascular Research Centre. For storage the CryoPure tube (Sarstedt, Nuembrecht, Germany) was used in the InGenious HyperCare study and Screwcap MCT (Alphalaboratories, Eastleight, UK) in the DiCADu and VASCAB study. Proteome analysis was carried out as batch analysis at Mosaiques Diagnostics, Hannover, Germany. Samples were sent frozen on dry ice with commercial couriers. This procedure was possible as no significant sample degradation is caused by long-term storage [351].

2.11.3.1 Sample preparation

For sample preparation 0.7 mL aliquots were thawed and 0.7 mL of a 2M urea, 10 mM NH_4OH and 0.02% sodium dodecyl sulfate were added. For removal of high molecular mass proteins the resulting solution was filtered using a Centrisart ultracentrifugation filter device and a 20 kDa molecular weight cut-off (Sartorious, Goettingen, Germany) at 3000 g for 45 minutes at 4 °C. To remove urea, electrolytes, salts and other interfering agents from the specimens, 1.1 ml of filtrate were applied onto a PD-10 desalting column (GE Healthcare, Uppsala,

Sweden) pre-equilibrated in 0.01% NH_4OH high performance liquid chromatography grade water (Roth, Karlsruhe, Germany). This decreased the matrix effect during mass spectrometry and enriches the polypeptides present. Samples were lyophilised in a Christ Speed-Vac RVC 2-18/Alpha 1-2 (Christ, Osterode a.H, Germany) and later resuspended with high performance liquid chromatography grade grade water to yield 0.8 g/L protein concentration as measured by bicinchoninic acid assay (Interchim, Montlucon, France).

2.11.3.2 Capillary electrophoresis mass spectrometry

The proteome was assessed by CE-MS. First a urine sample was separated according to the charge and frictional forces and hydrodynamic radius of its contents by capillary zone electrophoresis with a Beckman Coulter PAC/E system (Beckman Coulter Inc., Brae, USA). Fused-silica capillaries were provided by Beckman with an inner diameter/outer diameter of 75/360 µm and a length of 90 cm. The mobile phase contained 30% methanol and 0.5% formic acid in water. After a rinsing step with the mobile phase for 3 minutes, samples were injected for 20 seconds with a pressure of 1 pound per square inch (psi) allowing assessment of approximately 700 nL of sample. The separation was then performed by applying a charge of +30kV at the inlet of the capillary, resulting in a current of approximately 13µA. The capillary temperature was constantly 35 °C. After each sample the CE capillary was rinsed for 5 minutes with 0.1 mol/L sodium hydroxide followed by 5 minutes of water and 5 minutes of running buffer. The capillary electrophoresis was coupled to a electrospray ionization time of flight mass spectrometer (ESI-TOF-MS) which allowed to identify peptides and proteins by their mass/charge ratio with an electroionisation sprayer (Agilent Technologies Inc., Santa Clara, USA) and a micro TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). Both techniques were coupled via a grounded sheath-liquid interface, which used the same composites as the mobile phase of the CE applied at 5 µL/min. The flow was coaxial to the capillary. The ion-spray interface potential range was between -4.0 and -4.5 kV. A CE-MS run was executed for 30 minutes at 30 kV with 0.2 psi positive pressure. Mass spectrometry data was accumulated every 3 seconds over a range of mass/charge ratio (m/z) of 350-3000 and the mean was then calculated. In summary, samples were separated by migration time and mass/charge ratio as shown in Figure 2.18.

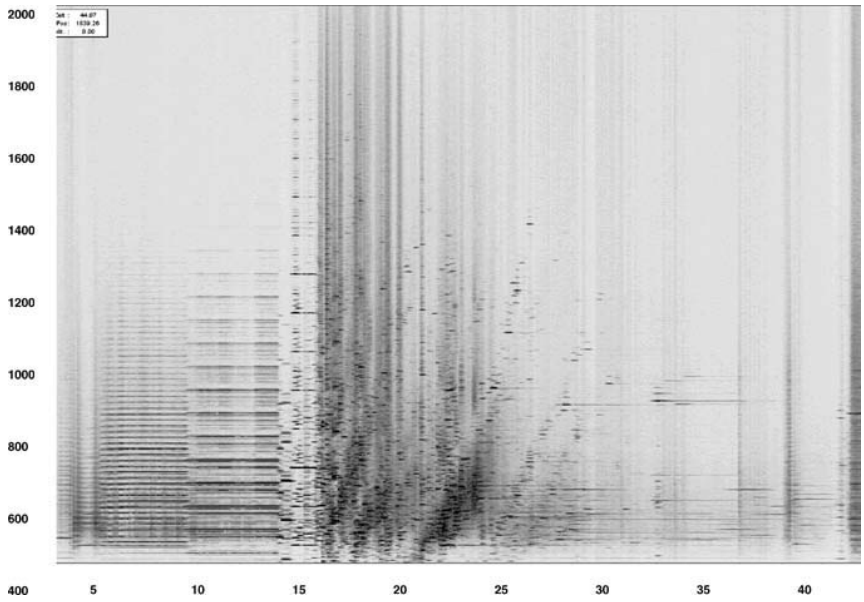


Figure 2.18 Example of CE-ESI-TOF-MS. Capillary electrophoresis coupled to electrospray ionization time of flight mass spectrometry (CE-ESI-TOF-MS) run of crude urine. The mass/charge ratio is shown on the y-axis and the migration time (min) is shown on the x-axis. Signal intensity is depicted by colour saturation. The illustration is taken from Kolch et al. [303].

2.11.3.3 Accuracy of CE-MS

Kolch et al. [303] extensively described characteristics of the CE-MS system. In brief, the average recovery of the sample preparation as described in 2.11.3.1 was approximately 85%. The detection limit was approximately 1 fmol. Monoisotopic mass signals were resolved for a charge (z) <6. Mass accuracy of the CE-ESI-TOF-MS method was determined to be less than 25 parts-per-million (ppm) for monoisotopic resolution and less than 100 ppm for unresolved peaks (z>6). Repeated measurements of the same aliquot and with different preparations of identical speciment were previously performed, to evaluate the method's precision [352]. Data sets were only acceptable if a minimum of 950 peptides or proteins were detectable with an MS resolution of at least 8000 and a minimum migration time interval of 10 minutes. Furthermore, the mean deviation of migration time had to be <0.35 minutes.

2.11.3.4 Data processing

Each CE-MS run provides a large amount of information, with over 1000 individual spectra for each sample. A number of steps are therefore required in order to accurately process the data. For this purpose the software MosaiquesVisu (mosaiques diagnostics and therapeutics AG, Hannover, Germany)

was employed. First it summarises mass spectral ion peaks representing identical molecules at different charges into single masses. Signals had to be observed on at least three consecutive spectra with a signal-to-noise-ratio of ≥4. Calculated charges of +1 were excluded to minimise interference with matrix compounds or drugs. The charge of each peak was then calculated, based on isotopic distributions and conjugated masses. The Mosaiques-Visu software deconvolutes the data afterwards. This results in a recording of mass spectral ion peaks from the same molecule at different charge states as a single mass. CE-migration time and ion signal intensity show a high variability. This is due to different concentrations of peptides and ions in the samples corresponding to the time of urine collection, hydration status of the patient etc. Therefore the data were normalised based on reference signals from 29 abundant "housekeeping" peptides generally present in urine [297]. These peptides are the result of normal biological processes, and do not appear to be affected by age, sex or disease state. Each of these peptides was present in more than 90% of all urine samples present. The individual sample data were calibrated with a local regression algorithm and the internal standard peptides as reference. The resulting peak list characterises each peptide by its molecular mass (in daltons), CE migration time, and signal intensity, providing a unique identification mark. After normalisation signal intensity was employed as a marker for relative quantity.

2.11.3.5 Cluster analysis

Data were then entered into a Microsoft SQL database, which allowed further analysis and comparison with individual samples. For identification of peptide/protein clusters, peaks in multiple samples were estimated as equal if the corresponding deviation was lower than ±50 ppm for 800 Da peptides, gradually increasing to ±75 ppm for 15 kDa peptides. Similarly, deviation of migration time was controlled to be below 0.35 minutes. Also cluster widths were increased by 2-5%, to adjust for analyte diffusion effects resulting in CE-peak widening with time. To eliminate peptides with sporadic appearance only peptides detected in more than 20% of the urine samples in a clinicaly defined cohort were further investigated. The last step reduces the quantity of peptides significantly to approximately 5% of the original number.

2.11.3.6 Sequencing of peptides

Peptides from urine were sequenced using capillary electrophoresis coupled to tandem mass spectrometry or liquid chromatography coupled to tandem mass spectrometry as published by Zurbig et al. [308]. MS/MS experiments were carried out on an Ultimate 3000 nano-flow system (Dionex/LC Packings, Bannockburn, USA) attached to an LTQ Orbitrap hyprid mass spectometer (Thermo Fisher Scientific, Germany) equipped with a nano-electrospray ion source. The mass spectrometer was operated in a data-dependent mode to automatically switch between MS and MS/MS acquisition. Survey full-scan MS spectra (from m/z 300-2000) were acquired in the Orbitrap. Ions were sequentially isolated from fragmentation in the linear ion trap using collision induced dissociation. General mass spectrometric conditions were: electrospray voltage, 1.6 kV; no sheat and auxiliary gas flow; ion transfer tube temperature, 225°C; collision gas pressure, 1.3 mTor; normalised collision energy, 32% for MS^2. Ion selection threshold was 500 counts for MS/MS. In addition samples were analyzed using electron transfer dissociation. Peptides were separated by nRP-HPLC (Agilent 1100; flow split by tee to ~60 nL/min) and introduced into an ETD-capable LTQ quadrupole linear ion trap (Thermo Fisher Scientific, San Jose, USA) via nESI, using previously described instrumental parameters [353]. All resultant MS/MS data were submitted to MASCOT (www.matrixscience.com) for a search against human entries (20413 sequences) in the Swiss-Prot database (Swiss-Prot Number 56.6) without any enzyme specificity. No fixed modification was selected, and oxidation of methionine and proline were set as variable modifications. Accepted parent ion mass deviation was 50 ppm; accepted fragment ion mass deviation was 500 ppm, accepted fragment ion mass deviation was 500 ppm. Only search results with a MASCOT peptide score of 20 or higher, which also met ion coverage stipulations as related to the main spectal features, were included. Data files from experiments performed on the ETD-enabled LTQ were searched against the IPI human non-redundant database using the Open Mass Spectrometry Search Algorithm (OMSSA, http://pubchem.ncbi.nlm.nih.gov/omssa), with an e-value cut-off of 0.01. For further validation of obtained peptide identifications, the strict correlation between peptide charge at the working pH of 2 and CE-migration time was utilised to minimise false-positive identification rates: Calculated CE-migration time of the sequence candidate based on its peptide sequence (number of basic

amino acids) was compared to the experimental migration time. Peptides were only accepted with a mass deviation below ± 25 ppm and a CE-migration time deviation below ± 2 min.

2.12 Statistics

Data were analysed using SPSS software, versions 15.0 and 19.0 (SPSS Inc., Chicago, USA). Normality of data distribution for all experiments was tested using the Kolmogorov-Smirnov test and visual inspection of Q-Q plots. Baseline data are expressed as mean ± standard deviation (SD) if normally distributed or median with 25-75% interquartile range if not normally distributed. Correlations were assessed by calculating Pearson's or Spearman's correlation coefficient for parametric and non-parametric data, respectively. Steiger's Z-test was used to compare bivariate correlations within a single population. Fisher's Z-test was used to compare bivariate correlations between populations. The two sample Student's t test or the Mann Whitney test were conducted as appropriate for the comparison of two groups of paired observations for continuous data. For comparison of categorical data of independent groups the Chi-squared test was employed. To compare two groups of paired categorical data the Wilcoxon test was performed. In general, a *P*-value of less than 0.05 was considered significant. Additional statistical tests will be discussed in relevant chapters.

Chapter 3 Association of central and peripheral pulse pressure with biomarkers of the intermediate cardiovascular phenotype

3.1 Introduction

The concept of the cardiovascular continuum implies the existence of early stages of target organ damage prior to the development of clinically overt cardiovascular disease. Such are expected in patients with higher CVD risk in early age groups. Young or middle aged hypertensive patients represent such a cohort. The prognostic value of CVD biomarkers in early stages of the cardiovascular continuum could therefore be conducted in such a population.

Treatment decisions in patients with hypertension are based on blood pressure levels, presence of other cardiovascular risk factors and evidence of end organ damage. Pulse pressure is an important predictor of cardiovascular risk in the elderly [354-356]. This has been recognised in the ESH/ESC guidelines on diagnosis and treatment of hypertension where pulse pressure features as one of the factors influencing prognosis in the elderly [357]. In contrast, in those younger than 55 years pulse pressure contributes only to small extent to risk prediction, as the contribution of pulse pressure to risk prediction in this age group is minimal, as shown in Table 3.1 [358].

Table 3.1 Different blood pressure indices as predictor of ischaemic heart disease mortality.

BP Index	Age (years) at risk				
	40-49	**50-59**	**60-69**	**70-79**	**80-89**
SBP	85%	91%	96%	95%	92%
DBP	84%	78%	70%	68%	69%
pPP	19%	35%	49%	51%	47%
MAP	100%	98%	96%	96%	97%

Depicted χ^2_1-values (%) represent the contribution of the specific BP indexes to risk prediction in certain age groups. The table was adapted from Lewington et al. [358]. SBP, systolic blood pressure; DBP, diastolic blood pressure; pPP, peripheral pulse pressure; MAP, mean arterial pressure.

Due to the increase of systolic pressure along the arterial system, aortic (central) pulse pressure is different from brachial (peripheral) pulse pressure [359]. The relationship between cPP and pPP, which can be expressed as pulse

pressure ratio [360] or pulse pressure amplification [361], is not only determined by blood pressure levels but depends on stiffness of conduit arteries and on pressure wave reflections [334, 362]. Antihypertensive therapy alters pulse pressure [363], and in hypertensive patients treated with an angiotensin receptor blocker based regimen the ratio between cPP and pPP was found to be lower than in patients on a beta blocker based regimen [364]. In longitudinal studies cPP readings derived from radial artery tonometry were reported to improve risk prediction in comparison to pPP in various cohorts [365-367]. We therefore investigated whether cPP in contrast to pPP could refine cardiovascular risk assessment also in a younger cohort.

Pulse pressure is strongly associated with end organ damage including carotid C-IMT and LVM [366, 368]. In a cross-sectional study we therefore investigated the relationship between cPP and pPP with intermediate cardiovascular phenotypes including LVMI, C-IMT, urinary albumin excretion and aortic PWV.

3.2 Material and Methods

3.2.1 Patients

All participants of this study were originally recruited into the InGenious HyperCare study, a family-based study into genetics and genomics of hypertension associated with microinflammation, oxidative stress and microalbuminuria. The recruitment criteria and process are described in chapter 2.1.3.

For this study we explored data from 1,589 participants recruited between 2008 and 2010 in 19 study centres. Measurement of central haemodynamics was optional. Analysis was restricted to patients with SBP ≤180 mmHg and DBP ≤110 mmHg, equivalent to 94% of the cohort. A total of 535 participants from 145 families fulfilled these criteria and had their cPP assessed. These patients were recruited in Brescia, Italy (n=45); Glasgow, United Kingdom (n=148); Krakow, Poland (n=127); Hechtel-Eksel, Noordenkempen, Belgium (n=78); Lund, Sweden (n=37); Nancy, France (n=25); Paris, France (n=12); and Valencia, Spain (n=63). Anthropometric data were collected according to local standards, and BSA was calculated using the Dubois & Dubois formula. All participants gave written

informed consent. The study was approved by local research ethics committees at each participating centre.

3.2.2 Blood pressure measurements

Standard blood pressure measurements in supine position are described in chapter 2.4. In the Belgian study centre blood pressure was measured with a standard mercury sphygmomanometer in both arms.

For measurement of blood pressure in supine position participants rested for at least 15 minutes, and the arm with higher sitting blood pressure was selected. After at least five additional minutes in supine position radial arterial pressure waveforms were recorded with a Millar piezo-resistive pressure transducer (Millar SPT 301, Millar Instruments, Houston, USA) coupled to a SphygmoCor device (AtCor Medical, Sydney, Australia). Supine blood pressure recorded with an automated digital oscillometric sphygmomanometer was employed for calibration, and with the obtained waveform the corresponding central (ascending aortic) waveform was generated using a generalised and validated [328] transfer function. Pulse pressure was defined as the difference between SBP and DBP. cPP and pPP were calculated based on aortic SBP and DBP and corresponding brachial readings in supine position, respectively. Mean arterial pressure in supine position was calculated with the integration of the radial waveform. The pulse pressure ratio was defined as cPP/pPP.

3.2.3 Biomarkers of the intermediate cardiovascular phenotype

Measurement of C-IMT was performed as described in chapter 2.6.1. Echocardiography followed a standardised procedure in all centres as described in chapter 2.7. According to ECG criteria LVH was present in 9% of study participants recruited in Glasgow. Data from other study centres were unavailable.

Aortic PWV was measured as described in chapter 2.6.5. Exceptions are the centres in Brescia and Paris who used the Complior (Colson, Garges-les-Gonesse, France), and the centre in Nancy who used the PulsePen device (DiaTecne s.r.l., Milan, Italy). The SphygmoCor and PulsePen devices used the subtracted distance approach to estimate travel distance, whereas the Complior device

used the direct carotid-femoral distance. PWV was determined from the foot-to-foot time interval of waveforms. PWV based on direct carotid femoral distance was converted to PWV based on subtracted distance.

Urinary albumin:creatinine ratio (ACR) was measured in local laboratories according to local practice. Measurements below the local detection limits were not further evaluated (n=85). Subjects with macroalbuminuria (ACR≥25 mg/mmol in men and ≥35 mg/mmol in women, n=2) were excluded from analysis. The eGFR was calculated with the MDRD formula.

3.2.3.1 Statistical analyses

Data were analysed using SPSS software (version 15.0). Correlations were assessed by calculating Pearson's correlation coefficient. We used Steiger's Z-test to compare bivariate correlations within a single population. To explore the effects of several influencing factors on the correlations between pulse pressure and intermediate phenotypes a two-step approach was used. First, multivariable regression models containing either cPP or pPP and other possible determinants of target organ damage were designed. Secondly, the independent variables were excluded if not significantly contributing to a regression model containing the pulse pressures. The corresponding models containing either cPP or pPP were compared using a one sample *t*-test on the differences between the residuals ($|RES_{cPP}|-|RES_{pPP}|$) of the respective regression models.

3.3 Results

3.3.1 Demographics

Clinical characteristics of the study cohort are shown in Table 3.2. Study centre distribution is illustrated in Figure 2.1. Clinical characteristics stratified by study centre are listed in Table 3.3, and chapter relevant biomarkers values (age, cPP, pPP, PWV and C-IMT) are illustrated for each study centre in Figure 3.1. Also the gender distribution is illustrated in Figure 3.2. There were no statically significant differences between the gender for age and the biomarkers cPP, PWV and C-IMT. Peripheral pulse pressure and LVMI were higher in men (P<0.001).

Table 3.2 Clinical characteristics.

	Hypertensive n=331	**Normotensive n=204**	***P*-value**
Age, years	51±15	39±16	<0.001
Sex, male/female	156/175	85/119	0.026
Height, cm	168±9	168±9	ns
Weight, kg	79.6 [70.0;91.0]	70.9 [61.5;82.2]	<0.001
BMI, kg/m²	27.7 [25.0;31.4]	24.9 [22.0;28.4]	<0.001
Sitting pSBP, mmHg	141±16	128±14	<0.001
Sitting pDBP, mmHg	85±10	78±9	<0.001
Sitting Heart rate, /min	69±12	71±10	ns
Coronary heart disease, %	4.4	2.0	0.02
Creatinine, µmol/L	78 [64;87]	72 [62; 83]	0.01
eGFR, ml/min	89±21	99±25	<0.001
Diabetes mellitus, %	10.0	3.0	<0.001
Supine pSBP, mmHg	135 [125;146]	126 [117;135]	<0.001
Supine pDBP, mmHg	81±11	76±9	<0.001
Supine mean arterial pressure, mmHg	100±12	93±11	<0.001
Supine cSBP, mmHg	123 [114;135]	113 [102;124]	<0.001
Supine cDBP, mmHg	82±11	77±10	<0.001
Supine pPP, mmHg	54 [46;63]	49 [43;57]	<0.001
Supine cPP, mmHg	42 [35;50]	34 [29;43]	<0.001
C-IMT, mm	0.62 [0.52;0.75]	0.55 [0.48;0.65]	<0.001
LVMI, $g/m^{2.7}$	41.6 [35.4;49.0]	35.4 [30.5;41.5]	<0.001
PWV, m/s	8.12 [7.08;9.70]	7.00 [6.10;7.88]	<0.001
ACR, mg/mmol	0.74 [0.48;1.28]	0.69 [0.39;1.17]	ns

All data are presented as mean ± standard deviation or median [interquartile range]. SBP, DBP and PP denote systolic blood pressure, diastolic blood pressure and pulse pressure, respectively, with "p" and "c" indicating peripheral (brachial) and central (aortic) readings. BMI, body mass index (weight/height^2); C-IMT, carotid intima-media thickness; LVMI, left ventricular mass index; PWV, aortic pulse wave velocity, ACR (n=338), albumin:creatinine ratio. Diabetes status was assessed by interview/questionnaire and random glucose where required

Table 3.3 Clinical characteristics stratified by study centre.

Centre	Brescia, Italy	Paris, France	Nancy, France	Glasgow, UK	Leuven, Belgium	Valencia, Spain	Lund, Sweden	Krakow, Poland	ANOVA, *P*-value
N	45	12	25	148	78	63	37	127	-
Hypertension, yes/no	28/17	12/0	18/7	85/63	37/41	42/21	21/16	88/39	0.004
Gender, male/female	21/24	5/7	9/16	60/88	37/41	34/29	19/18	54/73	ns
Age, years	49±16	59±14	47±19	44±16	50±14	45±15	56±15	41±15	<0.001
Sitting SBP, mmHg	130±16	135±16	146±18	138±16	133±15	134±18	142±17	136±15	<0.001
Sitting DBP, mmHg	82±9	82±7	85±9	82±10	84±9	79±12	83±10	82±10	ns
HR, /min	68±8	65±16	67±8	72±12	64±9	72±4	68±10	72±10	<0.001
Supine pSBP, mmHg	129±17	126±9	126±15	133±17	134±15	131±18	129±15	136±17	0.021
Supine pDBP, mmHg	77±4	70±7	68±8	79±9	83±9	78±12	77±9	81±11	<0.001
Supine cSBP, mmHg	119±18	117±12	112±15	121±18	125±16	120±17	118±15	123±19	0.023
Supine cDBP, mmHg	78±11	71±7	69±8	80±9	84±9	79±12	78±9	82±4	<0.001
Height, cm	167±10	166±9	170±9	169±9	169±10	165±11	172±10	169±9	ns
Weight, kg	73±14	74±13	75±15	79±19	80±18	82±18	78±16	76±16	ns
BMI, kg/m^2	26±4	27±5	26±5	28±6	28±5	30±6	27±5	27±5	0.001
Creatinine, µmol/l	70±22	82±23	95±16	76±15	88±29	78±19	75±14	67±12	<0.001
eGFR, ml/min	100±22	80±17	68±12	91±19	78±18	92±19	89±18	108±25	<0.001

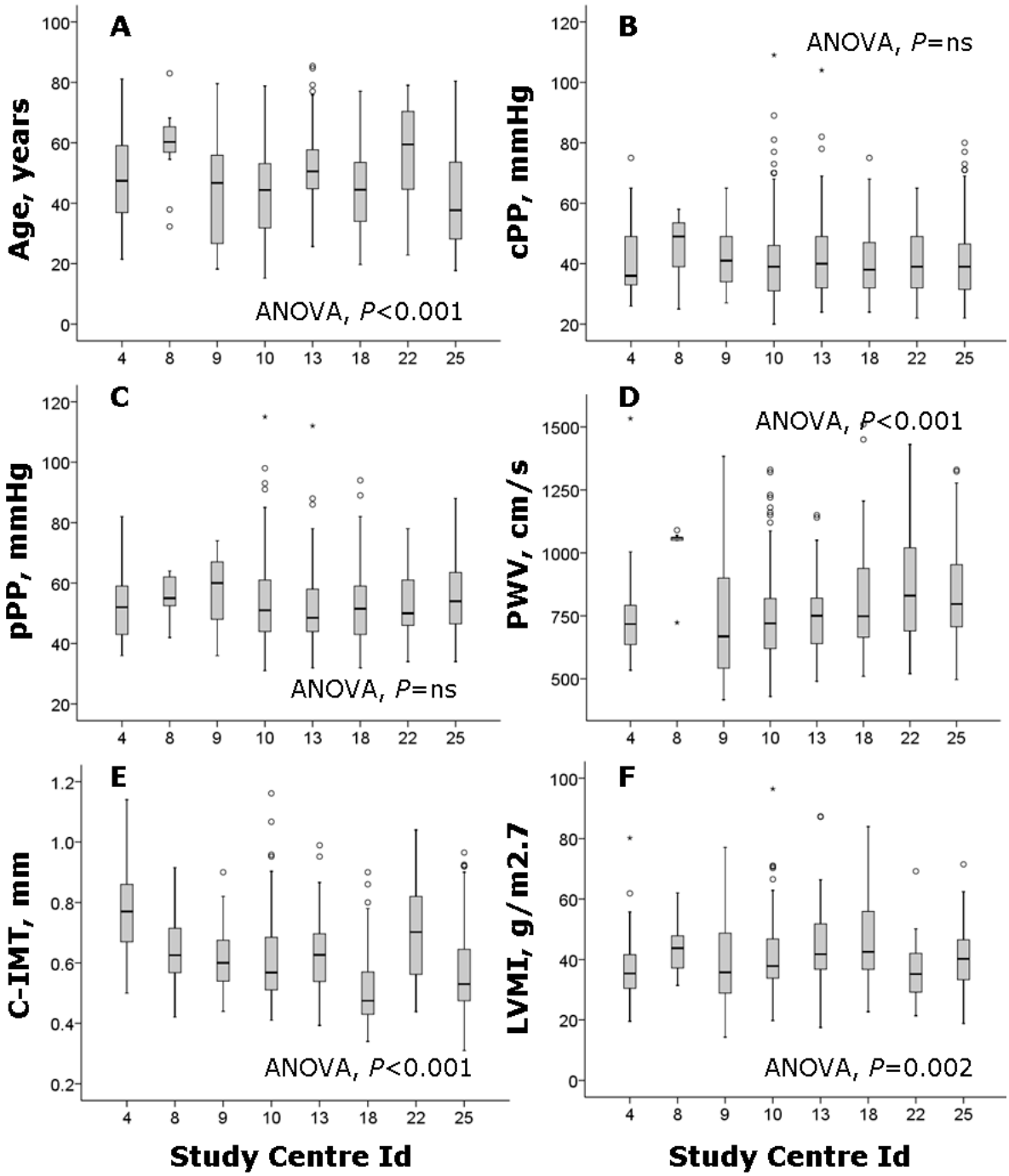


Figure 3.1 Comparison of surrogate biomarkers between study centres. Centres are depicted by their study identification number (4=Brescia, Italy; 8=Paris, France; 9=Nancy, France; 10=Glasgow, UK; 13=Leuven, Belgium; 18=Valencia, Spain; 22=Lund, Sweden; 25=Krakow, Poland).

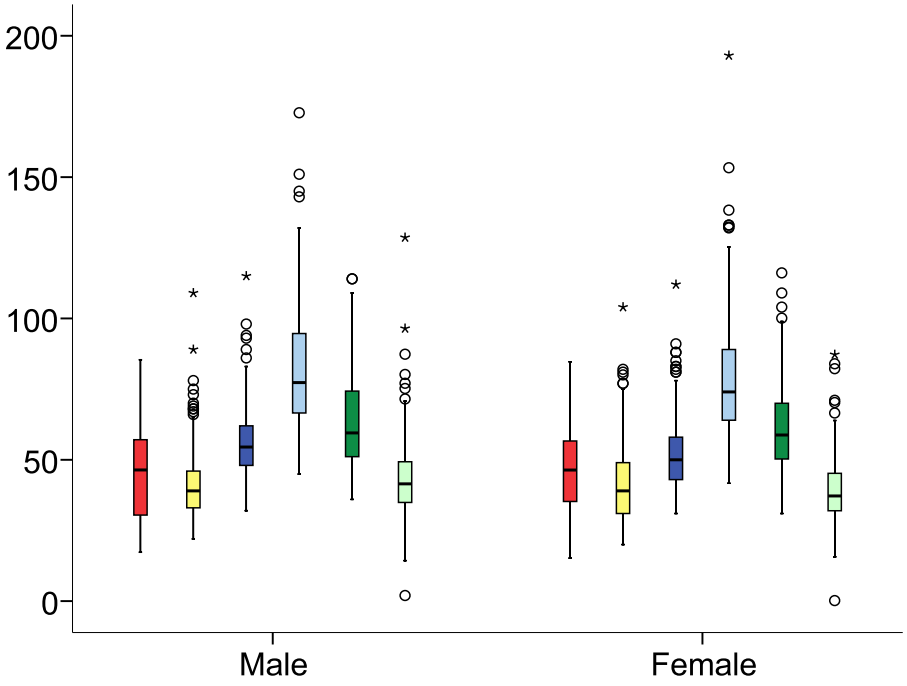


Figure 3.2 Surrogate biomarkers stratified by gender. From left to right are depicted age in years (red), cPP in mmHg (yellow), pPP in mmHg (dark blue), PWV in dm/s (light blue), C-IMT in 10^{-2} mm (dark green) and LVMI in g/$m^{2.7}$. Except higher pPP and LVMI in men (P<0.001), no significant differences between the genders were observed (P=ns).

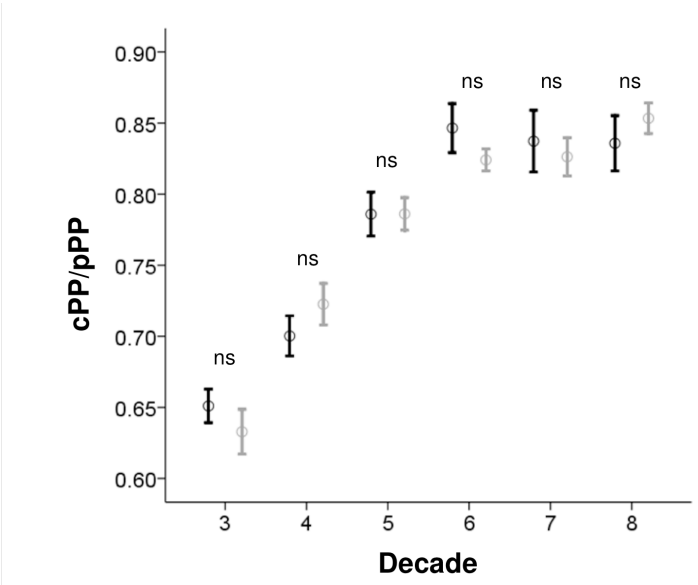


Figure 3.3 Pulse pressure ratios. Comparison of pulse pressure ratio (cPP/pPP) in hypertensive (grey) and normotensive (black) groups by decade. Error bars represent standard errors.

Hypertension was diagnosed in 79% (n=261) of study participants before the age of 50 years. In the hypertensive group 84% (n=273) were treated, of those 83% (n=227) were at least on two different drugs (70% with angiotensin-converting enzyme inhibitor or angiotensin receptor blocker, 46% with β-blocker, 38% with

calcium channel blocker, 52% with diuretics and 5% with other antihypertensive agents). Of the hypertensive subjects 52% had a SBP higher than 140 mmHg and 31% a DBP higher than 90 mmHg. Normotensive subjects in our study were characterised by higher PWV compared to reference values (Table 3.4). Pulse pressure ratio progressively increased through the first six decades of life, with no difference between normotensive and hypertensive subjects across all decades of age (Figure 3.3). Therefore the cohort was investigated as a whole.

Table 3.4 PWV according to age category in normotensive subjects.

Age category (years)	N	$PWV_{sphygmocor}$ Median (10-90 pc)	$PWV_{direct\_distance}$*0.8 Median (10-90 pc)	$PWV_{Reference}$ Median (10-90 pc)	Numerical comparison
<30	37	6.1 (4.8-7.0)	6.8 (5.6-7.7)	6.1 (5.3-7.1)	>
30-39	24	7.0 (5.8-7.8)	7.8 (6.3-8.7)	6.4 (5.2-8.0)	>
40-49	21	6.4 (5.3-7.1)	7.1 (5.9-7.8)	6.9 (5.9-8.6)	≈
50-59	8	7.5 (6.0-7.6)	7.9 (6.5-7.9)	8.1 (6.3-10.0)	≈

For comparison with $PWV_{Reference}$ values of a normal population with supine optimal or normal BP [369], PWV as measured by the Sphygmocor device was transformed according to the papers specifications ($PWV_{direct\ distance}$*0.8) and displayed in m/s. Only data from subjects with blood pressure <130/85 mmHg are displayed in this table as normal values were restricted to this BP range [369].

3.3.2 Univariate analysis

cPP, pPP, C-IMT, PWV, LVMI and ACR were not normally distributed and were log transformed to obtain normally distributed residuals for all regression models. Unless otherwise stated data on these phenotypes refer to log transformed values. cPP and pPP were strongly correlated (r=0.845; P<0.001). There were strong correlations between age and cPP (r=0.559; P<0.001) and pPP (r=0.277; P<0.001), PWV (r=0.563; P<0.001), C-IMT (r=0.652; P<0.001), LVMI (r=0.563; P<0.001) and eGFR (r=-0.517, P<0.001); the correlation between age and ACR was not statistically significant (r=0.101, P=0.063). The association between cPP and age was significantly stronger (Z=13.1, P<0.01) than that between pPP and age. Scatterplots of intermediate phenotypes vs age are depicted in Figure 3.4.

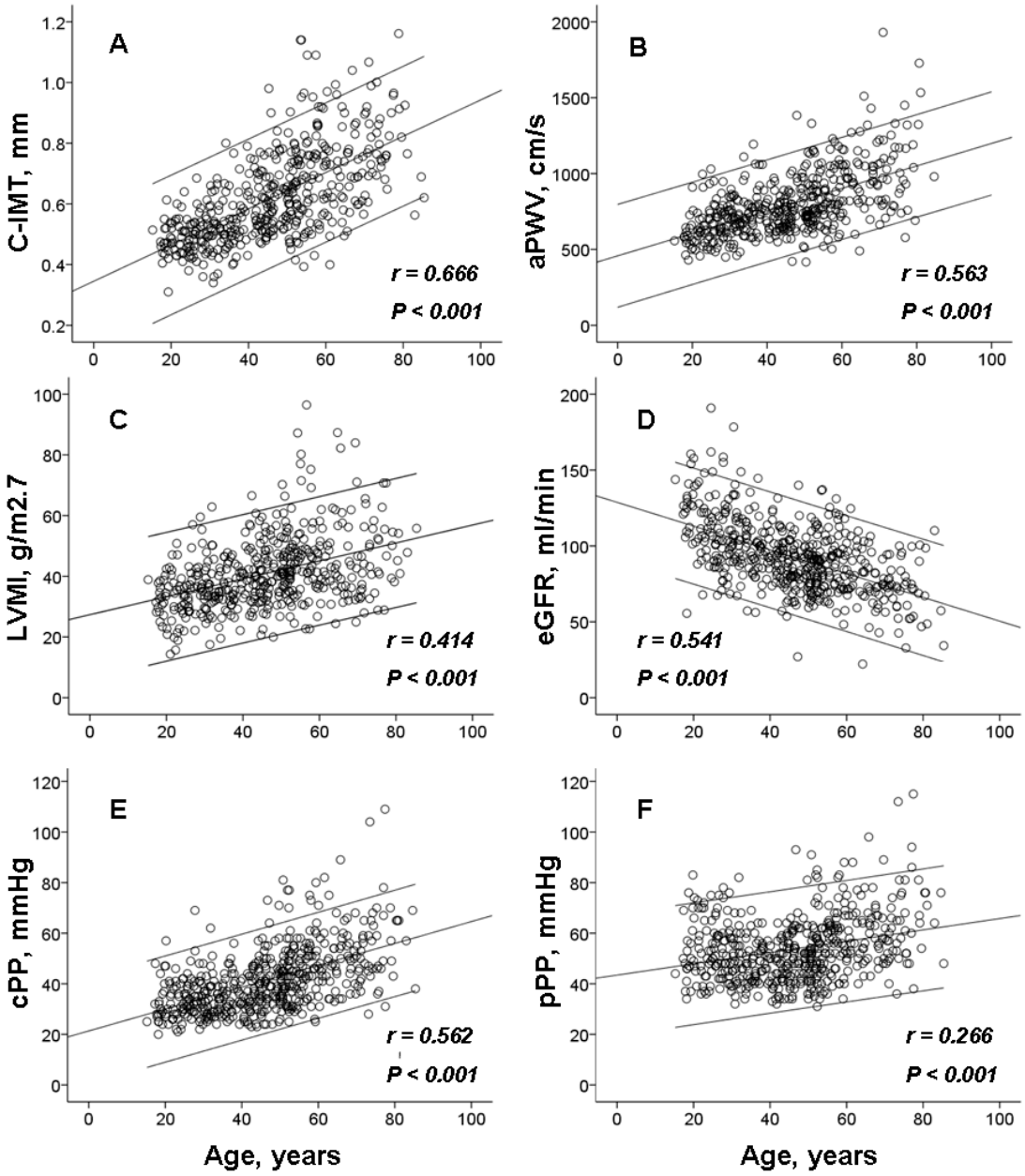


Figure 3.4 Relationship of age to CVD biomarkers. Shown are the relation of age to C-IMT (A), arterial PWV (B), LVMI (C), eGFR (D), cPP (E) and pPP (F). Depicted are 95% confidence intervals around linear fitting line as well as Spearman correlation coefficients. Only the association age to eGFR is represented with a Pearson correlation coefficient.

We then compared the correlations between cPP or pPP and intermediate cardiovascular phenotypes including PWV, C-IMT, LVMI and ACR (Figure 3.5). Correlations between pPP and eGFR were not statistically significant, and were not investigated further. With exception of ACR, correlations between cPP and intermediate phenotypes were stronger than those with pPP in these univariate analyses (Table 3.5). The findings were similar for the association of central or peripheral SBP with intermediate phenotypes (Table 3.5).

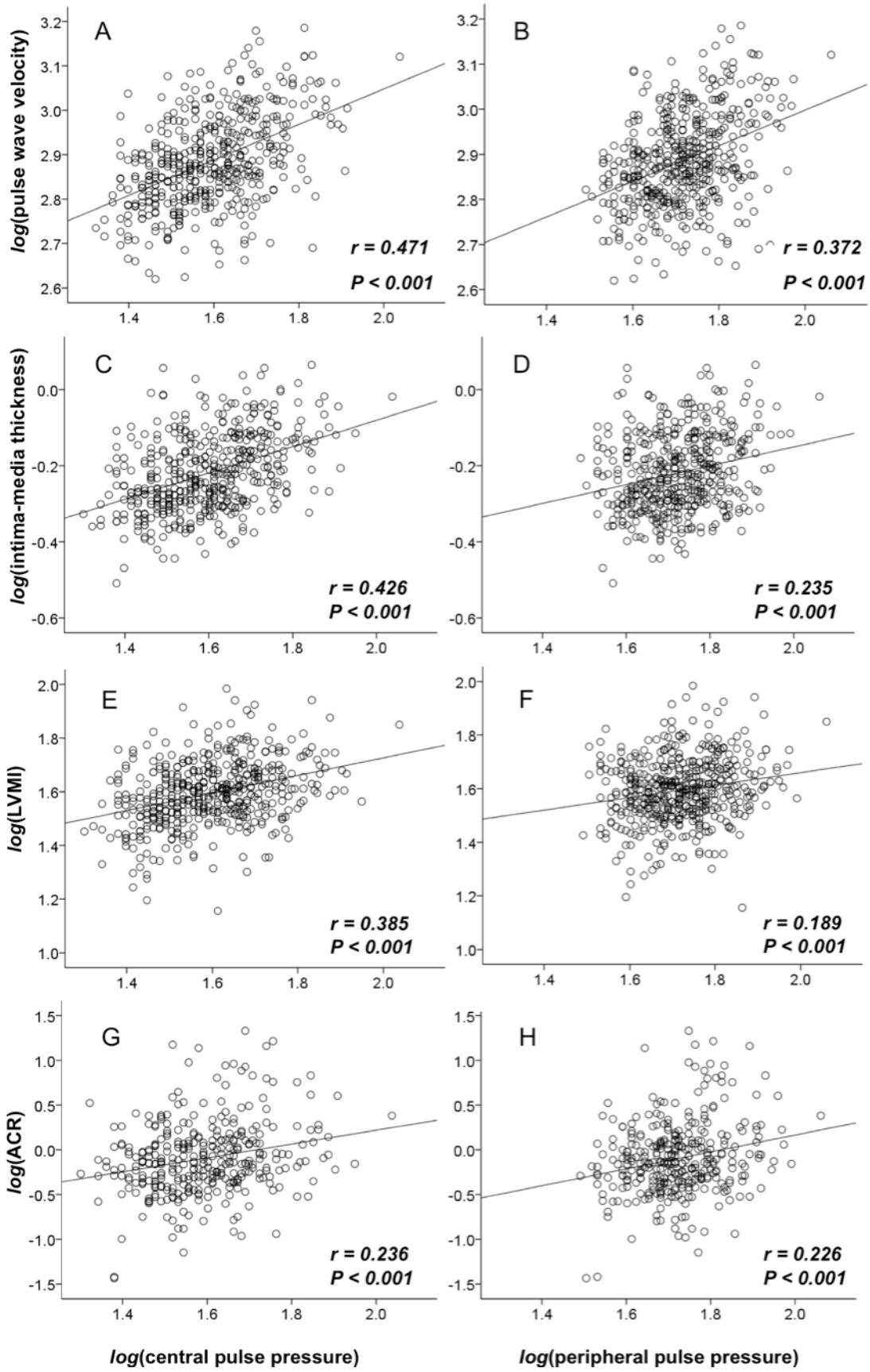


Figure 3.5 Relationship between pulse pressure and surrogate biomarkers. Scatterplots between log(pulse wave velocity) and either log(central pulse pressure) (A) or log(peripheral pulse pressure) (B). Scatterplots between log(carotid intima-media thickness) and either log(central pulse pressure) (C) or log(peripheral pulse pressure) (D). Scatterplots between log(LVMI) and either log(central pulse pressure) (E) or log(peripheral pulse pressure) (F). Scatterplots between log(albumin creatinine ratio) and either log(central pulse pressure) (G) or log(peripheral pulse pressure) (H).

Table 3.5 Correlations between pulse pressure and surrogate biomarkers.

	Pearson's correlation coefficient				Steiger's *Z*	*P*-value
	cPP	pPP	cSBP	pSBP		
C-IMT	0.426	0.235	-	-	7.789	< 0.01
	-	-	0.478	0.417	3.112	< 0.01
PWV	0.471	0.372	-	-	4.142	< 0.01
	-	-	0.326	0.193	5.868	< 0.01
LVMI	0.385	0.189	-	-	6.015	< 0.01
	-	-	0.391	0.297	4.445	< 0.01
ACR	0.236	0.226	-	-	0.3393	ns
	-	-	0.197	0.199	-0.0673	ns

Pearson's correlation coefficients are displayed in the table and were calculated on log transformed variables. All correlations were significant (*P* < 0.01). *Z* and *P*-values refer to the comparison between central (cPP) and peripheral pulse pressure (pPP) or central (cSBP) and peripheral systolic blood pressure (pSBP). C-IMT, carotid intima-media thickness; PWV, pulse wave velocity; LVMI, left ventricular mass index; ACR, albumin creatinine ratio.

3.3.3 Multivariate analysis

We accounted for other potential determinants in linear regression analysis (Table 3.7). Models contained hypertension status, mean arterial pressure, heart rate, gender, diabetes status and BSA and were reduced to those variables in addition to cPP or pPP that contributed significantly (P<0.05) to the model (Table 3.6). A regression model for pPP was developed using a stepwise approach to include and exclude potential determinants. The significant determinants were then forced into a regression model for cPP in order to directly compare the two models.

In these analyses the coefficients of variation were similar between models containing cPP or pPP, indicating similar predictive value of cPP and pPP when adjusted for relevant cofactors. To evaluate this formally, variability of the

residuals was compared between the models. The difference between the models containing cPP or pPP was borderline significant for prediction of PWV (P=0.066) with pPP having numerically the higher adjusted coefficient of determination (Table 3.6). Similarly, there was no difference between models to predict C-IMT (P=0.487) and LVMI (P=0.094) that contained cPP or pPP. We have further studied these models separately in individuals without β-blocker therapy (n=394; PWV, P=0.050; C-IMT, P=0.844; LVMI, P=0.867), in normotensive subjects (n=204; PWV, P=0.139; C-IMT, P=0.795; LVMI, P=0.917) and in hypertensive subjects (n=331; PWV, P=0.242; C-IMT, P=0.575; LVMI, P=0.989); and again no significant difference in the adjusted coefficients of determination between models with cPP and those with pPP was detected.

Table 3.6 Best fitting regression models containing either cPP or pPP for prediction of intermediate cardiovascular phenotypes.

	Central		***Peripheral***	
	β	***P*-value**	**β**	***P*-value**
		***log*(PWV)**		
R^2adj	0.399		0.413	
Hypertension, (yes/no)	0.112	0.003	0.086	0.020
Heart Rate, bpm	0.128	0.001	0.098	0.009
Age, yrs	0.460	<0.001	0.515	<0.001
MAP, mmHg	0.089	0.044	0.111	0.006
***log*(PP)**	0.226	<0.001	0.220	<0.001
		***log*(C-IMT)**		
R^2adj	0.429		0.428	
Age, yrs	0.598	<0.001	0.633	<0.001
***log*(PP)**	0.097	0.020	0.076	0.037
		***log*(LVMI)**		
R^2adj	0.181		0.170	
Age, yrs	0.318	<0.001	0.386	<0.001
***log*(PP)**	0.160	0.002	0.085	0.051

Regression models containing either *log*(cPP) or *log*(pPP) and determining *log*(PWV), *log*(C-IMT) and *log*(LVMI) are summarised. Adjusted coefficients of variation (R^2adj), β-coefficients and *p*-values corresponding to the independent variables of the regression models are listed. Full models are shown in Table 3.7.

Table 3.7 Linear regression models incorporating either cPP or pPP

	log(C-IMT)				log(PWV)				log(LVMI)			
Variables	B	SE	β	*p*-value	B	SE	β	*p*-value	B	SE	β	*p*-value
R^2adj (log cPP)	0.443				0.410				0.245			
Constant	-0.562	0.088	—	<0.001	2.346	0.094	—	<0.001	1.481	0.100	—	<0.001
log cPP	0.088	0.038	0.108	0.021	0.189	0.040	0.226	<0.001	0.043	0.052	0.045	0.402
BSA, m²	0.067	0.022	0.144	0.002	0.025	0.022	0.054	0.265	-	-	-	-
Hypertension, yes/no	-0.013	0.009	-0.060	0.140	0.022	0.009	0.106	0.009	0.019	0.012	0.075	0.106
MAP, mmHg	0.001	0.001	-0.049	0.256	0.001	0.001	0.034	0.431	0.002	0.001	0.156	0.001
Heart Rate, bpm	0.001	0.001	-0.053	0.165	0.001	0.001	0.139	<0.001	-0.001	0.001	-0.094	0.033
Age, years	0.004	0.001	0.619	<0.001	0.003	0.001	0.426	<0.001	0.002	0.001	0.266	<0.001
Gender, m/f	-0.003	0.009	-0.015	0.733	-0.010	0.010	-0.051	0.281	-0.034	0.010	-0.139	0.001
Diabetes mellitus, yes/no	-0.005	0.015	-0.012	0.737	-0.030	0.016	-0.069	0.070	-0.057	0.021	-0116	0.007
R^2adj (log pPP)	0.440				0.418				0.244			
Constant	-0.557	0.097	—	<0.001	2.233	0.102	—	<0.001	1.600	0.114	—	<0.001
log pPP	0.074	0.041	0.070	0.075	0.229	0.042	0.218	<0.001	-0.040	0.056	-0.032	0.480
BSA, m²	0.064	0.022	0.138	0.003	0.025	0.022	0.054	0.267	-	-	-	-
Hypertension, yes/no	-0.012	0.009	-0.057	0.159	0.023	0.008	0.109	0.007	0.019	0.012	0.075	0.106
MAP, mmHg	0.001	0.001	-0.034	0.411	0.001	0.001	0.041	0.330	0.002	0.001	0.181	<0.001
Heart Rate, bpm	-0.001	0.001	-0.071	0.057	0.001	0.001	0.109	0.004	-0.001	0.001	-0.104	0.015
Age, years	0.004	0.001	0.650	<0.001	0.003	0.001	0.487	<0.001	0.002	0.001	0.289	<0.001
Gender, m/f	-0.001	0.009	-0.006	0.895	-0.003	0.010	-0.014	0.771	-0.034	0.010	-0.141	0.001
Diabetes mellitus, yes/no	-0.006	0.016	-0.013	0.713	-0.026	0.016	-0.061	0.107	-0.062	0.021	-0.125	0.003

Regression analysis containing either log(cPP) or log(pPP) and determining log(PWV), log(C-IMT) and log(LVMI) with all variables forced into the models are summarised. Adjusted coefficients of variation (R^2adj), β-coefficients and *p*-values corresponding to the independent variables of the regression models are listed. Reduced models are shown in Table 3.6.

3.4 Discussion

3.4.1 Pulse pressures and intermediate phenotypes

Intermediate cardiovascular phenotypes reflect progression of cardiovascular disease and help to assess response to therapeutic intervention. Therefore, assessment of subclinical organ damage by measuring C-IMT, left ventricular hypertrophy and PWV contributes to cardiovascular risk stratification [172, 174, 370, 371].

PP is a strong determinant of subclinical and overt organ damage [368, 372, 373]. In univariate analysis we have demonstrated for the majority of intermediate cardiovascular phenotypes a stronger correlation with cPP than with pPP. For C-IMT, for example, the univariate models translate into an increase in C-IMT by 0.035 mm per 10 mmHg of cPP and 0.025 mm by 10 mmHg of pPP. This finding is in line with previous studies on C-IMT [366, 372] and LVMI [368] where local PP was found to be more strongly associated compared to peripheral PP. In our study, similar results were obtained for the relationships between central or peripheral SBP and subclinical organ damage. The equally strong association of cPP and pPP with ACR can be explained with the changes of PP along the arterial tree. It has been reported that the increase of PP from the central aorta to the aorta at the level of renal arteries [374] is similar to the difference between cPP and pPP [375].

Despite the apparently stronger association of cPP with end organ damage, multivariate models containing either cPP or pPP were equally strong in predicting intermediate cardiovascular phenotypes. As indicated by the β-coefficients, age is the major contributor to these models, and in line with previously reported data [375] we confirmed that cPP is stronger associated with age than pPP. Consequently, multivariate models containing pPP and age had similar coefficients of determination as those containing cPP. Similar observations were made previously for models predicting C-IMT from local and peripheral PP [372], and we have now extended these findings to other intermediate phenotypes in a relatively large cohort.

3.4.2 Specific characteristics of the study cohort

A large number of our study participants had early-onset hypertension and were treated for several years. Furthermore the majority of subjects had ≥ grade 2 hypertension and despite treatment only 48% of hypertensives had a normalised blood pressure owing to recruitment of the majority of index patients from clinics in tertiary referral centres. As the cohort mostly represents mixed systolic and diastolic hypertension the results do not cover rarer forms as isolated systolic hypertension in the young. The study design favoured inclusion of patients with a strong genetic component to their blood pressure.

This might explain why the PP ratio in our normotensive subjects was greater than that in a previous study [360]. These findings in our normotensive study participants could indicate early vascular ageing [376] and support a role of genetic factors in the development of vascular stiffness [377, 378]. Despite its cross-sectional nature the cohort displays different stages of the hypertension continuum.

Antihypertensive therapy leads to reduction of left ventricular hypertrophy [207], PWV [364] and C-IMT [379] which may affect some of our findings. It should further be noted that antihypertensive therapy exerts different effects on cPP and pPP. In particular β-blockers have been found to reduce cPP less substantially than calcium channel blockers in association with angiotensin-converting enzyme inhibitors or angiotensin receptor [363, 364]. We are, however, confident that the primary finding of our study is not affected by treatment as after exclusion of all subjects treated with β-blockers the variability of the residuals was not statistically significant between the regression models containing cPP or pPP.

3.4.3 Limitations

Our present study analysed data from several European centres that were generated according to predefined protocols but not read centrally in a core lab. We have therefore compared data and the spread of data between the centres. Although on formal testing there were differences between centres for some of the phenotypes the spread of data appeared acceptable; cPP and pPP were in

fact similar across all study centres. Moreover, the tight relationships between age and markers of organ damage and the lack of significant gender effects are reassuring of the quality of data. We do, however, acknowledge that this cross-sectional study does not prove causality and that the regression models only contain some of the potential determinants of target organ damage. For example, the etiology of left ventricular hypertrophy is complex and includes factors other than blood pressure.

3.4.4 Summary

Increased arterial stiffness and accelerated age-associated vascular changes are typical features in patients with diabetes mellitus [380], cardiovascular risk factors [381] and chronic renal failure [382]. Relationships between central haemodynamic parameters and intermediate phenotypes have been reported previously in cross-sectional studies [366, 368, 373]. Longitudinal studies focusing on populations with high prevalence of diabetes mellitus [366], CAD [383] and end-stage renal disease [367] showed that cPP is a better predictor of outcome than pPP. Furthermore, augmentation pressure and index, other central haemodynamic parameters, were found to improve prediction of major cardiovascular events in a cohort with high prevalence of CAD independently of pPP [176]. In contrast, a recent meta-analysis showed no significant advantage of central over peripheral PP in longitudinal studies [384]. Evidence for cPP being more predictive in elderly hypertensive patients [365, 385] is controversial.

In our middle aged cohort with high prevalence of hypertension cPP is more closely related to cardiovascular phenotypes than pPP. When adjusted for relevant cofactors, however, cPP does not provide additional information beyond pPP. Approximately 50% of hypertensive patients in this cohort were not treated to target. We therefore speculate that our findings may extend to untreated patients with hypertension. In these patients non-invasive assessment of cPP may therefore not provide additional information to brachial pulse pressure.

Considering the chronic progressive character of the cardiovascular continuum these findings imply that biomarkers with prognostic value in later stages of the

CVD process are not necessarily predictive in the earlier disease stages. Regarding the investigated biomarker cPP it remains uncertain at which point of the cardiovascular continuum the biomarker becomes predictive. Early onset hypertension and being of middle age however position the individual or study cohort on the cardiovascular continuum prior to this point.

Chapter 4 Plasma biomarkers in coronary artery disease

4.1 Introduction

Cardiovascular disease (CVD) is a chronic progressive condition. This includes early arterial dysfunction, different degrees of atherosclerosis and myocardial damage or stroke. Considering this wide range of disease stages Dzau and Braunwald [54] introduced the concept of the cardiovascular continuum implying more disease burden at later stages. This includes higher quantities of serologic circulating biomarkers. In this context a number of circulating biomarkers were shown to indicate a high risk for CAD in patients with stable angina like symptoms. Examples of such circulating biomarkers are troponin I [217], NT-pro BNP [218], CRP, IL-6 and ICAM-1 [108]. The accurate diagnosis of stable angina is clinically important as patients have an increased cardiovascular risk and benefit from secondary preventative measures.

These circulating biomarkers cover different pathophysiological aspects of atherosclerosis. We therefore hypothesised that assessment of multiple circulating biomarker could improve the diagnosis of CAD in stable angina patients, especially as the simultaneous assessment of multiple circulating biomarkers has been shown to improve cardiovascular risk prediction. For instance the combination of troponin I, NT-pro BNP, cystatin C and CRP can improve cardiovascular risk stratification by traditional risk factors in elderly men [61]. Also an approach with 9 circulating biomarkers in addition to the urinary albumin-to-creatinine ratio contributed to risk prediction in the Framingham study [60]. In contrast, in the HOPE study only NT-pro BNP out of 11 biomarkers improved risk prediction by traditional risk factors [62]. We therefore evaluated the usefulness of different circulating biomarkers for diagnosing CAD in two different cohorts in the following chapter. Both cohorts represent populations at different stages of the cardiovascular continuum. The cohort with the greater disease burden was investigated first to screen for potential markers.

4.2 Material and Methods

4.2.1 Study cohort

A number of circulating biomarkers were tested in a convenience sample of patients with established CAD and age and sex matched controls of the VASCAB study and a convenience sample of patients with angiographically confirmed CAD and age matched patients with angiographically excluded CAD of the DiCADu study. The VASCAB study was a cross-sectional cohort study described in detail in chapter 2.1.1. Cases in the study had either severe three vessels or left main stem CAD in accordance with contemporary guidelines [344-347]. The DiCADu study cohort was described in chapter 2.1.3. The West of Scotland Research Ethics Committee approved both studies. Details on both studies are provided in Chapter 2.

4.2.2 Plasma Biomarker Studies

Blood samples were collected after at least three hours of fasting. Blood was sampled with the Vacutainer® system (BD, Franklin Lakes, USA). Serum and EDTA plasma samples were centrifuged immediately after collection at 3000 G for 10 minutes and the supernatant was kept at -80 °C using 1.5 ml Screwcap MCT tubes (Alpha Laboratories, Eastleigh, UK). For both studies CRP and cholesterol levels, as well as uric acid for the DiCADU study, were analysed in the clinical biochemistry laboratories at Gartnavel General Hospital, Glasgow.

For the VASCAB study additional biomarkers were analysed using WideScreen® BeadPlex™ multiplex assays (Merck, Darmstadt, Germany) on a Luminex® xMAP® Technology platform (Luminex Corporation, Austin, USA) as detailed in chapter 2. Biomarkers were measured in serum and included IL-8, MCP-1, MIP-1β, TNFα, E-selectin, ICAM-1, leptin, P-selectin, sRAGE, adiponectin, cystatin C, en-RAGE, PAI-1 and VCAM-1.

In DiCADu study plasma samples a number of cardiac biomarkers were measured using a Randox Evidence Investigator and the producers Cardiac Array (Northern Ireland, United Kingdom). A sandwich chemiluminescent immuno - assay used a Randox Biochip containing an array of discrete test regions of immobilised antibodies specific to Troponin I, myoglobin, CK-MB, CA III, GPBB and hFABP. In

the DiCADu study IL-6 and NT-proBNP were also quantified as described in chapter 2.

All biomarkers are briefly summarised in Table 4.1.

Table 4.1 Plasma related biomarkers quantified in the VASCAB and DiCADu study.

Biomarker	Definition	Brief summary
1. Inflammation		
- CRP	C-reactive protein	Acute phase protein, synthesised in the liver
- IL-6	Interleukin-6	Interleukin amongst others secreted by vascular smooth muscle cells with pro-inflammatory properties
- IL-8	Interleukin-8	Chemokine inducing chemotaxis in granulocytes and macrophages, also a potent promoter of angiogenesis
- TNFα	Tumour necrosis factor alpha	Cytokine involved in systemic inflammation, stimulant of the acute phase reaction
- ICAM-1	Intercellular Adhesion Molecule 1	Endothelium and leukocyte associated transmembrane protein stabilizing cell-cell interaction and important in leukocyte endothelial transmigration
- VCAM-1	Vascular cell adhesion molecule 1	Expressed after cytokine stimulation of vascular endothelial cells, mediates the adhesion of lymphocytes, monocytes, eosinophils and basophils
- MCP-1	Monocyte chemotactic protein 1	Involved in recruitment of monocytes, memory T cells and dendritic cells to inflamed tissue caused by injury or infection
- MIP-1β	Macrophage inflammatory protein 1β	Chemoattractant for natural killer cells and monocytes
- E-selectin	aka. Endothelial-leukocyte adhesion molecule 1	Cell adhesion molecule expressed exclusively on endothelial cells after cytokine activation
- P-selectin	aka. Platelet activation dependent granule to external membrane protein	Essential component of the initial leukocyte recruitment to tissue inflammation, as well as for the recruitment and aggregation of platelets to sites of vascular injury
- sRAGE	Soluble receptor for advanced glycation end products	Involved in chronic inflammation with positive feed back loop
- enRAGE	Extracellular receptor for advanced glycation end product – binding protein	Secreted by neutrophils either in inflamed tissue or in the blood stream, transducing pro-inflammatory signals and functioning as a chemoattractant
2. Decreased fibrinolysis		
- PAI-1	Plasminogen Activator Inhibitor-1	Principal inhibitor of tissue plasminogen activator and urokinase produced by endothelial cells

3. Metabolic Hormones		
- Leptin	-	Appetite inhibitor predominantly secreted from white adipose tissue
- Adiponectin	-	Modulator of several metabolic processes including glucose regulation and fatty acid oxidation, exclusively secreted from adipose tissue
4. Kidney Function		
- Cystatin C	-	Ubiquitous protein, functions as an inhibitor of lysosomal proteinases and extracellular inhibitor of cysteine proteases. More precise marker for kidney function than creatinine.
5. Myocardial vulnerability		
- NT-proBNP	N-terminal prohormone of Brain Natriuretic Peptide	The 76 amino acid N-terminal fragment of BNP. Together with BNP secreted by ventricles in response to extensive stretching of heart muscle cells
- CK-MB	Creatine kinase isoenzyme MB	Enzyme catalyzing the conversion of creatinine and ATP to phospho-creatine and ADP, predominantly expressed in heart muscle
- Myoglobin	-	Iron and oxygen binding protein found in all muscle tissues, released from damaged muscle tissue
- GPBB	Glycogen phosphorylase isoenzyme B	Expressed in heart and brain tissue only, very early release after myocardial damage
- hFABP	Heart type fatty acid binding protein	Intracellular fatty acid transporter, released from myocytes following ischaemia
- Troponin I	-	Part of the troponin complex, released after myocardial damage
- CA III	Carbonic anhydrase 3	Metalloenzyme catalysing the hydration of CO_2, exclusive for muscle cells

Listed are abbreviations, full names and brief explanations for each marker. Markers are grouped according to their involvement in cardiovascular disease. ATP, adenosine triphosphate; ADP, adenosine diphosphate, CO_2, carbon dioxide; aka, also known as.

4.2.3 Statistics

As suggested by Lubin et al. [386], measurements below the detection limit were imputed with half of the detection limit when the quantity of such measurements was less than 10% of the total. In case 10-30% of biomarker levels were below the detection limit a random fill-in was chosen. If more than 30% of measurements were below the detection limit multiple imputation was used. In case of troponin I, values below the detection limit of <0.18 ng/ml were imputed using other markers of myocardial ischaemia as predictors.

We adjusted biomarkers for the contributing factors sex, BMI and diabetes in the DiCADu study. Biomarkers which were not normally distributed were log transformed to the base 10. In case the resulting data were normally distributed they were entered into a linear regression model with the additional independent variables sex, BMI and diabetes. Normally distributed data were directly entered in the regression model. Finally the residuals of the regression models of CAD and NCA patients were compared.

The two sample Student's t test or the Mann Whitney test were conducted as appropriate for the comparison of two groups of paired observations for continuous data. For comparison of categorical data of independent groups the Chi-squared test was employed. A stepwise binary logistic regression model was used to test the value of different biomarkers for prediction of CAD. The probability of CAD was calculated as $1/(1+e^{-x})$ where $x=a_1*x_1+a_2*x_2+….+B$ where a is the logistic regression coefficient corresponding the different phenotypes and B is the intercept term. Using corresponding probabilities a ROC-curve was drawn. In general, a *P*-value of less than 0.05 was considered significant. Data were analysed using SPSS software, version 19.0 (SPSS Inc., Chicago, USA).

4.3 Results

4.3.1 Study cohorts

In the VASCAB study 40 patients with severe CAD were age and if possible sex matched with 40 healthy control subjects. To enlarge the investigated cohort four additional CAD subjects with similar age distribution were added. In the DiCADu study 29 patients with significant CAD were age matched with 29 patients with normal coronary arteries. The age distribution of the remaining control subjects allowed to add ten further subjects to the control group. Clinical and demographic data of study participants are shown in Table 4.2.

Table 4.2 Demographic and clinical characteristics of VASCAB and DiCADu patients selected for circulating biomarker investigation.

	DiCADu			VASCAB		
	CAD, n=29	NCA, n=39	P-value	CAD, n=44	Control, n=40	P-value
Age, years	54±6	56±7	ns	62±10	62±9	ns
Sex, m/f	15/14	12/27	ns	42/2	32/8	0.03
BMI, kg/m^2	28±4	28±7	ns	29±5	26±3	0.008
SBP, mmHg	134±18	138±17	ns	136±20	136±19	ns
DBP, mmHg	80±10	81±9	ns	79±12	81±11	ns
Heart rate, /min	57±10	62±10	ns	65±12	67±11	ns
Total cholesterol, mmol/l	4.3 [3.6;5.6]	5.0 [4.3;5.7]	0.029	4.0 [3.5;4.9]	5.6 [4.8;6.2]	<0.001
LDL-cholesterol, mmol/l	2.4±1.1	2.9±1.3	ns	2.1±0.8	3.3±0.8	<0.001
HDL-cholesterol, mmol/l	1.2 [0.9;2.1]	1.2 [1.1;1.7]	ns	1.1±0.3	1.4±0.4	0.001
Triglycerides, mmol/l	1.4 [0.9;2.1]	1.6 [1.0;2.5]	ns	2.0 [1.3;2.7]	1.4 [1.0;2.3]	0.029
Hypertension History, %	68	57	ns	60	23	<0.001
Positive Family History, %	79	86	ns	34	48	ns
Diabetes History, %	17	13	ns	27	0	<0.001
Active smoking, %	30	47	ns	7	5	ns
Statin, %	90	49	<0.001	88	10	<0.001
Aspirin, %	93	36	<0.001	86	15	<0.001
Beta-blocker, %	86	21	<0.001	84	3	<0.001
ACEI/ARB, %	41	28	ns	60	8	<0.001

Data is displayed as means±SD and medians [IQR]. SD, standard deviation; IQR, interquartile range; CAD, coronary artery disease; NCA, normal coronary arteries; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; LDL-cholesterol, low density cholesterol; HDL-cholesterol, high density cholesterol.

4.3.2 Biomarker assessment

Circulating biomarkers in cases and controls of the VASCAB study are shown in Table 4.3. Levels of IL-8, TNFα, en-RAGE, VCAM-1, CRP, cystatin C and PAI-1 were significantly higher in patients with CAD compared to controls. There was no statistically significant difference in levels of MCP-1, MIP-1ß, E-selectin, ICAM-1, leptin, P-selectin, sRAGE and adiponectin. As several inflammatory biomarkers were highly significantly different between patients and controls ($P \geq 0.001$) in the VASCAB cohort measurement of only one or two inflammatory markers was considered reasonable in the DiCADu study to avoid redundancy. Considering costs and availability in local laboratories CRP was selected. IL-6 was previously measured in VASCAB samples, as shown in Table 2.7. Due to a very high number of samples with values below the detection limit multiple imputation appeared not reasonable and results are therefore not provided in Table 4.3. Considering the good evidence for IL-6 in prognosis of CVD, as described in subchapter 1.5.5, the biomarker measured with a different assay was selected for the DiCADu cohort.

Table 4.3 Circulating biomarker levels in the VASCAB cohort.

	CAD (n=44)	Controls (n=40)	p-value
Interleukin-8, pg/ml	3.3 [1.4;5.1]	1.9 [0.4;3.4]	0.009
MCP-1, pg/ml	96 [61;119]	99 [61;15-	ns
MIP-1ß, pg/ml	100 [58;149]	88 [70;128]	ns
TNFα, pg/ml	0.45 [0.03;0.79]	0.15 [0;0.49]	0.03
E-selectin, ng/ml	2.2 [1.3;3.4]	1.9 [1.2;2.4]	ns
ICAM-1, ng/ml	14 [13;18]	13 [11;14]	ns
Leptin, ng/ml	1.5 [0.9;2.4]	0.8 [0.4;1.7]	ns
P-selectin, ng/ml	32±12	32±12	ns
sRAGE, ng/ml	0.8 [0.5;1.6]	1.2 [0.8;1.5]	ns
Adiponectin, ng/ml	558 [175;1418]	322 [91;1015]	ns
Cystatin C, ng/ml	2090 [1088;3215]	760 [406;1607]	<0.001
en-RAGE, ng/ml	18 [5;35]	5 [0;14]	<0.001
PAI-1, ng/ml	60 [26; 143]	15 [10; 45]	<0.001
VCAM-1, ng/ml	422 [145;703]	107 [36;245]	<0.001
CRP, mmol/l	2.6 [1.4;5.9]	1.1 [0.8;2.6]	0.001

Compared are patients with coronary artery disease (CAD) to control subjects. Data is displayed as means±SD and medians [IQR]. SD, standard deviation; IQR, interquartile range; MCP-1, monocyte chemotactic protein 1; MIP-1ß, macrophage inflammatory protein 1ß; TNFα, tumour necrosis factor alpha; ICAM-1, intercellular adhesion molecule 1; VCAM-1, vascular cell adhesion molecule 1; sRAGE, soluble receptor for advanced glycation end products; en-RAGE, extracellular receptor for advanced glycation end product; PAI-1, plasminogen activator inhibitor 1; CRP, C-reactive protein; ns, non-significant.

Table 4.4 Circulating biomarker levels in the DiCADu cohort.

	CAD (n=29)	NCA (n=39)	*p*-value
Uric acid, mmol/l	0.30±0.08	0.31±0.09	ns
NT-proBNP, pg/ml	116 [56;159]	69 [41;139]	ns
CK-MB, ng/ml	128 [1;297]	146 [1;282]	ns
Myoglobin, ng/ml	616 [15;3501]	817 [17;2537]	ns
GPBB, ng/ml	49 [2;187]	108 [2;246]	ns
hFABP, ng/ml	66 [1;136]	74 [1;151]	ns
CA3, ng/ml	1070 [11;2252]	1418 [12;2191]	ns
Troponin I, ng/ml	0.025 [0.012;24]	22 [0.014;25]	ns
IL-6, pg/ml	0.7 [0.5;1.8]	0.9 [0.5;1.4]	ns
CRP, mmol/l	1.9 [1.0;4.3]	4.0 [2.0;7.4]	ns

Compared are patients with coronary artery disease (CAD) to those with normal coronary arteries (NCA). Data is displayed as means±SD and medians [IQR]. SD, standard deviation; IQR, interquartile range; BNP, brain natriuretic peptide; CK-MB, creatine kinase isoenzyme MB; GPBB, glycogen phosphorylase isoenzyme BB; hFABP, heart type fatty acid binding protein; CA3, carbonic anhydrase 3.

To adjust the circulating biomarkers in the DiCADu study for contributing factors data was log transformed. The corresponding data was only normally distributed for NT-proBNP (CAD vs. NCA; 2.0±0.4 vs. 1.9±0.4, P=ns), IL-6 (CAD vs. NCA; -0.06±0.42 vs. -0.05±0.32, P=ns) and CRP (CAD vs. NCA; 0.34±0.51 vs. 0.53±0.45, P=ns). The residuals of the linear regression models with log transformed NT-proBNP, IL-6 or CRP or the original urate levels as dependent variables and independent variables sex, BMI and diabetes are shown in Table 4.5.

Table 4.5 Circulating biomarker levels in the DiCADu cohort after adjustment for sex, BMI and diabetes.

	CAD	NCA	*p*-value
$RES_{Uric\ acid}$	-0.15±0.92	0.16±1.03	ns
$RES_{NT\text{-}proBNP}$	0.06±0.78	-0.04±1.09	ns
$RES_{IL\text{-}6}$	-0.02±1.15	0.01±0.86	ns
RES_{CRP}	-0.17±1.01	0.17±0.93	ns

Compared are patients with coronary artery disease (CAD) to those with normal coronary arteries (NCA). Listed are the residuals of linear regression models containing the named biomarker and sex, diabetes and body mass index. Data is displayed as means±SD and medians [IQR]. SD, standard deviation; IQR, interquartile range; BNP, brain natriuretic peptide; CRP, C-reactive protein; IL-6, interleukin 6.

4.3.3 Multiple marker approach

All biomarkers with a significant difference between CAD patients and controls in the VASCAB study, listed in Table 4.3, were entered into a stepwise regression model. The strongest model with the highest R-value (R^2 = 0.229) contained VCAM-1 and IL-8. The corresponding regression model parameters are listed in Table 4.5 as model 1. The ROC curves illustrating model 1 and its contributing biomarkers are depicted in Figure 4.1. The same figure also contains a list of related c-statistics values. The two biomarker model has an AUC of 0.770. The single markers have an AUC of 0.753 and 0.654 for VCAM-1 and IL-8, respectively.

Table 4.6 Regression models to predict CAD in the VASCAB study.

Biomarker	β-coefficient	*p*-value
	Model 1	
Constant	-	<0.001
VCAM-1	0.402	<0.001
IL-8	0.233	0.027
	Model 2	
Constant	-	<0.001
VCAM-1	0.342	0.001
BMI	0.306	0.004
IL-8	0.257	0.013

For model 1 IL-8, TNFα, cystatin C, en-RAGE, PAI-1, VCAM-1 and CRP were entered into a linear stepwise regression model. For model 2 sex, age, diabetes history, BMI and systolic blood pressure were added to the biomarkers in model 1 for a linear stepwise regression. IL-8, interleukin-8; TNFα, tumour necrosis factor alpha; VCAM-1, vascular cell adhesion molecule 1; en-RAGE, extracellular receptor for advanced glycation end product; PAI-1, plasminogen activator inhibitor 1; CRP, C-reactive protein; BMI, body mass index.

In a second step sex, age, history of diabetes, BMI and SBP were added to the circulating biomarkers with significant difference between VASCAB CAD patients and controls in a stepwise regression model. The strongest model (R^2 = 0.325) contained VCAM-1, BMI and IL-8. The corresponding regression model parameters are listed in Table 4.6 as model 2.

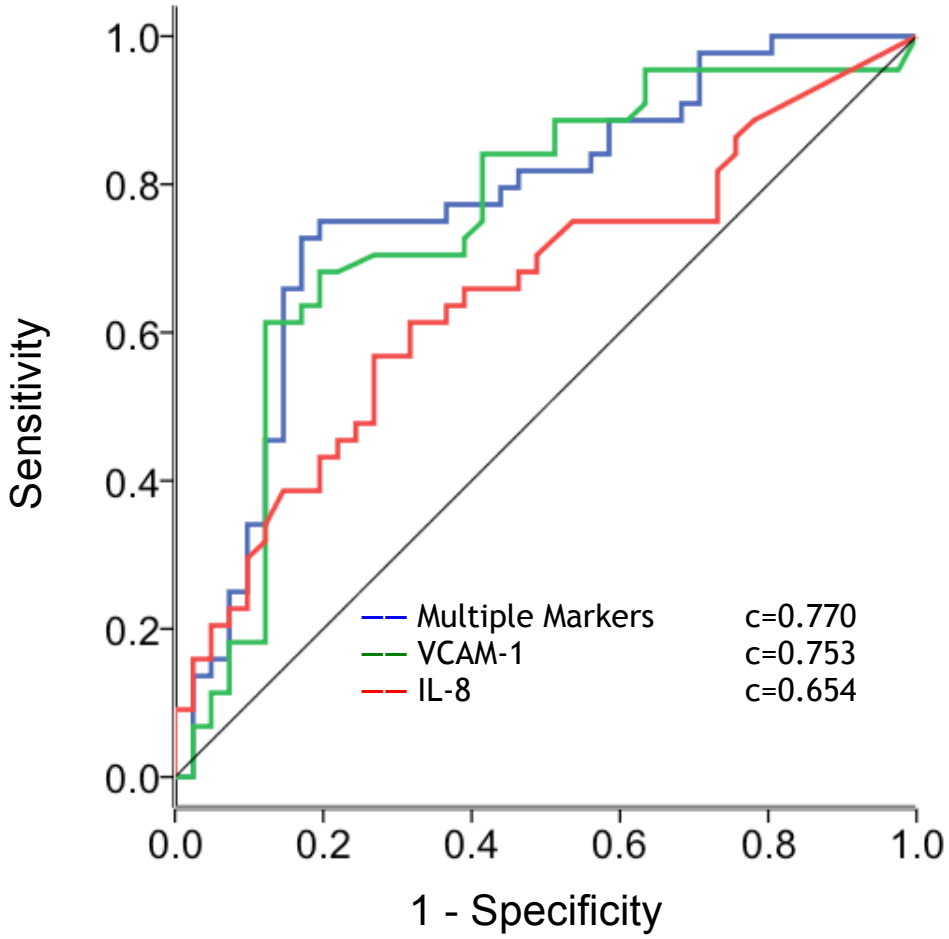


Figure 4.1 ROC curves for multiple biomarker regression model 1 and its components. C-statistic values are listed for the regression model as well as for its single components. ROC, receiver operating characteristic; VCAM-1, vascular cell adhesion molecule 1; IL-8, interleukin 8.

4.4 Discussion

4.4.1 The VASCAB and DiCADu studies

In the VASCAB study patient with symptomatic severe three vessel or left main stem CAD were compared with healthy control subjects lacking cardiac symptoms. The statistically significant differences of several circulating

biomarkers between cases and controls were measured despite clinical and demographic cohort similarities. Additionally circulating biomarkers contributed in stepwise regression models with or without addition of other contributing factors like sex, age, BMI, diabetes history and blood pressure. Although the experimental setting is artificial, this shows that an approach with multiple circulating biomarkers can be successful to differentiate between patients with CAD and healthy controls. The finding that regression model 2 contained circulating biomarkers shows their potential to contribute to CAD prediction models with traditional risk factors.

Patients in the DiCADu study had less severe CAD and controls were symptomatic suggesting functional alterations or microvascular disease. This might be due to the prevalence of traditional risk factors like diabetes, hypertension and smoking in the DiCADu control cohort. The DiCADu study was unable to provide evidence that circulating biomarker assessment differentiates stable angina patients with CAD from those with normal coronary arteries. Adjustment for covariables including sex, BMI and diabetes did not alter this finding. These covariables were chosen as they particulary effect BNP levels. BNP is decreased in the obese [387], in males [388] and in patients with insulin resistance [389]. Therefore, markers of inflammation and myocardial vulnerability were not significantly different between cases and controls in the DiCADu study. A multiple marker approach as conducted in the VASCAB cohort was therefore not reasonable.

In both studies patient were already on antianginal and secondary preventative therapy at the time of recruitment into the study. In the VASCAB study patients were medically treated and in the DiCADu study patient had percutaneous coronary interventions prior to recruitment in addition to medical treatment. Therefore both study cohorts are not suited to discover biomarkers that can be used in treatment naïve patients. In clinical practice however patients referred for chest pain assessment often receive antianginal and other therapies before further non-invasive test are carried out. The DiCADu study recruited therefore a clinically relevant cohort. The original hypothesis that a difference in circulating biomarker levels, as detected in the VASCAB study, would be reproducible in the DiCADu study on the basis of CAD or the absence of such could not be verified.

4.4.2 The origin of circulating biomarkers levels

CAD arises from atherosclerosis. Therefore its underlying pathogenesis involves an imbalance between lipid metabolism and a maladaptive immune response leading to a chronic inflammation of the artery wall. In both studies patients with CAD as part of their guideline conform secondary prevention [390] were more frequently treated with statins. This likely contributed to lower cholesterol levels in CAD patients in comparison to the control groups. Although no significant differences were detected for smoking, and blood pressure readings between cases and controls in the VASCAB study higher levels of inflammatory markers were detected in the CAD group. This suggests an ongoing inflammatory process despite secondary prevention therapy. The origin of these raised inflammatory markers could be a persisting local inflammatory unstable plaque, a reflection of the atherosclerotic burden with reduced marker quantity under treatment or a simple epi-phenomenon secondary to for instance immobility, forced sedentary lifestyle or caused by ischaemic episodes themselves. In regards of the latter it was previously shown that experimental induced ischaemia in the absence of atherosclerosis results in elevation of IL-6 and TNFα levels [391]. In patients with flow limiting CAD IL-6 also increases after dobutamin stress echocardiography [392] suggesting a direct link of transient myocardial ischaemia to inflammation. Patients in the VASCAB study had stable angina symptoms, therefore the observed difference in inflammatory markers between healthy subjects and angina patients is in line with previous reports [393, 394]. This finding was not reproducible in the DiCADu study. There was no significant difference in CRP and IL-6 levels between angina patients with or without CAD. The similarity in traditional risk factor distribution in the DiCADu cohort might be contributing. For instance the control group might have an atherosclerotic disease processes despite absence of CAD. The importance of angina in this context was investigated by Sels et al. [394]. The authors showed that angina patients in comparison to controls have higher levels of such biomarkers. The presence or absence of haemodynamic significance of CAD in these angina patients had however no effect on inflammatory biomarkers. This was reproducible when comparing the VASCAB controls with either DiCADu cases or controls.

Plasminogen Activator Inhibitor-1 is the dominant inhibitor of plasminogen activation. As a consequence it has a pro-thrombotic effect. In normal conditions only a small number of cells, such as smooth muscle cells, hepatocytes, adipocytes and platelets, release the protein into the circulation. The resulting plasma levels of 5-20 ng/ml are sufficient to control fibrinolysis. In pathologic conditions however tumour cells or the endothelium can secrete large quantities of PAI-1 after inflammatory cytokine stimulation. Therefore PAI-1 can be regarded as an indirect marker of inflammatory processes as for instance in atherosclerosis [395].

Cystatin C, an endogenous marker of kidney function, is more sensitive to detect mild to moderate decreases in glomerular filtration rate than creatinine [396]. It further is not affected by age, sex or muscle mass [397]. As a consequence cystatin C predicts all cause mortality better than creatinine or creatinine-based estimation equations [397]. The finding that cystatin C levels were increased in VASCAB patients in comparison to controls reflects therefore on the association of CAD with renal disease. CKD is an independent risk factor for the development of CAD, also underlined by the high prevalence of atherosclerosis in CKD [398]. As a renal function decline is associated with increased renal production of deleterious vasoactive substances like angiotensin II [216] CKD might indirectly contribute to atherosclerosis processes. Furthermore, traditional cardiovascular risk factors, such as diabetes mellitus, systolic hypertension, reduced high-density lipoprotein cholesterol or old age, are highly prevalent in CKD. Hypertension and diabetes are not only major risk factors for the development of CVD but also for CKD. For instance in the USA more than 35% of diabetic patients aged 20 or older as well as more than 20% of persons with hypertension have CKD [399]. Considering the risk factor distribution in the VASCAB cohort the increase in cystatin C in patients with severe CAD is therefore expected.

4.4.3 Limitations

The absence of haemodynamic significant flow limitation in the coronary artery system does not exclude atherosclerotic processes elsewhere. Aiming therefore at more specific cardiac makers, we hypothesised that biomarkers of myocardial vulnerability are elevated in angina patients with CAD in the DiCADu study. A

well-established marker for myocardial vulnerability is NT-pro BNP. Its largest body of evidence links the marker to heart failure, where it is usually released by the ventricular myocardium as a response to excessive stretching of the heart muscle. An elevation of NT-pro BNP can also be triggered by transient ischaemia. For instance percutaneous transluminal coronary balloon angioplasty can cause transient brain natriuretic peptide elevation [400]. If this is directly caused by myocardial ischaemia or secondary to transient ventricular dysfunction is controversial. Nevertheless, it suggests that angina patients with flow limiting CAD have transient fluctuations of NT-pro BNP levels. In this context, Weber et al. were able to show in patients with stable angina that NT-pro BNP level correlated with inducible myocardial ischaemia as assessed by single-photon emission computed tomography and the extent of CAD [218]. The finding that measured NT-pro BNP levels in the DiCADu study were predominately below 180 pg/mL, the 95th percentile in healthy individuals of the same age group, excludes co-morbidities like congestive cardiac failure. The statistically non-significant difference on the other hand makes a diagnostic value in the chosen experimental setting unlikely.

In the DiCADu study the investigated markers of myocardial ischaemia troponin I, CK-MB, myoglobin, GPBB, hFABP and CA3 have all in common that they are released into the blood stream due to myocardial injury. The largest body of evidence for such markers in stable angina exists for troponin. Additionally it is established that subclinical artherosclerosis as for instance in hyperglycaemic patients leads to higher troponin levels [401]. In stable angina patients Schulz et al. measured a statistical significant troponin I levels differences comparing patients with a ≥70 % stenosis with patients having a <50% stenosis [217]. Similar Sabatine et al. [402] showed a statistical significant difference of troponin I levels in stable angina patients who manifested with severe ischaemia on a nuclear myocardial perfusion scan in comparison to those with normal scans. The authors also demonstrated a significant and positive correlation of the extent of myocardial ischaemia and the duration of ischaemia with changes in troponin I levels. Considering the normalization of troponin levels 7 to 10 days after an ischaemic event increased baseline troponin levels of patients with haemodynamic significant CAD therefore likely reflect on transient ischaemic episodes. The DiCADu study was therefore unable to reproduce this finding as

patients were successfully treated with percutaneous coronary intervention prior to recruitment. This made ischaemia secondary to macrovascular flow limiting stenoses improbable.

4.4.4 Summary

We were able to verify the hypothesis that circulating biomarkers are able to differentiate angina patients with CAD and normal coronary arteries in the VASCAB study. Regression modelling suggested that circulating biomarkers contribute to traditional risk factors in the diagnosis of CAD. These results were however not reproducible in the DiCADu study. Biomarkers can therefore be diagnostic at more advanced stages of the cardiovascular continuum whilst the finding can not be reproduced at the next “lower” stage.

The DiCADu study result was probably influenced by the similarities in cardiovascular risk profiles between cases and controls and the successful treatment of CAD patients prior to recruitment. This might be different in treatment naïve patients. Existing literature for instance suggests that measuring biomarkers of myocardial injury [402] and wall stress [218] in patients undergoing stress testing could potentially aid in the diagnosis of haemodynamic significant CAD.

Chapter 5 Micro- and macrovascular biomarkers for the prediction of coronary artery disease

5.1 Introduction

The diagnosis of CAD in chest pain patients is a complicated process. Current guidelines [235, 343] suggest a diagnostic algorithm involving pre-test probability evaluation as well as non-invasive test modalities such as an exercise tolerance test. Especially in exercise tolerance tests risk stratification is essential for clinical guidance. Patients categorised as high or low cardiovascular risk are recommended to proceed to coronary angiography or medical management, respectively. Guidance for patients with intermediate risk is however inconclusive. Therefore restratification of these patients by non-invasive imaging or functional biomarkers could help in the decision process.

The cardiovascular continuum concept, as introduced by Dzau and Braunwald [54], highlights the progression of CVD through non-confined events. Later Dzau et al. [56] expanded the continuum to cardiovascular und renal pathophysiological processes. As illustrated in Figure 5.1 and 1.5 biomarkers of macro- and microvascular origin relate to intermediate steps of disease progression. As a consequence the peripheral vascular phenotype in CAD patients is worse than in the healthy population. Therefore biomarkers of the peripheral vasculature might provide additional diagnostic information.

Risk stratification used in the diagnosis of CAD was developed in prognostic studies. The DTS, for example, is an index developed from features of exercise treadmill testing. At first it was designed for outcome prediction [239], before Shaw et al. established that patients in the high-risk category had a high prevalence of flow limiting CAD [241]. This implies that risk stratification tools can support diagnosis. Macrovascular biomarkers as C-IMT [403], PWV measurement [174] and carotid distensibility [404] provide additional prognostic information in relation to classic cardiovascular risk factors. Similar invasive [405-407] and non-invasive assessment of endothelial function [189] improves outcome prediction. Considering this correlation and the diagnostic algorithm of

CAD we hypothesised that measurement of these biomarkers improves the diagnostic precision of the DTS.

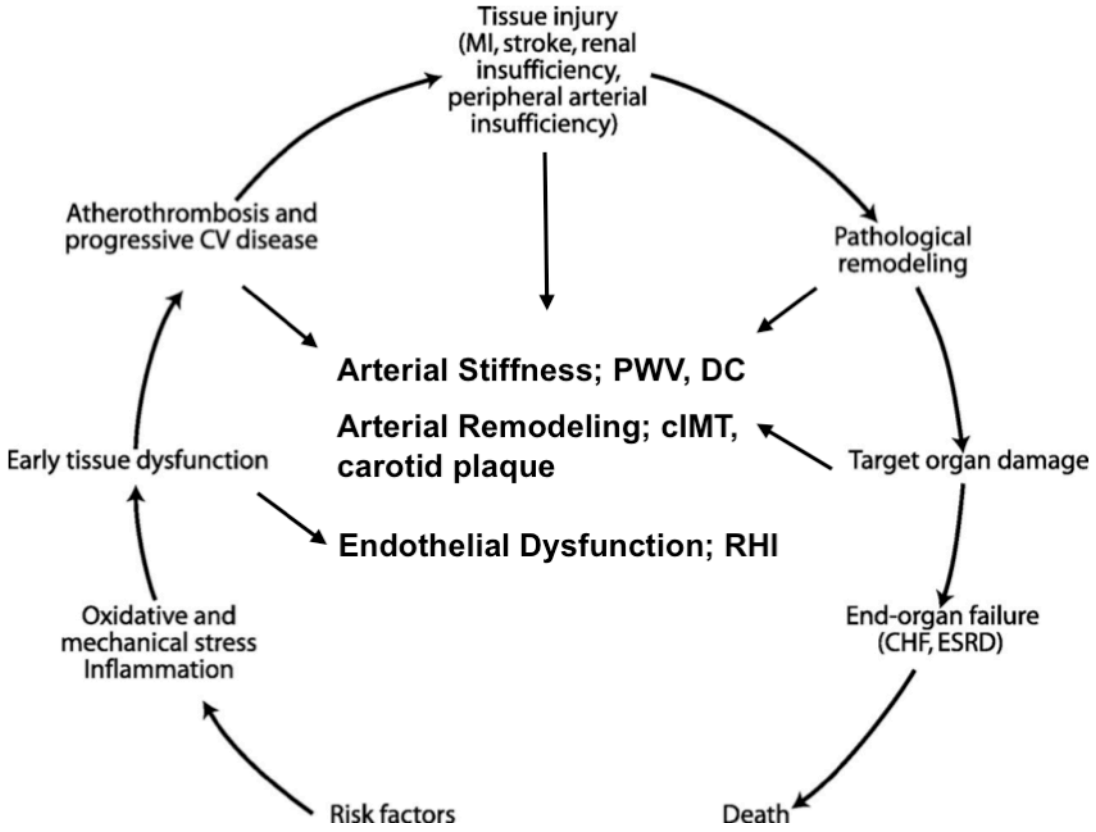


Figure 5.1 Cardiovascular continuum related to pathophysiological processes. Depicted is its link to non-invasive macro- and microvascular measurements. PWV, pulse wave velocity; DC; distensibility coefficient; cIMT, carotid intima-media thickness; RHI, reactive hyperaemia index.

5.2 Material and Methods

5.2.1 Medical history

The reported medical history was based on the DiCADu questionnaire, evaluation of casenotes and recordings on the study visit day when appropriate.

The DiCADu questionnaire section covering current limitations of every day activity was used to calculate an activity limitation index. For this purpose the limitation categories (“extremely limited” to “not at all limited”) per activity level (“dressing yourself” to “participating in strenuous sports”) were combined.

The category “limited for other reasons …” was set equal to “not at all limited” to cover chest pain only. Missing data was imputed by duplication of the next activity level grading. Data covering the gynaecologic history and angina history was also taken from the DiCADu questionnaire.

To calculate the probability of significant CAD according to Pryor et al. [408] the following criteria were used. Pathologic Q-waves were defined according to current guidelines [409] and as shown in Table 5.1. Hyperlipidaemia was defined as either fasting cholesterol of ≥6.5 mmol/l or a history of hyperlipidemia. Otherwise fasting cholesterol of ≥5.5 mmol/l was used as cut-off for the definition of hyperlipidaemia. For probability calculation smoking status was considered positive if currently or during the last 12 months a minimum of 10 cigarettes per day were consumed. This definition deviated slightly from the definition used by Pryor et al. [410]. Otherwise smoking status was positive when the participants had been smoking during the last week prior to the study visit. The diagnoses of diabetes mellitus, hypertension, cerebral vascular events and positive family history were based on questionnaires or medical records.

Table 5.1 Definition of pathologic Q waves according to current guidelines [409].

Q-wave in leads V2-3 ≥0.02 seconds or a QS-complex
Q-wave of QS-complex ≥0.03 seconds and >0.1 mV depths in leads I, II, aVL, aVF or V4-6 in two consecutive leads (e.g. I, aVL, V6; V4-6; II, III, aVF)
R-waves ≥0.04 seconds in V1-2 and a quotient of R- and S-wave ≥1 complemented by a positive T-wave and without a conduction defect

5.2.2 Cohort phenotyping

Characterization of the study cohort, including peripheral and coronary artery assessment, is described in detail in chapter 2. This includes measurement of anthropometric data, PWA, RM, PWV, C-IMT, carotid distensibility, RH-PAT, exercise treadmill testing and the Gensini score. PWA, RM, and PWV measurements were available on the basis of applanation tonometry with the Sphygmocor device. These markers of arterial stiffness were selected to evaluate the different aspects of arterial stiffness in the DiCADu cohort.

5.2.3 Statistics

A stepwise binary logistic regression model was used to test the value of diffeent biomarkers for prediction of CAD in the DiCADu cohort. The probability of CAD in the DiCADu cohort was calculated as $1/(1+e^{-x})$ where $x=a_1*x_1+a_2*x_2+….+B$ where a is the logistic regression coefficient corresponding to different biomarkers and B is the intercept term. ROC-curves were drawn using the resulting probabilities. The Net Reclassification Improvement [52] was calculated with the formula shown in Figure 5.2. Data were analysed using SPSS software, version 15.0 (SPSS Inc., Chicago, USA).

$$NRI \equiv \frac{\sum_{i\ in\ events}(p_{new}(i) - p_{old}(i))}{\#\ events} - \frac{\sum_{j\ in\ nonevents}(p_{new}(j) - p_{old}(j))}{\#\ non\ events}$$

Figure 5.2 Net reclassification improvement (NRI) formula. P_{new}, probability to identify event of new biomarker combination; P_{old}, probability to identify event of established/old biomarker combination; #nonevents; total number of nonevents. i, events (coronary artery disease); j, nonevents (normal coronary arteries). The first term quantifies the improvement in sensitivity. The second term quantifies the improvement in specificity.

5.3 Results

5.3.1 Study population

A total of 93 participants (46:47, CAD:normal coronary arteries) were recruited into the DiCADu study. Study visits took place 2 to 15 months (average of 9 months) after coronary angiography. Due to lack of chest pain history, acute infection or systemic inflammatory disease nine participants had to be excluded. Detailed analyses of anthropometric data and clinical history are summarised in Table 5.2. The CAD cohort contained more men and consecutively had a lower fat body mass percentage (each $P<0.05$). Drug treatment differed between both groups, although the percentage of vasoactive substance used, combining nicorandil, oral nitrate and calcium channel blockers together, was not significantly different, as shown in Table 5.3.

As shown in Table 5.4, there was no significant difference in angina type or cardiovascular risk factor frequency. Pre-test probability [343] and probability of significant CAD [408] were different between the CAD and the normal coronary artery group ($P<0.05$). Table 5.4 also contains questionnaire data covering a

relevant gynecologic history. In this regard a total of 82% of participants with normal coronary arteries compared to 94% in patients with CAD had either more than 3 symptoms suggestive of transition to menopause or reported being menopausal. The difference between both groups in this regard was not statistical significant (*P*>0.05).

Table 5.2 Anthropometric data.

	CAD (n=43)	NCA (n=41)	P-Value
Gender, m/f	26/17	13/28	0.008
Age, y	55.7±7.0	57.2±7.1	ns
Height, cm	167±8	167±12	ns
Weight, kg	78±14	78±23	ns
BMI, kg/m²	28.1±4.1	27.8±6.6	ns
WHR	0.91±0.08	0.92±0.07	ns
Body fat percentage, %	30.5±8.2	36.3±8.7	0.006

CAD, coronary artery disease; NCA, normal coronary arteries; m, male; f, female; ns, non-significant.

Table 5.3 Drug treatment on DiCADu study visit.

	CAD (n=43)	NCA (n=41)	*P*-value
ACEI, %	44	29	ns
CCB, %	28	26	ns
BB, %	91	22	<0.001
Statin, %	91	49	<0.001
Fibrates, %	2	0	ns
Oral Nitrat, %	9	7	ns
Nicorandil, %	28	7	0.014
Vasoactive Substances, %	43	34	ns

CAD, coronary artery disease; NCA, normal coronary arteries; ACEI, Angiontensin converting enzyme inhibitor; CCB, calcium channel blocker; BB, beta blocker; vasoactive substance, summary of nicorandil, oral nitrate and CCB therapy; ns, non-significant.

Table 5.4 Comparison of cardiovascular risk factors, gynaecologic and chest pain history.

		CAD (n=43)	NCA (n=41)	P-Value
Gynaecologic History				
	Menopausal, %	68	82	ns
	> 3 menopausal symptoms, %	23	21	ns
	Hysterectomy, %	35	39	ns
	Hormone replacement therapy, %	12	18	ns
Risk factors				
	Diabetes, %	23	17	ns
	Hypertension, %	65	59	ns
	Hyperlipidaemia, %	46	54	ns
	Positive family history, %	74	68	ns
	Current Smoker, %	28	17	ns
	Stroke/TIA, %	0	0	-
Symptoms				
	Typical angina, %	70	59	ns
	Atypical angina, %	26	29	ns
	Nonanginal chest pain, %	4	12	ns
	Activity limitation index	3.6 [2.7;4.7]	4.0 [3.3;5.0]	ns
Clinical Probability				
	Pre-test Probability [343]			<0.001
	>90%, n	22	15	
	10-90%, n	19	22	
	<10%, n	2	3	
	<5%, n	0	1	
	Significant CAD Probability [408]	0.68±0.24	0.50±0.25	0.001

CAD, coronary artery disease; NCA, normal coronary arteries; TIA, transitory ischaemic attack; ns, non-significant.

5.3.2 Exercise treadmill test data

Exercise treadmill test results were analyzed in 75 participants. 46 participants had positive results (27:19, CAD:normal coronary arteries) according to current guidelines [343]. Myocardial perfusion scans showed inducible ischaemia in five additional patients with normal coronary arteries. There was no significant difference for inducible ischaemia between the two groups (P=0.5). The DTS and the maximum ST depression during the exercise treadmill test were significantly different between the groups (P<0.05). Details of the exercise treadmill measurements are listed in Table 5.5.

Table 5.5 Exercise treadmill test differences between chest pain patients with CAD and normal coronary arteries prior to coronary angiography.

	CAD (n=38)	NCA (n=37)	P-Value
Exercise duration, seconds	408±130	420±144	ns
Peak Heart Rate, bpm	140±19	146±28	ns
Maximal ST-deviation, mm	1.7±1.1	1.1±0.9	0.013
Exercise chest pain, %	70.3	50.0	0.03
Duke treadmill score	-6.2±5.3	-1.2±5.9	<0.001
DTS risk of death (low/moderate/high)	0/28/10	6/29/2	0.01
Angina frequency per week prior to ETT (>4 per day/1-3 per day/≥3 per week/1-2 per week/ <1 per week), %	32/26/26/13/3	9/25/22/22/22	ns

CAD, coronary artery disease; NCA, normal coronary arteries; DTS, Duke treadmill score; ETT, exercise treadmill test; ns, non-significant.

5.3.3 Vascular phenotypes and their correlations

As summarised in Table 5.6, there was no significant difference between the CAD and normal coronary artery group regarding blood pressure, PWA, PWV and arterial stiffness. For PAT due to inappropriate measurements only 79

participants (CAD:normal coronary arteries; 40:39) could be evaluated. There was a significant difference between both groups for RHI and its related parameters, as shown in Table 5.6.

Table 5.6 Blood pressure and peripheral vascular biomarkers in groups with CAD and normal coronary arteries.

		CAD (n=43)	NCA (n=41)	P-Value
Blood Pressure				
	Sitting pSBP, mmHg	136±19	137±18	ns
	Sitting pDBP, mmHg	78±10	81±9	ns
	Supine pSBP, mmHg	130±15	134±14	ns
	Supine pDBP, mmHg	75±9	78±8	ns
	Supine mean arterial pressure, mmHg	95±12	99±9	ns
PWA/PWV				
	Supine pPP, mmHg	54 [48; 63]	56 [47; 64]	ns
	AIx, %	26±8	28±10	ns
	Reflection magnitude, %	1.7 [1.5; 1.8]	1.7 [1.5; 1.9]	ns
	Supine cSBP, mmHg	124±16	126±13	ns
	Supine cDBP, mmHg	76±10	79±8	ns
	Supine cPP, mmHg	47 [41; 52]	44 [38; 56]	ns
	PWV, m/sec	7.8±1.7	8.4±1.4	ns
PAT				
	RHI	1.9 [1.5; 2.3]	2.1 [1.8; 2.4]	0.03
	Average PAT^{b} Amplitude	781±456	570±365	0.03
	$PAT^{h}_{occluded}$	1.6 [1.1; 1.7]	1.8 [1.3; 2.2]	0.02
	$PAT^{h}_{control}$	1.0±0.3	1.0±0.3	ns
	Ratio $PAT^{h}_{occluded}$: $PAT^{h}_{control}$	1.6 [1.1; 2.0]	1.9 [1.4; 2.3]	ns
Carotid Ultrasound				
	C-IMT, mm	0.73±0.10	0.75±0.10	ns
	Distensibility Coefficient, 10^{-3}/kPa	3.8±1.2	3.4±0.9	ns
	Cross-sectional compliance, $m^2 \cdot kPa^{-1} \cdot 10^{-7}$	9.5 [7.0; 12.7]	8.5 [6.4; 11.6]	ns

CAD, coronary artery disease; NCA, normal coronary arteries; pSBP, peripheral systolic blood pressure; pDBP, peripheral diastolic blood pressure; cSBP, central systolic blood pressure; cDBP, central diastolic blood pressure; pPP, peripheral pulse pressure; cPP, central pulse pressure; AIx, augmentation index; C-IMT, carotid intima-media thickness; PAT, peripheral arterial tonometry; PAT^{b}, PAT signal at baseline; PAT^{h}, PAT signal during hyperaemia; ns, non-significant.

Focusing on participants with positive exercise treadmill test or positive myocardial perfusion scan results the RHI difference remained statistically

significant (CAD, 1.88 [1.54; 2.24]; normal coronary arteries, 2.15 [2.00; 2.45]; *P*=0.011). Correlations between RHI and macrovascular biomarkers, as well as amongst each other, are depicted in Figure 5.3.

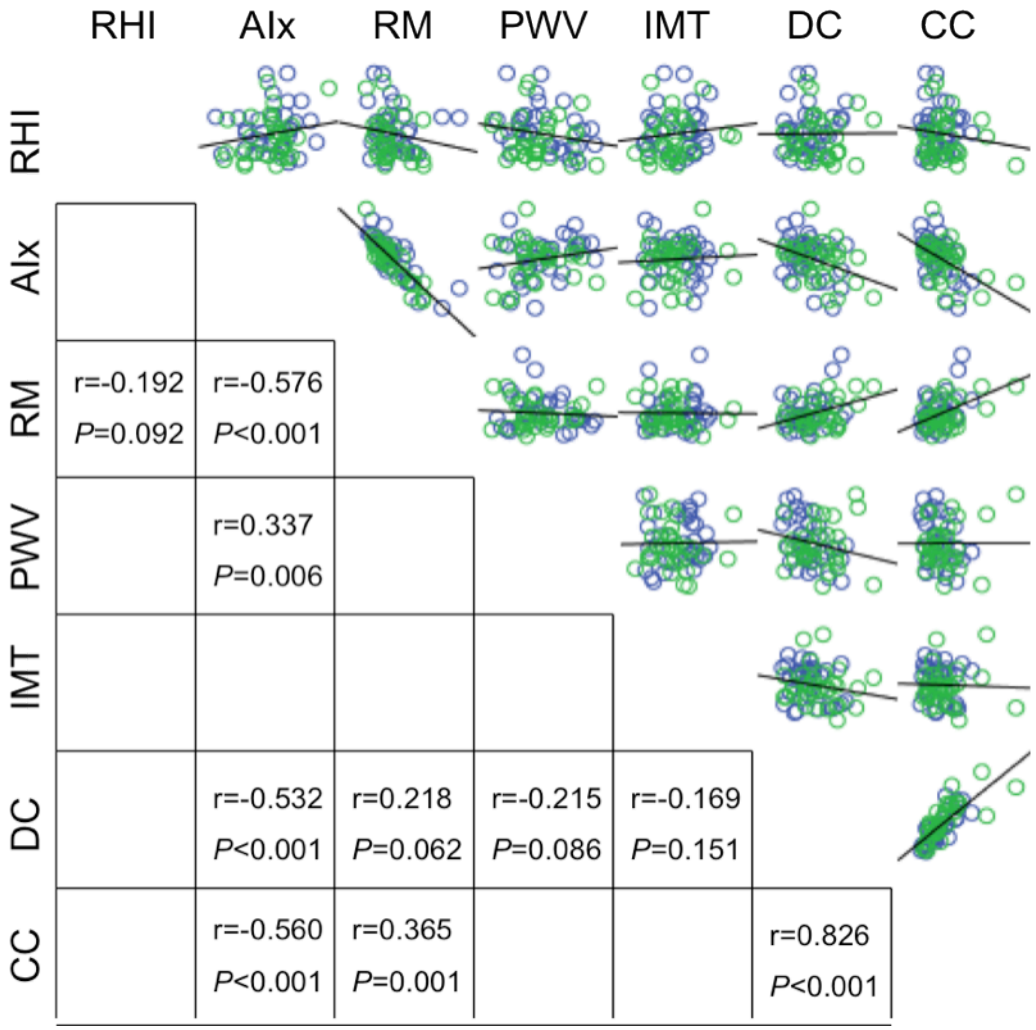


Figure 5.3 Correlations between micro- and macrovascular biomarkers. AIx (augmentation index), %; RM (reflection magnitude), %; IMT (intima-media thickness), mm; DC (distensibility coefficient), 10^{-3}kPa; CC (cross sectional compliance), m^2 kPa^{-1} 10^{-7}; blue circles, normal coronary arteries; green circles, coronary artery disease. With a 7x7 distribution, squares are mirrored on the intercept axis. R- and P-values correspond to the mirrored scatter plot. R-values represent either Pearson's correlation coefficient (PWV to AIx, DC to AIx, IMT to DC) or Spearman's rho. Correlations coefficients with a P>0.150 are not listed.

There were no significant correlations (for all, *P*>0.05) between DTS and the macrovascular biomarkers listed in Table 5.6. However age was significantly correlated with most macrovascular biomarkers, as shown in Table 5.7. Also the additionally investigated carotid plaque score was not correlated to any of the listed vascular phenotypes or age. However a borderline significant correlation between the carotid plaque score and the Gensini score (r=0.284, *P*=0.084) was

detected. When CAD patients were investigated exclusively, the Gensini score was not correlated to all mentioned peripheral vascular biomarkers or the DTS (for all, $P>0.05$). As illustrated in Figure 5.4, there was a significant correlation between RHI and the DTS, whereas the calculated probability for significant CAD did not related to either of them.

Table 5.7 Correlation of age and vascular phenotypes.

	Correlation coefficient (age)	P-value
RHI	0.213	0.060
C-IMT, mm	0.203	0.081
AIx, %	0.406	<0.001
RM, %	-0.222	0.051
PWV, cm/s	0.390	0.001
Distensibility Coefficient, 10^{-3}/kPa	-0.457	<0.001
Cross-sectional compliance, $m^2 \cdot kPa^{-1} \cdot 10^{-7}$	-0.446	<0.001
cPP, mmHg	0.319	0.004

RHI, reactive hyperaemia index; C-IMT, carotid intima-media thickness; AIx, augmentation index; RM, reflection magnitude; PWV, pulse wave velocity; cPP, central pulse pressure.

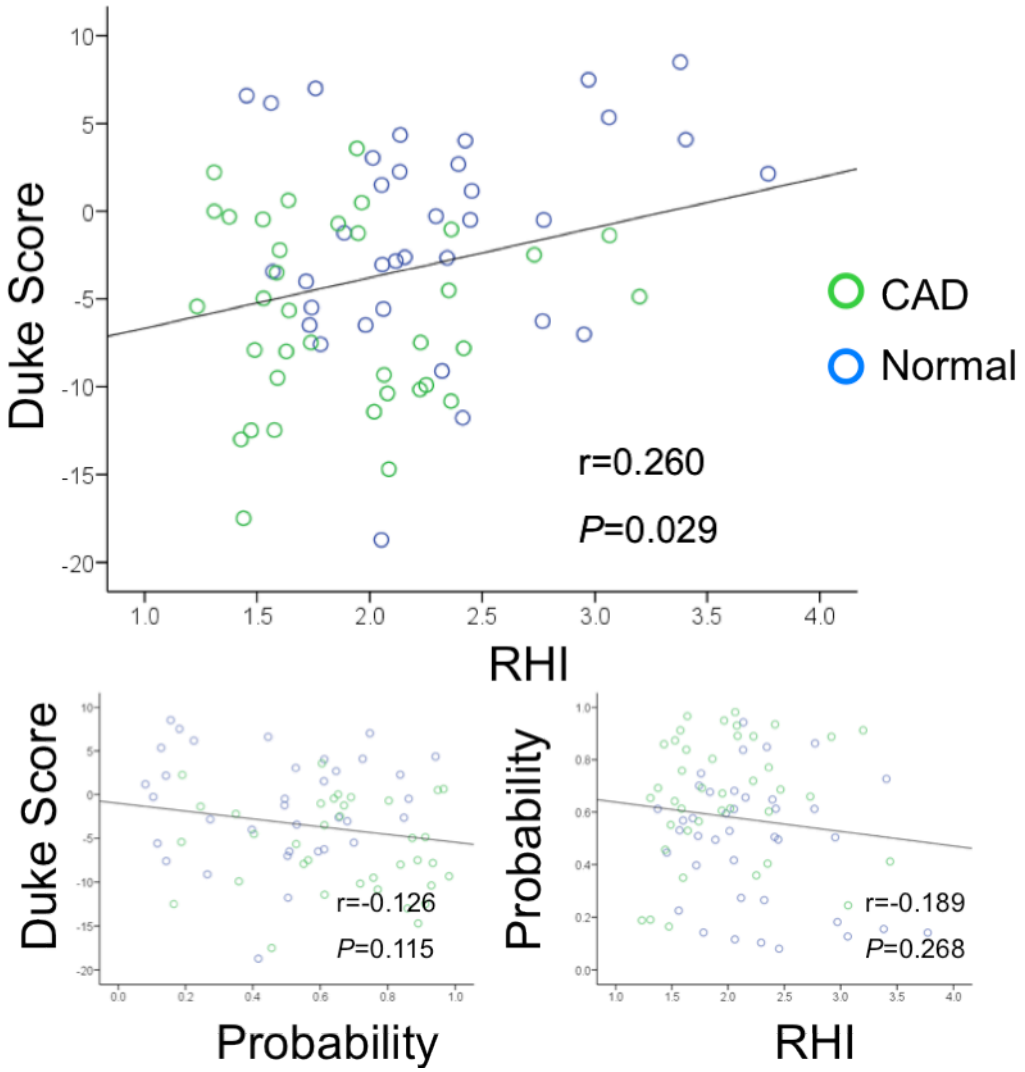


Figure 5.4 Correlations between reactive hyperaemia index (RHI), Duke treadmill score (Duke score) and significant coronary artery disease probability (probability) [408]. Green circles represent participants with significant coronary artery disease whereas blue circles represent participants with normal coronary arteries.

5.3.4 Analysis of parameters contributing to RHI

Baseline and hyperaemia PAT signals in both arms were assessed to calculate the RHI, the average baseline PAT amplitude and the ratio between hyperaemia PAT signals of the occluded and control arm. There was no significant difference between the baseline PAT amplitude of the right and left arm (696±417 vs.656±421, P=ns), as listed in Table 5.6. RHI and ratio between the hyperemia signals was significantly different between the CAD and normal coronary artery cohort (1.86 [1.53; 2.30] vs. 2.12 [1.76; 2.45], P=0.027 for RHI; 1.32 [1.08; 2.01] vs. 1.75 [1.39; 2.29], P=0.019 for ratio between hyperaemia PAT signal occluded:control arm). The average baseline PAT signal was higher (767±451 vs. 578±371, P=0.034) in the CAD group, whereas the difference in isolated post

hyperaemia signals was not significant (1370 [1183; 1718] vs. 1530 [1282; 2249], P=0.104). Consequently the baseline PWA was inversely correlated with the RHI (r=-0.509, P<0.001). A scatterplot of log transformed RHI vs. log transformed baseline PAT signal is shown in Figure 5.5. As the baseline PAT signal is gender dependent [411], a binary regression analysis was performed. Gender (P=0.017) and baseline PAT signal (P=0.026) remained significantly correlated to CAD status (overall, R^2=0.112, P=0.012).

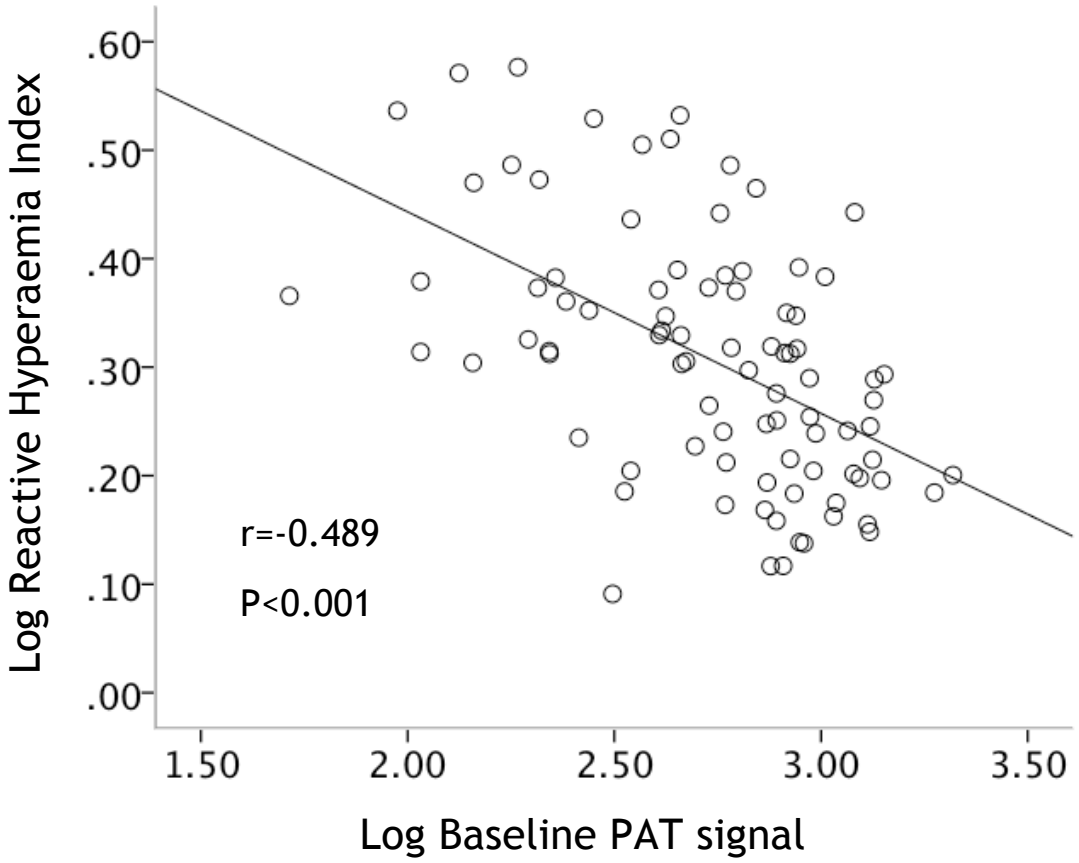


Figure 5.5 Correlation between log transformed RHI and average baseline PAT signal strength. PAT, peripheral arterial tonometry.

5.3.5 PAT in diagnosis of CAD

Addition of RHI to the components of the Pryor score [408] for diagnosis of significant CAD showed that RHI (*P*=0.049) next to gender (*P*=0.017) contributed significantly in a linear binary regression model. This finding persisted when gender and RHI were used alone in a linear regression model (Gender, *P*=0.023; RHI, P=0.071; overall, P=0.011). DTS and RHI contributed significantly (DTS, P=0.007; RHI, P=0.019; Overall, P=0.05) in a liner regression model for the

prediction of CAD. ROC-curve analyses were performed for DTS, RHI and DTS+RHI, as shown in Figure 5.6.

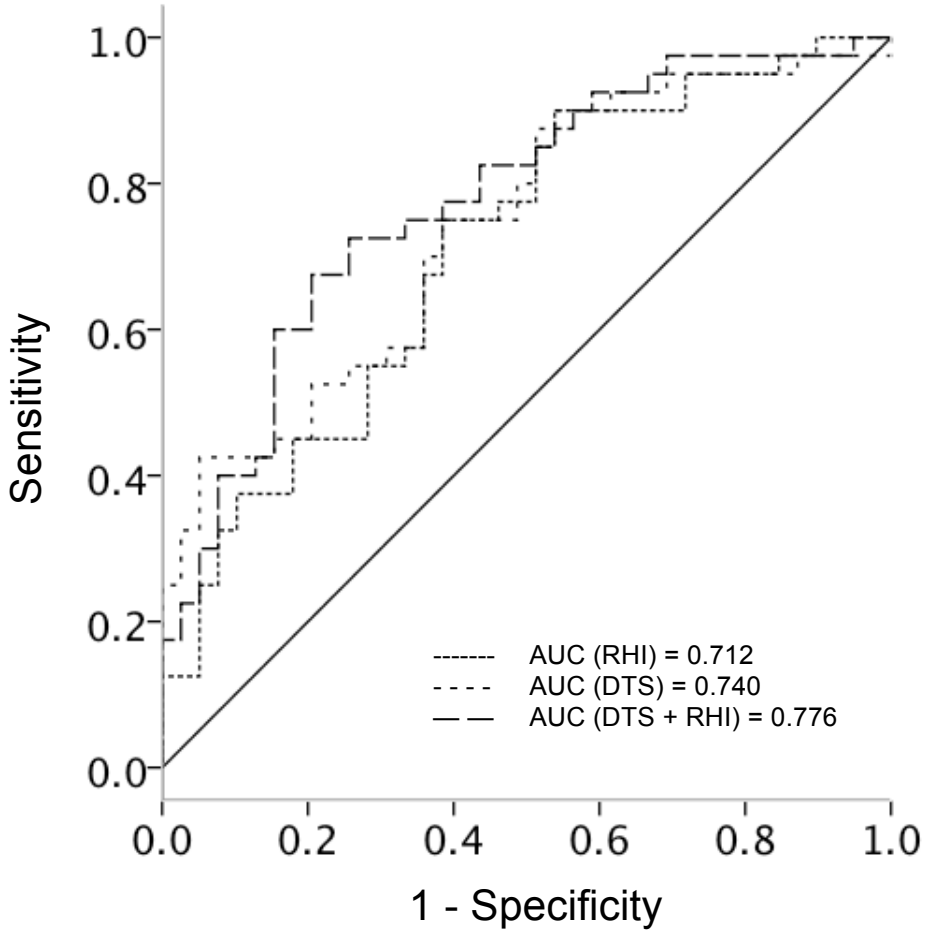


Figure 5.6 ROC curves for RHI (-----), DTS (- - -) and the combination of RHI and DTS (— —). Listed are AUC values. RHI, reactive hyperaemia index; DTS, Duke treadmill score.

Addition of macrovascular biomarkers to the DTS did not improve the AUC as shown in Table 5.8. The combination of RHI and DTS resulted in the highest AUC (c=0.776), as illustrated in Figure 5.6. Using the criteria of a positive exercise treadmill test and proven exercise induced ischaemia resulted in c=0.553 and c=0.508, respectively. The ROC curves of the combination RHI with DTS had a more convex shape compared to DTS alone. Consequently, sensitivity and specificity of RHI and DTS together, as shown in Table 5.9, were numerically higher compared to RHI or DTS alone. The related NRI was 7.6% comparing DTS alone with the combination of DTS and RHI. The NRI comparing exercise treadmill test results alone with the combination of an exercise treadmill test and RHI was 6.9%.

Table 5.8 AUC for macrovascular biomarkers.

	AUC (alone)	AUC (in addition to DTS)
DTS (n=75)	0.776	-
C-IMT, mm	0.610	0.683
AIx, %	0.545	0.714
RM, %	0.549	0.677
PWV, cm/s	0.566	0.687
Distensibility Coefficient, 10^{-3}/kPa	0.636	0.722
Carotid Cross sectional compliance, $m^2 \cdot kPa^{-1} \cdot 10^{-7}$	0.585	0.713
cPP, mmHg	0.545	0.682

Listed are c-values for carotid intima-media thickness (C-IMT), augmentation index (AIx), reflection magnitude (RM), pulse wave velocity (PWV), distensibility coefficient, carotid cross sectional compliance and central pulse pressure (cPP) for prediction of CAD alone and in addition to DTS.

Table 5.9 Test performances of CAD identification with RHI, DTS and RHI + DTS.

	Cut-off	Sensitivity	Specificity	PPV	NPV
RHI					
	1.85	0.48	0.69	0.61	0.44
	1.73	0.43	0.82	0.71	0.58
	1.58	0.28	0.90	0.73	0.45
DTS					
	-3.8	0.61	0.69	0.67	0.63
	-6.0	0.47	0.77	0.68	0.59
	-7.5	0.42	0.88	0.79	0.60
DTS+RHI					
	0.50	0.75	0.71	0.73	0.74
	0.64	0.55	0.80	0.74	0.64
	0.68	0.42	0.91	0.83	0.60

Listed are sensitivity, specificity, PPV (positive predictive value) and NPV (negative predictive value) with their corresponding cut-off points. RHI, reactive hyperaemia index; DTS, Duke treadmill score.

5.3.6 Carotid plaque score in diagnosis of CAD

The carotid plaque score was significantly different between participants with and without CAD (CAD vs. normal coronary arteries; 3.0 [1.5; 4.5] vs. 1.2 [0; 2.55], P<0.001). The c-statistic of the carotid plaque score in ROC analysis for the diagnosis of CAD was 0.716. As shown in Figure 5.7, addition of the carotid plaque score to a multiple biomarker model consisting of DTS and RHI improved the models c-statistic from 0.791 to 0.836. An NRI of 10.0 % reflects this improvement.

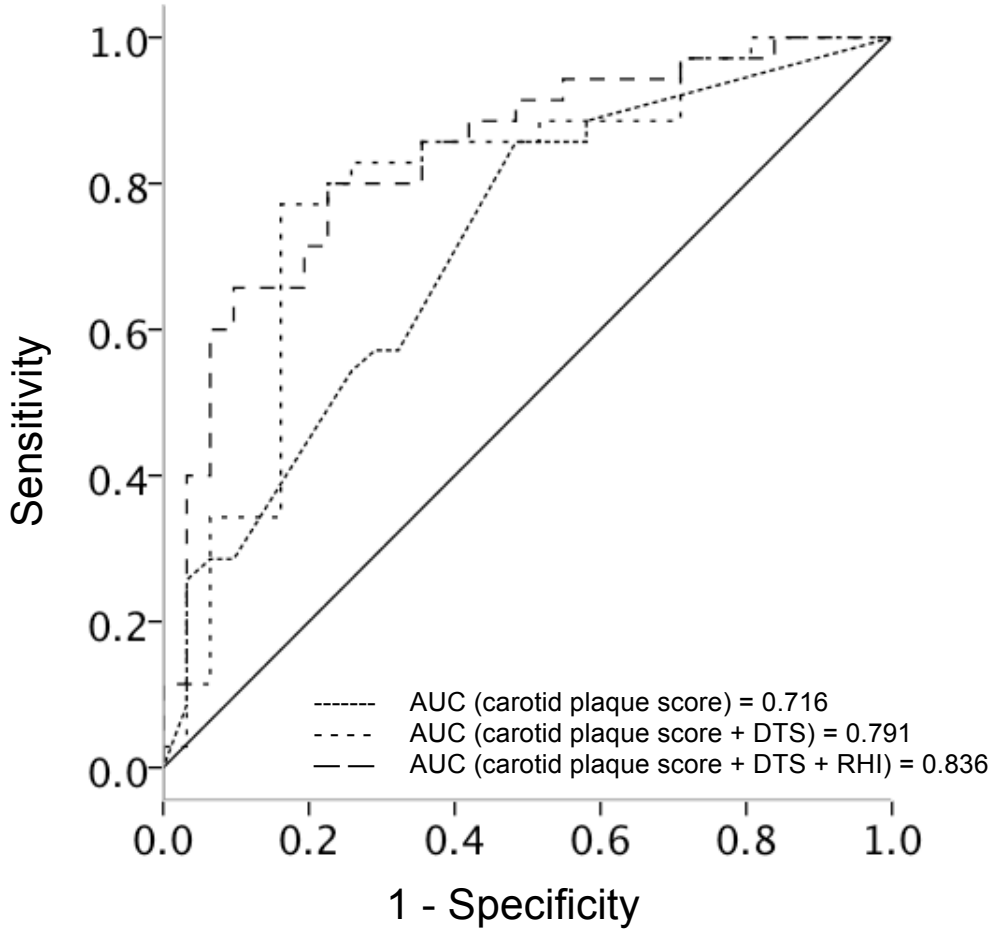


Figure 5.7 ROC curves for plaque score (----), the combination of DTS & plaque score (- - -) and the combination of DTS & RHI & plaque score (— —). Listed are AUC values. RHI, reactive hyperaemia index; DTS, Duke treadmill score.

5.4 Discussion

5.4.1 Study population

Based on the study inclusion criteria both subgroups were well matched for anthropometric features and cardiovascular risk profile. Gender differences can be explained with disease prevalence and therefore reduced availability of middle age women with CAD and men with exercise related chest pain despite normal coronary arteries. This gender mismatch contributed significantly to the CAD prediction by the Pryor score and questions therefore the potential of normal clinical features to improve prediction of significant CAD independent from gender in the DiCADu study. Evaluation of the pre-test probability showed that most patients were in the high risk or intermediate risk categories and therefore underwent exercise testing in accordance with current guidelines.

Considering the similarity of angina types between the CAD and normal coronary artery subgroup the difference in pre-test probability is due to gender.

No differences between female study participants with or without CAD could be detected regarding the gynaecological history. Typical chest pain with normal coronaries has a high prevalence in peri- or postmenopausal women [412]. In the DiCADu study female participants of the CAD and normal coronary artery subgroup were not different in this matter, possibly due to study inclusion criteria. Therefore consideration of the gynaecological history for further analysis or even measurements of oestrogen levels appeared pointless.

Differences in medication were caused by the retrospective study character and the indication of combined ACE-inhibitor, beta-blocker, aspirin and statin therapy in diagnosed CAD patients [235]. Therefore patients with CAD were more frequently treated with statins and anti-anginal medication including beta-blockers and nicorandil.

5.4.2 Micro- but not macrovascular features differentiate between CAD and normal coronary arteries

Micro- and macrovasculature differ in various aspects. Both are part of the cardiovascular system, but their anatomic structure, physiologic function and size are different. The microvasculature is composed of arterioles and capillaries. Arterioles are muscular and the primary side of vascular resistance. Their main purpose is the reduction of blood pressure and flow velocity to enable gas and nutrient exchange within the capillaries, succeeding the arterioles. Capillaries consist only of endothelial cells and have an average total surface area of 800 to 1000 m^2 per individual. Although the diameter of these vessels is in average only 7.5 µm, there is a large discrepancy between the total cross-sectional area between arterioles and capillaries favoring the latter. The blood pressure in the capillaries is therefore reduced. Considering their lack of muscular cells, capillaries however lack the capacity to alter blood flow and pressure directly. Therefore dynamic changes in the microvasculature are dependent to arteriolar function.

The macrovasculature on the other hand has a smaller total cross-sectional area and consists of arteries equipped with a large muscular layer. Although the elastic component of the aorta is important for the diastolic blood pressure the major arterial blood pressure regulation takes place in the resistance vessels, i.e. in the arterioles. Both, micro- and macrovasculature are lined with endothelial cells whose function can be assessed as discussed in chapter 1.6.1.1. Endothelial dysfunction in both leads to an alteration of smooth muscle cell tone and artery diameter. The functional implications of an impaired arterial dilatation capacity due to endothelial dysfunction however differ considering above mentioned range of physiological tasks.

In the investigated cohort cardiovascular risk factors were evenly distributed in participants with and without CAD. Especially blood pressure has a pathophysiological link to most macrovascular biomarkers. Also coronary perfusion takes place during diastole whereas mentioned macrovascular changes are predominant responses to SBP. Biomarkers such as PWV, PWA, C-IMT and carotid distensibility were therefore unable to differentiate between patients with CAD and normal coronary arteries in the DiCADu study. However RHI, a microvascular biomarker assessing endothelial function especially in the arterioles, was significantly different between both subgroups. Endothelial function measured by flow-mediated dilatation is influenced by gender until the age of 70 as shown by Benjamin et al. [413]. We therefore adjusted for gender differences in the study cohort and the RHI differences remained borderline significant. As flow mediated dilatation and RH-PAT measure the reactive hyperaemia response differently and in different locations, their indexes are not directly comparable, suggesting that the finding of Benjamin et al. does not excluded our result. Furthermore, Matsuzawa et al. [414] showed that RHI measured by EndoPAT is able to distinguish between CAD and normal coronary artery patients with typical chest pain. As the authors used an exclusive female population, the result was gender independent. Consequently, endothelial function measured by EndoPAT has the ability to differentiate between CAD and normal coronary artery patients in a gender independent manner.

5.4.3 Univariate correlations of vascular phenotypes

Macrovascular biomarkers such as carotid distensibility, PWA parameters and PWV were correlated with each other, underlining their shared aetiology arterial stiffening. As shown in chapter 3, the macrovascular biomarkers assessed in this study are highly age dependent. This finding could be reproduced in the DiCADu study with a strong correlation between age and all macrovascular biomarkers. The exception was the positive correlation of RHI and age. A high RHI value represents a normal endothelial function and therefore a negative correlation of RHI with age was anticipated, as aging is usually associated with endothelial dysfunction. The study selection criteria and the intention to age match cases with controls might have contributed to this finding. On an epidemiologic scale prevalence of symptomatic CAD is higher in older people. Increased cardiovascular risk profiles associated with endothelial dysfunction [139, 140] led however to CAD in younger age groups. Also several of the control subjects potentially suffered from microvascular angina [412] which is strongly correlated to endothelial dysfunction in the peripheral vasculature [148]. Therefore it is possible that above all younger DiCADu participants had an impaired endothelial function.

The Gensini score, a measurement of CAD extent and not of significant flow limiting CAD, was not correlated with any of the investigated macrovascular biomarkers with the exception of a borderline significant association with the carotid plaque score. This correlation however was not reproducible when CAD patients were investigated exclusively suggesting that the observation in the whole cohort only reflects on the significant carotid plaque score difference between patients with CAD and normal coronary arteries (Gensini score = 0). Furthermore the Gensini score was not correlated with the DTS underlining the association of the DTS with CAD severity [241] but not with CAD extent. The lack of a connection between the Gensini score and the macrovascular biomarkers suggests a dissociation of macrovascular and coronary pathophysiological processes and specific anatomical features, such as turbulent flow in the carotid arteries, supporting plaque development.

In RH-PAT measurements the baseline pulse wave amplitude is an important feature. Its inverse correlation with RHI suggests a limitation of the RH-PAT to

detect a post hyperaemia response. Considering the equal number of participants with and without CAD taking vasodilating medication this finding may be related to atherosclerotic arterial enlargement allowing increased blood flow at baseline.

DTS can be interpreted as a biomarker quantifying myocardial hypoperfusion under exercise conditions. Therefore myocardial dysfunction, microvascular or macrovascular CAD can reflect on a pathologic response. The finding that DTS and RHI are correlated underlines the connection of microvascular CAD with RHI [414] and the prevalence of microvascular dysfunction in CAD patients as suggested by the cardiovascular continuum. The RHI difference between CAD and normal coronary artery patients remained significant if patients with positive exercise tolerance tests were investigated exclusively. Therefore the mixture of patients with assumed cardiac microvascular angina and chest pain for other reasons had no effect on the finding.

5.4.4 Improvement of CAD diagnosis with non-invasive biomarkers

The diagnosis of CAD in patients with stable chest pain will become more reliant on imaging modalities such as magnet resonance imaging and computer tomography coronary angiography in the future [232]. Nevertheless less expensive and easy accessible test such as exercise treadmill tests still remain valuable, despite their suboptimal specificity and sensitivity [231]. We provided evidence that RH-PAT and carotid plaque score improves the diagnostic capacity of exercise treadmill tests. Although the investigated study cohort does not represent the average chest pain patient as documented by the relative high number of false positive exercise treadmill tests, especially this cohort characteristic is important, as successful reclassification of such test results would improve clinical care.

The cases and controls in the DiCADu study had a similar cardiovascular risk profile. Therefore differences in pretest probability [343] and probability of significant CAD [408] were gender dependent. Using the DTS for standardised evaluation of test results dispatches the test from this factor. The participants in the DiCADu study were randomly recruited from different hospitals of the

Greater Glasgow and Clyde area, and therefore the indication of exercise tests was based on standard clinical proceedings. Hence, improvement of the diagnostic algorithm can only derive from improvement of the test stratification. In this context we were able to show that an approach combining the DTS with RHI improved diagnosis with an NRI of 7.6%. As shown by the ROC-curves and corresponding tables this is due to reclassification of the intermediate risk category. Furthermore, addition of the carotid plaque score to both biomarkers provides an additional NRI of 10%. As RHI is an independent predictor of outcome [415] our finding supports the general strategy to use test results calibrated with outcome prediction for clinical guidance in CAD diagnosis [235, 343].

5.4.5 Study limitations

The retrospective character of the study and its consequences of treatment alteration post angiography and the delay between study visits and angiography limit the study. Especially the clinical situation at study visit was different from the situation when the decision was made to proceed to coronary angiography. However guideline conform treatment of risk factors in the CAD subgroup will lead to an irrelevant progression of atherosclerosis over an average of 9 months. Also, percutaneous coronary and drug interventions will favour clinical improvement in the CAD group and therefore remaining differences were attenuated by therapy, leading to an underestimation of the difference by current results. Another limitation of the current analysis is its focus on vascular phenotypes. For a study into multiple biomarkers a larger number of different biomarkers could have been assessed. Attempting a diagnostic approach with multiple biomarkers their quantity could be enlarged. Therefore adjustment for other risk factors or measurements of additional components of the cardiovascular continuum could have altered the results. It also remains unclear if the RH-PAT or carotid plaque score in this setting is superior to other non-invasive test modalities examining endothelial function or carotid plaque, respectively.

5.4.6 Summary

RHI and carotid plaque score contributed to diagnostic models with multiple biomarkers. This suggests that markers of different pathophysiological origin

might be additive in the diagnosis of CAD, similar to the additive effect of biomarkers in CAD prognostication. This proves that a limited system oriented approach could be valid for the diagnosis of CAD.

Within the cardiovascular continuum endothelial function represents a biomarker of early disease stages, as shown in Figure 1.5, wherease carotide plaque extent belongs to biomarkers quantifying subclinical atherosclerosis, an intermediate disease stage. This emphasises the value of early disease stage CVD biomarkers in cohorts covering later stages of the cardiovascular continuum.

Chapter 6 Urinary proteomics in the diagnosis of coronary artery disease

6.1 Introduction

Improvement of clinical care can be accomplished with the development of new biomarkers considering the limitations of existing CVD biomarkers. These can cover existing or new pathophysiological pathways of the cardiovascular continuum. Technological advances nowadays allow measurements of large numbersof molecules in biosamples. The resulting data sets make an unbiased inductive strategy for the development of new biomarkers possible. Such an approach can be carried out on different levels, such as the metabolite or protein level. The research field related to the latter is know as proteomics.

Proteomics, the analysis of a large number of proteins and polypeptides in body fluid or tissue, is useful for identification of unknown biomarkers [416]. Its goal is the comprehensive, quantitative description of protein expression. Modern proteomics platforms allow simultaneous assessment of large numbers of proteins and peptides. This allows the recognition of protein expression patterns, which can be used in disease diagnostics.

Urine has several advantages over blood in protein analysis, such as non-invasive sample collection, water soluble proteins, renal pre-selection of small size proteins, completed proteolysis at the time of sample collection and sample stability in storage. More importantly, urine contains polypeptides originating from all tissues with direct contact to the blood circulation since the glomerular barrier only restricts passage of large proteins. In healthy kidneys almost 30% of all urine protein derives from plasma [290]. The urine proteome represents therefore a variety of processes within the entire body. Our group therefore hypothesised that urine proteomics is capable to diagnose atherosclerotic vascular disease, in particular as a novel biomarker for the diagnosis of CAD. We demonstrated previously in a proof-of-concept study, further referenced as the Zimmerli study, that urine proteomics is able to differentiate between patients with severe CAD and healthy controls [323]. Additionally, the resulting proteome pattern was able to identify patients with an increased risk of adverse cardiovascular events [417].

In this chapter several steps in the development of novel biomarkers derived from urine proteomics for the diagnosis of CAD are presented. We tested the reproducibility of the Zimmerli study proteome pattern, recalibrated the urinary proteome pattern and analyzed the value of the resulting urine proteome pattern for the assessment of patients with chronic angina.

6.2 Material and Methods

Several study cohorts were investigated for the development of a CAD-specific urine proteome pattern. These included data or samples from three different studies: the Zimmerli study [323], the VASCAB study and the DiCADu study. For the Zimmerli study a total of 88 patients with CAD were enrolled from the pre-operative assessment clinic at the Western Infirmary, Glasgow. Thirty-two subjects with no history of angina, CAD, or peripheral artery disease were recruited as controls from a local health club and from surgical wards at Gartnavel General Hospital, Glasgow. Demographic and clinical data of the main study cohort is depicted in Table 6.2. Eleven patients with CAD were excluded due to missing (n=2) and insufficient (n=9) urine samples.

Furthermore, the VASCAB study contributed urine samples. The study compares patients with severe CAD prior to coronary artery bypass surgery with healthy volunteers. Healthy volunteers, both without any evidence of CAD according to history, ECG recording or other information, served as controls. The patients recruited into the study, who had elective surgery for removal of varicose veins, did not contribute. More study detail is described in chapter 2.1.1.

Samples from the DiCADu study were also investigated. Patients with or without CAD on coronary angiography were recruited. Control patients had normal coronary arteries whereas cases had at least one stenosis with >75% coronary artery lumen narrowing. The study is described in more detail in section 2.1.3.

Anthropometric data acquisition, study questionnaires, biochemistry, vascular phenotyping, assessment of exercise treadmill tests and CAD extent (Gensini score) are described in chapter 2.

We used CE-MS to measure the urinary polypeptide content, as described in chapter 2. Measurements were carried out at Mosaiques Diagnostics, Hannover, Germany. In the Zimmerli study [323] a CAD specific polypeptide pattern consisting of 15 different peptides was defined. For the purpose of this thesis the panel will be termed CAD_{15} score. As shown in Table 6.1, five of the polypeptides constituting the CAD specific panel were identified as fragments of collagen type α-1 (I) or (III).

Table 6.1 Peptide sequences in the CAD_{15} score. The list is adapted from Zimmerli et al. [323].

Peptide ID	Experimental Mass, Da	Migration time, min	Sequence	Protein name
16954	1435.72	28.86	SPhGSPGPDGKTGPPhGP	Collagen α-1 (I) chain
21244	1623.80	24.15	DGAPhGKNGERGGPhGGPhGP	Collagen α-1 (III) chain
25791	1834.90	31.15	GLPhGTGGPPhGENGKPhGEPGPh	Collagen α-1 (III) chain
27916	1933.95	21.63	GDDGEAGKPGRPhGERGPPhGP	Collagen α-1 (I) chain
53293	3158.60	29.69	GERGSPhGGPhGAAGFPhGARGLP-hGPhPGSNGNPGPPhGPh	Collagen α-1 (III) chain

Analysis of clinical parameters and CAD-specific urinary polypeptide scores were performed using SPSS software, version 15 (SPSS Inc., Chicago, Illinois, USA) as described in chapter 2.

6.3 Replication of the Zimmerli study

New biomarkers are often less accurate in an independent cohort as compared to the cohort of initial assessment. In a first step we therefore aimed to replicate the Zimmerli study [323]. Both studies had similar recruitment procedures and inclusion and exclusion criteria, thereby leading to a comparable cohort composition. The replication of the Zimmerli study appeared therefore possible.

6.3.1 Material and Methods

The Zimmerli study (section 6.2) and the VASCAB study (section 2.1.1) are described elsewhere. Corresponding demographic and clinical characteristics of both studies with available CE-MS data are depicted in Table 6.2. For both studies cases were recruited before coronary artery bypass surgery. Controls were individuals without history of CAD predominantly recruited in local fitness clubs (Zimmerli study) or via advert (VASCAB study).

A convenience sample of the VASCAB study was compiled with age and sex matched cases and controls (n=20/20) for the replication of the Zimmerli study. Only male subjects were selected to exclude gender specific differences. In the VASCAB study there were more male cases than male controls (CAD/healthy controls; 100/41). Therefore controls (all, 65.4±9.9 years) were matched manually with cases (all, 61.0±8.4 years) aiming for the best possible individual control to case agreement.

The polypeptide content of urine samples was measured with CE-MS and the CAD_{15} score was calculated as described in chapter 2 and in the Zimmerli study [323]. The classification threshold was defined as a CAD_{15} score = 13.

6.3.2 Results

The Zimmerli study and VASCAB study cohorts had similar characteristics (BMI, total and HDL cholesterol levels and CRP). Especially the cases were comparable with regard to age and medication. The control groups were however different for age, SBP and LDL cholesterol. Regarding the CAD_{15} score no significant difference was detected in the VASCAB study (CAD vs. control; 15.5±3.3 vs. 13.7±2.2, P=ns) in opposite to the Zimmerli study (CAD vs. control; 16.6±2.0 vs. 9.0±4.8, P<0.001). An apparent difference between the studies was therefore the “healthier” CAD_{15} score in Zimmerli study controls, in line with the younger age and generally more favorable risk profile of the controls in the Zimmerli study. The AUC of the CAD_{15} score was 0.940 in the Zimmerli study and 0.704 in the VASCAB sample. Sensitivity and specificity were 98% and 83% in the Zimmerli study and 54% and 58% in the VASCAB subgroup, respectively.

Table 6.2 Comparison of the Zimmerli study cohort with the VASCAB study cohort

	Zimmerli Study [323]			VASCAB Study		
	CAD, n=77	Control, n=32	P-value	CAD, n=20	Controls, n=20	*P*-value
Age, years	61±11	54±13	-	64±9	65±9	ns
Sex, m/f	56/21	21/9	ns	20/0	20//0	ns
BMI, kg/m2	26.2±4.8	25.3±3.1	ns	27.3±5.3	25.7±2.2	0.016
SBP, mmHg	132±20	123±12	<0.01	142±15	143±18	ns
DBP, mmHg	76±9	76±7	ns	81±12	82±10	ns
Heart rate, /min	-	-	-	66±15	66±11	ns
Total cholesterol, mmol/l	3.9±0.8	5.4±0.9	<0.001	3.7 [3.3; 5.0]	5.5 [4.7; 6.5]	<0.001
LDL-cholesterol, mmol/l	1.9±0.7	3.2±0.7	<0.001	1.9±0.8	3.4±1.0	<0.001
HDL-cholesterol, mmol/l	1.2±0.3	1.5±0.4	<0.01	1.0±0.2	1.4±0.4	0.001
Trilycerides, mmol/l	1.5 [1.8: 2.2]	1.3 [1.0; 2.7]	ns	2.2±1.1	1.7±0.8	ns
CRP, mg/l	2.6 [1.0; 6.3]	1.3 [0.3; 2.4]	<0.001	2.6 [1.6; 5.0]	1.1 [0.8; 2.5]	0.018
Active smoking, yes/no	14/63	3/29	<0.05	0/20	1/19	ns
Statin, yes/no	75/2	0/32	<0.001	15/5	3/17	<0.001
Aspirin, yes/no	-	-	-	18/2	5/15	<0.001
Beta-blocker, yes/no	-	-	-	17/3	0/20	<0.001
ACEI/ARB, yes/no	-	-	-	12/8	1/19	<0.001
eGFR (MDRD formula)	75±9	75±10	ns	-	-	
CAD_{15} score	16.55±2.0	9.04±4.8	<0.001	15.5±3.3	13.7±2.2	0.054

Listed is demographic data of both cohorts. Data from the Zimmerli study was adapted from Zimmerli et al. [323]. BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; LDL, low density lipoprotein; HDL, high density lipoprotein; CRP, C-reactive protein; ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; CAD, coronary artery disease; eGFR, estimated glomerular filtration rate.

6.3.3 Discussion

The results of the Zimmerli study were not reproducible in an independent, yet similar cohort. Several factors might have influenced this finding. First of all, the CAD_{15} score may not represent CAD. Instead it could reflect on cardiovascular risk, as the VASCAB study controls were older and had more cardiovascular risk factors. Secondly the score could be altered by exercise levels. Zimmerli et al. [323] observed that high physical activity contributes to a "healthier" CAD_{15} score. As the majority of the study's control subjects were recruited in a fitness club, their CAD_{15} score might represent their better physical fitness compared to the average population. The third factor is the possible presence of asymptomatic CAD in control subjects, which cannot be excluded especially in VASCAB controls due to their greater cardiovascular risk. The problem of presence of asymptomatic CAD was addressed by Snell-Bergeon et al. in the Coronary Artery Calcification in Type I Diabetes study (CACTI) [417]. The study is a prospective cohort study including 1,416 individuals asymptomatic for CAD at baseline. Six hundred fifty-two participants had type 1 diabetes mellitus. Participants developing clinical CAD (myocardial infarction, n=4; coronary artery bypass grafting, n=6; coronary angioplasty, n=9) occurring 1.4±1.3 years after study enrolment were matched for age, gender, diabetes status (n=4) and duration with patients with the lowest possible coronary calcium score as measured by computed tomography (score = 0, n=16; score 0-10, n=2; score = 51.7, n=1). According to the American Heart Association writing group [418] the presence of any atherosclerotic plaque is highly unlikely if the coronary calcium score is 0. Therefore control subjects had probably normal coronary arteries. Urine samples of the 38 participants were measured with CE-MS and the CAD_{15} score was calculated. The score difference was statistically significant (clinical CAD vs. control; 14.6±2.0 vs. 12.7±2.7, P=0.002) despite a similar cardiovascular risk and the lack of symptoms at baseline. This finding suggests that asymptomatic CAD instead of cardiovascular risk factor distribution contributed to the results in the VASCAB convenience sample.

6.4 Recalibration of Urine proteome pattern

As discussed results of urine proteomics are capable to differentiate CAD patients from "healthy" controls, suggesting detection of CAD processes. As the

CAD_{15} score was however not replicable in an independent cohort of similar origin refinement of polypeptide panel appeared necessary. We also enlarged and diversified the biomarker discovery cohort to minimise cohort specific bias. Therefore an increased number of cases and controls originating from several cohorts were used to recalibrate the CAD-specific polypeptide pattern [323, 417, 419-422].

6.4.1 Material and Methods

6.4.1.1 Biomarker discovery cohort

In the biomarker discovery cohort existing CE-MS data were compiled: the Zimmerli study [323], a study by von zu Muhlen et al. [419] (unstable angina pectoris (UAP) study), the CACTI study [417], an angiotensin converting enzyme inhibitor study [420], a fenofibrate treatment study [422] and additional healthy controls [323, 421]. This led to a total of 586 urine samples from existing cohorts comprising 408 patients and control subjects. The cohorts are summarised in Table 6.3 and described subsequently. Urine samples in all cohorts were investigated with CE-MS as described in chapter 2.

In the Zimmerli study [323] 151 CE-MS data sets from CAD patients were available: 77 urine samples collected prior to coronary artery bypass surgery and 74 urine samples collected on follow-up 14 months post surgery. Also 32 urine samples of control subjects were successfully assessed.

The unstable angina pectoris study included patients older than 18 years with typical angina symptoms combined with traditional cardiovascular risk factors [419] . Patients with renal disease, acute coronary syndromes with positive troponin I/T or significant CK/CK-MB elevation, acute inflammatory diseases, cardiogenic shock, congestive heart failure, significant calcific valve disease, atherosclerotic aneurysms, previous history of peripheral artery disease and known cerebrovascular disease were excluded. The CAD group (n=35) consisted of patients with at least two-vessel disease with lesions ≥75% on coronary angiography whereas controls had normal coronary arteries on coronary angiography (n=24).

Table 6.3 Cohort for urinary biomarker discovery.

Study cohort	Sample	CAD	Control	Primary Usage	Secondary Usage
Biomarker discovery	586	204	382		
Zimmerli study [323]	183	151	32	CAD markers	SVM modelling
UAP	59	35	24	SVM modelling	n.a.
CACTI	33	18	15	SVM modelling	n.a.
Additional controls	229	0	229	SVM modelling	n.a.
Ramipril, baseline	14	0	14	Medication markers	SVM modelling
Ramipril, follow-up	16	0	16	Medication markers	SVM modelling
Fenofibrate, baseline	26	0	26	Medication markers	SVM modelling
Fenofibrate, follow-up	26	0	26	Medication markers	SVM modelling
Blinded cohort, VASCAB	138	71	67	Validation	n.a.

UAP, unstable angina pectoris study (Study by von zur Muhlen et al. [419]); CACTI, Coronary Artery Calcification in Type I Diabetes [417]; VASCAB, vascular function in coronary artery disease study; SVM, support vector machine.

The CACTI study was already described in section 6.2.1.3.

The additional controls mentioned in Table 6.3 were collected at two sites. Forty men and 40 women, 18-55 years old were recruited at the University of North Dakota, USA, by newspaper, radio, TV and Internet advertisement [421]. Study volunteers were screened for eligibility including a general health assessment and standard clinical biochemistry. To rule out centre specific bias, samples from 73 new appointees at the University of Hannover, Germany with age older than 40 years who were free of self-reported illness were also analyzed.

To account for possible effects of angiotensin-converting enzyme inhibitors and lipid-lowering drug treatment two additional study cohorts were included.

Seventeen paired urine samples from age and sex-matched patients with hypertension, type 2 diabetes and lack of albuminuria were evaluated, before and 12 weeks after commencing treatment with the angiotensin-converting enzyme inhibitor Rampiril [420]. Also urine samples were acquired from 26 healthy individuals before and after 6 weeks of fenofibrate treatment [422].

6.4.1.2 Biomarker validation cohort

One hundred thirty-eight individuals recruited into the VASCAB study (section 2.1.1.) were used as a validation cohort. The cohort included 71 patients with severe CAD and 67 controls. Medical history, measurement of demographic and clinical parameters as well as blood and urine processing are described in chapter 2.

6.4.1.3 Treatment effect on the urine proteome

To test if short-term treatment with the angiotensin-2 receptor blocker irbesartan effects the CAD-specific urinary polypeptide pattern, urine samples of a study by Rossing et al. [423] were investigated. The authors collected urine samples from 55 patients with type 2 diabetes mellitus. All patients had microalbuminuria. After an 8 week washout period patients were treated with 300 mg irbesartan once daily for 2 weeks followed by 8 weeks of either 300 mgs, 600 mg or 900 mg once daily in a randomised, double blinded cross-over design. Urine samples were collected before and after 10 weeks of irbesartan treatment.

To assess the effect of long-term treatment with the angiotensin-2 receptor blocker irbesartan on the CAD-specific urinary polypeptide pattern, urine samples collected for the Irbesartan Microalbuminuria Type 2 Diabetes in Hypertensive Patients study (IRMA-2) [424] were assessed. All patients had type 2 diabetes mellitus. Urine samples were collected at baseline and after 2 years of treatment either with 300 mg irbesartan (n=11) or placebo (n=11) taken on a daily basis.

All studies mentioned in this chapter were approved by local ethics committees and are in accordance with the Declaration of Helsinki. All patients gave written informed consent.

6.4.2 Results

6.4.2.1 Biomarker discovery

In order to identify CAD specific urinary polypeptides, we compared the urine proteome measured with CE-MS of patients with CAD and controls in the biomarker discovery cohort. Polypeptides that were present in less than 50% of CAD and control samples were excluded. We found 398 potential candidates in the remaining panel of polypeptides. To ascertain significance of each polypeptide we repeated the statistical analysis (10 times) with random exclusion of 30% of the samples. Only the 265 polypeptides with significance in at least 7/10 permutations were investigated further.

As patients with CAD were usually treated with lipid lowering or angiotensin converting enzyme inhibitor agents, we adjusted for medication-specific polypeptide changes among the 265 candidates. Polypeptides were excluded from further analysis if the angiotensin converting enzyme inhibitor ramipril [420] or the lipid lowering drug fenofibrate [422] altered them significantly ($P<0.05$). Rampiril intake was indicated by 24 peptides and fenofibrate intake by 88. Among the medication specific polypeptides were 27 of the 265 CAD-specific candidates. Therefore the final set contained 238 CAD-specific biomarker candidates. A visualization of the polypeptide panel comparing the compiled data sets of cases and controls is depicted in Figure 6.1.

The 238 polypeptides were used in support vector machine (SVM) modelling. The final model ($C=3.79555$, $\gamma=0.004174$, and $\varepsilon=0.001$) was based on a total of 586 urine proteome profiles comprising 204 CAD and 382 control profiles in the biomarker discovery cohort. The final model is called CAD_{238} score in this thesis. The score classified the training set with an AUC of 0.95 in total cross validation. Using the classification threshold CAD_{238} score = -0.140, the pattern correctly identified CAD patients with a sensitivity of 88% and a specificity of 89%.

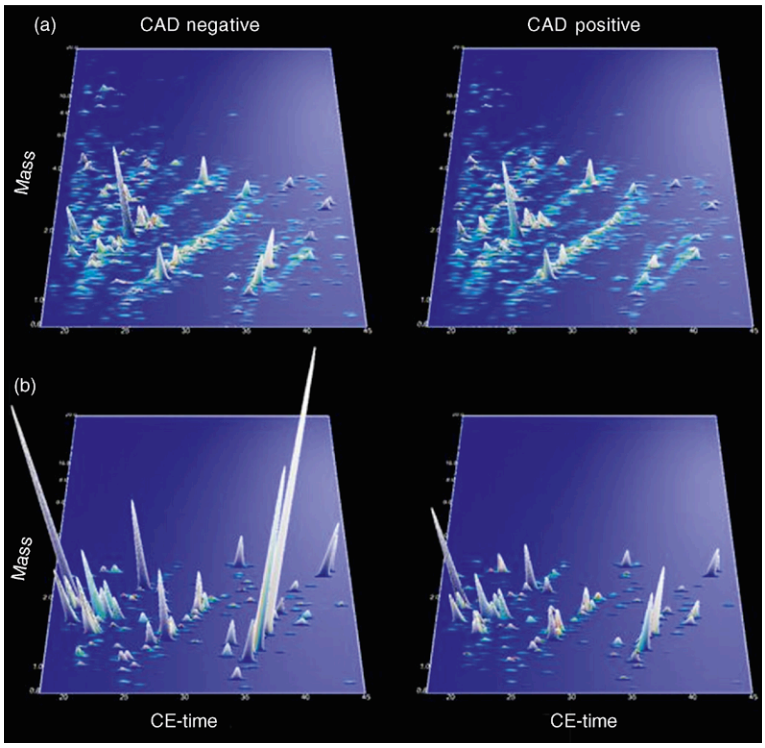


Figure 6.1 Urinary polypeptide panel specific for coronary artery disease (CAD). Capillary electrophoresis coupled to mass spectrometry measurement of urine (Panel A) resulted in the definition of 238 polypeptides constituting a CAD-specific polypeptide panel (Panel B). Normalised molecular weight of the peptides (800-20000 Da) (y-axis) in logarithmic scale is plotted against normalised capillary electrophoresis migration time (18-45 min) (x-axis). The mean signal intensity equivalent of the polypeptide peak is depicted 3-dimensionally (z-axis). The CAD-specific polypeptide pattern is magnified with a zoom factor of 4.5 (Panel B). Compiled data sets of patients with CAD and controls are shown.

6.4.2.2 Biomarker Validation

The model was then applied to the 138 urine samples of the VASCAB study in a blinded analysis (CAD vs. control; n=71 vs. n=67). This included urine samples of the age and sex matched VASCAB participants discussed in section 6.3. Receiver operating characteristics analysis of the score revealed an AUC of 0.87. The CAD_{238} score, using a classification threshold of CAD_{238} score equal to -0.140, correctly identified patients with CAD with a sensitivity of 79% and a specificity of 88%, corresponding to a positive and negative predictive value of 87% and 80%, respectively. The test performance of the panel was significantly improved in comparison to the CAD_{15} score ($P<0.0001$ in Fisher's exact test). Analysis of the 15-peptide panel in this enlarged cohort revealed an AUC of 0.68. The CAD_{15} score identified correctly patients with CAD with a sensitivity of 81.4% and a specificity of 48.5%.

Table 6.4 Cohort characteristics for the VASCAB study investigated for urine proteomics.

	CAD, n=71	Control, n=67	P-value
Gensini-Score	77 [56; 109]	-	
ACR (all > detection limit)	1.1 [0.7; 2.0]	0.9 [0.7; 1.6]	ns
Microalbuminuria, yes/no	6/49	5/57	ns
CAD_{238} score	0.1±0.4	-0.5±0.3	<0.001
Age, years	64.3±8.8	61.9±8.4	ns
Sex, m/f	56/15	41/26	0.023
BMI, kg/m2	29.5±5.0	26.0±3.5	<0.001
SBP, mmHg	139±25	138±19	ns
DBP, mmHg	78±12	82±11	ns
Heart rate, /min	64±12	68±13	0.039
Total cholesterol, mmol/l	4.1±0.8	5.7±1.2	<0.001
LDL-cholesterol, mmol/l	2.0±0.7	3.5±1.0	<0.001
HDL-cholesterol, mmol/l	1.2±0.3	1.5±0.4	<0.001
Trilycerides, mmol/l	1.8 [1.3; 2.6]	1.3 [1.0; 2.0]	<0.001
Hypertension History, yes/no	41/29	19/46	0.001
Positive Family History, yes/no	18/50	22/43	ns
Diabetes History, yes/no	17/53	0/67	ns
Active smoking, yes/no	7/64	5/62	ns
Statin, yes/no	63/8	8/59	<0.001
Aspirin, yes/no	61/10	9/58	<0.001
Beta-blocker, yes/no	59/12	5/62	<0.001
ACEI/ARB, yes/no	42/29	6/61	<0.001

Data was given as mean±SD or median±ICR as appropriate. P-values are from Student's t-test, Mann-Whitney U-test, Chi-square test or Fisher's exact test where appropriate. ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin-2 receptor blocker; CAD, coronary artery disease; NCA, normal coronary arteries; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

6.4.2.3 Short and long-term treatment effects

To test if the adjustment of the CAD_{238} score for a short-term ramipril effect made the score independent of treatment with agents interfering with the renin-angiotensin-aldosteron system, urine samples of a study by Rossing et al. [423]

were assessed. For all doses, the CAD_{238} score at baseline was compared with the respective value after a 10-week irbesartan administration. No significant effect was seen in any dose group (300 mgs, P=0.149; 600 mgs, P=0.709; 900 mgs, P=0.587).

Although the CAD_{238} score was adjusted for short-term treatment with agents interfering with the rennin-angiotensin-aldosteron system, the effect of long-term therapy on the score was unknown. Therefore 44 urine samples of the IRMA-2 study [424] were assessed. Urine samples at baseline and after 2 years of treatment with either irbesartan 300 mgs once daily (n=11) or placebo once daily (n=11) were analysed. Patients treated with irbesartan therapy for 2 years had a significant decrease (P=0.007) of the the CAD_{238} score towards a "healthier" biomarker panel compared with the placebo group.

6.4.2.4 CAD prediction with the CAD_{238} score

When the CAD_{238} score was applied to the 19 individuals developing clinical overt CAD and 19 individuals with almost no coronary artery calcium score in the CACTI study [417], the score significantly predicted the development of overt CAD (OR 2.2 [95% CI 1.3-.52] p=0.0016].

6.4.2.5 Peptide sequencing

Twenty-one percent of the 238 discriminatory peptides were sequenced (Table 6.5). In summary these included: fragments of α-1-antitrypsin, collagen type 1 and 3, granin-like neuroendocrine peptide precursor, membrane-associated progesterone receptor component 1, sodium/potassium-transporting ATPase gamma chain and fibrinogen-α chain. Polypeptides in the CAD_{238} score were not overlapping with polypeptides in the CAD_{15} score.

6.4.3 Discussion

6.4.3.1 The CAD-specific polypeptide panel

Although the CAD_{15} score was significantly associated with the presence of CAD, its test characteristics as seen in the VASCAB convenience sample (n=40) as well as a larger part of the VASCAB cohort (n=138) suggested room for further improvement. Consequently a new CAD-specific polypeptide panel was

established, using a greater number of patients and controls from several cohorts. We identified a urinary polypeptide panel based on 238 biomarkers, which was highly predictive of CAD. It does not reflect cholesterol-lowering therapy and is only influenced by longer-term treatment as opposed to short-term treatment with angiotensin-2 receptor blockers. This suggests that the CAD-specific polypeptide pattern is not influenced by drug therapy per se but is influenced by the long-term beneficial effects of medication. The panel is subject to changes over longer treatment periods and, therefore, not only reflects changes in the disease process but may also be a suitable surrogate marker to monitor treatment effects.

The CAD_{238} score is not yet ready of clinical application, yet it represents a first step in the development of a CAD-specific biomarker panel. To take the test a step further and to test if the score is suited for the diagnosis of CAD in patients with stable angina we designed the DiCADu study (section 2.1.3).

6.4.3.2 Peptide sequences

The identified polypeptides relate to atherosclerotic processes. For instance, an increased quantity of one particular α-1-antitrypsin fragment was found predominantly in the urine of CAD patients. Alpha-1-antitrypsin is a major acute phase protein with various anti-inflammatory and anti-apoptotic effects. As an acute phase protein its levels are raised several fold in inflammation [425]. Therefore the urine polypeptide might represent chronic inflammation in CAD. Also reduced levels of fibrinogen α-chain fragments in CAD were observed in the urine of patients with CAD. This may indicate fibrin formation with consecutive loss of free fibrinogen-α chains possibly caused by interaction between atherosclerotic plaque and the haemostasis system.

We also observed an up-regulation of specific collagen type 1 and 3 α chain fragments with a C-terminal GxPGP-motif. A database search (http://merops.sanger.ac.uk/) with cleavage sites at the C-terminal side of the proline residues specified matrix metalloprotease 2 (MMP-2), the A disintegrin and metalloproteinase with thrombospondin motifs 5 (ADAMTS5), the membrane Pro-x carboxypeptidase, and the prolyl oligopeptidase. Decreased activity or

levels of these proteases would explain the observed accumulation of C-terminal GxPGP-motif containing collagen fragments.

MMP-2 and ADAMTS5 are members of the metalloprotease family. For both proteins reduced circulating levels have been reported in unstable CAD or other forms of arteriosclerosis [426]. However in stable CAD Membrane Pro-x carboxypeptidase is a negative regulator of the vasopressor actions of renin-angiotensin system and provides a measure of endothelium relaxation due to its ability to set off nitric oxide and prostaglandin generation through Mas receptor activation [427].

Prolyl oligopeptidase, sometimes called post-proline cleaving enzyme, belongs to the serine peptidase family. Its activity to generate PGP tripeptide is confined to action on oligopeptides of less than 10 kD and it has an absolute requirement for the trans-configuration of the peptide bond preceding proline residues and is a two-step process involving MMP-9 as well [428]. The PGP tripeptide derived from collagen is reported to promote CXCR1/2 neutrophilic chemotactic migration [429].

The granin-like neuroendocrine peptide precursor possibly contributes to the neuroendocrine secretory pathway as a specific endogenous inhitor of proprotein convertase subtilisin/kexin type 1 inhibitor. It decreases the activity of the 84 kDa form but not the auto-catalytically derived 66 kDa form of the proprotein convertase subtilisin/kexin type 1 inhibitor. The latter is directly involved in the processing of hormones and other protein precursors including renin, enkephalin, somatostatin and insulin. Its link and the links of the remaining identified polypeptides to CAD are unclear.

Table 6.5 Sequences of polypeptides constituting the CAD_{238} score . Peptide ID are unique identifiers of CE-MS results.

Peptide ID	Experimental Mass, Da	Migration time, min	Sequence	Protein name
11413	981.59	24.8	VLNLGPITR	Uromodulin
13342	1,016.45	25.8	ApGDKGESGPS	Collagen α-1 (I) chain
21365	1,154.51	25.7	PpGEAGKpGEQG	Collagen α-1 (I) chain
22885	1,174.54	38.1	ADIAPSTDDLAS	Microfibrillar-associated protein 5
24117	1,194.55	26.7	SpGPDGKTGPPGp	Collagen α-1 (I) chain
27350	1,247.52	22.0	DKGETGEQGDRG	Collagen α-1 (I) chain
28561	1,265.59	27.1	SpGPDGKTGPpGPA	Collagen α-1 (I) chain
30575	1,297.58	27.4	SpGSpGPDGKTGPp	Collagen α-1 (I) chain
35339	1,378.61	28.8	ApGEDGRpGPpGPQ	Collagen α-1 (II) chain
36345	1,396.62	28.1	SpGERGETGPpGPAG	Collagen α-1 (III) chain
36988	1,408.66	39.1	GPPGppGPpGPPGPPS	Collagen α-1 (I) chain
38605	1,435.66	28.8	SpGSPGPDGKTGPpGP	Collagen α-1 (I) chain
38780	1,438.66	30.2	GLpGTGGPpGENGKpG	Collagen α-1 (III) chain
38879	1,439.66	29.8	TIDEKGTEAAGAMF	Alpha-1-antitrypsin
38910	1,440.56	24.3	DEAGSEADHEGTHS	Fibrinogen α chain
42304	1,485.67	23.8	DGQpGAKGEpGDAGAK	Collagen α-1 (I) chain
42404	1,487.65	29.6	GLSMDGGGSPKGDVDP	Sodium/potassium-transporting ATPase gamma chain
44464	1,521.69	30.5	GDSDDDEPPPLPRL	Membrane associated progesterone receptor component 1
48580	1,588.71	30.2	TGLSMDGGGSPKGDVDP	Sodium/potassium-transporting ATPase gamma chain
49958	1,608.73	30.9	SGDSDDDEPPPLPRL	Membrane associated progesterone receptor component 1
50008	1,609.75	30.2	TGSpGSpGPDGKTGPPGp	Collagen α-1 (I) chain
50840	1,623.73	24.1	DGApGKNGERGGpGGpGP	Collagen α-1 (III) chain

Peptide ID	Experimental Mass, Da	Migration time, min	Sequence	Protein name
51875	1,635.79	40.4	VGPpGPpGPpGPPGPPSAG	Collagen α-1 (I) chain
52100	1,638.73	20.2	AGSEADHEGTHSTKRG	Fibrinogen α chain
53744	1,666.78	30.7	KpGEQGVpGDLGApGPSG	Collagen α-1 (I) chain
53957	1,669.69	21.5	DEAGSEADHEGTHSTK	Fibrinogen α chain
55582	1,697.74	30.9	NGAPGNDGAKGDAGAPGAPG	Collagen α-1 (I) chain
55917	1,703.84	33.6	DHDVGSELPPEGVLGAL	granin-like neuroendocrine peptide precursor
57531	1,737.78	31.0	TGSpGSpGPDGKTGPPGpAG	Collagen α-1 (I) chain
58941	1,765.81	31.0	GPpGEAGKpGEQGVpGDLG	Collagen α-1 (I) chain
61573	1,825.79	20.1	DEAGSEADHEGTHSTKR	Fibrinogen α chain
63910	1,876.87	22.2	DDGEAGKPGRPGERGppGP	Collagen α-1 (I) chain
64256	1,882.80	20.2	DEAGSEADHEGTHSTKRG	Fibrinogen α chain
67097	1,931.90	31.5	APEAQVSVQPNFQQDKF	Prostaglandin-H2 D-isomerase; N-term.
67217	1,933.88	21.6	GDDGEAGKPGRpGERGPpGP	Collagen α-1 (I) chain
67386	1,936.88	32.2	GEKGPSGEAGTAGPpGTpGPQG	Collagen α-2 (I) chain
72868	2,055.14	33.4	VVVKLFDSDPITVTVPVEV	Clusterin
77018	2,133.96	27.8	DGQPGAKGEpGDAGAKGDAGPPGp	Collagen α-1 (I) chain
77763	2,149.96	27.8	DGQpGAKGEpGDAGAKGDAGPPGp	Collagen α-1 (I) chain
82026	2,226.99	26.3	GNSGEpGApGSKGDTGAKGEpGPVG	Collagen α-1 (I) chain
82509	2,233.04	20.5	GKNGDDGEAGKPGRpGERGPpGP	Collagen α-1 (I) chain
83577	2,249.04	20.5	GKNGDDGEAGKpGRpGERGpPGP	Collagen α-1 (I) chain
96875	2,529.14	28.3	GPPGADGQpGAKGEpGDAGAKGDAGpPGP	Collagen α-1 (I) chain
98089	2,559.18	19.4	DEAGSEADHEGTHSTKRGHAKSRP	Fibrinogen α chain
99746	2,583.20	28.3	AGPpGApGApGAPGPVGPAGKSGDRGETGP	Collagen α-1 (I) chain
100344	2,599.19	28.3	AGPpGApGApGApGPVGPAGKSGDRGETGP	Collagen α-1 (I) chain
104195	2,663.20	23.5	NRGERGSEGSPGHPGQPGPpGppGApGP	Collagen α-1 (III) chain
104786	2,679.20	23.5	NRGERGSEGSPGHpGQpGppGpPGAPGP	Collagen α-1 (III) chain
105352	2,695.20	23.5	NRGERGSEGSpGHpGQpGppGPPGAPGp	Collagen α-1 (III) chain

6.5 The CAD_{238} score in patients with stable angina

Considering the good discriminatory capacity of the CAD_{238} score we hypothesised that the score would differentiate CAD patients from patients with normal coronary arteries and angina like chest pain. To this goal the Diagnosis of Coronary Artery Disease with Urine proteomics (DiCADu) study was designed.

6.5.1 Material and Methods

Patients who underwent elective coronary angiography in the Golden Jubilee National Hospital (GJNH) in Clydebank were selected retrospectively on the basis of presence of severe CAD or complete absence of CAD as confirmed by angiography. Local cardiologists had referred the patients for further investigation of of typical symptom complexes and/or non-invasive test results. Study details are provided in chapter 2. The West of Scotland research ethics committee approved the study. In total 260 eligible patients were identified of whom 93 patients agreed to take part in the study.

CE-MS was performed in urine samples of 30 CAD patients and 30 individuals with normal coronary arteries, as described in chapter 2. The remaining urine samples were excluded due to insufficient sample quality or lack of age matches in the remaining participants. Assessment of DiCADu study participants including anthropometric and demographic data, standard laboratory measurements, Gensini score and vascular phenotypes were described in chapter 2.

As discussed in chapter 1, even a risk factor with low discriminatory power may reclassify the intermediate risk group [51, 52]. We therefore investigated if the CAD_{238} score improves classification by calculating the NRI as discussed in chapter 5. The NRI is a simple intuitive way of quantifying improvement offered by new biomarkers. We performed further statistical analysis of the CAD_{238} score with correlation assessment to a variety of potential contributing factors.

6.5.2 Results

6.5.2.1 DiCADu study

Characteristics of DiCADu participants in whom CE-MS analysis was performed are depicted in Table 6.6. Traditional cardiovascular risk factors were not significantly different between cases and controls.

Table 6.6 Cohort characteristics for the DiCADu study subgroup investigated for urine proteomics.

	CAD, n=30	NCA, n=30	P-value
Gensini-Score	40 [25; 61]	0	-
ACR (all > detection limit)	0.9 [0.6; 2.0]	1.6 [1.0; 3.4]	0.036
Microalbuminuria, yes/no	1/13	3/10	ns
CAD_{238} score	-0.5±0.3	-0.6±0.3	ns
Age, years	55.1±6.0	56.1±7.0;	ns
Sex, m/f	16/14	10/20	ns
BMI, kg/m2	27.9±4.2	28.8±7.5	ns
SBP, mmHg	138±17	138±19	ns
DBP, mmHg	78±10	81±9	ns
Heart rate, /min	57±9	59±9	ns
Total cholesterol, mmol/l	4.3 [3.8; 5.6]	4.8 [4.3; 5.7]	ns
LDL-cholesterol, mmol/l	2.1 [1.8; 3.3]	2.4 [2.0; 3.4]	ns
HDL-cholesterol, mmol/l	1.2 [0.9; 1.4]	1.3 [1.0; 1.6]	ns
Trilycerides, mmol/l	1.6 [1.1; 2.1]	1.7 [1.1; 2.5]	ns
Hypertension History, yes/no	22/7	16/12	ns
CAD Family History, yes/no	22/8	21/9	ns
Diabetes History, yes/no	6/24	4/26	ns
Active smoking, yes/no	7/23	5/25	ns
Statin, yes/no	26/4	17/13	0.02
Aspirin, yes/no	28/2	10/20	<0.001
Beta-blocker, yes/no	25/5	8/22	<0.001
ACEI/ARB, yes/no	12/18	10/20	ns

Data was given as mean±SD or median±ICR as appropriate. P-values are from Student's t-test, Mann-Whitney U-test, Chi-square test or Fisher's exact test where appropriate. ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; CAD, coronary artery disease; NCA, normal coronary arteries; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

Due to the confirmed diagnosis of CAD, cases were more frequently on secondary preventative therapy. In the DiCADu study CAD_{238} scores were not significantly different between cases and controls (CAD vs. normal coronary arteries; -0.5±0.3 vs. -0.6±0.3; P=ns). ROC curve statistical analysis revealed an AUC of 0.614 for the score. Using the previously defined CAD_{238} score threshold of -0.140, sensitivity was 20% and specificity was 93%.

6.5.2.2 Multiple biomarker model reclassification with the CAD_{238} score

As shown in Table 6.7 the CAD_{238} score added information to the approaches with multiple biomarkers discussed in chapter 5. The NRI was 1.2 % for the comparison of DTS and CAD_{238} score with DTS alone and 2.9% for the comparison DTS, carotid plaque score and CAD_{238} score with DTS and carotid plaque score alone. Also the CAD_{238} score added to the model consisting of DTS, RHI and carotid plaque score with a NRI of 2.0 %.

Table 6.7 Test qualities of different models with multiple biomarkers +/- CAD_{238} score.

Biomarker Panel	n (CAD/NCA)	AUC (Alone)	AUC (with CAD_{238} score)	NRI, %
DTS	51 (24/27)	0.663	0.680	1.2
DTS + carotid plaque score	48 (24/24)	0.765	0.795	2.9
DTS + carotid plaque score + RHI	47 (24/22)	0.786	0.813	2.0

DTS, Duke treadmill score; RHI, reactive hyperaemia index; CAD, coronary artery disease; NCA, normal coronary arteries; AUC, area under the curve; NRI, net reclassification index.

6.5.2.3 Comparison of the DiCADu and VASCAB cohorts

One purpose of the DiCADu study was evaluation of the CAD_{238} score in a cohort with less CAD extent in comparison to VASCAB cases. As represented by the Gensini score (DiCADu vs. VASCAB; 40 [25; 61] vs. 77 [56; 109]; P<0.001) this was the case. Also the CAD_{238} score in patients with CAD was different between both studies (DiCADu vs. VASCAB; -0.5±0.3 vs. 0.1±0.4; P<0.001) with the "healthier" value in the DiCADu study. Further differences between the cases of both studies were seen regarding age (DiCADu vs. VASCAB; 55.1±6.0 years vs. 64.3±8.8 years,

P<0.001), heart rate (DiCADu vs. VASCAB; 57±9 /min vs. 64±12 /min, P=0.006) and a positive family history for CAD (DiCADu vs. VASCAB; 73% vs. 27%; P<0.001).

The differences between control subjects of the DiCADu and the VASCAB study were more prominent. These involved age (DiCADu vs. VASCAB; 56.1±7.0 years vs. 61.9±8.4 years; P=0.002), BMI (DiCADu vs. VASCAB; 28.8±7.5 kg/m^2 vs. 26.0±3.5 kg/m^2; P=0.001), heart rate (DiCADu vs. VASCAB; 59±9 /min vs. 68±13 /min; P=0.01), total cholesterol (DiCADu vs. VASCAB; 4.8 [4.3; 5.7] mmol/l vs. 5.2 [4.3; 6.2] mmol/l; P=0.011), LDL cholesterol (DiCADu vs. VASCAB; 2.4 [2.0; 3.4] mmol/l vs. 2.9 [2.2; 3.6] mmol/l; P=0.001), history of hypertension (DiCADu vs. VASCAB; 53% vs. 29%; P=0.038), positive family history for CAD (DiCADu vs. VASCAB; 70% vs. 34%; P=0.001), statin intake (DiCADu vs. VASCAB; 57% vs. 12%; P<0.001), betablocker intake (DiCADu vs. VASCAB; 27% vs. 7%; P=0.011) and intake of an angiotensin converting enzyme inhibitor or angiotensin-2 receptor blocker (DiCADu vs. VASCAB; 33% vs. 9%; P=0.003). The CAD_{238} score was however not significantly different between the controls in both studies (DiCADu vs. VASCAB; -0.6±0.3 vs. -0.5±0.3; P=ns).

6.5.2.4 Correlations with the CAD_{238} score

When the DiCADu and VASCAB studies were combined the CAD_{238} score in patients with CAD (n=96) was correlated with the Gensini score (r=0.465, P<0.001), as shown in Figure 6.2.

To evaluate which covariates influence the score, the combined VASCAB and DiCADu control cohort was investigated. Due to differences in CAD severity and to avoid bias in this regard only controls were taken into account. Medication as beta-blocker, statin, calcium channel blocker or aspirin treatment did not impact on the CAD_{238} score, as none of the measured differences between treated and non-treated individuals was statistically significant (Figure 6.3). This was not the case for angiotensin converting enzyme inhibitor or angiotensin-2 receptor blocker treatment. Individuals on treatment had a higher CAD_{238} score, although the differences was only borderline statistically significant (therapy, yes/no (16/77); -0.4±0.3 vs. -0.6±0.3; P=0.053).

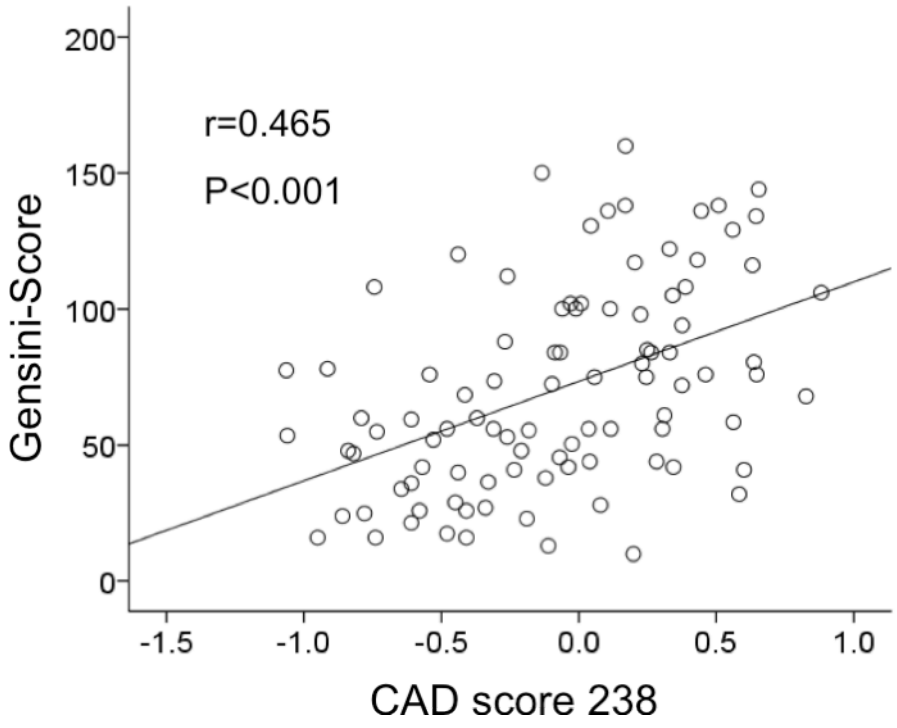


Figure 6.2 Correlation between CAD_{238} score (CAD score 238) and the Gensini score. The Gensini score (y-axis) is plotted against the CAD_{238} score (x-axis) for 96 patients.

The difference of the CAD_{238} score between the genders (male/female, n=50/47, -0.48±0.31 vs. -0.57±0.34, *P*=0.178) was not statistically significant. There was a significant correlation between the CAD_{238} score and age (r=0.253, P=0.013), as shown in Figure 6.4. BMI and urinary albumin:creatinine ratio were not correlated with the score. In patients with microalbuminuria the score was numerically lower (Microalbuminuria, yes/no (8/67): -0.58±0.26 vs. -0.49±0.34, *P*=0.494), yet the difference was not statistically significant.

When assessing the combined DiCADu and VASCAB control subjects the correlation between the CAD_{238} score and the macrovascular phenotypes C-IMT (n=70), PWV (n=68), AIx (n=87) and carotid plaque score was not statistically significant (P=ns). The carotid plaque score was only available in the DiCADu study. Investigating VASCAB and DiCADu participants with and without CAD together, the finding persisted with the exception of the borderline significant correlation of PWV with the CAD_{238} score (n=107, 8.3 [7.3; 9.7] m/s; r=0.184, P=0.058).

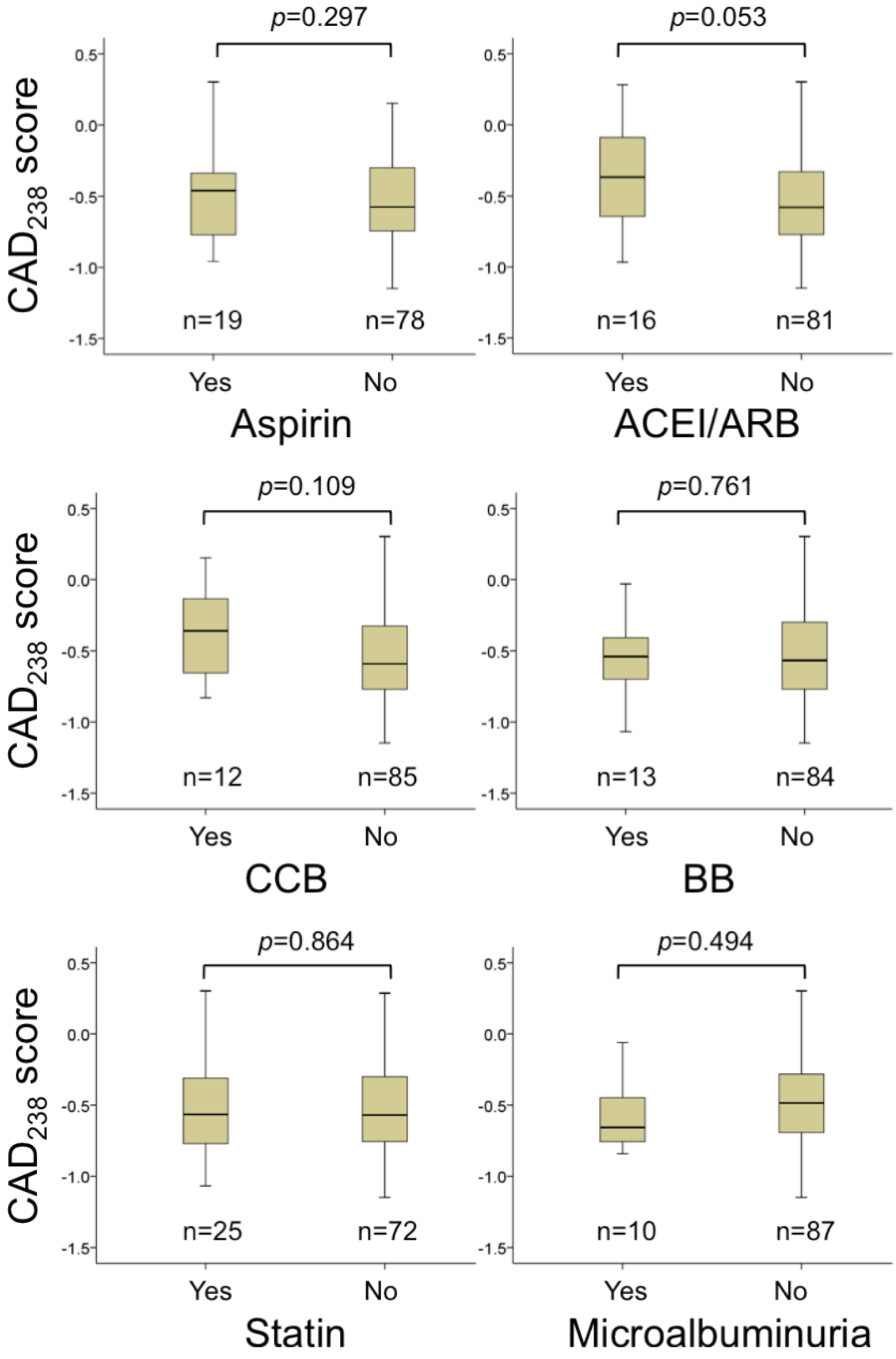


Figure 6.3 Treatment effect on the CAD_{238} score. For comparison the student t-test was used. ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotension receptor blocker; CCB, calcium channel blocker; BB, beta-blocker; CAD score 238, CAD_{238} score.

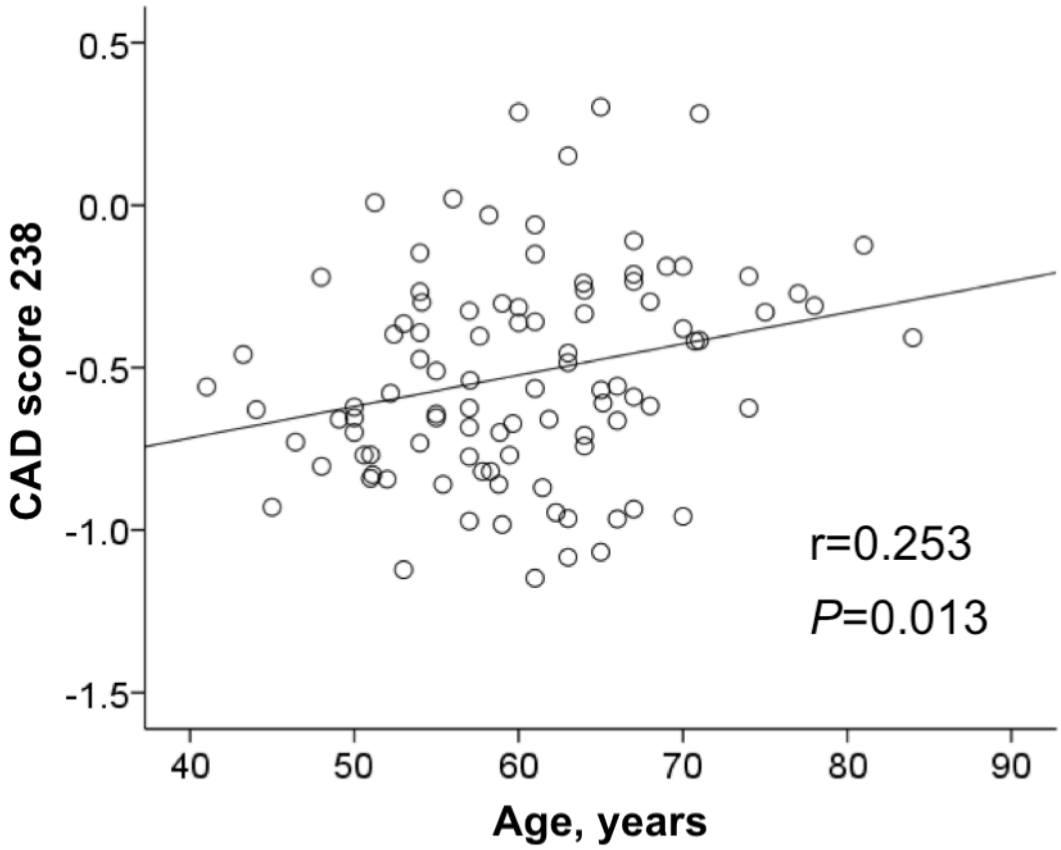


Figure 6.4 Correlation between age and CAD_{238} score (CAD score 238). Depicted are only control patients. Shown are the Pearson correlation coefficient and the corresponding P-value.

Regression models were calculated to evaluate if rather the CAD_{238} score than age, PWV and medication reflect on CAD extent. Age, PWV, angiotensin converting enzyme inhibitor or angiotensin-2 receptor blocker treatment and the CAD_{238} score were entered into the model to predict the CAD extent as assessed by the Gensini score. Due to lack of coronary angiography in VASCAB controls the modelling was restricted to cases of both studies and DiCADu controls (CAD/normal coronary arteries; n=93/30). Only the CAD_{238} score was a significant determinant of the Gensini score in a linear regression model containing the four factors (R^2adj=0.238, P<0.001; CAD_{238} score, ß=0.481, P<0.001; Age, ß=0.041, P=0.761; treatment, ß=0.036, P=0.764; PWV, ß=0.056, P=0.703). In stepwise regression modelling only the CAD_{238} score remained a determinant when all participants with known angiography results were taken into account (R^2adj=0.266, ß=0.526, P<0.001) or when only patients with CAD were investigated (R^2adj=0.187, ß=0.456, P=0.004).

6.5.3 Discussion

There was no statistically significant difference of the CAD_{238} score between the CAD and control group. The attempt to employ the CAD_{238} score in a setting with diagnostic uncertainty was therefore unsuccessful. As the score related to CAD in a variety of cohorts with different clinical situations it is probable that the factor connecting these studies is CAD. The CAD_{238} score therefore reflects on the disease. However several factors could contribute to the findings in the DiCADu study. To understand these better additional analyses were performed.

The clinical characteristics of cases in the Zimmerli and VASCAB studies were similar (section 6.3). In contrast there were significant differences between cases of the DiCADu and VASCAB studies cases. The Gensini score in DiCADu cases was smaller suggesting less coronary atherosclerosis. Additionally 60% of DiCADu cases had a percutaneous coronary intervention including a stent implantation. Therefore the blood contact with atherosclerotic plaque was greater in VASCAB study cases. This was likely the cause of the "healthier" CAD_{238} score in DiCADu study cases. There was no statistically significant difference between DiCADu and VASCAB controls for the CAD_{238} score. However several cohort characteristics were different. The prevalence of a positive family history for CAD, hypertension, diabetes and overweight was higher in DiCADu controls. This suggests that the CAD_{238} score reflects on CAD extent but is not sensitive enough to detect smaller differences of CAD extent.

A further argument supporting this hypothesis is the strong correlation between the Gensini score and the CAD_{238} score in the combined DiCADu and VASCAB CAD cohort independent of other factors. The Gensini score was designed to reflect on the number of diseased coronary arteries, their importance regarding dependent myocardial mass and the extent of arterial narrowing. It therefore indirectly relates to the extent of CAD. In extensive CAD the surface area of plaque will be larger. Therefore more arthrosclerosis related peptides enter the blood stream by for example diffusion. This will happen in each part of the artery system. The carotid plaque score was however not correlated with the CAD_{238} score suggesting that the identified proteome pattern is specific for cardiac atherosclerosis.

Another finding suggesting a connection between the CAD_{238} score and CAD extent is the positive NRI of the CAD_{238} score contributing to multiple biomarker models (section 5.4.4). This suggests that the CAD_{238} score represents pathophysiological CAD processes that are not covered by the other biomarkers in the model. As discussed in chapter 5, the small study cohort size does however no permit generalised conclusions.

The finding in the combined VASCAB and DiCADu controls that angiotensin converting enzyme inhibitor or angiontensin-2 receptor blocker therapy correlates to an "unhealthier" CAD_{238} score is in opposition to the previously mentioned long-term effect of such treatment. All DiCADu and VASCAB study controls on such therapy however had hypertension. The higher score might therefore reflect their increased cardiovascular risk.

6.6 Discussion

6.6.1 Collagen fragments and plaque

Most polypeptides in the panel of the CAD_{238} score were collagen fragments. As the collagen in artery walls increase with age [430], this might explain the score's correlation with age. An increased degradation of Type 1 and 3 collagens in atheromatous plaque [431] might also explain the predominance of collagen fragments, especially as atheromatous plaque burden increases with age [432]. In older subjects the collagen concentration in atheromatous plaque increases in areas close to the arterial lumen [14] and is therefore more frequently in contact with the blood stream.

Collagen type 1 and 3 are important constitutes of the arterial wall. Their highest concentration in the artery wall is in the intima (type 1 vs 3; 222 vs. 109 µg/mg dry weight) and in plaque (type 1 vs 3; 234 vs. 94 µg/mg dry weight) [433]. In the intima both collagen types occur mostly in the subendothelium where they are localised mainly in the deep layers in the younger population. In older subjects an increased amount of interstitial collagen can be found in surface intima regions [434]. Synthesis or degradation of collagens is slow in a healthy artery system [435]. This implies decreased levels of collagen fragments in the blood stream of healthy individuals. In atherosclerotic plaque the main

component of the fibrous cap, the region in contact with the circulating blood, consists of collagenes types 1 and 3 [434]. Also atherosclerosis causes an increased synthesis and degradation of many matrix components [436]. The majority of identified polypeptides in the urine were collagen type 1 and 3 α 1 chain fragments. This suggests that CAD-specific information is available in urine in form of degradation products of collagen linked to atherosclerotic plaque. As mentioned before the sequences identified by mass spectrometry and frequently occurring in CAD patients belonged to a C-terminal geranyl 6-O-xylopyranosyl-glucopyranoside motif amongst others specific for MMP-2. An increase in the identified collagen fragments suggests a higher activity or level of this metalloproteinase. A link between CAD and MMP-2 has been previously shown as the protease contributes to the development of atherosclerosis [437]. Also the CAD-specific polypeptide pattern changes induced by longer-term angiotensin-2 receptor blocker treatment in the IRMA-2 study [438] can be explained by the induction of MMP-2 through angiotensin 2 [439]. Arterial stiffening, as measured by aortic PWV, has been found to be associated with higher levels of MMP-2 [440]. Although this could explain the correlation between age and the CAD_{238} score, there was no significant correlation between the score and PWV. It remains therefore uncertain if arteries influencing PWV values, such as the aorta or carotids, contribute to the CAD_{238} score in addition to the coronary arteries. Further investigations to establish the exact origin of the collagen fragments in the urine are therefore required.

As recruitment criteria in the VASCAB and DiCADu study favour inclusion of patients with stable plaque, the dominance of MMP-2's cleavage products in CAD patients can be explained by the association of MMP-2 with such plaque [441]. The finding that exercise activity attenuates MMP-2 activity in pre-existing atherosclerotic plaque [442] might have contributed to "healthier" CAD_{15} score in the physical activity subgroup of the Zimmerli study [323].

6.6.2 Study limitations

The main limitation of urine proteomics in the diagnosis of CAD is its dependency on CAD extent and overall cardiovascular risk. Considering the results of the CACTI study [417] an increased overall cardiovascular risk might reflect on asymptomatic CAD (section 6.3.3). Therefore healthy controls of the VASCAB

study might have asymptomatic undetected CAD. This might explain why the Zimmerli pattern was unable to differentiate between cases and controls in the VASCAB study. Our findings also suggest that the CAD-specific urinary polypeptide pattern is related to CAD extent and not to the percentage of artery narrowing. It is therefore possible that a polypeptide pattern identifies coronary arthrosclerosis instead of flow limiting CAD. Also the exclusion of coronary arthrosclerosis by coronary angiography is incorrect. As discussed in section 1.1 “positive remodelling” of the artery wall can lead to a vessel wall plaque load of 40% before the cross-sectional luminal area is affected and CAD becomes detectable by coronary angiography.

In a prospective study investigating unstable angina patients [419] a CAD-specific polypeptide pattern was able to discriminate between patients with and without CAD. As urine samples in the DiCADu study were collected after coronary angiography including percutaneous coronary intervention in 60% of the cases the cohort was collected retrospectively. The design of the DiCADu study might have therefore contributed to the negative finding. Additionally study visits took place in average 9 months after the procedure. Therefore reendothialisation of stent surfaces were completed leading to a smaller contact area between plaque and blood and the CAD_{238} score could possibly differentiate between stable angina patients with and without CAD in a prospective study. However the DiCADu study results suggest a limitation of the CAD_{238} score in the diagnosis of significant coronary artery disease as opposed to severe three vessel or left main stem disease and to unstable angina [419]. The diagnostic success of the score in those clinical settings underlines the value of collagen turn over assessment in CAD diagnosis.

6.6.3 Summary

The observed sensitivity and specificity of the panel established in the Zimmerli study were not reproducible in a cohort of similar origin. A reason for this observation may be differences in cohort characteristics, especially in regards of the control group as Zimmerli and the VASCAB convenience sample cases had a similar CAD_{15} score. Consequently the CAD_{15} score was unable to differentiate between cases and controls of the VASCAB convenience sample, possibly due to asymptomatic CAD in controls subjects.

The CACTI study [417] and the study by von zur Muhlen et al. [419] supported urine proteomics in prognostication or diagnosis of CAD. We therefore aimed to refine the CAD-specific polypeptide pattern by enlarging the biomarker discovery cohort. Additionally the number of polypeptides in the proteome pattern was increased from 15 to 238. This allowed coverage of more pathophysiological processes involved in cardiovascular disease. The CAD_{15} score consisted predominantly of collagen 1 and 3 α chain 1 fragments whereas the CAD_{238} score contained for example fragments of α-1-antitrypsin, ProSAAS, Fibrinogen α chain and others (Table 6.1). The test performance of the CAD_{238} score in the validation cohort was significantly improved in comparison to the CAD_{15} score.

We then designed the DiCADu study to establish whether the CAD_{238} score could diagnose CAD in patients with stable angina. Probably due to smaller CAD extent in DiCADu cases this was not possible. In summary urine proteomics is a novel biomarker for CAD and has potential to add to risk stratification. The CAD-specific panel for the diagnosis of CAD requires further refinement in cohorts representing stable angina patients.

Considering the role of collagen fragments for the CAD_{238} score, the biomarker is probably related to atherosclerotic processes. Referring to the cardiovascular continuum the score therefore reflect on subclinical or clinical disease.

Chapter 7 Discussion

7.1 Summary of findings

The stages of the cardiovascular continuum can be investigated with a multitude of biomarkers. These can be circulating, functional and derived from different imaging modalities. Different biomarkers are appropriate for diagnosis and prognosis in the various stages of the cardiovascular continuum. The aim of this PhD was the assessment of CVD biomarkers at two stages, subclinical disease and clinical disease. Time constraints and limited resources allowed biomarker assessment in CVD diagnosis and to some extent in CVD prognostication as part of the PhD thesis. A marker of vascular stiffness, central pulse pressure, was investigated for prognostication in subclinical disease and a multiple biomarker approach was chosen for the the diagnosis of clinical disease. The latter included the development of a new biomarker. In summary the thesis covers biomarker discovery, proof of concept studies and prospective as well as diagnostic validation.

7.1.1 Central vs. peripheral pulse pressure

As current risk models are only predicting a small quantity of occurring cardiovascular events [27], the need for improvement remains. Aside from biomarker development for the general population, investigations of specific cohorts, such as patients with certain diseases and belonging to certain age groups or sharing specific biochemical features provided advances in the past. As CVD is a slowly progressing disease, risk assessment requires several years of follow up in studies with longitudinal design. We were however unable to measure risk prospectively. Consequently we had to choose a different approach. We correlated established risk prediction biomarkers with a standard and a new biomarker. To estimate which biomarker better predicts outcome the correlation strengths were compared. This strategy extends the concept of surrogate biomarkers into biomarker comparison for prognostication. It does not replace longitudinal studies but provides inside if such a study is likely to succeed or not. This is therefore comparable to a proof of concept study for CVD risk prediction.

The investigated biomarker was central pulse pressure (cPP) in comparison to peripheral pulse pressure (pPP). Considering the prognostic information of pPP in the elderly [354-356, 358], the hypothesis that central pulse pressure could improve risk prediction is comprehensible and was investigated as part of this thesis. Longitudinal studies focusing on populations with high prevalence of diabetes mellitus [366], CAD [383] and end-stage renal disease [367] had shown that cPP is a better predictor of outcome than pPP. These studies however covered disease processes with increased arterial stiffening and are therefore not transferable to other populations. Also a recent meta-analysis showed no significant advantage of central over peripheral pulse pressure [384] and data in elderly hypertensive patients is controversial [365, 385]. It is therefore uncertain if cPP is the better predictor of outcome in middle-aged hypertensive patients. We therefore compared cPP with pPP, the current gold standard in pulse pressure readings, by correlation with surrogate biomarker related to hypertension: left ventricular hypertrophy, carotid intima-media thickness, aortic pulse wave analysis and microalbuminuria. Especially as hypertension, one of the traditional risk factors, is strongly related to end organ damage represented by these biomarkers. Therefore, mentioned features of the cardiovascular system represent reasonable surrogate biomarkers for our analysis.

In univariate analysis the majority of these biomarkers were stronger correlated with cPP than with pPP. As soon as age was added to these biomarkers in multivariate analysis, it became apparent that age is the major contributor to these models. In univariate analysis cPP was also stronger associated with age than pPP. Consequently multivariate models containing pPP and age had similar coefficients of determination as those containing cPP. In our middle-aged cohort with high prevalence of hypertension cPP does not provide additional information beyond pPP when adjusted for relevant cofactors. As 50% percent of hypertensive patients in this cohort were not treated to target the finding might extend to untreated hypertensive patients, a group where risk stratification directly impacts on treatment. In these patients non-invasive assessment of cPP may therefore not provide additional information to brachial pulse pressure.

This shows that new biomarkers can have advantages over established biomarkers in certain cohorts, such as cPP over pPP in diabetic patients. Such

findings are however not extendable to other populations. Also our study underlined the importance of cofactor adjustment. Factors, such as age, are strongly associated with CAD. In case a new biomarker incorporates these factors better than the current gold standard its better biomarker characteristics might depend on these correlations.

7.1.2 Circulating biomarkers in the diagnosis of CAD

In addition to prognostication biomarkers can be used in diagnosis. As CAD is a multifactor disease we hypothesised that simultaneous assessment of several components of the CAD process could support its diagnosis. A variety of circulating biomarkers covers different CAD aspects. To some extend the diagnostic value of serologic biomarkers like troponin [217], N-terminal B-type natriuretic peptid [218, 443], CRP [108, 444] or oxidised LDL [445, 446], which all cover different aspects of atherosclerosis, were shown to identify patients with CAD. As a proof of concept study we therefore examined the discriminating capacity of several markers in the VASCAB cohort. This showed that the circulating biomarkers IL-8, TNFα, en-RAGE, VCAM-1, CRP, cystatin C and PAI-1 are capable to differentiate between patients with extensive CAD in comparison to healthy controls. Additionally we were able to show that these markers were superior to standard cohort characteristics in stepwise regression modelling. The VASCAB cohort however does not reflect a clinical relevant situation. We therefore tested the discriminating capacity of circulating biomarkers in the DiCADu study. There were however no statistical significant differences between angina patients with and without significant CAD for the investigated markers: urate, NT-proBNP, IL-6, Troponin I, CK-MB, myoglobin, GPBB, hFABP, CA3 and CRP. This suggests that these circulating biomarker do not provided additional diagnostic information in stable angina patients. However the number of investigated biomarkers was small and markers differentiating between CAD patients and healthy controls in the VASCAB cohort were, with the exception of CRP, different to the DiCADu study. The majority of CAD patients in the DiCADu cohort were successfully treated with percutaneous coronary interventions, leaving them without flow limiting coronary artery narrowing. The literature however suggest that higher levels of IL-6 [392], NT-proBNP [400] and troponin I [402] are related to myocardial ischaemia. The lack of flow limiting CAD or recently induced myocardial ischaemia at the timepoint

of blood collection might therefore prohibit the differentiation. This suggests that the success of circulating biomarkers in the diagnosis of CAD depends on the circumstances at blood collection; resting CAD patients might have lower levels than a patient finishing for instance an exercise treadmill test. Therefore the DiCADu study design likely contributed to the negative result. This implies that biomarker assessment covering CAD processes needs a specific study design when level fluctuations are expected.

7.1.3 Micro- and macrovascular biomarkers

Vascular biomarkers can reflect both functional and structural aspects of the micro-and macrovasculature. In contrast to larger arteries the microvasculature is limited to functional assessment due to restricted spatial resolution of non-invasive imaging. Furthermore, functional and structural changes of the peripheral vasculature resemble the coronary artery system. Amongst others, this has previously been shown for endothelial function [148] and arterial wall thickness [447]. In the DiCADu study one micro- and one macrovascular biomarker were able to distinguish between cases and controls: endothelial function as assessed by the RHI and a carotid plaque score.

Similar to our results, RHI measured by EndoPAT was able to distinguish between patients with CAD and normal coronary arteries in a study by Matsuzawa et al. [414]. Also RHI is an independent predictor of CAD morbidity and mortality [415]. RHI reflects on the endothelial function of the microvasculature. Endothelial function of the macrovasculature as measured by flow mediated dilatation also predicts cardiovascular outcome [145]. Endothelial function assessment might be therefore in general beneficial in the diagnosis of CAD.

Subclinical atherosclerosis, as assessed by the C-IMT and a carotid plaque score, is correlated with CAD extent as measured by the Synthax score [448]. In the study by Ikeda et al. the carotid plaque load was the better predictor of CAD extent also suggesting a role rather for carotid plaque than for C-IMT assessment in the diagnosis of CAD. This might account for the lack of C-IMT differences in the DiCADu study between cases and controls.

RHI and carotid plaque score contributed to diagnostic models with multiple biomarkers. This suggests that markers of different pathophysiological origin might be additive in the diagnosis of CAD, similar to the additive effect of biomarkers in CAD prognostication. This proves that a limited system oriented approach could be valid for the diagnosis of CAD. Such an approach attempts to position the individual as precisely as possible on the cardiovascular continuum. The number of biomarkers suitable for such modelling will however be limited. For instance the majority of macrovascular biomarkers related to arteriosclerosis were not significantly different in the DiCADu study. This underlines the differences between atherosclerosis and arteriosclerosis, suggesting that biomarkers are more likely to be diagnostic if they are specific for a disease process and not shared with others.

7.1.4 Urinary proteomics

We conducted several steps of the development of urinary polypeptide patterns for the diagnosis of CAD including biomarker identification and a diagnostic validation study. In the first step we tried to replicate results of a study [323] previously conducted by our group in an independent cohort. Despite strong similarities between this study and the replication cohort, the diagnostic precision of the CAD_{15} score was not reproducible. This underlines biomarkers are often less accurate in a second cohort in comparison to the cohort of initial assessment. Study replication in an independent cohort is therefore required before further steps of biomarker development, as described by Hlatky [50], are conducted.

Considering urine proteomic results in CAD of other studies [417, 419], we concluded that the CAD_{15} score was not specific enough and a new attempt to define a CAD-specific urinary polypeptide pattern was necessary. The biomarker discovery cohort was enlarged to 204 urine samples of CAD patient and 382 urine samples of controls, including samples from different locations and covering different clinical CAD presentations. This strategy was chosen to reduce the influence of local cohort characteristics and of specific CAD presentations. As CAD patients are often treated with lipid-lowering agents and angiontensin converting enzyme inhibitors, the CAD specific urinary polypeptide pattern was adjusted for treatment effects. The resulting CAD_{238} score correctly identified

patients with CAD with a sensitivity of 79% and a specificity of 88% in the validation set. These test characteristics are comparable with other established non-invasive tests (Table 1.4). In case similar results would be reproducible in a diagnostic validation study (Table 1.1) urine proteomics could compete with test as stress-echocardiography and myocardial perfusion scans in clinical practice. We therefore identified a clinical situation where the CAD diagnosis is relevant, patients with stable angina. The DiCADu study was designed to test the CAD_{238} score in such patients. The score was unable to differentiate between patients with significant flow limiting CAD and patients with normal coronary arteries on angiography. The biomarker characteristics in the validation cohort were therefore not reproducible in one clinical relevant scenario. This however does not exclude its relevance in other situations such as an acute coronary syndrome.

The CAD_{238} score was strongly correlated with the extent of CAD, suggesting that the score is unable to detect smaller differences in CAD extent in stable patients. In our study the CAD_{238} score was only able to differentiate extensive CAD from control subjects. This shows that biomarker can be specific for a disease process; yet their diagnostic capacity is limited to the extremer end of the disease spectrum.

7.2 Limitations

The described research had several limitations. The number of study participants in the VASCAB and DiCADu study was small and does not permit generalized conclusions. Also the cohort composition of the studies was disparate prohibiting reproduction of results. For instance the definition of control subjects in chapter 6 was quite divergent complicating the design of biomarker for a specific clinical scenario. Also the cases in the biomarker development panel derived from different CAD cohorts: patients with unstable angina [417], asymptomatic patients who were developing clinically overt CAD on follow-up [419] and patients with stable symptoms and severe CAD [323]. This allowed to isolate the shared CAD-specific urinary polypeptide pattern, however the resulting CAD score was not diagnostic in stable angina patients.

Especially the DiCADu study had a small number of cases and controls. Amongst others this might lead to greater influence of outliers on analysis results. Furthermore the DiCADu study had a retrospective design. This possibly impacted on circulating biomarker or urine proteomics results. CAD specific circulating biomarkers were likely reduced in cases as flow limiting CAD was no longer present after successful therapy and the CAD_{238} score was likely "healthier" as the blood to plaque surface contact area was diminished after percutaneous coronary intervention.

Also some of the test procedures could be improved. For instance non-standardised test were used such as the Luminex platform (section 2.10.3) or the Randox investigator (section 2.10.4). Furthermore, none of the imaging biomarkers were assessed for inter- or intraobserver variability. Also not all of the functional biomarkers have been as well standardised as the EndoPAT measurements (section 2.6.7). Especially data collected via medical records, such as angiography results and exercise treadmill test results, fall into this category.

7.3 General conclusions

All aspects of the cardiovascular continuum can play a role in CAD screening, prediction of disease recurrence, therapeutic monitoring, CAD diagnosis and prognostication. This is independent from the stage of the cardiovascular continuum. As we were able to show, early processes such as endothelial dysfunction or later processes such as plaque formation can support the diagnostic process. However, biomarkers assessing processes related to several diseases, such as arterial stiffness in arteriosclerosis and arthrosclerosis, might not be helpful in diagnosis. In this regard markers of arterial stiffness were unable to differentiate between patients with and without CAD in the DiCADu study. This implies that biomarkers exclusively covering the CAD pathophysiology are more useful in its diagnosis. Also biomarkers can be useful in some specific patient collectives but not necessarily in all cohorts. In end-stage renal disease cPP is the better cardiovascular outcome predictor [367] whereas in middle aged hypertensive patients it appears not to be.

Instead of applying established biomarkers, also new biomarkers can be developed. Urine proteomics showed great promise in this regard, as polypeptide patterns have been developed for several diseases: the diagnosis of renal disease [291], kidney transplant rejection [292], CKD [293] and urothelial cancer [289]. Urinary polypeptide patterns can also reflect CAD as we were able to show. Although the CAD_{15} and the CAD_{238} score were unable to reproducibly differentiate between patients with severe CAD and healthy controls or with and without CAD, respectively, our data suggests a strong link of the urinary polypeptide with CAD. These peptides are predominantly composed of collagen fragments. CAD specific collagen breakup processes have not been considered as biomarkers so far. Therefore urine proteomics discovered an atherosclerotic disease process not yet established in CAD diagnosis or prognostication. The CAD-specific urinary polypeptide pattern will require further refinement before a CAD score can be established in clinic. Yet the findings described in this thesis are already promising.

7.4 Future directions

Two positive findings of this PhD thesis merit further investigations: the improvement of the discriminatory capacity of ETTs by endothelial function and carotid plaque assessment; the correlation between the CAD_{238} score and CAD extent.

The finding that endothelial function measurement with Endo-PAT and semiquantitative carotid plaque assessment with carotid ultrasound differentiate between angina patients with and without CAD has the potential to improve standard established care protocols in a time and cost effective manner. Especially the C-statistic and net reclassification improvement of exercise treadmill tests with both biomarkers is promising. Further studies should, however, be carried out prospectively. This would have several advantages compared to the strategy employed in this thesis. Prospective patient recruitment for instance conducted in rapid access chest pain or cardiology clinics would allow selection of patients with positive ETTs prior to coronary angiography. Such a study design would allow the direct translation of findings into clinical practice. Investigations prior to coronary angiography would also exclude the influence of percutaneous coronary interventions and the

consequential improvement in exercise capacity. This could have beneficial effects, as endothelial function is expected to be worse in inactive individuals. Additionally a prospective design would lead to homogeneous medical treatment as angina patient prior to coronary angiography receive a standardized treatment. In the DiCADu study the similarities in medical management were on the other hand conincidental. A prospective study could be powered utilizing the cohort differences of the DiCADu study and the standard deviations of mentioned biomarkers. This would allow to determine an appropriate sample size for a prospective study.

Also the question which biomarker of endothelial function and carotid plaque load assessment has the greatest capacity to differentiate between angina patients with and without CAD could be addressed. As discussed in the introduction several markers for assessment of endothelial function are available. Furthermore direct quantification of carotid plaque load such as 3-dimensional ultrasonography or magnet resonance imaging could be tested.

In the DiCADu study the CAD_{238} score was not able to differentiate between angina patients with and without CAD. Taking into account previously discussed contributing factors to urinary polypeptide patterns, such as CAD extent, exercise levels and medication a prospective study as drafted above might however show different results. On the other hand, as the CAD_{238} score is associated with CAD extent, instead of or in addition to differentiating between angina patients with and without CAD it could have prognostic value.

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Dear Study Participant,

UNIVERSITY of GLASGOW

VAScular function in Coronary Artery Bypass patients – VASCAB Study

We would like you to answer a few questions. Ideally you might do this at home before your appointment visit. However, if you need assistance we will go through the list together at your appointment visit.

Please read the questions, then look at the options and tick the most appropriate answer in the answer box. If you are unsure of anything, put a mark beside it and discuss it with us at your appointment visit. If there is a question you prefer not to answer, please simply put a mark beside it so that we know.

For example:

No.	Question	
1	Sex	Male ☑1 Female ☐2
2	Date of Birth	___ / ___ / ___ Day Month Year
4	How many children have you ever had?	(insert number of children) ___2___
12	Which of the following best describes your main work status over the last 12 months?	Full-time employee ☐1 Part-time ☐2 Retired / at home ☐3

Remember your name is not recorded on any of the pages of the main questionnaire to help maintain your privacy.

VASCAB Study Team

7.4.1 Participant Main Questionnaire

7.4.2 ***Section 1 - Demographics and Family***

The background of a person has a substantial effect on an individual's risk of heart disease. In this first section we would like to find out a bit about you, your living circumstances and your family.

No.	Question		
1	Sex	Male Female	☐1 ☐2
2	Date of Birth	_____/_____/_____ Day Month Year	
3	Marital status	Single (never married) Married Living with partner Divorced or separated Widowed	☐1 ☐2 ☐3 ☐4 ☐5
4	How many children have you ever had?	(insert number of children)	_____
5	How many children are alive now?	(insert number)	_____
6	Are you one of a twin?	No Yes, identical Yes, non-identical	☐1 ☐2 ☐3
7	How many brothers do you have (all live births)?	(insert number of brothers)	_____
8	How many brothers are alive now?		_____
9	How many sisters do you have? (all live births)	(insert number of sisters)	_____
10	How many sisters are alive now?		_____

No.	Question	
11	What is the highest level of education you have completed?	Primary school completed ☐1 Secondary school completed ☐2 Technical college completed ☐3 University completed ☐4 Post graduate degree ☐5
12	Which of the following best describes your main work status over the last 12 months?	Full-time employee ☐1 Part-time ☐2 Retired / at home / unemployed ☐3
13	Which of the following best describes your racial background?	European or Caucasian ☐1 Other: Please specify: ☐2 _____
14	Would you say that in general **your quality of life** is -	Excellent ☐1 Very Good ☐2 Good ☐3 Fair ☐4 Poor ☐5
15	Would you say that in general **your health** is -	Excellent ☐1 Very Good ☐2 Good ☐3 Fair ☐4 Poor ☐5

The next questions are about your family.

No.	Question	
F1	Have any of your relatives had a **heart attack**?	Yes ☐1 No (skip the next question and go to question F3) ☐2
F2	If **yes** in question F1, **who** has had a heart attack and **how old** were they at heart attack? (Please enter "not known" if you are not sure. Please indicate if you know approximate ages but not exact ages.)	Mother Yes ☐1 No ☐2 Age __ Father Yes ☐3 No ☐4 Age __ Sister Yes ☐5 No ☐6 Age __ Brother Yes ☐7 No ☐8 Age __ Son/Daughter Yes ☐9 No ☐10 Age __ Other Yes ☐11 No ☐12 Age __
F3	Have any of your relatives had a **stroke**?	Yes ☐1 No (skip the next question and go to question F5) ☐2
F4	If **yes** in question F3, **who** has had a **stroke** and **how old** were they at stroke? (Please enter "not known" if you are not sure. Please indicate if you know approximate ages but not exact ages.)	Mother Yes ☐1 No ☐2 Age __ Father Yes ☐3 No ☐4 Age __ Sister Yes ☐5 No ☐6 Age __ Brother Yes ☐7 No ☐8 Age __ Son/Daughter Yes ☐9 No ☐10 Age __ Other Yes ☐11 No ☐12 Age __
F5	Is there any relative in your family who has or had **high blood pressure**?	Yes ☐1 No (skip the next question and go to question F7) ☐2
F6	If **yes** in question F5, **who** has or had **high blood pressure** and **how old** were they when high blood pressure was diagnosed? (Please enter "not known" if you are not sure. Please indicate if you know approximate ages but not exact ages.)	Mother Yes ☐1 No ☐2 Age __ Father Yes ☐3 No ☐4 Age __ Sister Yes ☐5 No ☐6 Age __ Brother Yes ☐7 No ☐8 Age __ Son/Daughter Yes ☐9 No ☐10 Age __ Other Yes ☐11 No ☐12 Age __

No.	Question	
F7	Is there any relative in your family who has or had **diabetes** (high blood sugar)?	Yes ☐1 No (skip the next question and go to the next section) ☐2
F8	If **yes** in question F5, **who** has or had **diabetes** and **how old** were they when diabetes was diagnosed? (Please enter "not known" if you are not sure. Please indicate if you know approximate ages but not exact ages.)	Mother Yes ☐1 No ☐2 Age __ Father Yes ☐3 No ☐4 Age __ Sister Yes ☐5 No ☐6 Age __ Brother Yes ☐7 No ☐8 Age __ Son/Daughter Yes ☐9 No ☐10 Age __ Other Yes ☐11 No ☐12 Age __

7.4.3 Section 2 - Life Style Factors

In this section, there are questions about your lifestyle. A person's lifestyle can give us important clues as to the cause of their heart disease.

The first questions are about how much alcohol you drink.

No.	Question	
A1	Have you ever consumed a drink that contains alcohol?	Yes ☐1 No (skip this section and go to the next section) ☐2
A2	Have you consumed alcohol in the **past 12 months**?	Yes ☐1 No (skip this section and go to the next section) ☐2
A3	In the past 12 months, **how frequently** have you had at least one drink?	Daily ☐1 3 to 4 days per week ☐2 Weekly ☐3 Fortnightly ☐4 Monthly or on special occasions only ☐5
A4	When you drink alcohol, **on average,** how many drinks do you have during one day?	Number of drinks per day: (A drink is equal to 1 small glass of wine, a half pint of beer, 1 shot of spirits or liqueur.) _____

These questions are about smoking and use of tobacco.

No.	Question		
S1	Have you **ever smoked** any tobacco products?	Yes, currently smoke Yes, but stopped within past 12 months Yes, but stopped more than 12 months ago No (skip this question and go to the next section)	☐1 ☐2 ☐3 ☐4
S2	How **old** were you when you **first started** smoking daily?	(Give age in years)	_____
S3	What is the **maximum number** you have smoked per day for as long as a year	(insert number of cigarettes / cigars / hand made cigarettes per week / oz. of tobacco)	_____
S4	**PAST SMOKERS – only** Why did you give up smoking?	On doctor's advice Other reason	☐1 ☐2
S5	**PAST SMOKERS – only** For roughly how many years did you smoke?	Less than 5 years 5 to 10 years 10 to 20 years More than 20 years	☐1 ☐2 ☐3 ☐4

These questions are about your diet.

No.	Question		
D1	In a typical week, on **how many days** do you eat **fruit**?	(Insert number of days)	_____
D2	Approximately **how many pieces/ servings** of fruit do you eat on one of those days?	(Insert number of servings/ pieces)	_____
D3	In a typical week, on **how many days** do you eat **green leafy vegetables**? (e.g. spinach, salad leaves)	(Insert number of days)	_____
D4	Approximately **how many servings/ meals** would you have **green leafy vegetables** on one of those days?	(Insert number of servings/ meals)	_____

These questions are about your regular exercise and physical activity.

No.	Question	
P1	On average, how much physical activity do you do each day **during working hours**? (if retired or at home, this refers to during the day)	Lots (e.g. heavy lifting, digging, going up & down stairs) ☐1 Medium (e.g. light lifting, walking, light house-work, shopping, painting) ☐2 Light activity (e.g. standing, occasional working) ☐3 Almost none (e.g. desk job, sitting, driving) ☐4
P2	On average, how much physical activity do you do each day **after working hours**? (if retired, this refers to evenings and weekends)	Lots (e.g. competitive sports, aerobics, multiple times a week) ☐1 Medium (e.g. Casual sports, going to gym, regular walks 1-2 times per week) ☐2 Light activity (e.g. occasional working or bowls) ☐3 Almost none (e.g. Watching TV, listening to music, cooking, driving) ☐4

7.4.4 Section 3 - Current Medical conditions and risk factors

This final section is about your medical conditions and treatments.

No.	Question	
M1	Have you ever been told by a doctor or other health worker that you have **high blood pressure** or **hypertension**?	Yes ☐1 No, my blood pressure was always normal (skip the next question and go to question M3) ☐2 No, I have never had my blood pressure taken (skip the next question and go to question M3) ☐3
M2	***If yes,*** about **how long ago** were you first told by a doctor that you had high blood pressure?	(insert number of years) _____

No.	Question		
M3	Have you ever been told by a doctor or other health worker that you have **diabetes** (high blood sugar)?	Yes No, my blood sugar was always normal (skip the next question and go to question M5) No, I have never had my blood sugar taken (skip the next question and go to question M5)	☐1 ☐2 ☐3
M4	***If yes,*** about **how long ago** were you first told by a doctor that you had diabetes (a high blood sugar)?	(insert number of years)	_____
M5	Have you had a medical diagnosis of a **heart attack/ myocardial infarction**?	Yes No	☐1 ☐2
M6	Have you had a medical diagnosis of a **Stroke/ transient ischaemic attack**	Yes No	☐1 ☐2
M7	Have you had a medical diagnosis of **blood vessel disease in your legs/ peripheral vascular disease**	Yes No	☐1 ☐2
M8	Have you had a medical diagnosis of a **weak heart/ heart failure**	Yes No	☐1 ☐2
M9	Have you had a medical diagnosis of **kidney disease/ renal failure**	Yes No	☐1 ☐2
M10	Have you had a medical diagnosis of **lung/chest problems? e.g. bronchitis/emphysema/COPD/Asthma**	Yes No	☐1 ☐2
M11	Do you have or have you ever been given a diagnosis of **cancer**? *If yes* what type:_____	Yes No	☐1 ☐2
M12	Do you have **rheumatoid arthritis**? (inflammation of joints)	Yes No	☐1 ☐2
M13	Do you have **osteoarthritis** ?(wear and tear arthritis)	Yes No	☐1 ☐2

No.	Question	
M14	Do you have any other long standing medical conditions that are not already listed?	Yes ☐1 No ☐2
	If yes what are these conditions? *(you may leave blank if you prefer not to answer)*	

The next 2 questions are for women only

No.	Question	
W1	Have you gone through the **menopause**? i.e. have your periods stopped	Yes ☐1 No ☐2
W2	Have you ever taken the oral contraceptive pill (OCP) **or** hormone replacement therapy (HRT)?	Yes currently ☐1 Yes previously but now stopped ☐2 (Number of years stopped ☐3 ____) No never

The next 3 questions are for patients with diabetes only.

No.	Question	
CD1	Have you ever been told you have **damage to your eyes** (retinopathy) from having diabetes?	Yes ☐1 No ☐2
CD2	Do you have any **foot problems** due to diabetes (neuropathy)? e.g. ulcers, numbness, have missing /lost toes due to diabetes	Yes ☐1 No ☐2
CD3	Have you ever been told that your **kidneys** have been damaged from having diabetes (nephropathy)?	Yes ☐1 No ☐2

Please write the name of your current medications as they are labelled from the medicine box, or your script.
It may be easier for you just to bring a current medication list issued by your doctor or by your chemist with you. If you have such a list please leave the following box blank.

	Name of medication	**Dose of medication**	**How many times medication is taken per day?**	**How long have you been taking this medication?**
T1	_____			(please insert ____ years/months)
T2	_____			(please insert ____ years/months)
T3	_____			(please insert ____ years/months)
T4	_____			(please insert ____ years/months)
T5	_____			(please insert ____ years/months)
T6	_____			(please insert ____ years/months)
T7	_____			(please insert ____ years/months)
T8	_____			(please insert ____ years/months)
T9	_____			(please insert ____ years/months)
T10	_____			(please insert ____ years/months)

University of Glasgow

Dear Study Participant,

InGenious HyperCare Study **Study-Nr.: P10 ____ ___ 1A**

We would like you to answer a few questions. Ideally you might do this at home before your appointment visit. However, if you need assistance we will go through the list together at your appointment visit.

Please read the questions, then look at the options and tick the most appropriate answer in the answer box. If you are unsure of anything, put a mark beside it and discuss it with us at your appointment visit. If there is a question you prefer not to answer, please simply put a mark beside it so that we know.

For example:

No.	**Question**	
1	Sex	Male ☑1 Female ☐2
2	Date of Birth	___/___/___ Day Month Year
4	How many children have you ever had?	(insert number of children) ___2
12	Which of the following best describes your main work status over the last 12 months?	Full-time employee ☐1 Part-time ☐2 Retired / at home ☐3

Remember your name is not recorded on any of the pages of the main questionnaire to help maintain your privacy.

InGenious HyperCare Team

7.4.5 Questionnaire

7.4.6 *Section 1 - Demographics and Family*

The background of a person has a substantial effect on an individual's risk of heart disease. In this first section we would like to find out a bit about you, your living circumstances and your family.

No.	Question		
1	Sex	Male Female	☐1 ☐2
2	Date of Birth	_____/_____/_____ Day Month Year	
3	Marital status	Single (never married) Married Living with partner Divorced or separated Widowed	☐1 ☐2 ☐3 ☐4 ☐5
4	How many children have you ever had?	(insert number of children)	_____
5	How many children are alive now?	(insert number)	_____
6	Are you one of a twin?	No Yes, identical Yes, non-identical	☐1 ☐2 ☐3
7	How many brothers do you have (all live births)?	(insert number of brothers)	_____
8	How many brothers are alive now?		_____
9	How many sisters do you have? (all live births)	(insert number of sisters)	_____
10	How many sisters are alive now?		_____

No.	Question	
11	What is the highest level of education you have completed?	Primary school completed ☐1 Secondary school completed ☐2 Technical college completed ☐3 University completed ☐4 Post graduate degree ☐5
12	Which of the following best describes your main work status over the last 12 months?	Full-time employee ☐1 Part-time ☐2 Retired / at home / unemployed ☐3
13	Which of the following best describes your racial background?	European or Caucasian ☐1 Other: Please specify: ☐2 _____
14	Would you say that in general **your quality of life** is -	Excellent ☐1 Very Good ☐2 Good ☐3 Fair ☐4 Poor ☐5
15	Would you say that in general **your health** is -	Excellent ☐1 Very Good ☐2 Good ☐3 Fair ☐4 Poor ☐5

The next questions are about your family.

No.	Question	
F1	Have any of your relatives had a **heart attack**?	Yes ☐1 No (skip the next question and go to question F3) ☐2
F2	If **yes** in question F1, **who** has had a heart attack and **how old** were they at heart attack? (Please enter "not known" if you are not sure. Please indicate if you know approximate ages but not exact ages.)	Mother Yes ☐1 No ☐2 Age __ Father Yes ☐3 No ☐4 Age __ Sister Yes ☐5 No ☐6 Age __ Brother Yes ☐7 No ☐8 Age __ Son/Daughter Yes ☐9 No ☐10 Age __ Other Yes ☐11 No ☐12 Age __
F3	Have any of your relatives had a **stroke**?	Yes ☐1 No (skip the next question and go to question F5) ☐2
F4	If **yes** in question F3, **who** has had a **stroke** and **how old** were they at stroke? (Please enter "not known" if you are not sure. Please indicate if you know approximate ages but not exact ages.)	Mother Yes ☐1 No ☐2 Age __ Father Yes ☐3 No ☐4 Age __ Sister Yes ☐5 No ☐6 Age __ Brother Yes ☐7 No ☐8 Age __ Son/Daughter Yes ☐9 No ☐10 Age __ Other Yes ☐11 No ☐12 Age __
F5	Is there any relative in your family who has or had **high blood pressure**?	Yes ☐1 No (skip the next question and go to question F7) ☐2
F6	If **yes** in question F5, **who** has or had **high blood pressure** and **how old** were they when high blood pressure was diagnosed? (Please enter "not known" if you are not sure. Please indicate if you know approximate ages but not exact ages.)	Mother Yes ☐1 No ☐2 Age __ Father Yes ☐3 No ☐4 Age __ Sister Yes ☐5 No ☐6 Age __ Brother Yes ☐7 No ☐8 Age __ Son/Daughter Yes ☐9 No ☐10 Age __ Other Yes ☐11 No ☐12 Age __

No.	Question	
F7	Is there any relative in your family who has or had **diabetes** (high blood sugar)?	Yes ☐1 No (skip the next question and go to the next section) ☐2
F8	If **yes** in question F5, **who** has or had **diabetes** and **how old** were they when diabetes was diagnosed? (Please enter "not known" if you are not sure. Please indicate if you know approximate ages but not exact ages.)	Mother Yes ☐1 No ☐2 Age __ Father Yes ☐3 No ☐4 Age __ Sister Yes ☐5 No ☐6 Age __ Brother Yes ☐7 No ☐8 Age __ Son/Daughter Yes ☐9 No ☐10 Age __ Other Yes ☐11 No ☐12 Age __

7.4.7 Section 2 - Life Style Factors

In this section, there are questions about your lifestyle. A person's lifestyle can give us important clues as to the cause of their heart disease.

The first questions are about how much alcohol you drink.

No.	Question	
A1	Have you ever consumed a drink that contains alcohol?	Yes ☐1 No (skip this section and go to the next section) ☐2
A2	Have you consumed alcohol in the **past 12 months**?	Yes ☐1 No (skip this section and go to the next section) ☐2
A3	In the past 12 months, **how frequently** have you had at least one drink?	Daily ☐1 3 to 4 days per week ☐2 Weekly ☐3 Fortnightly ☐4 Monthly or on special occasions only ☐5
A4	When you drink alcohol, **on average,** how many drinks do you have during one day?	Number of drinks per day: (A drink is equal to 1 small glass of wine, a half pint of beer, 1 shot of spirits or liqueur.) _____

These questions are about smoking and use of tobacco.

No.	Question		
S1	Have you **ever smoked** any tobacco products?	Yes, currently smoke Yes, but stopped within past 12 months Yes, but stopped more than 12 months ago No (skip this question and go to the next section)	☐1 ☐2 ☐3 ☐4
S2	How **old** were you when you **first started** smoking daily?	(Give age in years)	_____
S3	What is the **maximum number** you have smoked per day for as long as a year	(insert number of cigarettes / cigars / hand made cigarettes per week / oz. of tobacco)	_____
S4	**PAST SMOKERS – only** Why did you give up smoking?	On doctor's advice Other reason	☐1 ☐2
S5	**PAST SMOKERS – only** For roughly how many years did you smoke?	(Give number of years)	_____

These questions are about your diet.

No.	Question		
D1	In a typical week, on **how many days** do you eat **fruit**?	(Insert number of days)	_____
D2	Approximately **how many pieces/ servings** of fruit do you eat on one of those days?	(Insert number of servings/ pieces)	_____
D3	In a typical week, on **how many days** do you eat **green leafy vegetables**? (e.g. spinach, salad leaves)	(Insert number of days)	_____
D4	Approximately **how many servings/ meals** would you have **green leafy vegetables** on one of those days?	(Insert number of servings/ meals)	_____

These questions are about your regular exercise and physical activity.

No.	Question	
P1	On average, how much physical activity do you do each day **during working hours**? (if retired or at home, this refers to during the day)	Lots (e.g. heavy lifting, digging, going up & down stairs) ☐1 Medium (e.g. light lifting, walking, light house-work, shopping, painting) ☐2 Light activity (e.g. standing, occasional working) ☐3 Almost none (e.g. desk job, sitting, driving) ☐4
P2	On average, how much physical activity do you do each day **after working hours**? (if retired, this refers to evenings and weekends)	Lots (e.g. competitive sports, aerobics, multiple times a week) ☐1 Medium (e.g. Casual sports, going to gym, regular walks 1-2 times per week) ☐2 Light activity (e.g. occasional working or bowls) ☐3 Almost none (e.g. Watching TV, listening to music, cooking, driving) ☐4

7.4.8 Section 3 - Current Medical conditions and risk factors

This final section is about your medical conditions and treatments.

No.	Question	
M1	Have you ever been told by a doctor or other health worker that you have **high blood pressure** or **hypertension**?	Yes ☐1 No, my blood pressure was always normal (skip the next question and go to question M3) ☐2 No, I have never had my blood pressure taken (skip the next question and go to question M3) ☐3
M2	***If yes,*** about **how long ago** were you first told by a doctor that you had high blood pressure?	(insert number of years) _____

No.	Question		
M3	Have you ever been told by a doctor or other health worker that you have **diabetes** (high blood sugar)?	Yes No, my blood sugar was always normal (skip the next question and go to question M5) No, I have never had my blood sugar taken (skip the next question and go to question M5)	☐1 ☐2 ☐3
M4	***If yes,*** about **how long ago** were you first told by a doctor that you had diabetes (a high blood sugar)?	(insert number of years)	_____
M5	Have you had a medical diagnosis of a **heart attack/ myocardial infarction**?	Yes No	☐1 ☐2
M6	Have you had a medical diagnosis of a **Stroke/ transient ischaemic attack**	Yes No	☐1 ☐2
M7	Have you had a medical diagnosis of **blood vessel disease in your legs/ peripheral vascular disease**	Yes No	☐1 ☐2
M8	Have you had a medical diagnosis of a **weak heart/ heart failure**	Yes No	☐1 ☐2
M9	Have you had a medical diagnosis of **kidney disease/ renal failure**	Yes No	☐1 ☐2
M10	Have you had a medical diagnosis of **lung/chest problems? e.g. bronchitis/emphysema/COPD/Asthma**	Yes No	☐1 ☐2
M11	Do you have or have you ever been given a diagnosis of **cancer**? *If yes* what type:_____	Yes No	☐1 ☐2
M12	Do you have **rheumatoid arthritis**? (inflammation of joints)	Yes No	☐1 ☐2
M13	Do you have **osteoarthritis** ?(wear and tear arthritis)	Yes No	☐1 ☐2

No.	Question	
M14	Do you have any other long standing medical conditions that are not already listed?	Yes ☐1 No ☐2
	If yes what are these conditions? *(you may leave blank if you prefer not to answer)*	

The next 2 questions are for women only

No.	Question	
W1	Have you gone through the **menopause**? i.e. have your periods stopped	Yes ☐1 No ☐2
W2	Have you ever taken the oral contraceptive pill (OCP) **or** hormone replacement therapy (HRT)?	Yes currently ☐1 Yes previously but now stopped ☐2 (Number of years stopped ☐3 ____) No never

The next 3 questions are for patients with diabetes only.

No.	Question	
CD1	Have you ever been told you have **damage to your eyes** (retinopathy) from having diabetes?	Yes ☐1 No ☐2
CD2	Do you have any **foot problems** due to diabetes (neuropathy)? e.g. ulcers, numbness, have missing /lost toes due to diabetes	Yes ☐1 No ☐2
CD3	Have you ever been told that your **kidneys** have been damaged from having diabetes (nephropathy)?	Yes ☐1 No ☐2

Please write the name of your current medications as they are labelled from the medicine box, or your script.
It may be easier for you just to bring a current medication list issued by your doctor or by your chemist with you. If you have such a list please leave the following box blank.

	Name of medication	**Dose of medication**	**How many times medication is taken per day?**	**How long have you been taking this medication?**
T1	_____			(please insert _____ years/months)
T2	_____			(please insert _____ years/months)
T3	_____			(please insert _____ years/months)
T4	_____			(please insert _____ years/months)
T5	_____			(please insert _____ years/months)
T6	_____			(please insert _____ years/months)
T7	_____			(please insert _____ years/months)
T8	_____			(please insert _____ years/months)
T9	_____			(please insert _____ years/months)
T10	_____			(please insert _____ years/months)

University of Glasgow | BHF Glasgow Cardiovascular Research Centre

Study-Nr.: ___-_____-___

Dear Sir or Madam,

DiCADU Study
Diagnosis of coronary artery disease with urinary proteomics

We would like you to answer a few questions. Ideally you might do this at home before your appointment visit. However, if you need assistance we will go through the list together at your appointment visit.

Please read the questions, then look at the options and tick the most appropriate answer in the answer box. If you are unsure of anything, put a mark beside it and discuss it with us at your appointment visit. If there is a question you prefer not to answer, please simply put a mark beside it so that we know.

For example:

No.	Question	
1	Sex	Male ☑1 Female ☐2
2	Date of Birth	___/___/___ Day Month Year
4	How many children have you ever had?	(insert number of children) ___2___
12	Which of the following best describes your main work status over the last 12 months?	Full-time employee ☐1 Part-time ☐2 Retired / at home ☐3

Remember your name is not recorded on any of the pages of the main questionnaire to help maintain your privacy.

The DiCADu Team

7.4.9 Questionnaire

7.4.10 ***Section 1 - Demographics and Family***

The background of a person has a substantial effect on an individual's risk of heart disease. In this first section we would like to find out a bit about you, your living circumstances and your family.

No.	Question		
1	Sex	Male Female	☐1 ☐2
2	Date of Birth	_____/_____/_____ Day Month Year	
3	Marital status	Single (never married) Married Living with partner Divorced or separated Widowed	☐1 ☐2 ☐3 ☐4 ☐5
4	How many children have you ever had?	(insert number of children)	_____
5	How many children are alive now?	(insert number)	_____
6	Are you one of a twin?	No Yes, identical Yes, non-identical	☐1 ☐2 ☐3
7	How many brothers do you have (all live births)?	(insert number of brothers)	_____
8	How many brothers are alive now?		_____
9	How many sisters do you have? (all live births)	(insert number of sisters)	_____
10	How many sisters are alive now?		_____

No.	Question	
11	What is the highest level of education you have completed?	Primary school completed ☐1 Secondary school completed ☐2 Technical college completed ☐3 University completed ☐4 Post graduate degree ☐5
12	Which of the following best describes your main work status over the last 12 months?	Full-time employee ☐1 Part-time ☐2 Retired / at home / unemployed ☐3
13	Which of the following best describes your racial background?	European or Caucasian ☐1 Other: Please specify: ☐2 _____
14	Would you say that in general **your quality of life** is -	Excellent ☐1 Very Good ☐2 Good ☐3 Fair ☐4 Poor ☐5
15	Would you say that in general **your health** is -	Excellent ☐1 Very Good ☐2 Good ☐3 Fair ☐4 Poor ☐5

The next questions are about your family.

No.	Question	
F1	Have any of your relatives had a **heart attack**?	Yes ☐1 No (skip the next question and go to question F3) ☐2
F2	If **yes** in question F1, **who** has had a heart attack and **how old** were they at heart attack? (Please enter "not known" if you are not sure. Please indicate if you know approximate ages but not exact ages.)	Mother Yes ☐1 No ☐2 Age __ Father Yes ☐3 No ☐4 Age __ Sister Yes ☐5 No ☐6 Age __ Brother Yes ☐7 No ☐8 Age __ Son/Daughter Yes ☐9 No ☐10 Age __ Other Yes ☐11 No ☐12 Age __
F3	Have any of your relatives had a **stroke**?	Yes ☐1 No (skip the next question and go to question F5) ☐2
F4	If **yes** in question F3, **who** has had a **stroke** and **how old** were they at stroke? (Please enter "not known" if you are not sure. Please indicate if you know approximate ages but not exact ages.)	Mother Yes ☐1 No ☐2 Age __ Father Yes ☐3 No ☐4 Age __ Sister Yes ☐5 No ☐6 Age __ Brother Yes ☐7 No ☐8 Age __ Son/Daughter Yes ☐9 No ☐10 Age __ Other Yes ☐11 No ☐12 Age __
F5	Is there any relative in your family who has or had **high blood pressure**?	Yes ☐1 No (skip the next question and go to question F7) ☐2
F6	If **yes** in question F5, **who** has or had **high blood pressure** and **how old** were they when high blood pressure was diagnosed? (Please enter "not known" if you are not sure. Please indicate if you know approximate ages but not exact ages.)	Mother Yes ☐1 No ☐2 Age __ Father Yes ☐3 No ☐4 Age __ Sister Yes ☐5 No ☐6 Age __ Brother Yes ☐7 No ☐8 Age __ Son/Daughter Yes ☐9 No ☐10 Age __ Other Yes ☐11 No ☐12 Age __

No.	Question	
F7	Is there any relative in your family who has or had **diabetes** (high blood sugar)?	Yes ☐1 No (skip the next question and go to the next section) ☐2
F8	If **yes** in question F5, **who** has or had **diabetes** and **how old** were they when diabetes was diagnosed? (Please enter "not known" if you are not sure. Please indicate if you know approximate ages but not exact ages.)	Mother Yes ☐1 No ☐2 Age __ Father Yes ☐3 No ☐4 Age __ Sister Yes ☐5 No ☐6 Age __ Brother Yes ☐7 No ☐8 Age __ Son/Daughter Yes ☐9 No ☐10 Age __ Other Yes ☐11 No ☐12 Age __

7.4.11 Section 2 – Chest pain, chest tightness or angina

In this section, there are questions about your chest pain symptoms. The quality and quantity of chest pain helps us better to understand the cause of your heart disease.

No.	Question	
C1	Over the past 4 weeks, on average, how many times have you had **chest pain, chest tightness or angina**?	4 or more per day ☐1 1-3 times per day ☐2 3 or more times per week, but not every day ☐3 1-2 times per week ☐4 Less than once a week ☐5 None over the past 4 weeks ☐6
C2	If you had no **chest pain, chest tightness or angina** during the past 4 weeks, what caused it to disappear?	Medical treatment (pills) ☐1 Angiography with Stent ☐2 Other: Please specify: ☐3 _____
C3	Did any of the following treatments improve your **chest pain, chest tightness or angina** in the past?	Medical treatment (pills) ☐1 Angiography with Stent ☐2 Other: Please specify: ☐3 _____

C4	Over the <u>past 4 weeks</u>, if you had **chest pain, chest tightness or angina**, how long, on average, did an episode last?	Less than 5 minutes 5 to 10 minutes 10 to 15 minutes More than 15 minutes	☐1 ☐2 ☐3 ☐4
C5	Over the <u>past 4 weeks</u>, on average, how may times have you had to take nitroglycerin (nitroglycerin tablets or spray) for you **chest pain, chest tightness or angina**?	4 or more per day 1-3 times per day 3 or more times per week, but not every day 1-2 times per week Less than once a week None over the past 4 weeks	☐1 ☐2 ☐3 ☐4 ☐5 ☐6
C6	If you had to use nitroglycerin spray or tablets <u>over the past 4 weeks</u>, in average, how long did it take until you noticed a change?	Less than 1 minutes 1 to 5 minutes 5 to 10 minutes More than 10 minutes No effect	☐1 ☐2 ☐3 ☐4 ☐5
C7	Under which circumstances did you suffer from **chest pain, chest tightness or angina**? (If more than one option, please circle the dominant)	Rest Emotional stress On physical exertion	☐1 ☐2 ☐3
C8	How would you describe the onset of your **chest pain, chest tightness or angina**?	Gradual Rapid	☐1 ☐2
C9	Where did your **chest pain, chest tightness or angina** mainly appear in relation to your body?	Centre of chest Upper part of stomach Left shoulder Other: Please specify: _____	☐1 ☐2 ☐3 ☐4

The following is a list of activities that people often do during the week. Although for some people with several medical problems it is difficult to determine what it is that limits them, please go over the activities listed below and indicate how much limitation you have had **due to chest pain, chest tightness or angina.** If you suffered from chest pain over the past 4 weeks please refer to this period.

No.	**Activity**	**Extremely limited**	**Quite a bit limited**	**Moderately limited**	**Slightly limited**	**Not at all limited**	**Limited for other reasons or did not do the activity**
Y1	Dressing yourself	☐1	☐2	☐3	☐4	☐5	☐6
Y2	Walking indoors on level ground	☐1	☐2	☐3	☐4	☐5	☐6
Y3	Showering	☐1	☐2	☐3	☐4	☐5	☐6
Y4	Climbing a hill or a flight of stairs without stopping	☐1	☐2	☐3	☐4	☐5	☐6
Y5	Gardening, vacuuming or carrying groceries	☐1	☐2	☐3	☐4	☐5	☐6
Y6	Walking more than a block at a brisk pace	☐1	☐2	☐3	☐4	☐5	☐6
Y7	Running or jogging	☐1	☐2	☐3	☐4	☐5	☐6
Y8	Lifting or moving heavy objects (e.g. furniture, children)	☐1	☐2	☐3	☐4	☐5	☐6
Y9	Participating in strenuous sports (e.g. swimming, tennis)	☐1	☐2	☐3	☐4	☐5	☐6

Section 3 - Life Style Factors

In this section, there are questions about your lifestyle. A person's lifestyle can give us important clues as to the cause of their heart disease.

The first questions are about how much alcohol you drink.

No.	Question		
A1	Have you ever consumed a drink that contains alcohol?	Yes No (skip this section and go to the next section)	☐1 ☐2
A2	Have you consumed alcohol in the **past 12 months**?	Yes No (skip this section and go to the next section)	☐1 ☐2
A3	In the past 12 months, **how frequently** have you had at least one drink?	Daily 3 to 4 days per week Weekly Fortnightly Monthly or on special occasions only	☐1 ☐2 ☐3 ☐4 ☐5
A4	When you drink alcohol, **on average,** how many drinks do you have during one day?	Number of drinks per day: (A drink is equal to 1 small glass of wine, a half pint of beer, 1 shot of spirits or liqueur.)	_____

These questions are about smoking and use of tobacco.

No.	Question		
S1	Have you **ever smoked** any tobacco products?	Yes, currently smoke Yes, but stopped within past 12 months Yes, but stopped more than 12 months ago No (skip this question and go to the next section)	☐1 ☐2 ☐3 ☐4
S2	How **old** were you when you **first started** smoking daily?	(Give age in years)	_____

S3	What is the **maximum number** you have smoked per day for as long as a year	(insert number of cigarettes / cigars / hand made cigarettes per week / oz. of tobacco)	_____
S4	**PAST SMOKERS – only** Why did you give up smoking?	On doctor's advice Other reason	☐1 ☐2
S5	**PAST SMOKERS – only** For roughly how many years did you smoke?	(Give number of years)	_____

These questions are about your diet.

No.	Question		
D1	In a typical week, on **how many days** do you eat **fruit**?	(Insert number of days)	_____
D2	Approximately **how many pieces/ servings** of fruit do you eat on one of those days?	(Insert number of servings/ pieces)	_____
D3	In a typical week, on **how many days** do you eat **green leafy vegetables**? (e.g. spinach, salad leaves)	(Insert number of days)	_____
D4	Approximately **how many servings/ meals** would you have **green leafy vegetables** on one of those days?	(Insert number of servings/ meals)	_____

These questions are about your regular exercise and physical activity.

No.	Question		
P1	On average, how much physical activity do you do each day **during working hours**? (if retired or at home, this refers to during the day)	Lots (e.g. heavy lifting, digging, going up & down stairs) Medium (e.g. light lifting, walking, light house-work, shopping, painting) Light activity (e.g. standing, occasional working) Almost none (e.g. desk job, sitting, driving)	☐1 ☐2 ☐3 ☐4

P2	On average, how much physical activity do you do each day **after working hours**? (if retired, this refers to evenings and weekends)	Lots (e.g. competitive sports, aerobics, multiple times a week) Medium (e.g. Casual sports, going to gym, regular walks 1-2 times per week) Light activity (e.g. occasional working or bowls) Almost none (e.g. Watching TV, listening to music, cooking, driving)	☐1 ☐2 ☐3 ☐4

7.4.12 Section 4 – Current Medical conditions and risk factors

This final section is about your medical conditions and treatments.

No.	Question		
M1	Have you ever been told by a doctor or other health worker that you have **high blood pressure** or **hypertension**?	Yes No, my blood pressure was always normal (skip the next question and go to question M3) No, I have never had my blood pressure taken (skip the next question and go to question M3)	☐1 ☐2 ☐3
M2	***If yes,*** about **how long ago** were you first told by a doctor that you had high blood pressure?	(insert number of years)	_____

No.	Question		
M3	Have you ever been told by a doctor or other health worker that you have **diabetes** (high blood sugar)?	Yes No, my blood sugar was always normal (skip the next question and go to question M5) No, I have never had my blood sugar taken (skip the next question and go to question M5)	☐1 ☐2 ☐3
M4	***If yes,*** about **how long ago** were you first told by a doctor that you had diabetes (a high blood sugar)?	(insert number of years)	_____
M5	Have you had a medical diagnosis of a **heart attack/ myocardial infarction**?	Yes No	☐1 ☐2
M6	Have you had a medical diagnosis of a **Stroke/ transient ischaemic attack**	Yes No	☐1 ☐2
M7	Have you had a medical diagnosis of **blood vessel disease in your legs/ peripheral vascular disease**	Yes No	☐1 ☐2
M8	Have you had a medical diagnosis of a **weak heart/ heart failure**	Yes No	☐1 ☐2
M9	Have you had a medical diagnosis of **kidney disease/ renal failure**	Yes No	☐1 ☐2
M10	Have you had a medical diagnosis of **lung/chest problems? E.g. bronchitis, emphysema, asthma**	Yes No	☐1 ☐2
M11	Do you have or have you ever been given a diagnosis of **cancer**? *If yes* what type:_____	Yes No	☐1 ☐2
M12	Do you have **rheumatoid arthritis**? (inflammation of joints)	Yes No	☐1 ☐2
M13	Do you have **osteoarthritis** ?(wear and tear arthritis)	Yes No	☐1 ☐2

No.	Question	
M14	Do you have any other long-standing medical conditions that are not already listed?	Yes ☐1 No ☐2
	If yes what are these conditions? *(you may leave blank if you prefer not to answer)*	

The next 2 questions are for women only

No.	Question	
W1	Have you gone through the **menopause**? i.e. have your periods stopped	Yes (skip the next question and go to question W3) ☐1 No ☐2
W2	Have you recently noticed any of the following symptoms?	Hot flushes ☐1 Breast tenderness ☐2 Fatigue ☐3 Irregular periods ☐4 Vaginal dryness (e.g. discomfort during intercourse) ☐5 Urinary urgency (a pressing need to urinate more frequently) ☐6 ☐7 Mood swings ☐8 Difficulty sleeping
W3	Have you ever taken the oral contraceptive pill (OCP) **or** hormone replacement therapy (HRT)?	Yes currently ☐1 Yes previously but now stopped ☐2 (Number of years stopped ____) ☐3 No never
W4	Did you ever undergo an operation on your reproductive organs (e.g. hysterectomy)?	Yes, please specify: ☐1 _____ No ☐2

The next 3 questions are for patients with diabetes only.

No.	Question	
CD1	Have you ever been told you have **damage to your eyes** (retinopathy) from having diabetes?	Yes ☐1 No ☐2
CD2	Do you have any **foot problems** due to diabetes (neuropathy)? e.g. ulcers, numbness, have missing /lost toes due to diabetes	Yes ☐1 No ☐2
CD3	Have you ever been told that your **kidneys** have been damaged from having diabetes (nephropathy)?	Yes ☐1 No ☐2

Please write the name of your current medications as they are labelled from the medicine box, or your script.
It may be easier for you just to bring a current medication list issued by your doctor or by your chemist with you. If you have such a list please leave the following box blank.

	Name of medication	**Dose of medication**	**How many times medication is taken per day?**	**How long have you been taking this medication?**
T1	_____			(please insert _____ years/months)
T2	_____			(please insert _____ years/months)
T3	_____			(please insert _____ years/months)
T4	_____			(please insert _____ years/months)
T5	_____			(please insert _____ years/months)
T6	_____			(please insert _____ years/months)
T7	_____			(please insert _____ years/months)
T8	_____			(please insert _____ years/months)
T9	_____			(please insert _____ years/months)
T10	_____			(please insert _____ years/months)

Appendix D

Relevant publications